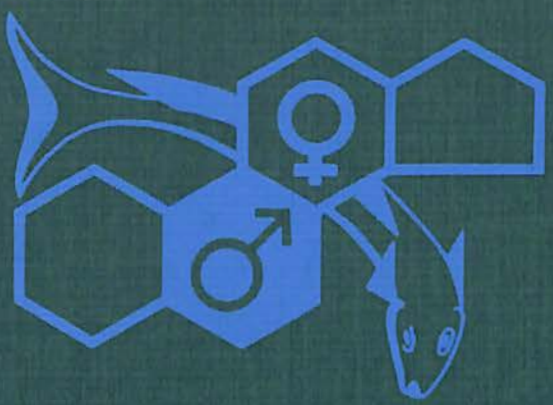
REPRODUCTIVE PHYSIOLOGY OF FISH



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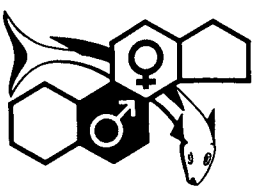

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C.J.J. Richter and H.J.Th. Goos (compilers)

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CONTENTS

This book contains the text of about 100 papers and posters presented at the Second International Symposium on Reproductive Physiology of Fish, held at Wageningen, the Netherlands, from 2-6 August 1982.

Modern fish farming is a highly specialized, intensive industry, more and more requiring possibilities to manipulate reproduction. Altering sexual cycles and sex ratios, induction of maturation of gametes, ovulation and spermation, storing over longer periods of time of eggs and sperm and artificial fertilization are techniques commonly used for several species of commercial fish but still under development for others. Such techniques can be achieved by trial and error and some results are impressive. However, a better understanding of the reproductive physiology of fish might help to more and faster progress.

The aim of the symposium was to bring together those concerned with applied aspects of fish reproduction and daily problems of fish farming, with researchers interested in the more fundamental problems of the reproductive physiology of fish.

The generous financial support from :
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was a great help to organize this symposium.

Thanks are due to the staff of the International Agricultural Centre, Wageningen, for housing the symposium.

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C.J.J.Richter,
H.J.Th.Goos.

REPRODUCTIVE PHYSIOLOGY AND FISH CULTURE

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A good knowledge of the mechanisms regulating the reproductive function in fish is requisite to the development of aquaculture. Once acquired, this knowledge makes it possible to exercice varying degrees of control on the different stages of reproduction, to adapt the techniques to fit new situations, and then to teach and propagate these techniques to extention officers and fish culturists, Reproductive physiology has progressed considerably during the last few years owing to the development of new and varied techniques; it has proven its ability to synthesize all the data from many diversified and complex fields such as endocrinology, cytology and molecular biology, Some of the crucial problems is reproductive physiology have thus been studied and partly resolved. Research has usually been carried out on laboratory animals in very artificial conditions mimicking traditional research in animal husbandry. But this is changing; there is now a trend toward studying the animal in its natural environment, where it is submited to a larger variety of environmental factors.

On some basic problems of physiology interesting fish culturists.

The control of reproductin in fish farming involves control of sex, the reproductive cycle and the release of gametes as well as the management of gametes and embryos. Controlling reproduction implies a knowledge of the following major problems.

1) Sex determinism and differentiation. We must determine the dynamics of these processes, the factors responsible, the origin of somatic cells, the relationship between germ cells and somatic cells and the development of these cells.
2) Puberty. It is desirable to be able to advance, delay or inhibit puberty. We must study the initiation of the functioning at central, pituitary and gonadal levels and how these are related to growth.
3) Gametogenesis and gametes release. We must acquire a knowledge of the dynamic (cycles), the endocrine (endocrine and neuroendocrine regulation as related to environment) and metabolic (nutrition) aspects of gametogenesis as well as of its genetic side (ecophysiological strains and breeds). The ovarian and hepatic aspects of vitellogenesis are crucial as well as the final phase of oocyte maturation and spermiation which often has to be induced in numerous cultured species. It is necessary to know when and how this phase can be induced and the consequences that may result from a given treatment (possibility of aneuploidy or subsequent sterility). A good knowledge of the flux of energy at these stages (part used for hydration and remaining part for embryogenesis) is important and decide what strategy should be used for larvae rearing. We must also study the optimal environmental conditions for obtaining good-quality gametes during gametogenesis. The gamete release is a key point in fish culture and an improvement of the techniques of induced spawning requires a better knowledge of the neuroendocrine mechanisms of regulation which are at least in some species either stimulatory or inhibitory as shown recently. This open now the possibility (chemical or environmental) to overcome this inhibition which probably strongly linked to the environment.
4) Spawning. A simple way to obtain fertilized eggs is to put males and females together in a tank or pond and let them reproduce naturally. In these conditions, the behavior of the fish and the environmental cues (physical, chemical and social) are primordial and can, in certain cases, resolve the problem of induced spawning.
5) Gametes physiology. When artificial insemination is used, it is essential to understand the physiology of the gametes, their energy requirements and their long and short-term storage as well as the composition of their companion fluids. In order to mix the gametes at an optimal rate during artificial insemination, the quality of egg and sperm and change in time must first be assessed.

6) Fertilization and embryogenesis. The environmental conditions in which the gametes are associated at artificial insemination, fertilization, and the onset of development may have different effects on embryonic development, particularly on pregastrulation stages. It is therefore imperative to define such conditions as, for example, salinity, temperature, pH, ionic composition and dilution rate, as accurately as possible. The quality of embryonic development, which also depends of the environmental conditions may be used as a criterion of gametes quality. All these factors are interdependent and must be considered along with other functions such as nutrition, osmoregulation, respiration and behavior.

Such detailed data cannot be obtained on a large number of species and therefore research has to be limited to a few species serving as representative models of intensive

rearing (aquaculture of transformation; e.g. salmonids) or extensive rearing (aquaculture of production; e.g. cyprinids and cichlids which often need a hatchery).

However, the idea of a model species is not a complete answer to the problem of obtaining complex data because there are wide divergences between species of the same family and even within a species: dynamic of oogenesis is different in tench and in roach LH-RH is much more active in phytophagous species than in carp, HCG induces ovulation or spermiation in goldfish but not in carp, the sculpin is a hatch-spawner in Dorset chalk streams but lays only a single batch of eggs in Teesdale streams. Data cannot be applied directly but must always be adapted to cultured fish whose reproduction is to be controlled.

On the necessity of new managing fish species in the wild and the need for original data in the field of reproductive physiology

The approach described above, which seeks to acquire detailed knowledge of a small number of species, is simply a copy of the approach used in animal husbandry, i.e. domestication trials with control of the main functions and monoculture usually in intensive conditions in tanks or in a relatively well controlled ecosystem. This idea has been applied to aquaculture with some success but with many failures owing to the fact that fitness for domestication varies a great deal among species, and we do not have enough data from other fields.

At present there is another demand in aquaculture concerning the management of the natural or quasi-natural environment; this means managing (understanding, protecting, exploiting) complex aquatic ecosystems considered in their entirely with different trophic levels and a wide range of species. In this case, monoculture and domestication cannot be used; a new type of knowledge is needed. We must develop research in which animals, as plants, will be returned to that complex, interrelated system known as the trophic chain. The main functions, studied in relation to the environment, will include a variety of physical-chemical, climatic, biological and social factors. As concerns reproduction, we must be able to estimate and predict the fecundity, prolificness and reproductive periods of different species in relation to the food available to brood fish and larvae with special reference to climatic conditions such as temperature. The ecological conditions for reproduction should be analysed for a large number of species. The ecology of spawning grounds needs to be studied, particularly from the point of view of oxygenation, physical-chemical composition of the environment, turbidity, conditions attractive brood fish, aeration of eggs and the importance of predation during juvenile stages. We shall then know how to protect eggs and fry and manage spawning areas and their annexes (nests, egg substrate), We must be able to estimate brood fish populations as well as their egg and larval production and , if necessary, extra brood fish or juveniles.

For polyculture, it is necessary to determine the conditions in which reproduction occurs in the wild and how these conditions change in response to an increase in the diversification of the number of species.

The reproductive physiologist should also be able to analyse how and why the changes in the environment have consequences on the reproduction and the recruitement of the species as for instance the drastic modifications in the age composition of the atlantic salmon fisheries and the considerable increase of the male parr and female grilses.

Conclusions

If research is to contribute to fish culture, the knowledge we must acquire in the field of reproductive physiology is of two kinds: (i) knowledge of the mechanisms governing each of the reproductive stages, the potentialities of reproduction and their manifestation in cultured fish and (ii) knowledge of the mechanisms regulating reproduction in the wild. In the first case, mecanisms and potentialities should be studied in a variety of species and sometimes even independently of their interest to culturists; some species are more fitted than others to the study of a particular problem. It will then be necessary to adapt the results to cultured species. In the second case, we must favor ecophysiological research because this approach particularly concerns species to be raised in polyculture and may involve a relatively large number of them.

In any case, the fish physiologist must continually inform himself of new developments in methods and techniques and of the state of research in progress in others animal species. Fish species are numerous and diversified and some well studied species in other classes may serve as models.

Finally, it is not enough that the culturist understand reproduction; that knowledge must be associated and integrated with knowledge in other fields such as nutrition, genetics and pathology. We must associate and synthetize in experimental fish farm laboratory and experimental informations comming from all fields of research.

SOME PERSPECTIVES ON FISH GONADOTROPINS

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Key words: Gonadotropins, isolation, biological action, measurement, assay.

Opening remarks

The weight of evidence continues to favour multiple gonadotropins (GtH) in the few teleosts which have been examined in detail. It is important to distinguish between multiple forms of "similar" carbohydrate-rich GtH's (e.g. isohormones) and hormones which differ sufficiently in their carbohydrate composition or content to be separated on immobilized lectins such as wheat germ or Concanavalin A-Sepharose.

For the purposes of this address I shall use the term vitellogenesis to refer to the synthesis of vitellogenin by the liver and the uptake into the gonad "i.e. exogenous vitellogenesis" and any synthesis or assembly of yolk proteins that may take place in the ovary at this time "i.e. endogenous vitellogenesis".

Let us now turn to the action of fish gonadotropins on fish and particularly the evidence relating to gonadotropins that are atypical in the sense that they differ from the classical gonadotropins described for other life forms, at least until very recently. I am referring to the gonadotropins that are rather poor in carbohydrate and not retained on Concanavalin A-Sepharose. The other terms applied to these hormones are Con AI GtH's to distinguish them from the more conventional Con AII type which are adsorbed on the immobilized lectin. Finally, these hormones, which have been found in each teleost we have examined to date, have been called vitellogenic hormones since their biological actions have only been demonstrated during this important phase of reproduction.

The scope of this manuscript is confined to teleosts. The listener is referred to reviews by Fontaine & Burzawa-Gerard, 1978; Idler & Ng, 1980; Crim, 1982; Ball, 1981, and Burzawa-Gerard, 1982 for rhythms and other aspects not covered here. The action of mammalian hormones on fish will not be discussed.

Isolation of gonadotropins

In various investigations pituitary glands were collected fresh from fish whenever possible, or as soon as possible after death, to prevent postmortem degradation of hormones. This was done in the case of salmonids (Breton et al., 1976; Donaldson et al., 1972; Idler et al., 1975a; Yoneda & Yamazaki, 1976) winter flounder (Ng & Idler, 1979) Tilapia (Farmer & Papkoff, 1977). Acetone powder of carp pituitaries was obtained from a commercial source by Burzawa-Gerard (1971). Acetone powder from pike eel pituitaries was similarly prepared by Huang and associates (1981).

Solvent fractionation and salting out was a purification step in the isolation of gonadotropins from pituitaries of carp by Burzawa-Gerard (1971); chinook salmon by Donaldson and coworkers (1972); sturgeon by Burzawa-Gerard and associates, (1975a); chum salmon by Yoneda & Yamazaki (1976); Tilapia by Farmer & Papkoff, (1977), and Hyder et al., (1979); and pike eel by Huang's group (1981). Alcoholic percolation as described by Bates and others (1959) and extraction with ethanolic ammonium acetate buffer followed by precipitation with ethanol, that is the Stockell Hartree procedure of 1966, was employed in these studies.

Idler and his group (1975a, b) innovated the use of affinity chromatography on Con A-Sepharose in their scheme of isolation of gonadotropins. Con A-Sepharose chromatography was adopted by Pierce and others (1976) and Breton and associates (1978). Chum salmon pituitary extract yielded a broad peak unadsorbed on Con A-Sepharose (designated Con AI fraction) and an adsorbed fraction (designated Con AII fraction) which was eluted as a sharp peak by inclusion of α-methyl-D-glucoside in the buffer. The protease inhibitor Trasylol was used and divalent cations including calcium, magnesium and manganese assure efficient adsorption. The immobilized lectin selectively adsorbs glycoprotein with specific structures (Goldstein et al., 1965), leaving behind in the unadsorbed fraction proteins with a low carbohydrate content or not having the sugars requisite for binding to the lectin. Rechromatography of the Con AI fraction on Con A-Sepharose is frequently necessary to eliminate contamination with Con AII material. Idler & Hwang (1978) found that the inclusion of ethylene glycol(50% v/v) in the elution buffer optimized the recovery, from Con A-Sepharose, of maturational hormone which was contained in the Con AII fraction.

Other immobilized lectins with different carbohydrate binding specificities in the isolation of teleost gonadotropins have been tested by Idler & Ng (1979). Lentil lectin Sepharose has a carbohydrate specificity

similar to that of Con A-Sepharose but it has a lower affinity according to Stein & associates, 1971. Wheat germ lectin Sepharose and Helix pomatia lectin Sepharose are specific for N-acetyl-glucosamine and N-acetylgalactosamine respectively. It was found that Con AII fraction was adsorbed on Lentil lectin-Sepharose, wheat germ lectin Sepharose and Helix pomatia lectin-Sepharose. Breton in 1981 showed that wheat germ lectin adsorbed the gonadotropin which was adsorbed on Con A-Sepharose. Thus, both groups found the same gonadotropin fraction to be adsorbed on the immobilized lectins.

Gel filtration separates proteins mainly on the basis of molecular size. Sephadex G-100 or G-75 was used by most investigators although some workers employed Ultrogel AcA 54 or AcA 44. In the case of a species, such as flounder where the peak following the void volume is relatively high molecular weight (62,000) then Ultrogel allows a better separation than Sephadex G-75. The buffer used was either the volatile NH_4HCO_4, which lends itself to lyophilization or Tris-Cl at a near neutral pH.

Diethylaminoethyl (DEAE) cellulose or a DEAE ion exchanger such as DEAE Bio-Gel A or DEAE-Sephadex A 50 was used to purify teleost gonadotropins on the basis of charge properties.

The buffer used was either NH_4HCO_3, tris buffer or sodium glycinate buffer. A concentration gradient was used to elute the adsorbed material. The virtues of NH_4HCO_3 have been noted for purifying mammalian gonadotropins. It is easy to remove and results in good stability of the preparations. However, it has also given trouble with DEAE Bio-Gel A. For example, from time-to-time protein which is normally held on the column will appear in the unadsorbed fraction from Con AI. This fraction must be checked, say by electrophoresis, and run again on DEAE Bio-Gel A if any bands faster than Rf 0.2 are observed (Idler, 1981). This problem caused us to sometimes find a higher Rf for prolactin than 0.14 and 0.18. We now elute prolactin and then reverse the flow to obtain the other peptides which are than isolated by preparative PAGE.

Carboxymethyl ion exchanger chromatography was used by Pierce & associates (1976), by Idler & Ng (1979) and by Hyder & coworkers (1979) employing ammonium acetate buffer at an acidic pH. Amberlite CG-50 was employed by Farmer & Papkoff (1977).

Preparative PAGE was one of the steps in the purification protocol used by Burzawa-Gerard (1971) with carp and by Huang & coworkers (1981) with pike eel.

A variety of bioassays have been utilized to monitor gonadotropic activity during isolation. Testosterone output by rat Leydig cells *in vitro* was employed as an assay for Tilapia gonadotropin by Farmer & Papkoff (1977). Pike eel gonadotropin was also active in this assay but much less so than the Tilapia hormone. The day-old cockerel testicular radiophosphate uptake assay was used in the isolation of salmon gonadotropin by Donaldson, Idler, Pierce & associates. The pike eel gonadotropin was also active in this avian assay. However, Ishii & Yamamoto (1976) found that a salmon pituitary extract was inactive in stimulating hypertrophy of chick Sertoli cells. The discrepancy in their findings may have been due to the dosage or to the assay parameters employed.

Amphibian assays involving spermiation and oocyte maturation were used to monitor gonadotropic activity during isolation of carp and sturgeon gonadotropins by Burzawa-Gerard.

There was also a variety of isolation procedures utilizing teleost assays. Yoneda & Yamazaki (1976) purified chum salmon gonadotropin with the goldfish spermiation assay of Yamazaki & Donaldson (1969). Stimulation of testicular growth by Schmidt *et al.*, 1965 and gonadal cAMP by Idler *et al.*, 1975a in immature trout, stimulation of lipovitellin production by Grönlund (1969) oocyte maturation *in vitro* by Breton *et al.*, 1976 and by Hirose, 1980 and *in vivo* by Ng & Idler (1978a) and Sundararaj & Nayyar (1976), testicular interlobular and intralobular histology by Hyder *et al.*, 1979, testicualr steroidogenesis by Huang, and vitellogenesis by Sundararaj & Idler's group have been used. Gonadotropin induced a depletion of free cholesterol in the mature ovary of murrel (C. punctatus) and this formed the basis of an assay ranging from 1-10 µg, Mukherjee & Bhattacharya, 1981.

It is advisable to use, wherever possible, the same or a closely related species in biological studies, to circumvent the prolem of biological specificity. When Fontaine *et al.*, 1972 compared the potencies of their carp gonadotropic preparation with salmon gonadotropin SG-G100 of Donaldson in stimulating adenylate cyclase activity in the goldfish, he found that the carp gonadotropin was 36 times more potent than SG-G100. The possibility has to be taken into account that the carp gonadotropic preparation and SG-G100 may be of different degrees of purity, but it is very likely that the same picture would emerge after the differences in the extent of purification have been allowed for. Thus *Cyprinus carpio* maturational hormone was about five times more active than salmon maturational hormone in stimulating cAMP production by grass carp ovaries *in vitro* according to Idler & Ng, 1979. Carp gonadotropin was more active than catfish gonadotropin in stimulating the production of cAMP in eel ovary and both were much more active than was sturgeon gonadotropin conclude Dufour & associates, 1979. It has been the interest of

many comparative endocrinologists to test the effect of mammalian gonadotropins on fish reproduction. There is a discrepancy between the actions of mammalian and teleost gonadotropins on vitellogenesis in the catfish. Induction of vitellogenin into the circulation was achieved by mammalian gonadotropins but no incorporation of the yolk precursor into the oocytes resulted (Nath & Sundararaj, 1981). This phenomenon again illustrates the principle of zoological specificity of gonadotropins. In the assay of maturational activity using the germinal vesicle breakdown of Oryzias latipes oocytes in vitro as the assay parameter, it was found that ovine LH was less potent than SG-G100 and the slopes of the dose-response curves were different (Hirose, 1980). Carp gonadotropin and mammalian LH differed in their stimulation of eel ovarian cAMP (Fontaine et al., 1981).

Salmon gonadotropin SG-G100 had no effect on the processes of luteinization and progesterone secretion in cultured monkey granulosa cells according to Channing, et al., 1974. Burzawa-Gerard (1974) found carp gonadotropin was not active in the rat HCG argmentation test for mammalian FSH or the rat ovarian ascorbic acid depletion test for LH. Huang reported pike eel gonadotropin was much more active in stimulating testosterone production in vitro by carp testes than by rat Leydig cells. In general, mammalian bioassays could seldom be utilized to monitor gonadotropic activities during the isolation of teleost gonadotropins.

For assay of teleost gonadotropins using teleosts the most popular assays have been migration of the germinal vesicle in vitro, spermiation, uptake of glycolipophosphoprotein, steroidogenesis in vitro and in vivo, estrogen secretion, vitellogenin production, and cyclic AMP production in vitro. Of course, the radioimmunoassay for the carbohydrate-rich gonadotropin has been the most used of all the assays. It may well be that phosphorus uptake into the day-old chick will prove to be the least specific as to species in a quantitative sense for Con AII type gonadotropins; this can be useful.

Technical considerations

If advances on gonadotropins of teleosts have been slower than we all would wish the problem lies in several areas. Fish pituitaries are relatively small, weighing less than a milligram in small teleosts and 40-50 mg in an 8-10 pound salmon. Thus, the work of my group alone has required more than 60,000 flounder and 80,000 salmon pituitaries in the past 9 years and there has seldom been enough purified hormones on hand to do bioassays at more than one or two levels. The elapsed time to process less than 1000 pituitaries to the point of homogeneity of most of the peptides on PAGE is about 3 weeks with our quite good walk-in cold room facilities. This can be accelerated by use of solvent or chemical precipitation and freeze-drying to name two procedures which we have elected not to include until more is known about stability of the fish hormones. The overall yield through 6 major purification steps and dialysis and concentration through molecular sieves generally does not exceed 15% even though individual steps will range from 50-90% for most components of the fraction not adsorbed on Concanavalin A-Sepharose. The relative death of appropriate homologous bioassays has been a bottleneck particularly for studies on ovarian growth. The uptake of radioactive phosphorus and amino acids, during the most active phase of vitellogenesis has been studied in some detail. The interpretation of results depends on how the analyses are performed and this ranges from counting pieces of gonads to precipitation with trichloroacetic acid to isolation of the glycolipophosphoprotein fraction by dialysis or it could be done with Concanavalin A-Sepharose. The recent advent of a mild iodination procedure (Iodogen) which permits the satisfactory labelling of vitellogenin offers greater specificity.

Removal of the pituitary generally is essential if the fish is vitellogenic; this is feasible for some species such as catfish and flounder but very tricky and tedious for most salmonids.

The stunted landlocked Atlantic salmon (Ouananiche) is a valuable experimental animal because the male matures at 1+ years and the female at 2+ years and even at 3+ the weight is only about 100 grams; thus hormones and physical facilities can be conserved.

Action of fish gonadotropins on fish

Hypophysectomy brings about a decrease in gonadosomatic index and a diminished incorporation of yolk into the gonad (Campbell & Idler, 1976). Thus exogenous vitellogenesis is under control of the pituitary. This finding confirmed the observation of many investigators that vitellogenesis is arrested by ablation of the pituitary.

Wiegand & Peter (1980) demonstrated that salmon gonadotropin SG-G100 increases the plasma concentrations of triglycerides and total cholesterol in goldfish with small ovaries. The lipid mobilization may be mediated by estrogen produced by the ovary under gonadotropic stimulation. The same treatment produces a decrease in the plasma concentrations of lipids in fish with big ovaries, probably due to a gonadotropin-stimulated uptake of the lipids into ovary.

Vitellogenic activity in the teleost gonadotropin preparation SG-G100 and carp GtH has been noted by 3 research groups on 3 continents. A similar gonadotropin

preparation has been shown to stimulate vitellogenesis in the eel by Fontaine & associates (1976) and Nath & Sundararaj (1981) noted that SG-G100 and carp GtH stimulated vitellogenin and yolk granule production in catfish.

Dependency of ovarian estrogen production on the pituitary has been established by various investigators. In vitro evidence was obtained by Yaron & Barton (1980) using plaice ovaries and homologous pituitary extract. In vivo evidence was obtained by Crim & Idler (1978) administering salmon pituitary extract into trout, and by Fostier & associates (1979) using the homologous carp system. Bhattacharya (1981) reported at Hong Kong that a freshwater perch responded to SG-G100 with elevated ovarian 17β-hydroxysteroid dehydrogenase activity; this is the enzyme that regulates the conversion of estradiol into estrone.

In our laboratory juvenile female rainbow trout had an extremely low plasma concentration of estrogen and as a consequence the plasma vitellogenin level was negligible. The production of both estrogen and vitellogenin in these fish was boosted by treatment with a salmon pituitary Con AII hormone. The Con AI fraction (unabsorbed on Concanavalin A-Sepharose) immunologically purified by adsorption with an antiserum to the maturational hormone, manifests no such activity. Male trout treated with a salmon pituitary Con AII fraction do not respond by producing vitellogenin as in the female because of the inability of their gonads to produce estrogen (Idler & Campbell, 1980).

In 1972 Rudolph Reinboth stated "Biochemical work and a large number of histophysiological studies are at variance with regard to the question whether one or two gonadotropic hormones exist in teleosts". Perhaps a few of us believe without reservation that this question now has been answered in the affirmative but certainly this viewpoint is not shared by some investigators.

I wish to emphasize the question of unity or multiplicity of gonadotropins in some teleost fish since generally little attention has been given to this subject in other reviews. I have intentionally changed "duality" as originally used by Reinboth (1972) in his excellent discussion of the topic for "multiplicity" so as not to suggest that there must be either one or two gonadotropins in the pituitary. It is my contention that there is adequate published evidence that more than one gonadotropin exists in the few species of teleosts which have been studied from this viewpoint. It is required only that one accept that a fish pituitary polypeptide that is involved with gonad development during the phase of active vitellogenesis is a gonadotropin whether it does or does not bear a close chemical resemblance to LH and FSH of other life forms.

One group who have supported the concept of a single gonadotropin observed in 1978 with reference to carp and salmon preparations "each purified hormone was able to stimulate every pituitary dependent stage of sexual development in various teleosts" (Fontaine & Burzawa-Gerard). Let us briefly examine this statement. The term "Purified" is not very specific and we know that the salmon preparation (SG-G100) was a good one, given the stage of the art at the time, but work reported in 1976 by a group that included the discoverer clearly established that it contained large quantities of peptide not adsorbed on Concanavalin (Pierce, et al.,) and Dr. Burzawa-Gerard has recently shown that a carp GtH preparation contained 5% of such protein (Burzawa-Gerard, 1982).

Campbell & Idler (1976) discovered that the Con AI (Unadsorbed on Concanavalin A-Sepharose) fraction of the pituitary extract of American plaice stimulated incorporation of radiophosphate labeled plasma yolk into the ovary of winter flounder. Ng & Idler (e.g 1978a) confirmed this finding by extending the study to the Con AI fractions of winter flounder, chum salmon and carp pituitaries.

I now wish to summarize some of the data which supports the presence of carbohydrate-rich and carbohydrate-poor gonadotropins in pituitaries of these species. Our principal experimental animal frequently is the winter flounder, either hypophysectomized or treated with antibodies to fish gonadotropins in order to tie up endogenous gonadotropin(s). Most of the assays have been conducted on hormones isolated from Pacific salmon and winter flounder. Reproduction in oviparious female teleosts, as in other oviparous vertebrates, are comprised of two phases about which we have most knowledge of GtH action: (a) vitellogenesis and (b) oocyte maturation and ovulation. The elevation of plasma 11-ketotestosterone in hypex winter flounder by partially purified and purified Con AII GtH is quite dramatic. By contrast even the crude Con AI fraction containing vitellogenic gonadotropin was inactive at a dose reflecting the difference in abundance of the Con AI and Con AII fractions (Ng & Idler, 1980).

When Con AII GtH and Con AI GtH are administered to hypex flounder and oocytes are biopsied and examined at intervals only the Con AII GtH stimulates migration of the germinal vesicle as evidenced by the maturation rating (Ng & Idler, 1978a). In retrospect, perhaps the first hint that something might be wrong with Con AII GtH doing everying was the failure of others to detect changes, or to barely detect Con AII hormone, in plasma of fish undergoing active vitellogenesis (e.g. Crim et al., 1975). This is the reason I emphasized the word administered.

The first firm evidence for multiple gonadotropins came with the discovery of a fraction, in the Con AI subfraction, which like the Con AII hormone could stimulate the uptake of phosphorus and leucine into the glycolipophosphoprotein fraction of ovaries of hypex flounder. The isolation of the glycolipophosphoprotein fraction is based on the salt solubility and water insolubility of this material in ovary as shown by Plack, et al., 1971. Similar data have been published for Con AI gonadotropins of the four species which we have studied. To further convince ourselves that we were looking at the uptake of vitellogenin, radioactive Vg was prepared by giving estradiol and $^{33}PO_4$ to a male flounder; after dialysis to remove $^{33}PO_4$ and low molecular weight phosphorus compounds the plasma was injected into hypex flounder (Ng & Idler, 1980). The uptake into the ovary was stimulated by the most highly purified flounder Con AI gonadotropin available. The salmon Con AI gonadotropin occurs in two different molecular weight forms. Another piece in the puzzle was provided by an in vitro study conducted on trout oocytes by Chris Campbell and reported at Paimpont (1978). Campbell prepared radioactive trout vitellogenin by reductive methylation and compared the stimulation of uptake by crude pituitary extract, the Con AII, and Con AI fraction. Con AI fraction produced a significant stimulation of Vg uptake into the oocyte while Con AII did not.

The vitellogenic activity of Chinook salmon pituitary Con AI fraction was also inferred by the finding of Upadhyay & associates (1978) who showed that Breton's highly purified Con AII type salmonid maturational hormone was incapable of inducing vitellogenin incorporation in the immature trout whereas the whole pituitary extract produced ultrastructural changes in the ovary charcteristic of exogenous vitellogenesis.

Vitellogenic hormone administered to estrogenized or non-estrogenized flounder, hypophysectomized when they were actively undergoing vitellogenesis, stimulates incorporation of vitellogenin into the ovary, but was not able to do so in non-estrogenized flounder at an earlier phase of vitellogenesis in September. Presumably the difference in results is due to the low plasma titer of vitellogenin in fish hypophysectomized at the earlier phase with the consequence that the stimulatory effect of vitellogenic hormone on vitellogenin incorporation is not obvious. Fish actively undergoing vitellogenesis before hypophysectomy would be expected to continue producing vitellogenin at an appreciable, albeit decreased, rate after hypophysectomy (e.g. Idler & Ng, 1979).

There are potential problems of interpretation when hypophysectomized fish are used to study vitellogenin uptake since hormones are removed in addition to the one under study. With this in mind, we turned to the use of antibodies to the Con AI and Con AII gonadotropins. We have evidence that flounder and salmon hormones purified through the carboxymethyl cellulose step gave a CMI fraction which is free of hormones that stimulate the thyroidal system (Ng, et al., In Press).

Ng & associates (Ng, et al., 1980a) established that vitellogenic Atlantic salmon treated with an antiserum to maturational hormone have lower plasma levels of vitellogenin and estradiol than those treated with an antiserum to vitellogenic hormone or normal rabbit serum. A decreased incorporation of radioactive phosphate into gonadal yolk occurs in fish treated with either type of gonadotropin antiserum. An antiserum to vitellogenic hormone arrests vitellogenesis and induces follicular atresia in vitellogenic flounder, while an antiserum to maturational hormone does not have any appreciable effect on the histological appearance of the ovary (Ng, et al., 1980b) When taken together these findings suggest that maturational hormone is responsible for initiating vitellogenesis by inducing ovarian estrogen secretion and hence hepatic vitellogenin synthesis, but its role in promoting gonadal vitellogenin incorporation is relatively minor compared with that of vitellogenic hormone. The inhibitory effect of the maturational hormone antiserum on gonadal vitellogenin incorporation in salmon may be a consequence of its influence on plasma estradiol and vitellogenin rather than a direct effect.

These roles of vitellogenic hormone and maturational hormone are supported by immunofluorescent localization of the hormones in the ovary. The hormones are detected, probably bound to their respective receptors, in specific regions of the ovarian section. Vitellogenic hormone is found in the ooplasma of both large immature and vitellogenic oocytes and in follicular envelopes of vitellogenic oocytes, implying that the hormone prepares or primes the large immature oocytes for the vitellogenic phase and it stimulates the vitellogenic oocytes to incorporate vitellogenin. Maturational hormone is located in follicular envelopes in interstitial tissue and large immature oocytes suggesting its role in steroidogenesis and vitellogenesis through estrogen and vitellogenin production. However, it cannot be located in the ooplasma of vitellogenic oocytes (Ng, et al., 1980b).

Let us now consider the pituitary from female winter flounder in August when vitellogenesis is underway (Burton, et al., 1981). Consecutive sections were made of the rostral pars distalis and the proximal pars distalis. When sections were treated with antibody to flounder maturational

hormone prior to use of the fluorescent second antibody no fluorescence was detected and therefore no maturational hormone was detected in these regions of the pituitary. By contrast, the proximal pars distalis area fluoresced when antibody to vitellogenic hormone was used. This was clear also when consecutive sections through the proximal pars distalis were photographed and only the antibody to the vitellogenic hormone resulted in fluorescence. Let us now examine the pituitary of a female flounder in April which is close to spawning time. The antibody to the maturational hormone localizes in the rostral lobe while the antibody to the vitellogenic hormone localized in the proximal pars distalis as it did in the pituitary from the vitellogenic flounder.

To sum up, the process of vitellogenesis in teleosts has been shown to be similar to that operating in other oviparous vertebrates. The contribution of autosynthetic processes (endogenous vitellogenesis) to the yolk mass in teleost ovary, relative to exogenous yolk acquired by incorporation of vitellogenin, has not been estimated. Exogenous vitellogenesis can be considered to consist of two phases. The first phase involves the induction of hepatic vitellogenin production under stimulation of ovarian estrogen. During the second phase vitellogenin is taken up from the blood stream and incorporated into ovarian yolk proteins. In salmonids maturational gonadotropin occurs at high levels in plasma around spawning time but is near the lower limit of the radioimmunoassay during the phase of active incorporation of vitellogenin and there appears to be a small increase in maturational gonadotropin coincident with an increase in estradiol in trout plasma early in vitellogenesis. Wiegand & Idler (In press) found recently that antibody to maturational gonadotropin inhibits ovarian growth immediately prior to the rapid and massive increase in the ovarian weight. Thus, there appears to be a low level of maturational hormone present when the fish resumes ovarian development after spawning but it is sufficient to establish vitellogenin production by the liver.

Sundararaj & associates (1982) found very recently that carp carbohydrate-rich gonadotropin induced some "vitellogenic oocytes" and at higher doses some yolky oocytes in post-spawned hypophysectomized catfish and concluded that low levels maintained the sensitive system. It is not known what role, if any, the 5% Con AI protein, recently reported by Dr. Burzawa-Gerard in carp gonadotropin plays in the processes. If I have correctly read the abstracts, we shall learn that the Con AI component of carp gonadotropin stimulated pinocytosis in another teleost, Gobius niger. Certainly we found carp Con AI gonadotropin has similar biological action to other teloest Con AI gonadotropins but the Con AI is not likely to occur in the highly purified carbohydrate-rich carp gonadotropin. Given the current state of knowledge we suggest that low levels of maturational gonadotropin initiate vitellogenesis through its action on ovarian estrogen secretion which in turn augments hepatic vitellogenin synthesis although other actions on ovarian growth are not precluded. Ovarian yolk deposition and growth is then primed and maintained primarily by vitellogenic gonadotropin.

Interpretation can only be done with more confidence when Con AII gonadotropin free of Con AI and vica versa are employed.

Other supportive data to distinguish Con AI vitellogeninic from Con AII maturational hormones reported by Ng & associates were various amino acid and carbohydrate analyses and the very poor cross-reactivity of antibodies produced to each gonadotropin.

Let us return briefly to the Con AII maturational gonadotropins. There are multiple molecular weight forms of gonadotropin from more than one species. In 1975 our group isolated two fractions on DEAE Bio-Gel A from the Con AII fraction of chum salmon. One acted preferentially to stimulate cAMP in immature male trout testes while the other preferentially stimulated the ovary (Idler, et al., 1975b). Breton & associates (1978) employing similar chromatographic procedures found that the male chinook salmon pituitary Con AII fraction contained a maturational hormone which differed from its female counterpart in biological specific activity of stimulating oocyte maturation in female trout. α Amino butyric acid was found in the preparation from male but not female pituitaries. There also may be a gonadotropin in sturgeon, according to Goncharov & associates (1980) which has some sex specificity.

If I may be permitted a reminiscence, it has been 20 years since we isolated 11-ketotestosterone and the female sex steroid, 20β-dihydro-17α-hydroxy progresterone from plasma of Pacific salmon. I am very pleased that others have clearly demonstrated their importance in regulating reproductive processes (e.g. Jalabert, 1976). Both are controlled by gonadotropin according to Ng & Idler (1980) and Fostier & associates (1981). We shall learn more about the naturally occurring levels of these hormones in plasma at this meeting.

Antibody cross-reactivities and the subunits

The glycoprotein hormones have a common quaternary structure which is shared, but each hormone has an α and β subunit which are not identical. The subunits of mammalian gonadotropins are not biologically active and both carp GtHα and GtHβ have little or no biological activity in four test systems. The amino acid sequences of α subunits are

very similar and the differences in biological action reside in the β subunits (Fontaine & Burzawa-Gerard, 1978). When one considers immunological cross-reactivities the situation is not so predictable. For example, there is excellent recognition of human glycoprotein hormones by an antibody to hHCGα but antibody to hLHα failed to recognize the α-subunit of αLH's from three mammalian non-primates (for discussion see Vaitukaitis, 1978). Surprisingly there was complete recognition of the three non-primate β-subunits by the antibody to human LHβ. However, antibodies to other glycoprotein β-subunits resulted in incomplete cross-reaction with β-subunits of other species.

Turning to fish Burzawa-Gerard & associates have studied the immunoreactivity of α and β subunits from mammalian and fish carbohydrate-rich gonadotropins with antibodies to their carp gonadotropin and its α and β subunits. On this criteria, they conclude that the carp β subunit is more closely related to LHβ than to FSHβ. The antibody to the carp α subunit recognized the Cyprinoform teleosts (carp and catfish) GtH but not GtH from eel and sturgeon. The zoological specificity of the β-subunit was much weaker and all fish gonadotropins and even bovine LH competed with carp GtHβ for the antibody. Given the present state of knowledge, biological and immunological activity do not seem to have a high degree of correlation.

The human pituitary contains a large reserve of LHα but little, if any, of the β-subunit according to Vaitukaitis. Fontaine & Burzawa-Gerard (1978) report that cGTH contains of the order of 20% subunits. Antibodies to the carp α-subunit exhibited no cross-reaction with the β-subunit and 20-50% with cGtH. Similarly the antibody to the β-subunit exhibited no recognition of the α-subunit and 25% with cGtH. Finally, cGtH antibody recognized the β-subunit 100% but reacted less than 0.1% with the α-subunit. Thus, it would seem that free α-subunits in either the pituitary or blood should not interfer very much with the determination of cGtH using the cGtH antibody; there is still the question of thyroid stimulating hormones to consider.

Pituitary extracts of 14 species of fish from the North Atlantic were tested in the rainbow trout and carp glycoprotein gonadotropin systems and it was concluded that even fish in the same order did not preferentially react with the expected antibody (Bye, et al., 1980). However, there were possible complicating factors (proteolytic enzymes and stage of the reproductive cycle) that prevent firm conclusions. Tan & Dodd (1978) investigated pituitary extracts and plasma from 35 species using a salmon (SG-G100) antibody in a salmon-salmon homologous system and a salmon-carp heterologous system. They concluded that immunological properties of fish gonadotropins may not correspond to known phylogenetic relationships but parallelism was established for some salmonid systems as it was by Crim & associates earlier (1975). Generally speaking the cross-reactivity of antibodies have been unpredictable among species in radioimmunoassays.

Radioimmunoassays and forms of hormones

In conclusion I would like to address the question of what is being measured by RIA or what should be measured. Fish endocrinologists might note that there is a growing body of evidence that mammalian gonadotropins as isolated from the pituitary are not always identical with the hormones circulating in the blood. There is even evidence of qualitative differences in FSH's secreted by the pituitary and these may be related to discrepancies between bioassay and radioimmunoassay results (Bogdanove & Nansel, 1978). There is a recent report of an FSH in blood that does not bind to Concanavalin A-Sepharose and which may be the form secreted by the hamster pituitary (Chappel et al., 1982).

There is now considerable evidence that teleost pituitary gonadotropins from the same species can have different molecular weights, or are separable on ion exchangers, PAGE and isoelectric focusing. Most of the research has been done on the "carbohydrate-rich" Con AII type gonadotropins but there is some similar evidence for the "carbohydrate-poor" Con AI gonadotropins. Stuart-Kregor et al., 1981 observed dramatic changes in plasma testosterone and 11-keto-testosterone levels in precocious male Atlantic salmon at a time when there is "little or no variation in circulating GtH" and yet we know that pituitary Con AII GtH greatly increases the plasma level of these steroids (Ng & Idler, 1980). Is it possible that the RIA is not measuring the form of the hormone present in blood at this time? One alternate explanation is that the change is in the receptors rather than in the blood hormone levels. I reported at the recent symposium on fish migration and reproduction in Tokyo that an antibody to pituitary prolactin from chum salmon gave parallelism with pituitary extracts but not with plasma (Idler, 1981). As antibodies are produced to the various "isohormone" of the teleost gonadotropins it will be interesting to see which cross-react with blood and how well, but more interesting will be their biological actions both qualitative and quantitative as homologous bioassays are developed for more species. It will not be an easy task to sort out the active from the inactive forms and the various interferences in plasma but a good start has been made in the mammalian field and those of us who work on poikilotherms will have to come to grips with the problems.

In my view heterologous assays should be avoided except for specific comparative

studies. Thus, the considerable knowledge of the structure and function of gonadotropins based on pituitary hormones may undergo substantial modification as information is gained about the circulating forms which act on the target tissues. Perhaps it is appropriate to reiterate a plea that A.S. Parks made in opening a 1957 Ciba coloqium that we should remember "a hormone is something that goes around in the blood to act in another part of the body" and he urged more research on the nature of hormones in the blood. This does not, of course, preclude transformation of a hormone enroute to, or at its target.

References

Ball, J.N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians and reptiles. Gen. Comp. Endocrinol. 44: 135-170.

Bates, R.W., R.W. Garrison & T.B. Howard, 1959. Extraction of thyrotropin from pituitary glands, mouse pituitary tumors and blood plasma by percolation. Endocrinology 65: 7-17.

Bhattacharya, S. Sen, & S. Deb, 1981. Hormonal regulation of ovarian 17β-hydroxysteroid dehydrogenase in teleost. Abstract of papers. Ninth International Symposium on Comparative Endocrinology. Hong Kong. p. 30.

Bogdanov, E.M., & D.D. Nansel, 1978. Biological and immunological distinctions between pituitary and serum LH in the rat. In: "Structure and Function of the Gonadotropins" (K.W. McKerns, ed.), pp. 415-430. Plenum Press. New York.

Breton, B., 1981. A study of salmon (Oncorhynchus tshawytscha) pituitary gonadotropin dissociation. Gen. Comp. Endocrinol. 45: 147-152.

Breton, B., B. Jalabert & P. Reinaud, 1976. Purification of gonadotropin from rainbow trout (Salmo gairdnerii Richardson) pituitary glands. Ann. Biol. anim. Biochim. Biophys. 16(1): 25-36.

Breton, B., P. Prunet & P. Reinaud, 1978. Sexual differences in salmon gonadotropin. Ann. Biol. anim. Biochem. Biophys. 18(4): 759-765.

Burton, M.P., D.R. Idler & T.B. Ng, 1981. The immunofluorescent location of teleost gonadotropins and thyrotropins in flounder pituitary. Gen. Comp. Endocrinol. 43: 135-147.

Burzawa-Gerard, E., 1974. Etude biologique et biochimique de l'hormone gonadotrope d'une poisson téléostéen, la Carpe (Cyprinus carpio L.). Mem. Mus. Nat. Hist. Nat. Ser. Zool. 86: 1-77.

Burzawa-Gerard, E., 1982. Chemical data on pituitary gonadotropins and their implication to evolution. Can. J. Fish. Aquat. Sci. 39: 80-91.

Burzawa-Gerard, E., B.F. Goncharov & Y.A. Fontaine, 1975a. L'Hormone gonadotrope hypophysaire d'un poisson chondrostéen, l'esturgeon (Acipenser stellatus Pall.). I. Purification. Gen. Comp. Endocrinol. 27: 289-295.

Bye, V.J., B. Breton & R. Billard, 1980. Immunological cross-reaction between pituitary gonadotropin from North Atlantic fisheries. Gen. Comp. Endocrinol. 41: 130-134.

Campbell, C.M., 1978. In vitro stimulation of vitellogenin incorporation into trout oocyte by salmon pituitary extracts. Ann. Biol. anim. Biochem. Biophys. 18(4): 1013-1018.

Campbell, C.M. & D.R. Idler, 1976. Hormonal control of vitellogenesis by hypophysectomized winter flounder (Pseudopleuronectes americanus Walbaum). Gen. Comp. Endo. 28:143

Channing, C.P., P. Licht, H. Papkoff & E.M. Donaldson, 1974. Comparative activities of mammalian, reptilian and piscine gonadotropins in monkey granulosa cell culture. Gen. Comp. Endocrinol. 22: 137-145.

Chappel, S.C., C. Coutifaris & S.J. Jacobs, 1982. Studies on the microheterogeneity of follicle-stimulating hormone present within the anterior pituitary gland of ovariectomized hamsters. Endocrinology 110: 846-854.

Crim, L.W., 1982. Environmental modulation of annual and daily rhythms associated with reproduction in teleost fishes. Can. J. Fish. Aquat. Sci. 39: 17-21.

Crim, L.W., & D.R. Idler, 1978. Plasma gonadogropin, estradiol and vitellogenin and gonad phosvitin levels in relation to the seasonal reproductive cycle of female brown trout. Ann. Biol. anim. Biochim. Biophys. 18: 1001-1005.

Crim, L.W., E.G. Watts & E.M. Donaldson, 1975. The plasma gonadotropin profile during sexual maturation in a variety of salmonid fishes. Gen. Comp. Endocrinol. 27: 62-70.

Donaldson, E.M., F. Yamazaki, H.M. Dye & W. W. Philleo, 1972. Preparation of gonadotropin from salmon (Oncorhynchus tsawytscha) pituitary glands. Gen. Comp. Endocrinol. 18: 469-481.

Dufour, S., E. Burzawa-Gerard & Y.A. Fontaine, 1979. Evolution des hormones glycoprotéiques hypophysaires:Données radioimmunologiques sur les sousunités de l'hormone gonadotrope de la carp (Cyprinus carpio L). C.R. Acad. Sci. Paris 289: 137-140.

Farmer, S.W., & H. Papkoff, 1977. A teleost (Tilapia mossambica) gonadotropin that resembles luteinizing hormone. Life Sci. 20: 1227-1232.

Fontaine, Y.A., & E. Burzawa-Gerard, 1978. Biochemical and biological properties of fish gonadotropins and their subunits: comparison with mammalian hormones. In: "Structure and Function of the Gonadotrop-

ins" (M.W. McKerns ed.). pp. 361-380. Plenum Press. New York and London.
Fontaine, Y.A., E. Lopez, N. Delerue-Labelle, E. Fontaine-Bertrand, F. Lalier & C. Salmon, 1976. Stimulation gonadotrope de l'ovarie chez l'anguille (Anguilla anguilla L.) hypophysectomisée. J. Physiol. 72: 871-892.
Fontaine, Y.A., C. Salmon, E. Fontaine-Bertrand, E. Burzawa-Gerard & E.M. Donaldson, 1972. Comparison of the activities of two purified fish gonadotropins on adenyl cyclase activity in the goldfish ovary. Can. J. Zool.50: 1673-1676.
Fostier, A., B. Breton & B. Jalabert, 1979. Stimulation hypophysaire de la sécrétion oestradiol-17β chez la carpe commune, Cyprinus carpio L. Ann Endocrinol. 40: 83-84.
Fostier, A., B. Jalabert, C. Campbell, M. Terqui & B. Breton, 1981. Cinétique de liberation de 17α hydroxy-20β dihydroprogestérone par des follicules de Truite arc-en-ciel, Salmo gairdnerii. C.R. Acad. Sci. Paris Ser. III. 292: 777-780.
Goldstein, I.J., C.E. Hellerman & J.M. Merrick, 1965. Protein-carbohydrate interaction. I. The interaction of polysaccharides with Concanavalin A. Biochim. Biophys. Acta 97: 68-76.
Goncharov, B.F., E. Burzawa-Gerard & A.A. Kuznetsov, 1980. A study on the heterogeneity of the pituitary gonadotropin from sturgeon (Acipenser stellatus Pall.). Gen. Comp. Endocrinol. 40: 342.
Grönlund, W., 1969. Biological assay and partial characterization of the gonadotropic factors of the pituitary gland of Pacific salmon (Oncorhynchus) M.Sc. Thesis University of Washington.
Hirose, H., 1980. A bioassay for teleost gonadotropin using the germinal vesicle breakdown (GVDB) of Oryzias latipes oocyte in vitro. Gen. Comp. Endocrinol. 41: 108-114.
Huang, F.-L., C.-J. Huang, S.-H. Lin, T.-B. Lo and H. Papkoff, 1981. Isolation and characterization of gonadotropin isohormones from the pituitary gland of pike eel (Muraenesox cinereus). Int. J. Peptide Protein Res. 18: 69-78.
Hyder, M., A.V. Shah & A.S. Hartree, 1979. Methallibure studies on Tilapia III. Effects of Tilapian partially purified pituitary gonadotropic fractions on the testes of methalibure-treated Sarotherodon spirulus (=Tilapia nigra). Gen. Comp. Endocrinol. 39: 475-480.
Idler, D.R., 1981. Salmon prolactin and its radioimmunoassay. ORI Symposium on Fish Migration and Reproduction. Abstracts. Tokyo
Idler, D.R., L.S. Bazar & S.J. Hwang. 1975a. Fish gonadotropin(s). II. Isolation of gonadotropin(s) from chum salmon pituitary glands using affinity chromatography. Endocrine Res. Commun. 2(3): 215-235.
Idler, D.R., L.S. Bazar & S.J. Hwang, 1975b. Fish gonadotropin(s). III. Evidence for more than one gonadotropin in chum salmon pituitary glands. Endocrine Res. Commun. 2(3): 237-249.
Idler, D.R., & S.J. Hwang, 1978. Conditions for optimizing recovery of salmon gonadogropin activity from Con A-Sepharose. Gen. Comp. Endocrinol. 36: 642-644.
Idler, D.R., S.J. Hwang & L.S. Bazar, 1975c. Fish gonadotropins(s). I. Bioassay of salmon gonadotropin(s) in vitro with immature trout gonads. Endocrine Res. Commun. 2(3):199-213.
Idler, D.R., & T.B. Ng, 1979. Studies on two types of gonadotropins from both salmon and carp pituitaries. Gen. Comp. Endocrinol. 38: 421-440.
Idler, D.R. & C.M. Campbell, 1980. Gonadotropin stimulation of estrogen and yolk precursor synthesis in juvenile rainbow trout. Gen. Comp. Endocrinol. 41: 384-391.
Idler, D.R., & T.B. Ng, 1980. Pituitary gonadotropins of bony fish. Isolation and biological action. Ninth International Congress on Animal Reproduction and Artificial Insemination. (Madrid) 2: 339-345.
Ishii, S., & K. Yamamoto, 1976. Demonstration of follicle stimulating hormone (FSH) activity in hypophyseal extracts of various vertebrates by the response of the Sertoli cells of the chick. Gen. Comp. Endocrinol. 29: 506-510.
Jalabert, B, 1976. In vitro oocyte maturation and ovulation in rainbow trout (Salmo gairdneri) northern pike (Esox lucius) and goldfish (Carassius auratus) J. Fish. Res. Board Can. 33: 974-988.
Nath, P., & B.I. Sundararaj, 1981. Induction of vitellogenesis in the hypophysectomized catfish, Heteropneustes fossilis (Bloch): Effects of piscine and mammalian hormones. Gen. Comp. Endocrinol. 43: 191-200.
Ng, T.B., & D.R. Idler, 1978a. "Big" and "Little" forms of plaice vitellogenic and maturational hormones. Gen. Comp. Endocrinol. 34: 408-420.
Ng, T.B., & D.R. Idler, 1978b. A vitellogenic hormone with a large and a small form from salmon pituitaries. Gen. Comp. Endocrinol. 35: 189-195.
Ng, T.B., & D.R. Idler, 1979. Studies on two types of gonadotropins from both American plaice and winter flounder pituitaries. Gen. Comp. Endocrinol. 38: 410-420.
Ng, T.B., & D.R. Idler, 1980. Gonadotropic regulation of androgen production in flounder and salmonids. Gen. Comp. Endocrinol. 42: 25-38.
Ng, T.B., C.M. Campbell & D.R. Idler, 1980a. Antibody inhibition of vitellogenesis and oocyte maturation in salmon and flounder. Gen. Comp. Endocrinol. 41: 233-239.
Ng, T.B., D.R. Idler & M.P. Burton, 1980b.

Effects of teleost gonadotropins and their antibodies on gonadal histology in winter flounder. Gen. Comp. Endocrinol. 42: 355-464.

Ng, T.B., D.R. Idler & J.G. Eales, 1982. Pituitary hormones that stimulate the thyroidal system in teleost fishes. (In Press). Gen. Comp. Endocrinol.

Pierce, J.G., M.R. Faith & E.M. Donaldson, 1976. Antibodies to reduced S-carboxymethylated alpha subunit of bovine luteinizing hormone and their application to study of the purification of gonadotropin from salmon (Oncorhynchus tshawytscha) pituitary glands. Gen. Comp. Endocrinol. 30: 47-60.

Plack, P.A., D.J. Pritchard & N.W. Fraser, 1971. Egg proteins in cod serum. Natural occurence and induction by injections of oestradiol-3-benzoate. Biochem. J. 121: 847-856.

Reinboth, R., 1972. Hormonal control of the teleost ovary. Amer. Zool. 12: 307-324.

Stein, M.D., I.K. Howard & H.J. Sage, 1971. Studies on a phytohemagglutinin from the lentil. IV. Direct binding studies of Lens culinaris hemagglutinin with simple saccharides. Arch. Biochem. Biophys. 146: 353-355

Stockell Hartree, A., 1966. Separation and partial purification of the protein hormones from human pituitary glands. Biochem. J. 100: 754-761.

Sundararaj, B.I., P. Nath & E. Burzawa-Gerard, 1982. Synthesis of vitellogenin and its uptake by the ovary in the catfish, Heteropneustes fossilis (Bloch) in response to carp gonadotropin and its subunits. Gen. Comp. Endocrinol. 46: 93-98.

Sundararaj, B.I., & S.K. Nayyar, 1976. Effects of carp gonadotropin on ovarian maintenance, maturation and ovulation in hypophysectomized catfish, Heteropneustes fossilis (Bloch). Gen. Comp. Endocrinol. 30: 472-476.

Upadhyay, S.N., B. Breton & R. Billard, 1978. Ultrastructural studies on experimental induced vitellogenesis in juvenile rainbow trout. Ann. Biol. anim. Biochim. Biophys. 18: 1019-1025.

Vaitukaitis, J.L., 1978. Glycoprotein hormones and thier subunits - immunological and biological characterization. In: "Structure and Function of the Gonadotropins" (K.W. McKerns, ed.). Plenum Press. New York. pp. 339-360.

Wiegand, M.D., & D.R. Idler (In Press). Impairment of early ovarian growth in landlocked Atlantic salmon by an antibody to carbohydrate-rich gonadotropins. Gen. Comp. Endocrinol.

Wiegand, M.D. & R.E. Peter, 1980. Effects of salmon gonadotropin (SG-G100) on plasma lipids in the goldfish, Carassius auratus. Can. J. Zool. 58: 967-972.

Yaron, Z., & C. Barton, 1980. Stimulation of estradiol-17β output from isolated ovarian fragments of the plaice (Pleuronectes platessa) by homologous pituitary extract. Gen. Comp. Endocrinol. 42: 151-154.

Yondeda, T., & F. Yamazaki, 1976. Purification of gonadotropin from chum salmon pituitary glands. Bull. Jap. Soc. Sci. Fish. 42: 343-350.

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GONADOTROPIC FUNCTION DURING THE REPRODUCTIVE CYCLE OF THE FEMALE RAINBOW TROUT, SALMO GAIRDNERI, IN RELATION TO OVARIAN STEROID SECRETION : IN VIVO AND IN VITRO STUDIES

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Summary

An in vivo study demonstrated considerable changes, during oogenesis, in the short-term (circadian and ultradian) plasma profiles of the maturational gonadotropin, GtH. The related profiles of circulating estradiol-17β (E_2-17β) and 17α-hydroxy-20β-dihydroprogesterone (17α, 20β-OH-P) were monitored. The functional significance of the different plasma GtH patterns was further explored in vitro using an ovarian open-perifusion system. It has been concluded that at early ovarian recrudescence high-amplitude plasma GtH pulses, constituting the spring GtH rise, might be related to the regulation of E_2-17β production as well as to early stages of gamete development. During exogenous vitellogenesis the appearance of plasma GtH pulses of moderate amplitude might regulate the increase in circulating E_2-17β levels and their daily cyclicity. Around the time of oocyte maturation and ovulation, continuously high GtH levels (and possibly their daily cycles) might be obligatory signals, adjusted to the modified ovarian steroidogenic potential, for the maintenance of the production of maturation-inducing steroid (17α, 20β-OH-P) and perhaps for the continuous inhibition of enzyme activities (aromatase, 17-20 lyase) interfering with such a production.

Keywords: rainbow trout, GtH, sex steroids, daily fluctuations, control of steroidogenesis.

Introduction

The pituitary control of ovarian development in salmonids involves maturational gonadotropin (GtH) modulation of ovarian steroidogenesis. Exposure of the ovaries to GtH in vivo or in vitro enhances the secretion of estradiol-17β (E_2-17β) during vitellogenesis (Billard et al., 1978; Breton et al., 1982a; Nagahama et al., 1982), or earlier (Idler & Campbell, 1980), and of 17α-hydroxy-20β-dihydroprogesterone (17α, 20β-OH-P) before the onset of oocyte maturation (Fostier et al., 1981a; Suzuki et al., 1981; Scott et al., 1982)

The study of seasonal variations of plasma GtH and sex steroids throughout the reproductive cycle has demonstrated a temporary increase in GtH at early stages of ovarian recrudescence (Billard et al., 1978; Breton et al., 1982a,b), while E_2-17β concentrations were still low and either stable (Whitehead et al., 1978; Scott et al., 1980; van Bohemen & Lambert, 1981) or increasing slightly (Billard et al., 1978; Breton et al., 1982b). While some studies reported a moderate increase in plasma GtH levels throughout vitellogenesis (Billard et al., 1978; Breton et al., 1975, 1982b), another study noted an initial increase followed by a continuous decline (Bromage et al., 1982). During the same process the levels of plasma E_2-17β and testosterone rose considerably, then declined progressively from the final stages of vitellogenesis (Billard et al., 1978; Fostier et al., 1978; Whitehead et al., 1978; Scott et al., 1980, 1982; van Bohemen & Lambert, 1981; Breton et al., 1982b); this decrease was accompanied by marked elevations in the GtH and the 17α, 20β-OH-P levels which induce oocytes to undergo maturation and ovulation (Billard et al., 1978; Fostier et al., 1978, 1981b; Breton et al., 1982b; Bromage et al., 1982; Scott et al., 1982). Whereas 17α, 20β-OH-P decreases regularly after ovulation (Fostier et al., 1981b), GtH continues to rise for 2 to 3 more weeks (Jalabert & Breton, 1980).

Such long term hormonal profiles might reflect only partially the gonadotropic function (Hontela & Peter, 1978; Zohar et al., 1982) and its relation to ovarian steroidogenesis. The present paper summarizes a series of experiments in which we studied, at certain characteristic stages of the reproductive cycle of the female rainbow trout (1) the short term profiles of plasma maturational GtH, E_2-17β and 17α, 20β-OH-P levels using free swimming fish fitted with a catheter in the dorsal aorta (Zohar, 1980) and (2) the regulation of ovarian steroidogenesis by salmon maturational GtH using an in vitro ovarian open-perifusion system. With such an approach, we hoped to obtain additional data on the gonadotropic function and its relation to ovarian steroid secretion.

Results and discussion

A. Early ovarian recrudescence (March)

The ovaries of the studied fish contained germ cells at developmental stages ranging from oogonia to primary oocytes undergoing early exogenous vitellogenesis. Bleeding the cannulated females at 4 or 1-hr intervals for 24 hrs revealed short-term (1 to 2 hrs), high amplitude (ΔGtH = up to 100 ng/ml) pulses in plasma GtH levels (fig. 1a) in 12 out of 26 fish; in the others these levels remained low and stable. In most cases, only one pulse was observed during the 12-hr sampling periods.

E_2-17β levels studied at the same time were low and stable (fig. 1a). However, when *in vitro* perifused ovarian pieces were exposed to GtH pulses similar to those recorded *in vivo* (amplitude: 50 or 100 ng/ml; duration: 1.5-2 hrs), we observed an immediate and continuous amplification of the E_2-17β secretion rate, followed by its slow decrease. E_2-17β release was amplified up to at least 8 hrs after GtH application.

The plasma GtH pulses, which were not synchronous among the fish, might constitute the brief spring GtH rise in female trout sampled monthly or every 2 weeks (see Introduction). Our *in vitro* data suggest the stimulatory role of these pulses in regulating E_2-17β production. The stable *in vivo* E_2-17β levels, together with the prolonged *in vitro* enhancement of E_2-17β secretion in response to a GtH pulse might indicate maintenance by the GtH pulses of constant circulating concentrations of E_2-17β over short periods. The high-amplitude GtH pulses could also play a role in the numerous oogonial mitoses and oocyte meioses observed in the ovaries of the studied fish and thought to require gonadotropin support.

B. Exogenous vitellogenesis (June and September-October)

At earlier stages of exogenous vitellogenesis (June), GtH (1-3 ng/ml) and E_2-17β (0.5-1.5 ng/ml) levels were low and constant. At more advanced stages of exogenous vitellogenesis (September-October) blood samples taken at intervals of 4, 1 or 0.5 hrs showed that GtH levels fluctuated again in most of the females while basal GtH levels were only slightly higher than those recorded in June. These fluctuations included short-term (1-3 hrs) random GtH pulses (fig. 1b) of moderate amplitude (ΔGtH = up to 5 ng/ml) occurring with a relatively high frequency (up to 5 per 12 hrs). Although no regular synchronous daily pattern of GtH levels was noted, most of the GtH pulses were observed during the photophase and early scotophase. The appearance of GtH pulsatility during vitellogenesis was accompanied by a large increase in plasma E_2-17β up to levels ranging from 6 to 30 ng/ml. Individual E_2-17β profiles revealed continuous, relatively synchronized daily variations overriding the GtH pulses (fig. 1b). A gradual increase in the E_2-17β levels was observed during the day in all studied females, followed by a decrease starting at late photophase or during the scotophase, depending on the fish.

Under *in vitro* perifusion conditions, a single GtH pulse induced an extended amplification of the ovarian E_2-17β output; this response was reproduced after each of three successive GtH pulses. When the frequency of the pulses increased, mimicking intervals observed *in vivo* (8, 4, 3 hrs), the successive ovarian responses became superimposed, leading to a constantly elevated (fig. 2), or even to a continuously increasing, rate of E_2-17β production.

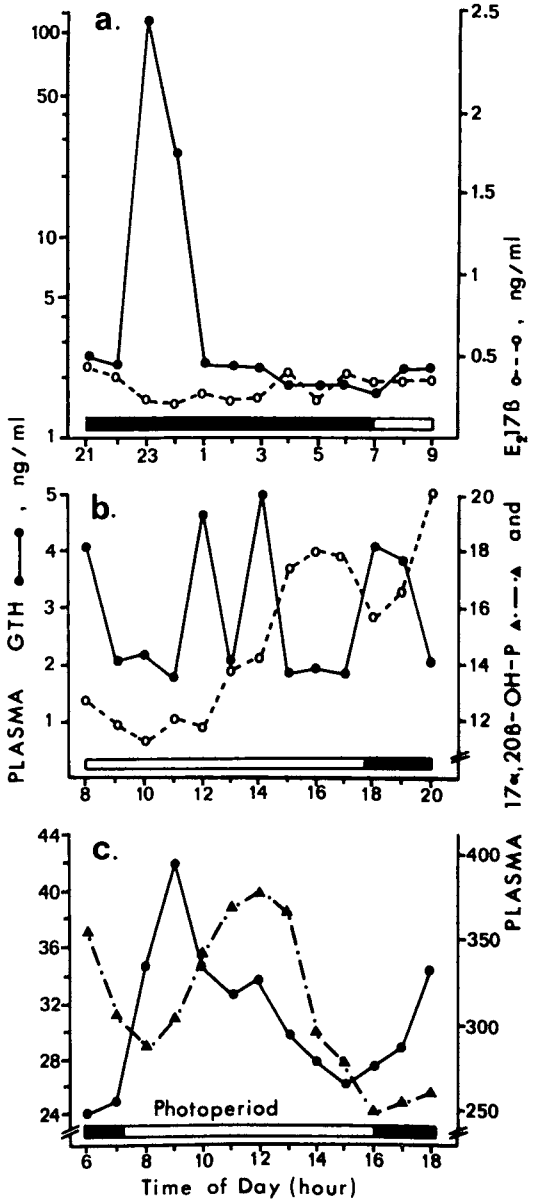

Figure 1. Isolated examples of individual profiles of plasma GtH and E_2-17β or 17α, 20β-OH-P levels in female rainbow trout at different characteristic stages of sexual development: a. early ovarian recrudescence (March) b. advanced exogenous vitellogenesis (October) and c. periovulation: maturation and ovulation occurred during a 48-hr period which included the sampling times.

Taken together, our *in vivo* and *in vitro* results indicate that during exogenous vitellogenesis, the short-term pulses in the plasma GtH levels are detected by the ovaries which respond by amplifying the secretion rate

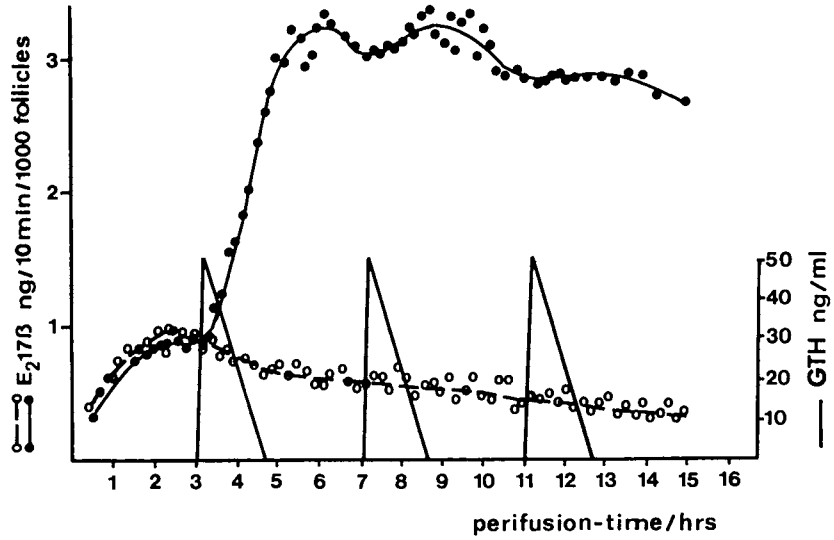

Figure 2. Profiles of E_2-17β secretion from an in vitro perifused rainbow trout ovary undergoing exogenous vitellogenesis; ovarian fragments were either exposed to 3 successive GtH pulses at 4-hr intervals (•——•) or not stimulated (o---o).

of E_2-17β for a much longer time than the duration of the GtH pulse. The frequency of the GtH pulses might determine whether the E_2-17β concentration in the plasma would increase (at higher frequencies), remain constant or even decrease (at lower frequencies). In any case, the changes in the E_2-17β levels would be gradual rather than abrupt. Therefore, as proposed for the goldfish by Hontela & Peter (1978), the changes in the short-term pattern of GtH levels during vitellogenesis in the female rainbow trout are probably essential to the modulation of E_2-17β secretion and of ovarian development. The appearance of GtH pulsatility could regulate the important gradual augmentation in plasma E_2-17β levels throughout exogenous vitellogenesis, and an unequal distribution of plasma GtH pulses over the 24-hr period might regulate the daily cycle of E_2-17β at advanced exogenous vitellogenesis.

C. Oocyte maturation and ovulation (December-January)

The germinal vesicle in the oocytes of the studied females was terminating migration or the oocytes were undergoing meiotic maturation. In some cases ovulation was completed no more than 72 hrs prior to the beginning of sampling.

The profiles of gonadotropin were quite different compared to previous stages. GtH levels were higher and fluctuated in a continuous rather than in an episodic manner (fig. 1c). A consistent daily cycle was observed in all sampled fish, whatever the sampling frequency (1, 3 or 4-hr intervals). A relative synchronization appeared among individual profiles. Two distinct GtH surges occurred, one at early photophase (fig. 1c) and the other during the mid-scotophase. The onset of the GtH increases was closely related to the beginning of the photophase and the scotophase, respectively (fig. 1c), suggesting the regulatory role of the photoperiod. In females undergoing oocyte maturation and in ovulated females, 17α, 20β-OH-P levels were increasing or high, showing daily fluctuations that were either synchronized with the GtH changes or somewhat phase-shifted in relation to them (fig. 1c).

Parallel to the onset of the change in the in vivo GtH profiles (when the germinal vesicle approached the oocyte periphery), a marked modification was observed in ovarian steroidogenic capacity and responsiveness to GtH: (1) The continuous in vitro exposure of follicles to physiological levels of GtH (25-50 ng/ml) did not result, as at previous stages of oocyte development, in prolonged elevation of E_2-17β output; instead, E_2-17β secretion increased briefly, then dropped rapidly. (2) Androgen release, which was only slightly stimulated at earlier stages probably due to efficient aromatase activity, was strongly enhanced by GtH. Androgen output was maximal while E_2-17β production decreased. (3) Continuous administration, not a pulse, of GtH initiated a 17α, 20β-OH-P release 12 to 17 hrs after the beginning of the stimulation. At that time, androgen production rate decreased, reaching a low level, while the 17α,20β-OH-P output continued its dramatic increase which was followed by oocyte maturation.

These data suggest that increasing GtH levels in vivo act on follicles ready to undergo maturation (1) by stimulating the production of androgens whose aromatisation to E_2-17β is depressed, possibly due to the GtH rise and (2) by modulating the enzyme system impli-

cated in the synthesis of the maturation-inducing steroid (17α,20β-OH-P), perhaps involving the activation of 20β-hydroxysteroid dehydrogenase (Suzuki et al., 1981) and the diminution of 17-20 lyase activity. The functional significance of the daily cycles in circulating GtH should be studied in relation to ovarian steroidogenesis and to oocyte maturation and ovulation.

While the secretion of androgens by perifused follicles drops rapidly as 17α,20β-OH-P output increases, circulating androgen levels during oocyte maturation are still high, though decreasing (Campbell et al., 1980; Scott et al., 1982). The maintenance of high levels of androgens and low levels of E_2-17β might contribute to the establishment of an adequate steroid environment for the synthesis of 17α,20β-OH-P and for oocyte maturation. In fact, the efficiency of the in vitro induction of both processes by GtH is enhanced by testosterone and reduced by E_2-17β (Jalabert, 1975; Jalabert & Fostier, this symposium).

References

Billard, R., B. Breton, A. Fostier, B. Jalabert & C. Weil, 1978. Endocrine control of the teleost reproductive cycle and its relation to external factors: Salmonid and Cyprinid models. In: P.J. Gaillard & H.H. Boer (Ed.): Comparative Endocrinology. Elsevier/North-Holland, Amsterdam. P. 37-48.

Bohemen, Ch. G., van & J.G.D. Lambert, 1981. Estrogen synthesis in relation to estrone, estradiol and vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 45: 105-114.

Breton, B., B. Jalabert, A. Fostier & R. Billard, 1975. Etude sur le cycle reproducteur de la truite arc-en-ciel et de la tanche. Effet de variations expérimentales de la température. J. Physiol. Paris. 70: 561-564.

Breton, B., Y. Zohar & R. Billard, 1982a. Photoperiod and gonadotropin control of the reproductive cycle in the female rainbow trout. Proceeding of the 9th International Symposium on Comparative Endocrinology, Hong-Kong. In press.

Breton, B., A. Fostier, Y. Zohar, P.Y. Le Bail & R. Billard, 1982b. Gonadotropine glycoprotéique maturante et oestradiol 17β pendant le cycle reproducteur chez la truite fario (Salmo trutta) femelle. Gen. Comp. Endocrinol. In press.

Bromage, N.R., C. Whitehead & B. Breton, 1982. Relationships between serum levels of gonadotropin, oestradiol 17β and vitellogenin in the control of ovarian development in the rainbow trout: II. The effects of alternations in environmental photoperiod. Gen. Comp. Endocrinol. In press.

Campbell, C.M., A. Fostier, B. Jalabert & B. Truscott, 1980. Identification and quantification of steroids in the serum of rainbow trout during spermiation and oocyte maturation. J. Endocr. 85: 371-378.

Fostier, A., B. Jalabert, C. Campbell, M. Terqui & B. Breton, 1981a. Cinétique de libération in vitro de 17α-hydroxy-20β-dihydroprogestérone par des follicules de truite arc-en-ciel, Salmo gairdnerii. C.R. Acad. Sci. Paris Ser. III, 293: 777-780.

Fostier, A., B. Breton, B. Jalabert & O. Marcuzzi, 1981b. Evolution des niveaux plasmatiques de la gonadotropine glycoprotéique et de la 17α-hydroxy-20β-dihydroprogestérone au cours de la maturation et de l'ovulation chez la truite arc-en-ciel, Salmo gairdenii. C.R. Acad. Sci. Paris Ser. III, 293: 817-820.

Fostier, A., C. Weil, M. Terqui, B. Breton & B. Jalabert, 1978. Plasma estradiol-17β and gonadotropin during ovulation in rainbow trout (Salmo gairdneri R.). Ann. Biol. anim. Bioch. Biophys. 18: 929-936.

Hontela, A. & R.E. Peter, 1978. Daily cycles in serum gonadotropin levels in the goldfish : effects of photoperiod, temperature and sexual condition. Can. J. Zool. 56: 2430-2442.

Idler, D.R. & C.M. Campbell, 1980. Gonadotropin stimulation of estrogen and yolk precursor synthesis in juvenile rainbow trout. Gen. Comp. Endocrinol. 41: 384-391.

Jalabert, B., 1975. Modulation par différents stéroïdes non maturants de l'efficacité de la 17α-hydroxy-20β-dihydroprogestérone ou d'un extrait gonadotrope sur la maturation intrafolliculaire in vitro des ovocytes de la truite arc-en-ciel Salmo gairdnerii. C. R. Acad. Sci. Paris Ser. D, 281: 811-814.

Jalabert, B. & B. Breton, 1980. Evolution de la gonadotropine plasmatique t-GtH après l'ovulation chez la truite arc-en-ciel (Salmo gairdneri R.) et influence de la rétention des ovules. C.R. Acad. Sci. Paris Ser. D, 290: 799-801.

Jalabert, B. & A. Fostier, 1982. Physiological basis of practical means to induce ovulation in fish. Proceeding of the international symposium on reproductive physiology of fish, Wageningen, Netherlands.

Nagahama, Y., H. Kagawa & G. Young, 1982. Cellular sources of sex steroids in teleost gonads. Can. J. Fish. Aquat. Sci. 39: 56-64.

Scott, A.P., V.J. Bye & S.M. Baynes, 1980. Seasonal variations in sex steroids of female rainbow trout (Salmo gairdneri Richardson). J. Fish. Biol. 17: 587-592.

Scott, A.P., E.L. Sheldrick & A.P.F. Flint, 1982. Measurement of 17α, 20β-dihydroxy-4-pregnen-3-one in plasma of trout (Salmo gairdneri Richardson): Seasonal changes and response to salmon pituitary extract. Gen. Comp. Endocrinol. 46: 444-451.

Suzuki, K., B.I. Tamaoki & K. Hirose, 1981. In vitro metabolism of 4-pregnenes in ovaries of a freshwater teleost, the ayu (Plecoglossus altivelis): Production of 17α, 20β-dihydroxy-4-pregnen-3-one and its 5β reduced metabolites, and activation of 3β-

and 20ß-hydroxysteroid dihydrogenases by treatment with a fish gonadotropin. Gen. Comp. Endocrinol. 45: 473-481.
Whitehead, C., N.R. Bromage & J.R.M. Forster, 1978. Seasonal changes in reproductive function of the rainbow trout (Salmo gairdeni) J. Fish. Biol. 12: 601-608.
Zohar, Y., 1980. Dorsal aorta catheterization in rainbow trout (Salmo gairdneri) I. Its validity in the study of blood gonadotropin patterns. Reprod. Nutr. Develop. 20: 1811-1823.
Zohar, Y., B. Breton & R. Billard, 1982. Short-term profiles of plasma gonadotropin levels in the female rainbow trout throughout the reproductive cycle. Gen. Comp. Endocrinol. 46: 396.

EXISTE-T'IL PLUSIEURS GONADOTROPINES (GTH) CHEZ LES POISSONS ? DONNEES BIOCHIMIQUES ET VITELLOGENESE EXOGENE.

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Summary

Since 1963, it has been suggested that one glycoproteic gonadotropin (cGTH) is present in the carp pituitary. Further biological and biochemical data have confirmed this hypothesis. However, if cytological data on GTH cells in the pituitary, circulating level during the reproductive cycle and exogenous vitellogenesis are considered, the hypothesis of one GTH is controversial. Affinity chromatography had led to the separation of two distinct GTHs. One hormone, poor in carbohydrate, acts on the vitellogenin incorporation in the oocyte. The second, rich in carbohydrate is able to stimulate several aspects of the gametogenesis. Such a discrepancy will be review relative to the biochemical data of two fish GTHs and to the activity of cGTH on exogenous vitellogenesis. Biochemically fish GTHs have shown a high degree of homology with mammalian gonadotropins especially with the lutropin. In some hypophysectomized fish, cGTH initiates the synthesis of vitellogenin and its incorporation in the oocyte by pinocytosis. In the eel, a (Vg) radioimmunoassay has been developped. Plasma level of vitellogenin can be determined as well as the rate of yolk incorporation in the oocyte. Thus, cGTH is potent on exogenous vitellogenesis. A pituitary factor, able to act on ovarian growth which is not strictly speaking a gonadotropin, make up a working hypothesis.
Keywords : number of fish GTH, biochemical characterizations, exogenous vitellogenesis, eel vitellogenin RIA.

Introduction

L'hypothèse de l'existence d'une seule hormone gonadotrope chez la carpe a été proposée en 1963 par Fontaine et Gérard. Le développement des recherches biochimiques et biologiques à partir de la glycoprotéine gonadotrope hypophysaire (cGTH) a toujours apporté des éléments en faveur de cette hypothèse chez la carpe *(Cyprinus carpio)* et chez l'esturgeon *(Acipenser stellatus)* (Burzawa-Gérard et Fontaine, 1965 ; Burzawa-Gérard, 1974 ; Burzawa-Gérard et al., 1975 ; Fontaine et Burzawa-Gérard, 1978). Si l'on considère les données cytologiques relatives aux cellules gonadotropes de l'hypophyse, la détermination des taux circulants de GTH au cours du cycle de reproduction, ainsi que l'étude de la vitellogénèse exogène (réf. dans Burzawa-Gérard, 1981) l'hypothèse d'une gonadotropine unique ne se révèle pas toujours satisfaisante. La séparation par chromatographie d'affinité d'une gonadotropine pauvre en carbohydrate, l'hormone vitellogénèse et d'une gonadotropine riche en carbohydrate, l'hormone de maturation, à partir d'hypophyses de différents poissons téléostéens (Idler et Ng, 1979 ; Ng et Idler, 1979) suggère au contraire, l'existence de deux gonadotropines. L'hormone de maturation présente un spectre d'activité gonadotrope très étendu sur la gamétogénèse incluant, chez la femelle, la vitellogénèse, la maturation et l'ovulation. L'hormone de vitellogénèse serait nécessaire à une étape très précise de celle-ci qui est l'incorporation de la vitellogénine dans l'ovocyte. Elle serait, en revanche, inactive sur la stéroïdogénèse, la maturation et l'ovulation.

Une disproportion importante apparaît donc entre les potentialités gonadotropes de ces deux hormones et l'on peut s'interroger sur sa signification. Dans le présent article, nous nous proposons d'éclaircir le problème de l'existence d'une ou plusieurs gonadotropines en posant deux questions : (1) les données biochimiques structurales sur la GTH de carpe mettent-elles en évidence la présence d'une deuxième hormone ? (2) la cGTH possède-t-elle intrinsèquement une activité sur la vitellogénèse exogène?

I - Données biochimiques sur les GTH de carpe (cGTH) et l'esturgeon (aci-GTH)

a) Structure

La cGTH et l'aci GTH sont des glycoprotéines de poids moléculaire de 28000 à 32000 daltons. La composition globale en acides aminés distingue ces deux hormones. Deux sous-unités, respectivement identifiées en type α et β sont associées par des liaisons non-covalentes pour former le dimère hormonal biologiquement actif. Un degré d'isologie élevé a pu être mis en évidence par la comparaison des séquences partielles d'amino-acides entre la cGTH α et la sous-unité α d'origine mammalienne d'une part, et la cGTH β et la LH β bovine d'autre part (Jollès et al., 1977). La parenté de la cGTH β avec une sous-unité LH β est confirmée par des recherches radioimmunologiques réalisées à partir d'anti sérum cGTH α et cGTH β (Burzawa-Gérard et al, 1980).

b) Site d'association des sous-unités

(1) Des associations à partir de sous-unités hétérologues ont été obtenues (Burzawa-Gérard et Fontaine, 1976 ; Burzawa-Gérard et Goncha-

rov, 1979). Entre les sous-unités de cGTH et d'aci GTH, une double hybridation est possible. Les mêmes schémas stériques sur ces couples α et β sont donc présents pour permettre les liaisons hydrogènes d'une structure quaternaire stable. Une unique hybridation est seulement possible entre la sous-unité LH α et les sous-unités β de cGTH ou d'aci GTH. Il est difficile de définir si l'absence d'hybridation opposée est due à un empêchement stérique ou à d'autres facteurs comme l'environnement ionique du site d'association de l'une ou l'autre sous-unité. Il paraît impératif que la structure du site d'association de la sous-unité α reste complémentaire de celui de la sous-unité β pour que l'hormone gonadotrope puisse être formée. Une évolution synchrone des sites d'association sur α et β a dû se produire (Fontaine et Burzawa-Gérard, 1978). (2) Des informations relatives à l'influence de l'une ou l'autre sous-unité au cours de l'association ont été apportées par la molécule hybride (bLH α - cGTH β). Des études par radioimmunologie ont suggéré que la nature de la sous-unité α pourrait jouer un rôle sur la conformation de la sous-unité β dans le dimère (Dufour, 1980). Les deux sous-unités sont par contre impliquées pour déterminer la vitesse d'association à une température donnée (Marchelidon et al., 1979). Une activité gonadotrope intense de la molécule hybride (bLH α - cGTH β) est obtenue chez un amphibien. Elle est 4 à 6 fois plus active que la cGTH ou que la LH bovine, mais chez un téléostéen, son activité gonadotrope est équipotente à celle de cGTH (Fontaine-Bertrand et al,1981).

c) Micro-hétérogénéité de la cGTH et de l'aci GTH.

(1) Le comportement de la cGTH en chromatographie d'affinité par la concanavaline met en évidence une affinité variable de l'hormone pour la lectine. Quatre fractions peuvent être séparées dont une (Fraction I-cGTH) qui n'est pas liée ; elle représente environ 5 % des protéines. La partie glycanique de la cGTH peut donc être considérée comme variable (Burzawa-Gérard, 1982).

(2) Différentes préparations d'aci GTH possèdant le même poids moléculaire et les mêmes amino-acides N-terminaux ont été étudiées par isoélectrofocalisation. De nombreux composants apparaissent dont les points iso-électriques s'étendent de 4,0 à 6,5. Ils sont tous actifs sur le test de maturation des ovocytes de crapaud in vitro. Il ne s'agit pas d'un artéfact de purification car l'hétérogénéité de la charge des molécules hormonales est aussi observée dans les extraits hypophysaires. Ces différences ne sont pas dues non plus à une différence dans le nombre de résidus acide sialique (Goncharov et al., 1979 ; Kuznetsov et al., 1982).

Les données structurales que nous venons de résumer établissent une relation d'homologie avec les gonadotropines des Vertébrés et plus particulièrement avec la gonadotropine de type LH. Jusqu'à aujourd'hui, l'existence d'une telle relation n'a pu être montrée avec l'hormone de vitellogénèse pauvre en carbohydrate décrite par Idler et ses collaborateurs. La mise en évidence de la présence d'une structure quaternaire de cette hormone, avec une SU " β de vitellogénèse" serait un argument majeur pour prouver l'existence de deux gonadotropines.

L'hétérogénéité vis-à-vis de la charge ou du contenu glycanique existe aussi pour les gonadotropines LH et FSH (Réf. dans Wakabayashi, 1980) ; elle semble être chez les poissons beaucoup plus importante. L'étude de la séquence au N-terminal de la sous-unité α de la cGTH a montré, qu'en fait, cette partie de l'hormone existait sous deux formes, l'une ne possèdant pas le nonapeptide au N-terminal par lequel commence la séquence de l'autre. Cette caractéristique est aussi signalée pour les sous-unités α mammaliennes (Réf. dans Jollès et al., 1977). Les GTHs sont obtenues à partir d'un grand nombre d'hypophyses dont le contenu en GTH pourrait être à des stades variables de synthèse. L'hétérogénéité des préparations hormonales obtenues par les différents auteurs en serait peut-être le reflet. La structure de l'hormone libérée au moment de la sécrétion hypophysaire chez les poissons n'a pas été étudiée. Elle peut être différente de sa forme de stockage, des informations dans ce sens pourraient être intéressantes.

II - Données sur l'action de la cGTH sur la vitellogénèse exogène.

L'activité de la cGTH sur la vitellogénèse exogène a été recherchée sur divers receveurs.

(1) Chez *Carassius auratus* hypophysectomisé, le traitement par la cGTH fait apparaître un accroissement important de l'ovaire. L'analyse cytologique montre l'apparition d'un ou plusieurs anneaux de vésicules vitellines (Burzawa-Gérard, 1974).

(2) Chez *Heteropneustes fossilis* hypophysectomisé, l'effet de la cGTH sur la vitellogénèse exogène a été plus particulièrement étudié. Les taux circulants de vitellogénine, mesurés par le dosage du phosphore alkali-labile dans le sérum, augmentent et des ovocytes au stade III, caractéristique de la présence de vitellus, apparaissent. Des doses faibles d'hormone ont ici été utilisées (0,08 à 0,45 µg/g, de poids corporel/injection) (Sundararaj et al., 1981).

(3) Chez *Gobius niger* hypophysectomisé, l'action de la cGTH, ainsi que celle de la Fraction I-cGTH (non liée sur la concanavaline) ont été recherchés sur l'ovaire à la même dose de 0,4 µg par g de poids corporel et par injection. L'ovogénèse est fortement

stimulée dans les deux cas. L'activité relative à la vitellogénèse exogène a été caractérisée par l'apparition de la vitellogénine circulante (immunoprécipitation avec un sérum antivitellogénine de *Gobius niger*) et par l'identification de nombreuses vésicules de pinocytose, signe d'internalisation de Vg dans les ovocytes parvenus au stade II^+ définie par Le Menn (1979). Les traitements par cGTH ou par la Fraction I-cGTH induisent la même stimulation sur la vitellogénèse (Le Menn et Burzawa-Gérard, 1982).

(4) Chez *Anguilla anguilla*, la vitellogénèse exogène a été recherchée par des techniques biochimiques. Elles ont eu pour objectif la mise au point d'un dosage radioimmunologique (RIA) de la vitellogénine (Vg) de l'anguille afin de mettre en évidence l'apparition de la Vg plasmatique après stimulation de l'hormone gonadotrope et de déterminer le taux d'incorporation dans l'ovaire par l'apparition de vitellus (immunoréactif).

- La vitellogénine d'anguille a été purifiée à partir de sérums d'anguilles traitées à l'oestradiol 17 β qui a pour effet de stimuler la synthèse de Vg par le foie. Deux étapes de purification ont été nécessaires (1. filtration du sérum sur Ultrogel A4 ; 2. chromatographie sur hydroxylapatite HA Ultrogel en tampon phosphate pH 6,8 des protéines exclues pour K_D 0,4 à 0,6). La position de la vitellogénine au cours de ces fractionnements a été obtenue par le dosage du phosphore alkali-labile selon la technique décrite par Wallace et Jared, 1968. La Vg est obtenue après application d'un deuxième gradient (tampon phosphate pH 6,8 de 0,2 M à 0,5 M). L'électrophorèse analytique (PAGE) de la fraction obtenue qui révèle une unique bande de R_f = 0,20 - 0,21 met en évidence un état de pureté satisfaisant. Ce matériel a été utilisé pour obtenir des antisérums chez le lapin.

= Un RIA de la vitellogénine a été mis au point après marquage de la Vg à l'iode (^{125}I) selon les techniques classiques. Des sérums d'anguille [(1) traitement par 15 mg d'oestradiol 17 β - (2) 100 mg d'oestradiol 17 β] sont capables d'inhiber la liaison ^{125}I Vg ; AS Vg. Les sérums des animaux témoins l'inhibent faiblement pour les femelles et pas du tout pour les mâles (Fig. 1). De très faibles quantités de Vg pourraient être présentes chez les anguilles femelles argentées. Des quantités très importantes sont en revanche obtenues pour les traitements à l'oestradiol 17 β (Tableau 1). Des extraits d'ovaires

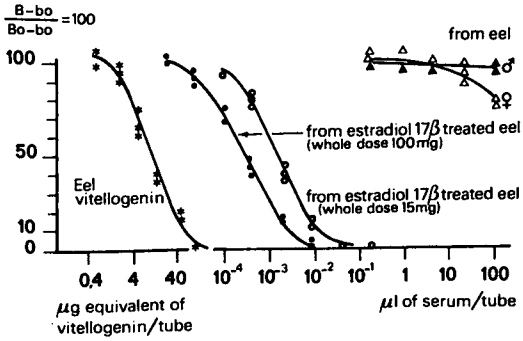

Figure 1 - Inhibition de la liaison ^{125}I Vg ; AS Vg par différents sérums d'anguille.

Tableau 1. Taux circulant de Vg dans le sérum d'anguilles témoins et traitées à l'oestradiol 17 β .

Sérums	Vg g équivalent/µl
témoins ♂	⩽ 0,0005
témoins ♀	⩽ 0,05
oestradiol 17 β (15mg)	241,57 (207,97-280,60)
oestradiol 17β(100mg)	688,6 (609,41-778,15)

d'anguille ayant des RGS de 1,4 (témoin) et de 12,8 ou 50 (ayant reçu des injections d'extraits hypophysaires de carpe) ont été préparés après ultracentrifugation. L'inhibition de la liaison (^{125}I-Vg ; AS Vg) est incomplète, mais la réaction croisée entre le vitellus et la Vg est importante. L'extrait d'ovaire témoin n'entraîne pas d'inhibition.

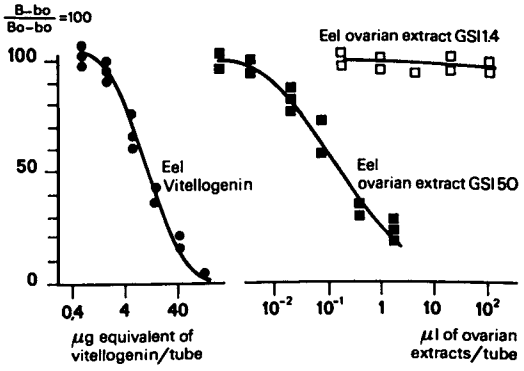

Figure 2 - Inhibition de la liaison (^{125}I Vg; AS Vg) par des extraits d'ovaire d'anguille.

Une estimation du taux d'incorporation de la Vg dans l'ovaire est possible (Tableau 2).

Tableau 2. Estimation de la quantité de vitellogénine incorporé dans l'ovaire en fonction du RGS.

Extrait d'ovaire d'anguille pour des RGS de :	Vg (µg équivalent/g d'ovaire)
1,4	⩽ 0,2
12,8	99170 (90227-122785)
50	925900 (745000-1132000)

Des résultats préliminaires ont été obtenus chez l'anguille après des traitements à la cGTH d'une part, et à l'oestradiol 17 β d'autre part. La mesure du taux de Vg plasmatique de l'incorporation de Vg dans l'ovocyte ont confirmé d'une part la validité du RIA, et d'autre part la compétence de la cGTH sur la vitellogénèse exogène.

Conclusion

Ces derniers résultats montrent que la cGTH est active sur la vitellogénèse, de plus l'ensemble des données résumées ici sont toutes en accord avec l'hypothèse de la présence d'une hormone gonadotrope unique, chez la carpe. L'existence présumée d'une seconde gonadotropine ayant sur la gonade une action aussi limitée que l'est celle de l'incorporation dans l'ovocyte peut être interprétée dans le sens d'une action complémentaire de l'hypophyse;dans ce cas,l'hormone de vitellogénèse ne serait pas une gonadotropine au sens strict . Des recherches concernant les taux circulants de l'hormone de vitellogénèse doivent être réalisées afin de mieux comprendre son rôle physiologique.

Références

Burzawa-Gérard, E., 1974. Mémoires du Muséum, série A, Zool., 86, 1-77.

Burzawa-Gérard, E., 1981. Océanis, 6, 655-676.

Burzawa-Gérard, E., 1982. Can. J. Fish. Aquat. Sci., 39, 80-91.

Burzawa-Gérard, E. et Y.A. Fontaine, 1965. Gen. Comp. Endocrinol., 5, 87-95.

Burzawa-Gérard, E. et Y.A. Fontaine, 1976. C. R. Acad. Sci., 282, série D, 97-100.

Burzawa-Gérard, E. et B.F. Goncharov, 1979. Gen. Comp. Endocrinol., 40, 338.

Burzawa-Gérard, E., S. Dufour et Y.A. Fontaine, 1980. Gen. Comp. Endocrinol., 41, 199-211.

Burzawa-Gérard, E., B.F. Goncharov et Y.A. Fontaine, 1975. Gen. Comp. Endocrinol., 27, 296-304.

Dufour, S., 1980. Thèse de 3e cycle, Paris.

Fontaine, Y.A. et E. Burzawa-Gérard, 1978. In "Structure and function of the gonadotropins". K. Mac Kerns ed., 361-380.

Fontaine, Y.A. et E. Gérard, 1963. C. R. Acad. Sci., 256, 5634-5637.

Fontaine-Bertrand, E., J. Marchelidon, C. Salmon et Y.A. Fontaine, 1981. C. R. Acad. Sci., 292, 507-510.

Goncharov, B.F., E. Burzawa-Gérard et A.A. Kuznetsov, 1979. Gen. Comp. Endocrinol., 40, 342.

Idler, D.R. et T.B. Ng, 1979. Gen. Comp. Endocrinol., 38, 421-440.

Jollès, J., E. Burzawa-Gérard, Y.A. Fontaine et P. Jollès, 1977. Biochimie, 59, 893-898.

Kuznetzov, A.A., B.F. Goncharov et E. Burzawa-Gérard, 1982. Gen. Comp. Endocrinol. (sous presse).

Le Menn, F., 1979. Comp. Biochem. Physiol., 62A, 495-500.

Le Menn, F. et E. Burzawa-Gérard, 1982. Soumis à Gen. Comp. Endocrinol.

Marchelidon, J., R. Salesse, J. Garnier, E. Burzawa-Gérard et Y.A. Fontaine, 1979. Nature, 281, n° 5729, 314-315.

Ng, T.B. et D.R. Idler, 1979. Gen. Comp. Endocrinol., 38, 410-420.

Ng, T.B., D.R. Idler et M.P. Burton, 1980. Gen. Comp. Endocrinol., 42, 355-364.

Sundararaj, B.I., P. Nath, E. Burzawa-Gérard, 1982. Gen. Comp. Endocrinol.

Wakabayashi, K., 1980. In : S. Ishii et al. Ed. Hormones, adaptation and evolution, Jap. Sci. Press Tokyo, 271-279.

Wallace, R.A. et D.W. Jared, 1968. Can. J. Biochem., 46, 953-959.

POSITIVE TESTOSTERONE FEEDBACK ON GONADOTROPIC HORMONE IN THE RAINBOW TROUT

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Summary

Testosterone increases the gonadotropic hormone (GtH) content of pituitaries in the juvenile rainbow trout. A silastic capsule was the most effective method of administering testosterone compared with cocoa butter or castor oil. When pituitary GtH reached the microgram level (equivalent with the pituitary GtH level during the spawning season) GtH release and stimulation of the testes in the immature male was observed.

Previously, Crim & Evans, 1979 showed that in the juvenile trout intraperitoneal (IP) testosterone(T) administration in cocoa butter increases the pituitary GtH content. However, in comparison with the GtH content of pituitaries obtained from precocious salmon parr (Crim & Evans, 1978) we realized that the stimulation of GtH by T in the immature trout was relatively small (nanograms of GtH accumulated). In the present investigation methods of administering testosterone were studied to determine a more effective manner of stimulating GtH production in the immature trout. Three different methods of administering T to yearling trout were examined:

1) multiple IP injections of T in castor oil.
2) a single IP implant of T in cocoa butter.
3) a single IP implant of T in a silastic capsule.

Figure 1 shows that a small increase in pituitary GtH followed T administration in both cocoa butter and castor oil. T treatment via silastic capsule produced a much greater increase in the pituitary GtH content (micrograms vs nanograms). In addition to the effect of T upon pituitary GtH, the influence of steroid administration on plasma GtH and gonadal changes were also studied. If pituitary GtH reached only the nanogram level no increase in plasma GtH was observed and the immature gonads remained undeveloped. In contrast, the dramatic increase in pituitary GtH resulting from the T treatment via silastic capsule was associated with GtH release (at 16 weeks plasma GtH increased >3-fold compared with sham control values - data not shown). Onset of spermatogenesis and a significant increase in gonadosomatic index of immature males receiving T-silastic capsule was correlated with the elevated plasma GtH level; in females, however, ovarian stimulation was not observed in conjunction with the GtH release indicating a deficiency in the endocrine milieu necessary for premature oocyte development.

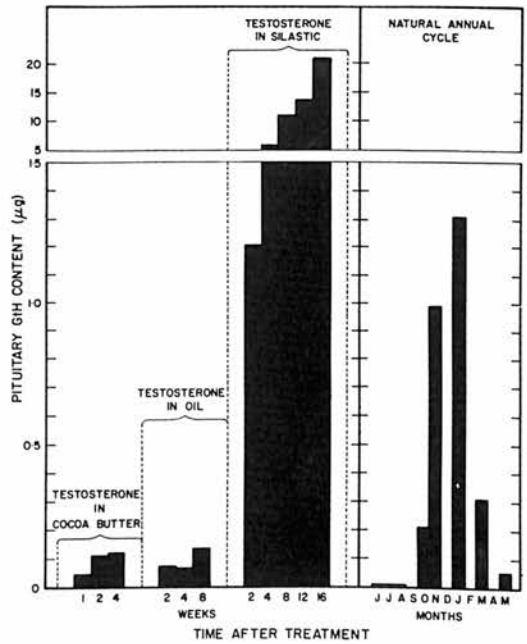

fig. 1. Pituitary GtH content in juvenile trout following T administration in cocoa butter (20 µg T), castor oil (bi-weekly injections of 20 µg T), and silastic capsules containing approximately 3 mg T. The seasonal profile for pituitary GtH content in the precocious male salmon parr is provided for reference.

References

Crim, L.W., D.M. Evans, 1978. Can. J. Zool. 56: 1550-1555.

Crim, L.W., D.M. Evans, 1979. Gen. Comp. Endocrinol. 37: 192-196.

PURIFICATION AND BIOLOGICAL CHARACTERIZATION OF SALMON PROLACTIN

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Keywords: chinook salmon, prolactin, osmoregulation.

Introduction

The existence in the teleost pituitary of a protein similar to mammalian prolactin has been well established. This hormone is endowed with osmoregulatory activity (see review Ensor, 1979; Clarke & Bern, 1980) and lactogenic activity in the rabbit mammary gland (Prunet et al., 1979; Houdebine et al., 1981). In this communication we present the purification and the characterization of prolactin from chinook salmon (Oncorhynchus tschawytscha).

Results

Purification and biochemical characterization

Prolactin from chinook salmon pituitaries was purified by acid acetonic extraction, followed by saline precipitation, isoelectric focusing on ion exchange column and gel filtration.

The purity of the final product was assessed by NH_2-terminal aminoacid analysis revealing the presence of only one amino acid (performed by professor H. Papkoff, Hormone Research Laboratory, University of California San Francisco, USA), and by SDS electrophoresis which showed only one band. Salmon prolactin appeared to have a molecular weight of 22.000 daltons. In electrophoresis at pH 4.5 this hormone migrated as a major band (R_f=0.5) followed by a second band of lower intensity, whereas at pH 9.3 salmon prolactin gave no band. The demonstration of the purity of the salmon prolactin preparation by the above described technics allowed us to consider the second band obtained in electrophoresis at pH 4.5 as another form of prolactin rather than a contaminant. The electrophoresis of the rostral part of coho salmon pituitaries in the presence of SDS or at pH 4.5 revealed the existence of a major protein running at the same position as the purified chinook salmon prolactin.

Biological characterization

Throughout the purification, prolactin bioactivity was estimated by radioreceptor assay for lactogenic hormone (Prunet et al., 1977). However, considering the heterologous nature of this assay, it was necessary to confirm the lactogenic activity of this hormone in the mammary gland culture system (Prunet et al., 1979; Houdebine et al., 1981). Chinook salmon prolactin exhibited a clear lactogenic activity, 20 times lower than ovine prolactin activity (performed by Dr. L.M. Houdebine, INRA Laboratoire de Physiologie de la Lactation, Jouy-en-Josas, France).

The sodium-retaining activity of chinook salmon prolactin was assessed in the Fundulus bioassay which was demonstrated to be specific of prolactin activity (Grau, E.G.; Prunet, P.; Nishioka, R.S.; Gross, T.; Bern, H.A.; submited for publication). Salmon prolactin showed a maximum activity for injected doses as low as 4 ng/ml and appeared to be 100 times more potent than ovine prolactin. However, at higher doses the response obtained was much lower, although it was significantly higher than the control.

Conclusion

Chinook salmon prolactin which appeared to be a basic protein (pI > 9) was obtained in a highly purified form. The prolactin character of this protein was established by its lactogenic activity in the rabbit mammary gland and its sodium-retaining activity in Fundulus heteroclitus.

References

Clarke, W.C. & H.A. Bern, 1980. Comparative endocrinology of prolactin in "Hormonal protein and peptides" (C.H. Li, ed.), 8:105-197. Academic Press, New York.

Ensor, D.M., 1978. "Comparative endocrinology of prolactin". Chapman and Hall, London.

Houdebine, L.M., S.W. Farmer & P. Prunet, 1981. Induction of rabbit casein synthesis in organ culture by tilapia prolactin and growth hormone. Gen. Comp. Endocrinol., 45: 61-65.

Prunet, P., J. Djiane & B. Breton, 1977. Application of a radioreceptor assay for lactogenic hormone to the detection of fish prolactin like hormone. J. Endocrinol., 73: 391-392.

Prunet P., L.M. Houdebine, C. Delouis, & B. Breton, 1979. Stimulation of milk synthesis in the rabbit by fish pituitary extract. J. Endocrinol., 83:393-400.

MORPHOMETRIC ANALYSIS OF STRUCTURAL CHANGES IN *Rhamdia hilarii* GONADOTROPHIC CELLS DURING THE REPRODUCTIVE CYCLE

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Hypophyseal gonadotrophic cells (GTH cells) from *Rhamdia hilarii* in different phases of the gonadal cycle exhibit conspicuous structural variations when analysed in thin section. Such qualitative analysis clearly shows dilation of the rough endoplasmic reticulum (RER) cisternae but does not reveal variation in the relative volumes of secretory granules (SG) and of the remaining cytoplasmic elements.

GTH cells from animals with maturing, mature and spent gonads were studied by morphometric analyses in thick (0.25 µm) and thin araldite sections (Table I).

In all stages, the Golgi apparatus and mitochondria occupy a very small fraction of the cytoplasmic volume. During the maturing gonadal phase the majority of GTH cells contain many SG occupying the greater part of cytoplasmic volume. The RER cisternae are moderately distended. In the mature gonadal phase, the mean number of SG increases slightly while the RER cisternae are more dilated (Figure 1). In the spent gonadal phase both the number and relative volume of the SG decrease while the relative volume of the RER increases. From maturing to spent gonadal stages surface-to-volume ratio (specific density) analysis shows that the RER cisternae become extremely dilated while its total surface area remains unchanged.

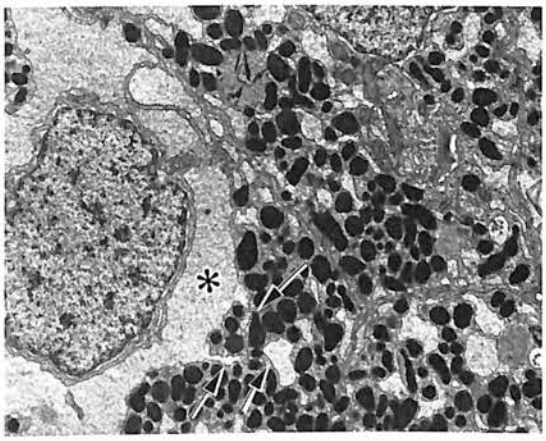

Fig.1 Hypophyseal GTH cells from *Rhamdia hilarii* with *mature* gonads. (*) denotes RER cisternum in the perinulear region which probably coalesce with the "small", juxtaposed RER elements (arrows). Note polymorphic secretory granules. X 12,000.

Table I. Morphometric data on gonadotrophic cells from Rhamdia hilarii ($\bar{x} \pm S\bar{x}$).

	Gonadal stages			Statistical analysis	
	maturing	mature	spent	§§	§§§
Nuclear V (μm^3)	78.3 ± 6.36	73.6 ± 7.76	70.2 ± 4.74	0.40	27.8
Cytoplasmic V (μm^3)	262.8 ± 20.86	264.7 ± 18.61	360.7 ± 38.65	4.13	119.5
V_V of hyaloplasm (%)	20.2 ± 1.38	17.4 ± 1.27	15.5 ± 1.48	3.11	5.9
§ V_V of mitochondria (%)	1.3 ± 0.10	1.2 ± 0.18	1.7 ± 0.53	0.85	1.4
§ V_V of Golgi complex (%)	0.5 ± 0.24	0.4 ± 0.08	0.5 ± 0.03	0.13	0.6
V_V of SG (%)	38.4 ± 0.10	36.8 ± 1.48	23.3 ± 3.24*	16.21	8.9
V_V of RER (%)	36.7 ± 1.16	42.4 ± 0.26	57.0 ± 2.97*	32.07	8.0
S_V of SG (μm^2SG/μm^3cytoplasm)	5.3 ± 0.20	5.0 ± 0.10	3.5 ± 0.34*	16.85	0.4
S_V of RER (μm^2RER/μm^3cytoplasm)	3.1 ± 0.04	2.8 ± 0.16	2.2 ± 0.37*	31.40	0.4
S_V/V_V of SG ($\mu m^2/\mu m^3$SG)	13.7 ± 0.51	13.6 ± 0.26	15.1 ± 0.78	2.20	2.4
S_V/V_V of RER ($\mu m^2/\mu m^3$RER)	8.4 ± 0.32	6.7 ± 0.39	3.9 ± 0.60	25.06	2.0
S of SG (μm^2)	1387.2 ± 141.15	1332.1 ± 68.88	1230.8 ± 48.35	0.68	411.8
S of RER (μm^2)	807.7 ± 67.40	753.9 ± 82.78	824.6 ± 183.91	0.09	532.8
N of SG	3.1 ± 0.01	3.2 ± 0.05	2.9 ± 0.03*	14.77	0.1

RER, rough endoplasmic reticulum; N, log number of secretory granules; S, total surface area; S_V, surface density; S_V/V_V, surface-to-volume ratio; SG, secretory granule; V, volume; V_V, volume fraction.
§ percentile values for these parameters were less than 2% and are not considered further
§§ F values for differences between maturing, mature and spent glands.
§§§ least significant difference obtained with the Tuckey test (p = 0.05).
* significant at p = 0.05

A STUDY OF METHODS OF ADMINISTERING LHRH ANALOGS: ADVANCEMENT OF SPERMIATION IN THE LAND-LOCKED SALMON (SALMO SALAR)

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Summary

During an experimental period of 11 days, LHRH analogs administered by injection or by implant accelerated the onset of spermiation and increased the amount of collectable milt compared with control fish. This increase in collectable milt volume is associated with an increase in spermatozoa production.

LHRH has been previously used to advance the spawning period in a variety of fishes (Lam, T. 1982. Canad. J. Fish. Aq. Sci. 39: 111-137). However, further work is required to optimize LHRH or LHRH analog treatment schedules especially if the aim is to provide LHRH stimulation for extended periods of time. The purpose of the present study was to evaluate practical and effective ways of administering LHRH analogs to salmon to reduce frequent handling of the fish and maximize the period of biological effectiveness of the peptide hormone. Spermiation in prespawning landlocked salmon was used as a biological test. For this purpose LHRH analogs were administered by intraperitoneal injections or by intraperitoneal implant in fish weighing 90 ± 5 g, maintained a 8°C under simulated natural photoperiod. The experiment lasted 11 days.

Intraperitoneal injections of D-Ala^6 LHRH ethylamide

a) Group Sa - a total dose of 0.750 mg/kg b.w. was dissolved in saline and administered on alternative days (total of 6 injections).

b) Group Pg - a total dose of 0.750 mg/kg b.w. was dissolved in 40% propylene glycol and administered twice, 6 days apart (total of 2 injections).

Intraperitoneal implants

a) Group Si - a single implant of silicone rubber containing 150 µg of D-Ala^6 LHRH ethylamide (1.5 mg/kg b.w.) was administered at the beginning of the experiment.

b) Group Ch - a single implant of compacted cholesterol powder containing 25 µg of D-Nal(2)^6 LHRH (Syntex, Inc.) was administered at the beginning of the experiment (dose 250 µg/kg b.w.).

After these four LHRH treatments, the volume of sperm released was determined by milt stripping every two days and compared to that monitored in controls (group C).

All four methods of applying LHRH analogs accelerated the onset of spermiation in comparison with controls. The initiation of spermiation occurred 6 days earlier than in the controls after LHRH injections in saline or following the silicone and cholesterol implants. LHRH injections in propylene glycol were less effective, spermiation beginning just 4 days earlier than in the control group. All types of LHRH treatments increased the amount of collectable milt compared with control fish during the experimental period (Fig. 1).

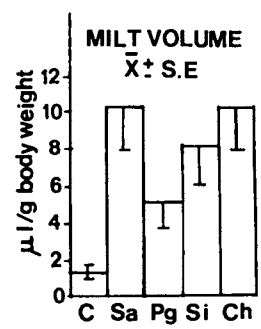

fig. 1. Influence of LHRH analogs treatment on the total amount of milt collected throughout the duration of the 11-day experimental period.

Study of the spermatocrit indicated that the increase in milt volume provoked by LHRH analogs is associated with an advancement in spermatozoa production and not just due to sperm dilution.

In conclusion, LHRH analogs can be administered in variety of vehicles to accelerate the onset of spermiation and possibly other reproductive events in salmon. Multiple injections in saline or single silicone or cholesterol implants have comparable effectiveness. The implantation methods have the advantages of minimizing the costs of labor and eliminating the need for frequent handling of fish which can be a practical problem in fish easily stressed.

APPLICATION OF AN *IN VITRO* BIOASSAY FOR FISH GTH IN IMPROVING HARVEST OF CARP PITUITARIES

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Harvest of carp pituitaries, as a spawning-inducing agent, is currently restricted in Israel to live donor fish of 1 kg bw or more (Rothbard,1981). The present study is an attempt to utilize to a greater extent carp pituitaries available at a fish-processing plant while adopting the timetable of the plant.

The gonadotrophic potency of the pituitaries was assessed by an *in vitro* bioassay recently developed in our laboratory (Yaron et al., in press). Aqueous extract of the tested pituitaries was added to ovarian incubates of *Sarotherodon aureus* at concentrations equivalent to 10^{-6} - 10^{-2} gland/ml. The resulting increase in estradiol (E_2) output was compared to that elicited by standard fish gonadotrophins (SG-G100, Donaldson,or cGTH, Burzawa-Gerard). All tests were performed on pools of 10 glands or more.

Post mortem loss of gonadotrophic potency

The gonadotrophic potency *in vitro* of pituitaries harvested from carp heads kept 24 h *post mortem* at 4° did not differ from that of glands taken immediately after decapitation of live carps (Fig.1).

The suitability of the *post-mortem* glands for spawning induction was verified by injecting homogenized glands into female carps (1 gland/kg) according to the procedure of Rothbard(1981). None of the controls, but 5/6 of the treated fish spawned successfully.

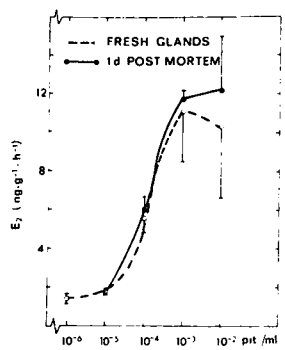

Fig.1 Gonadotrophic activity in vitro *of 1 day* post-mortem, *and fresh carp pituitaries.*

GTH activity in pituitaries of small and large fish

Gonadotrophic potency measured *in vitro* in 10 glands taken from small (<300 g) but ripe fish was similar to that taken on the same day from larger fish (1000 - 1400 g; Fig.2).

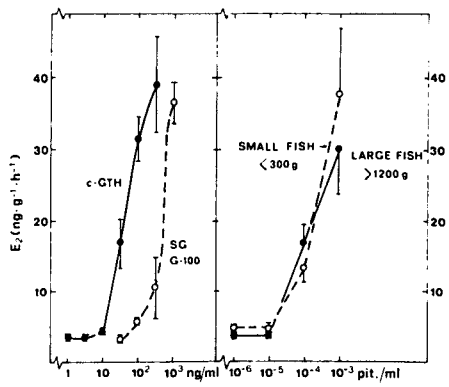

Fig.2 Gonadotrophic activity in vitro *of pituitaries of small and large carps compared with that of cGTH and SG-G100.*

Conclusions

The harvest of carp pituitaries can be improved by using heads of fish one day after death and by utilizing also fish of smaller size.

References

Rothbard, S. 1981. Induced reproduction in cultivated cyprinids - the common carp and the group of chinese carps. Bamidgeh 33: 103-121.

Yaron, Z., A. Bogomolnaya and E.M. Donaldson, in press. The stimulation of estradiol secretion by the ovary of *Sarotherodon aureus* as a bioassay for fish gonadotrophin. Hong Kong University Press.

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COMPARISON OF PURIFIED PITUITARY GONADOTROPINS OF MALE AND FEMALE PINK SALMON (Oncorhynchus gorbuscha Walb.)

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Summary

Purified gonadotropic hormone (GTH) preparations have been isolated separately from acetone dried pituitaries of male and female pink salmon. In the comparative bioassay using frog Rana temporaria oocyte maturation test and the adenyl cyclase activity measurements in homogenates of mud-loach Misgurnus fossilis ovaries a pronounced discrepancy in the activity was found between both GTH preparations. The conclusion about duality and sex specificity of pink salmon GTH was made.

Pituitary glands from male and female salmons were collected during the fish spawning in autumn 1980 at the Sokolovsk fish-plant (Sakhalin) and acetone dried. Isolation of the GTH was performed as described by Donaldson et al.(1972). At the ion-exchange chromatography on DEAE-cellulose four separate protein fractions were isolated from the pituitaries of the either sex and no differences were found in the chromatographic distribution between protein fractions extracted from male and female glands.

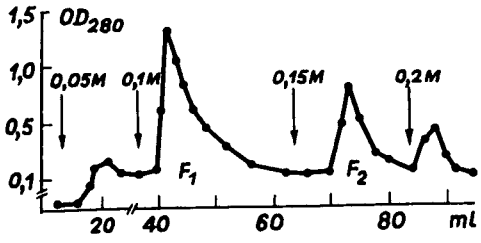

fig.1. Chromatography on DEAE-cellulose DE-32 Whatman. The column (1.7x9.5 cm) was equilibrated in 0.02 M Na-Gly buffer pH 9.3. Stepwise gradient of NaCl was used.

The two active fractions of female fish pituitaries F_1 and F_2 as well as corresponding male preparations M_1 and M_2 formed bands with R_f 0.38-0.52 and 0.82, respectively, when analysed on 7.5% polyacrilamide gel (pH 8.3).

F_1 revealed activity in frog oocyte maturation at minimum concentration of 12 µg/ml while M_1 gave no response in this biotest. Inducing mud-loach ovulation F_1 was active at the minimum dose of 60 µg per fish while M1 gave no response even at 180 µg/fish. Discrepancy between F_1 and M_1 was found in adenyl cyclase activity measurements as well.

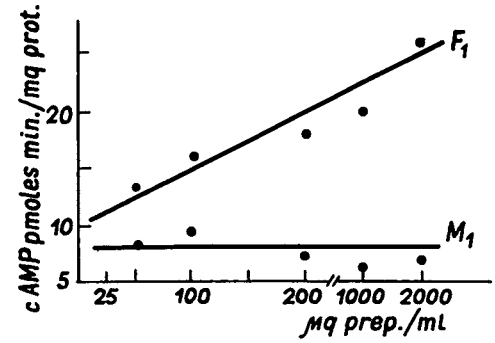

fig.2. Adenyl cyclase activity induced by F_1 and M_1 in homogenates of mud-loach ovary.

F_2 and M_2 showed a coinciding gonadotropic activity both on the oocytes (11 µg/ml) and the mud-loach ovulation (80 µg/fish).

Partial antigenic identity (spur) between M_1 and F_1 and M_2 and F_2 was found in double-immunodiffusion cross-reactions in 1% agar gel with antisera raised in rabbits against each of the preparations.

So it seems very likely that pituitary gland of pink salmon produces two GTH-s. The GTH preparation of male and female fish with R_f 0.38-0.52 revealed the most pronounced sex specificity in the used test systems.

References

Donaldson E.M., Yamazaki F., Dye H.M., & Phillеo W.W. 1972. Preparation of gonadotropin from salmon (Oncorhynchus tshawytscha) pituitary glands. Gen. Comp. Endocrinol., 18, 469-481.

NATURE, LOCALIZATION AND ACTIONS OF NEUROHORMONES REGULATING GONADOTROPIN SECRETION IN TELEOSTS

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Summary

The gonadotropin (GtH) releasing hormone (GnRH) of teleosts is similar to luteinizing hormone-releasing hormone (LH-RH), perhaps differing by amino acid substitutions at positions 7 and 8. Perikarya immunoreactive for LH-RH have been localized in the ventral-lateral nucleus preopticus periventricularis (NPP) and the posterior nucleus lateral tuberis (NLT), as well as other areas. Localization of immunoreactive fiber tracts from cells in the NPP and NLT to the pituitary gland suggests that these areas are the origin of the endogenous releasing hormone. Functional evidence from lesioning studies has strongly implicated the NLT in the regulation of GtH secretion via GnRH, but the evidence for involvement of the preoptic region in GnRH secretion is conflicting. LH-RH and superactive analogues (LRH-A) stimulate GtH release in teleosts. There is evidence for self-potentiation and self-suppression of the action of LRH-A and LH-RH. In certain species the action of LRH-A lasts much longer than LH-RH; analogues can be used to manipulate certain phases of the reproductive cycle in teleosts.

A GtH release inhibitory factor (GRIF) has been found in goldfish. Lesioning studies indicate that GRIF probably originates from the anterior-ventral NPP. Dopamine has GRIF activity in goldfish to block spontaneous release of GtH and modulate the action of LRH-A. It is difficult to induce ovulation in goldfish by injection of LRH-A; however, by blocking the actions of dopamine by injection of a receptor antagonist, the GtH release-response to LRH-A can be potentiated and ovulation induced more readily.

In addition to the classic negative feedback effect of sex steroids in sexually developing teleosts, estrogens, and androgens that can be aromatized to estrogens, have a positive feedback effect in sexually immature salmonids and eels. Such a positive feedback effect may be an important part of the mechanism inducing onset of sexual development.

Introduction

With the increasing importance of fish culture on a world-wide basis, there is an increasing necessity for regulation of reproduction of traditionally cultured fish, as well as newly cultured species. To assure maximal production from culture operations, it is usually necessary to induce ovulation and spermiation for production of fry. Synthetic analogues of luteinizing hormone-releasing hormone (LH-RH) have been successfully used to induce spawning of several carp species in China (Anonymous, 1977 a, b; Jiang et al., 1980) and to induce ovulation of salmonids in North America (Donaldson et al., 1981/1982, 1982). Essential for the efficient application of neurohormones in the regulation of reproduction of cultured fish is knowledge of the nature and actions of the neurohormones. In this paper I will review the nature, brain localizations and actions of neurohormones regulating gonadotropin (GtH) secretion. In addition, I will also discuss the role of these factors, and sex steroids, in the regulation of certain events in the reproductive cycle.

Gonadotropin Releasing Hormone (GnRH)

Nature

GnRH activity has been demonstrated in crude extracts of the hypothalamus and telencephalon of several species of teleost (Breton et al., 1971; Crim et al., 1976; Crim & Evans, 1980). Crim et al. (1976) and Crim & Evans (1980) found that hypothalamic and telencephalic extracts had more GnRH activity per brain segment, than other regions tested (cerebellum and medulla), suggesting that there is a differential distribution of GnRH in the brain. Studies have not been done to determine the specific GnRH activity of various brain regions.

King & Millar (1979, 1980) studied the crossreactivity of hypothalamic extracts from a wide range of vertebrates, including tilapia (Sarotherodon mossambicus), with four antisera, each reacting with different regions of the LH-RH molecule. The extracts from rat and amphibia were parallel to each other and with LH-RH, whereas the extracts from tilapia, an elasmobranch, birds and

reptiles were parallel to each other but not with LH-RH. When run on cation exchange, affinity and high performance liquid chromatography columns, the movement of tilapia immunoreactive material was always similar to that of birds and reptiles, but different from LH-RH and the immunoreactive material in hypothalamic extracts from mammals and amphibia. Tilapia hypothalamic extract, as well as the extracts from other vertebrates, had GnRH activity on cultured ovine anterior pituitary cells. These studies suggest there is conservation of the biologically active region of GnRH throughout vertebrate phylogeny, but that there are at least two different forms of GnRH in vertebrates.

Brain extracts of cod (Gadus morhua morhua) were found to contain three different LH-RH-like immunoreactive moieties (Barnett et al., 1982). Based on the elution patterns of the materials from ion exchange, gel and high performance liquid chromatography columns, two of the LH-RH-like materials were found to be similar in size to LH-RH. The third was a "big" LH-RH-like material, suggested to be a precursor of the authentic GnRH extended at the N-terminal. On the basis of cross-reactivity with antisera with known antigenic requirements, it was suggested that cod GnRH's differ from LH-RH by amino acid substitution at position 8 (-Arg^8-), or, less likely, position 7 (-Leu^7-). This supports the findings by King & Millar (1979, 1980), based on crossreactivity of tilapia hypothalamic extract with LH-RH antisera with known specificity, that tilapia GnRH most likely differs from LH-RH by substitution at position 7 or 8. Both of these studies indicate that the CO_2-terminal and NH_2-terminal of tilapia and cod GnRH are identical to LH-RH (p Glu^1-His^2-Trp^3-;-Pro^9-Gly^{10}-NH_2), and that the central part of the molecule may also be similar to LH-RH (-Ser^4-Tyr^5-Gly^6-).

Studies by Idler and Crim (1982), published in preliminary form, indicate that the winter flounder (Pseudopleuronectes americanus) has two forms of LH-RH-like immunoreactive material, one that is similar in size to LH-RH, and another that is larger. Chromatography studies done on the winter flounder material indicate that the smaller molecule is similar to LH-RH in size, supporting the studies reviewed above.

Localization: Anatomical and Functional Evidence

The localization of LH-RH immunoreactive material in sections of the brain and pituitary from several species of teleosts has been investigated. Unfortunately there is some confusion in this area due to lack of confirmation from one investigation to another on the same species, probably due to differences in specificity of antisera, and incorrect description of the localization of the reactive material.

Immunocytochemical techniques have localized LH-RH-like material in the proximal neurohypophysis, in the region of the GtH cells, in rainbow trout (Salmo gairdneri) (Dubois et al., 1979), platyfish (Xiphophorus maculatus, X. helleri, and X. sp.) (Schreibman et al., 1979; Münz et al., 1981), Japanese eel (Anguilla japonica) and puffer (Fugu niphobles) (Nozaki & Kobayashi, 1979).

Goos & Murthanoglu (1977) found small perikarya immunoreactive for LH-RH scattered in the area dorsalis pars medialis of the telencephalon of rainbow trout. In addition, they found immunoreactive fibers scattered in the anterior hypothalamus, in the region of the horizontal commissure, apparently continuous with fibers ending dorsal to the pituitary stalk. The fibers could not be traced beyond this point. Other investigators have not been able to detect immunoreactive perikarya or fibers in the brain of rainbow trout (Dubois et al., 1979; Nozaki & Kobayashi, 1979).

Immunoreactive fibers, but no perikarya, were found in the ventral preoptic region, telencephalon, infundibular nucleus (=nucleus lateral tuberis), habenular nucleus, and optic tectum, as well as in the neurohypophysis of the proximal lobe of the pituitary of the Japanese eel (Nozaki & Kobayashi, 1979). In carp (Cyprinus carpio), Pan et al. (1979) described LH-RH immunoreactive fibers amongst nucleus preopticus (NPO) perikarya, and also in the lateral forebrain bundles, ventral to the preoptic recess, the optic tectum, and the lateral hypothalamic region. These workers also described immunoreactive perikarya in the NPO; however, these cells appeared to be in the meninges located lateral to the preoptic region and ventral to the telencephalon (R. Peter, personal observations).

Schreibman et al. (1979) described reactive perikarya in the area ventralis pars ventralis and pars lateralis, and the NLT of platyfish; however, the localization designated as being in the ventral telencephalon was actually along the lateral extensions of the preoptic recess in the ventral-lateral nucleus preopticus periventricularis (NPP) (R. Peter & M.P. Schreibman, unpublished observations; Schreibman et al., 1982). Münz et al. (1981) also described reactive perikarya in the ventral-lateral NPP, designated the nucleus preopticus basalis lateralis, but not the NLT in platyfish. Schreibman et al. (1979) and Münz et al. (1981) described reactive

fibers, presumably from the NPP perikarya, dorsal to the optic chiasma, in the region of the horizontal commissure, dorsal to the pituitary stalk, amongst NLT cells and in the pituitary stalk. Münz et al. (1981) also found reactive perikarya in the anterior-ventral telencephalon bordering the olfactory bulbs, designated the nucleus olfacto-retinalis telencephali, and in the dorsal midbrain just posterior of the posterior commissure. The nucleus olfacto-retinalis projects to the olfactory bulb via the olfactory tracts, the retina via the optic chiasm and optic nerve, and to the dorsal telencephalon ipsilaterally and contralaterally via the anterior commissure (Münz et al., 1981, 1982). Immunoreactive fibers were also found in most telencephalic and mesencephalic regions, with a prominent projection to the optic tectum (Münz et al., 1981).

Münz et al. (1982) found that cichlid and centrarchid fish possessed LH-RH reactive cells in the nucleus olfacto-retinalis telencephali, similar to the platyfish (a poecilid); however, cyprinid (including goldfish), notopterid and anabantid fish did not.

In goldfish (Carassius auratus), LH-RH immunoreactive perikarya were found along the borders of the ventral-lateral preoptic recess in the NPP, and in the NLT posterior of the pituitary stalk (Kah et al., 1982a). Finding reactive cells in this particular part of the NLT fits well with results from lesioning experiments on goldfish (Peter, 1970; Peter & Crim, 1978; Peter & Paulencu, 1980; R. Peter, unpublished results), that destruction of the NLT in the pituitary stalk region and posterior of the pituitary stalk caused onset of gonadal regression and/or blockage of gonadal regression. It was suggested, on the basis of the lesioning studies, that this part of the NLT was involved in regulation of GtH secretion via release of GnRH. However, no decrease in serum GtH levels was found in the fish with NLT lesions (Peter & Crim, 1978; Peter & Paulencu, 1980). Resolving this conflict, Peter (1982) reported that NLT lesions caused the daily cycle in serum GtH to disappear, without causing an overall decrease in the mean serum GtH levels. A daily cycle in serum GtH is usually present in goldfish that are undergoing ovarian recrudescence or that have completed ovarian development preparatory to spawning (Hontela & Peter, 1978, 1980), and disappearance of the daily cycle is associated with the onset of gonadal regression (Hontela & Peter, 1980; Hontela, 1982). Hontela & Peter (1978) hypothesized that a daily cycle in blood levels of GtH is of importance for stimulation of ovarian development; this was supported by subsequent findings (Hontela & Peter, 1980; Hontela, 1982). On this basis, the role of the NLT may be to promote GtH release on a cyclic basis via secretion of GnRH.

Injection of goldfish with monosodium L-glutamate caused edema and hypertrophy, up to about 48 hours after injection, in the NLT from the anterior margin of the pituitary stalk through to the posterior end of the NLT, and also in a smaller region of tissue in the anterior-ventral NPP (Peter et al., 1980). This was followed, five days after injection, by necrosis of most of the perikarya in the affected region of the NLT and at least some of the perikarya in the NPP. Correlating with the initial period of edema and hypertrophy, and the following stage of necrosis, serum GtH levels were significantly increased for up to 48 hours after injection, and were similar to normal levels at 5, 7 and 8 days after injection. At 31 days after injection there was a dose dependent decrease in gonadosomatic index (GSI; R. Peter and C. Nahorniak, unpublished results). In the long term experiments, a significant decrease in serum GtH was found in goldfish injected with a high dose of glutamate, but not with the lower doses, even though the lower doses were also effective in causing a decrease in GSI. These results provide confirmation that the NLT is involved in regulation of GtH secretion and gonadal recrudescence.

Kah et al. (1982b) found that injection of glutamate in goldfish caused degeneration of only type B aminergic-like fibers in the proximal neurohypophysis. Since the degenerating fibers would have originated mainly from the NLT in the pituitary stalk region and posterior of the pituitary stalk, these results indicate involvement of this particular region of the NLT in regulation of activity of the GtH cells and growth hormone cells in the proximal pars distalis.

In lesioning studies on paar of Atlantic salmon (Salmo salar) (Dodd et al., 1978) and killifish (Fundulus heteroclitus) (Pickford et al., 1981), destruction of part of the NLT caused gonadal regression or blockage of gonadal recrudescence.

While the above evidence clearly implicates the NLT in regulation of GtH secretion via GnRH, immunocytological studies also implicate the preoptic region, specifically the ventral-lateral NPP. Lesions in the preoptic region of female goldfish caused a significant decrease in GSI at 42 days postoperatively (Peter & Crim, 1978), suggesting some involvement in the maintenance of gonadal activity. However, these results have not been confirmed (Peter, 1970; Peter & Paulencu, 1980; R. Peter, unpublished results). Perhaps since the lesioning current was high and the lesions large in the study by Peter & Crim (1978), the lesions may have interfered with a GtH release inhibitory factor thought to originate from the preoptic region (Peter & Paulencu, 1980; see below), as well as GnRH originating from the

NPP. It would be of great interest to selectively lesion the area of the NPP in which the LH-RH immunoreactive cells are located to determine the effects on gonadal activity and GtH secretion, and compare the results to the effects of lesions in the NLT. Since there are probably two populations of GnRH perikarya involved in regulation of GtH secretion, it may be that there are differences in function between the two;the NLT has been implicated in regulation of daily cycles of GtH release in goldfish.

Actions

It is well established that hypothalamic extracts from both teleosts and mammals, synthetic LH-RH and analogues of LH-RH that are superactive in mammals can stimulate GtH release from teleost pituitaries in vivo and in vitro (Ball, 1981; Peter, 1982). Until synthetic teleost GnRH is available it will be impossible to directly compare its potency in a teleost with LH-RH. Nevertheless, data by King & Millar (1980) suggests that tilapia GnRH has similar potency to LH-RH in stimulating LH release from sheep pituitary cells in vitro. Given that teleost GnRH's and LH-RH are similar in structure, synthetic LH-RH can be used in studies on fish to gain information on the actions of endogenous GnRH.

Peter (1980) found that a pair of injections of LH-RH, or the superactive analogue des-Gly^{10}[D-Ala^{6}] LH-RH ethylamide (LRH-A), were more effective in stimulating increased serum GtH levels than a single injection of similar dosage. This indicates that potentiation of the GtH release-response can occur. A pair of injections of LRH-A at a low dosage caused, in some cases, a greater increase in serum GtH levels than injections at a higher dosage, indicating that suppression of the release-response can occur due to excess hormone. There were no significant differences in the peak serum levels of GtH induced by similar dosages of LH-RH and LRH-A; however, LRH-A caused a more prolonged release-response under certain conditions.

Lin et al. (1982) reported that the length of the interval between a pair of injections and the acclimation temperature of goldfish interact to influence the GtH release-response in goldfish. At a warm temperature (20°C) injections with an interval of 3 hours induced higher serum GtH levels than when the interval was 9 hours. At a cold temperature (12°C), injections at a low dosage (0.01µg LRH-A/g body weight) were as effective as a higher dosage (0.1µg/g BWt) at an interval of 3 hours. With a 9 hour interval, however, the low dosage was much less effective than the high dosage. These preliminary studies demonstrate that the relationship between the dosage of LRH-A, the interval between injections, temperature, and the GtH release-response is complex. Investigators clearly need to take great care in defining the conditions under which LH-RH or its analogues are tested.

A seasonal variation in the in vivo GtH release-response to LH-RH or LRH-A has been found in brown trout (Salmo trutta) (Crim & Cluett, 1974), rainbow trout (Weil et al., 1978) and goldfish (Lin et al.,1982). In each species the greatest GtH release-response occurred in fish at the time of spawning, when gonadal development was complete or nearing completion preparatory for spawning.

Crim et al. (1981a) demonstrated that analogues of LH-RH that block the action of the authentic molecule in mammals (inhibitory analogues, iLRH-a), also block the action of LH-RH on GtH release in vivo in brown trout. Superactive analogues were not more active than LH-RH in vivo; in vitro LRH-A was somewhat more active than LH-RH, but other superactive analogues had a similar potency to LH-RH.

In coho salmon (Oncorhynchus kisutch) LRH-A is clearly more active than LH-RH in stimulating plasma GtH and inducing ovulation (G. Van der Kraak, H.-R. Lin, E.M. Donaldson, H.M. Dye and G.A. Hunter, personal communication). A single injection of LRH-A (20µg/kg BWt) caused the same magnitude of increase in plasma GtH as 200 µg LH-RH/kg BWt. In addition, the effect LRH-A persisted for at least 96 hours, whereas the increase in plasma GtH following LH-RH injection lasted for only about 24 hours. Both LH-RH and LRH-A accelerated the time of ovulation, although LRH-A was much more effective (Donaldson 1981/1982, 1982). Although LRH-A can be a highly effective agent for inducing ovulation in some species, such as the coho, clearly the responsiveness to this analogue, and others also, can vary greatly between species.

A chronic increase in plasma GtH, lasting at least 4 weeks, was induced in landlocked Atlantic salmon implanted with pellets that gave a continuous release of a superactive analogue of LH-RH (Crim et al., 1982). The pellets were effective in stimulating GtH release, and in causing increased pituitary GtH content, in salmon that were in regressed, recrudescing and prespawning gonadal conditions. The effects on gonadal condition were complex. In sexually regressed male salmon there was no effect on GSI, but histological examination of the testes suggested stimulation of testicular recrudescence. In recrudescing females there was an increase in GSI, suggesting stimulation of vitellogenesis. In recrudescing males, however, GSI was decreased compared to sham controls. The testes of the analogue treated males contained predominantly spermatids and spermatozoa,

whereas the sham implanted fish had spermatocytes and spermatids; the analogue apparently induced maturation of the testes, without stimulating additional growth. Prespawning females were all induced to ovulate, in some cases considerably earlier in the season than normal. Prespawning males were induced to spermiate considerably earlier than usual, or to have increased sperm volume if spermiation had already commenced. In addition to demonstrating that chronic exposure to analogues of LH-RH can accelerate gonadal recrudescence and the time of ovulation and spermiation in Atlantic salmon, the results also show that there is no down-grading of the GtH release-response to a normal or less than normal level, as would be the case in a mammal. However, whether some suppression of the GtH release-response occurred cannot be determined from the data presented.

In early studies, large doses of LH-RH were used to induce ovulation in several teleost species, including goldfish (for review, Peter, 1982) and common carp (C.H. Pan, personal communication). LRH-A has been found to be highly effective in inducing ovulation (> 80%) in cultured grass carp (Ctenopharyngodon idellus), black carp (Mylopharyngodon piceus), silver carp (Hypophthalmichthys molitrix) and spotted silver carp (Aristichthys noblis) (Anonymous 1977a, b; Jiang et al., 1980). Grass carp ovulated within 12 to 22 hours of a single injection of LRH-A ranging from 1 to 100µg/kg BWt; two injections with a 7 to 10 hour interval reduced the time to ovulation to 3 to 14 hours after the second injection. Silver carp artificially spawned for the first time with LRH-A were more sensitive (required one injection of 2 to 20µg/kg BWt to induce ovulation) than fish that had been induced previously (required two injections totaling 10µg/kg BWt). Black carp were relatively less responsive, requiring two or three injections with dosages up to 400µg/kg BWt. These results indicate that LRH-A can have wide application for inducing ovulation in carp; however, the results are difficult to evaluate because they are a compendium of experiments done under different conditions by different workers at many different fish farms. In my own experience on goldfish, ovulation did not occur in more than half of the test fish in experiments in which dosages from 10µg to 1mg/kg BWt were used (R. Peter, unpublished results). However, a high frequency of ovulation occurred when goldfish were injected with LRH-A (100µg/kg BWt) and pimozide, a dopamine antagonist (Chang & Peter, 1982 a, b, c). Clearly, LRH-A can be a highly effective agent for inducing ovulation in cyprinids, although there is a wide variability in sensitivity. However, in those species which are less responsive, the combination of LRH-A with a dopamine blocking agent to reduce the GtH release-inhibitory action of dopamine (see below), can be used to induce ovulation.

The cellular mechanism of action of GnRH has received little attention in teleost fish. Following injection of mud carp (Cirrhrinus molitorella) with ^{125}I-LRH-A, Pan et al. (1981) found, by ultrastructural autoradiography, that label appeared over the cell membrane, Golgi body, secretory granules, mitochondria and nucleus of the GtH cells. A greater uptake of label was observed when the mud carp were injected with a potentiating dose of cold LRH-A ten minutes before administration of the label. In goldfish held at 12°C, administration of a potentiating dose of LRH-A 12 hours before injection of ^{125}I-LRH-A caused a greater uptake of label into the pituitary gland by 20 to 30 minutes, and a lower content of label at 24 hours postinjection (O. Bres, R.E. Peter, H.-R. Lin and C.S. Nahorniak, unpublished results). This confirms that potentiation induces a greater uptake of GnRH, and suggests that it also causes a more rapid depletion or turnover of GnRH in the pituitary gland. Although these results require confirmation, perhaps further investigations on teleosts may provide information on the dynamics of GnRH metabolism in the pituitary.

Gonadotropin Release-Inhibitory Factor (GRIF)

Nature, Localization and Actions

The presence of a GRIF in goldfish was suggested by Peter et al. (1978) as the explanation for the dramatic increase in serum GtH levels found in mature female goldfish following large lesions in NLT-pituitary stalk region. The increase in serum GtH lasted for at least 12 days, although levels had decreased to near normal by this time. In addition, nearly all the lesioned females ovulated.

Peter and Paulencu (1980) found that destruction of the pituitary stalk, and not the NLT, was the cause of the dramatic increase in GtH secretion. This indicates that removal of the pituitary gland from hypothalamic control, by lesioning the pituitary stalk, allows spontaneous release of GtH to occur, confirming the presence of GRIF. Additional lesioning studies in male as well as female goldfish indicated that the origin of GRIF is the anterior-ventral preoptic region, in the area of the NPP that is ventral to the anterior commissure (Peter & Paulencu, 1980). This area is more anterior than the location of LH-RH immunoreactive perikarya in the lateral-ventral NPP. Tracts for GRIF are located bilaterally in the lateral preoptic

region, and the lateral-anterior hypothalamic region, converging to enter the pituitary stalk. Nagahama & Peter (1982) confirmed that lesioning the preoptic region and the pituitary stalk cause elevated serum GtH and ovulation in mature female goldfish. In addition, ultrastructure studies of the pituitary from lesioned fish revealed depletion of secretory granules, and extensive development of the Golgi and dilation of the endoplasmic reticulum, suggesting that secretion and synthesis were both occurring in GtH cells.

Pars distalis transplant studies in goldfish, summarized in a preliminary report by Peter et al. (1982), support the presence of GRIF. Transplanting the pars distalis from one goldfish to either beside the brain ("juxta" location), into the third ventricle in the preoptic region, or the ventricular region ventral to the optic tectum resulted in an increase in serum GtH levels in the recipient fish. This increase in serum GtH in the recipient fish is due to the spontaneous release of GtH from the transplanted pars distalis; the serum GtH levels return to normal levels within 24 hours after removal of the transplanted pars distalis from the "juxta" location. Fish with the transplant in the "juxta" location had higher serum GtH levels than those with the transplant in the brain ventricles. This indicates there is some factor in the brain, presumably present in the cerebrospinal fluid, that suppresses the spontaneous release of GtH.

No attempts have been made to extract and isolate GRIF. However, strong evidence has been presented that dopamine (DA) acts as a GRIF in goldfish (Chang et al., 1982; Chang and Peter, 1982 a, b, c). Chang et al. (1982) found that treatment of goldfish with 6-hydroxy-dopamine, a catecholaminergic neurotoxin, caused an increase in serum GtH levels, suggesting that catecholaminergic neurons inhibit GtH release. Treatment with α-methyl-p-tyrosine, which blocks the synthesis of dihydroxyphenylalamine (L-DOPA) the precursor of DA, and blocking the conversion of L-DOPA to DA with carbidopa, both caused an increase in serum GtH levels. On the other hand, clonidine, an α-noradrenergic agonist, caused an increase in serum GtH, and treatment with diethyldithiocarbamate (DDC), which blocks the conversion of DA to norepinephrine, had no effect on serum GtH levels. These results indicate that DA has an inhibitory effect, whereas norepinephrine has a stimulatory effect on GtH release. However, these studies do not indicate the level of action of DA or norepinephrine.

Chang and Peter (1982b) found that intraperitoneal (ip) injection of DA and apomorphine, a DA agonist, caused a decrease in serum GtH levels in intact goldfish, whereas pimozide, a DA antagonist, caused a significant increase. Injection of DA into the third ventricle had no significant effects on serum GtH, suggesting that the actions of DA to decrease serum GtH were outside the blood brain barrier. Injection of DA and apomorphine both caused a marked decrease in the elevated serum GtH levels of goldfish lesioned in the preoptic region to abolish endogenous GRIF. Furthermore, DA and APO blocked the effects of LRH-A to cause GtH release, and blocked the high rate of GtH that had previously been induced by LRH-A. These results indicate that DA acts at the level of the pituitary to block spontaneous release of GtH, and also to block, or modulate, the actions of GnRH.

In a preliminary report, Crim (1982) indicated that DA could diminish the GtH release-response to LH-RH and decrease the spontaneous release of GtH by rainbow trout pituitaries in vitro. This suggests that the GRIF activity of DA in teleosts may be widespread.

As reported above, injection of LRH-A into mature female goldfish resulted in a relatively low rate of induced ovulation ($\leq$ 50%) (R. Peter, unpublished results). Chang & Peter (1982a, c) found that injection of mature female goldfish held at 12°C with primozide, a DA agonist, at the time of the first or the second of two injections of LRH-A 12 hours apart potentiated the GtH release-response, and resulted in an ovulation rate of 87% or higher; injection of pimozide at the same time as a single injection of LRH-A, or 12 hours ahead of the injection of LRH-A resulted in an ovulation rate of 35% and 70% respectively. Notably, these ovulation rates were obtained in spite of the fish being held at 12°C, a temperature at which goldfish do not normally undergo spontaneous ovulation. At temperatures of 18-20°C, a more usual temperature for spawning, pimozide is even more effective at potentiating LRH-A effects on GtH secretion and ovulation (M. Sokolowska, R. Peter, C.H. Pan, C.S. Nahorniak & J.P. Chang, unpublished results). The results indicate that blocking the GRIF activity of DA greatly facilitates the application of LRH-A in induced ovulation of goldfish, and suggests that the normal preovulatory GtH surge in goldfish is regulated by removal of DA inhibition on GtH release and stimulation of GtH secretion by GnRH. Whether any other factors have GRIF activity and can modulate the action of GnRH is not known.

Steroid Feedback

A negative feedback effect of sex steroids on GtH release in teleosts undergoing or at a prespawning stage of gonadal development has been demonstrated (for review, Peter, 1982). Both the pituitary and the

NLT are involved in the negative feedback action of sex steroids, although the pituitary is apparently the most important direct feedback site (Billard & Peter, 1977).

Sexually immature salmonids show a positive feedback effect of sex steroids. A marked increase in the GtH content of the pituitary in male and female Atlantic salmon parr occurred after testosterone implantation in the pituitary and NLT, but not when testosterone was implanted in the preoptic region or optic tectum (Crim & Peter, 1978). Immature rainbow trout were shown to have an increase in the pituitary content of GtH after injection of testosterone intraperitoneally in a cocoa butter vehicle to give slow release (Crim and Evans, 1979). Recently it was demonstrated that the positive feedback effect on pituitary GtH was in response to estrogenic steroids (estradiol, estrone and estriol) and androgenic steroids that can be aromatized to estrogens (testosterone propionate, 17α-methyltestosterone, androstenedione), whereas non-aromatizable androgens (5α-dihydrotestosterone, 11-ketotestosterone, 11β-hydroxytestosterone) and progestins (17α-hydroxyprogesterone, 17α-hydroxy-20β-dihydroprogesterone) were ineffective (Crim et al., 1981b). This was supported by the additional finding that the aromatase inhibitor 1, 4, 6-androstatrien-3, 17-dione blocked the action of testosterone on pituitary GtH.

van den Hurk (1982) reported that shortly after hatching, rainbow trout exhibit an increase in immunocytochemically detectable GtH in the pituitary after treatment with methyl testosterone, progesterone and 17α-hydroxyprogesterone. This indicates that the positive feedback effect can occur at an early age, and is not just a phenomenon that occurs at or near the onset of sexual development. In addition, these results indicate that progestins as well as aromatizable androgens are active at this stage. Since the progestins do not have a positive feedback effect at a later age (Crim et al., 1981b), it would be of interest to determine the changes in steroid responsiveness as development proceeds.

On the basis of light microscope studies, Olivereau & Chambolle (1978) and Olivereau & Olivereau found that estradiol treatment of immature eels (Anguilla anguilla) caused stimulation of GtH cells.

Although aromatizable androgens and estrogens can induce synthesis of GtH in the pituitary of immature salmonids and eels, in order for this to be considered a truly functional positive feedback effect, there must also be release of GtH from the pituitary. Crim and Evans (1980) found that there was greater spontaneous release of GtH in vitro from pituitaries taken from testosterone treated immature rainbow trout as compared to control fish, and that LH-RH and synthetic superactive analogues could stimulate GtH release in vitro from the pituitaries of the testosterone treated trout. This demonstrates that the GtH accumulated in the pituitary of the immature rainbow trout can be released; however, evidence that release occurs in vivo after accumulation is not clear. Crim and Peter (1978) found that spermatogenesis was advanced in immature Atlantic salmon with a testosterone pellet implanted in the pituitary and NLT, although plasma GtH remained undetectable. Crim and Evans (1982) reported that Atlantic salmon parr implanted with a long release capsule containing testosterone had onset of spermatogenesis and, increased pituitary and plasma GtH. Whether the appearance of GtH in the plasma reflects GtH release stimulated by endogenous GnRH or spontaneous release is not known. Nevertheless, these results suggest that a positive feedback by estrogens or aromatizable androgens may be an important part of the mechanism of onset of sexual development or puberty. In support, the aromatase enzyme is present in relatively high concentrations in the hypothalamus of all teleosts investigated (Callard, 1982). However, the changes in aromatase activity during a reproductive cycle of any one species have not been investigated.

Concluding Remarks

There are many basic problems remaining; the identity of GnRH and the function of the various brain regions where GnRH has been tentatively localized are obvious. However, although there seems to be a reasonable amount of information available, much more needs to be known about the actions of LH-RH, analogues of LH-RH, and, ultimately, native GnRH. There is no set of comprehensive studies available for any one species to enable understanding the role of the releasing hormone in a reproductive cycle, or the effects on pituitary and gonadal activities of acute and chronic exposure to range of dosages of synthetic releasing hormone. Without development of such information for at least some representatives of the families within which there are commercially important species, the attempts at tinkering with various phases of the reproductive cycle with synthetic releasing hormones may be futile, or produce results which could even be subject to misinterpretation. In spite of these pessimistic cautions, there has been important success in the application of LRH-A for induced ovulation of salmon and certain carp species. However, the fact that there are major differences in the response to LRH-A between closely related species makes the need for basic

research in this area more poignant.

Perhaps the most intriguing development in this area has been the discovery of GRIF, and that DA has GRIF activity in goldfish. The finding that DA can modulate the response to LRH-A, and by blocking DA activity with a receptor antagonist the goldfish is more responsive to LRH-A and more subject to induced ovulation, indicates a way of regulating ovulation in those species that seem to be "resistant" to the effects of the releasing hormone. The presence of GRIF, the actions of DA on GtH secretion throughout reproductive cycles, and whether other factors have GRIF activity requires investigation in teleosts in general. The investigation of the interactions of native teleost GnRH and DA on GtH secretion could lead to important developments in the regulation of various phases of the reproductive cycle, particularly ovulation and spermiation.

The finding that there is a positive feedback effect of estrogens and aromatizable androgens in sexually immature salmonids and eels changes the perspective generally held on the effects of sex steroids on GtH secretion. The most recent finding that the onset of spermatogenesis can be stimulated through this mechanism raises interesting prospects for the manipulation of this phase of the reproductive cycle; the presence of such a positive feedback effect needs to be investigated in teleosts in general.

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References

Anonymous, 1977a. A new highly effective ovulating agent for fish reproduction. Scientia Sinica 20:469-474.

Anonymous, 1977b. A further investigation on the stimulatory effect of a synthetic analogue of hypothalamic luteinizing hormone releasing hormone (LRH-A) on spawning in "domestic fishes". Acta Biochem. Biophys. Sinica 9:15-24.

Ball, J.N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians and reptiles. Gen. Comp. Endocrinol. 44:135-170.

Barnett, F.H., J. Sohn, S. Reichlin & I.M.D. Jackson, 1982. Three luteinizing hormone-releasing hormone like substances in a teleost fish brain: none identical with the mammalian LH-RH decapeptide. Biochem. Biophys. Res. Commun. 105:209-216.

Billard, R., & R.E. Peter, 1977. Gonadotropin release after implantation of anti-estrogens in the pituitary and hypothalamus of goldfish, Carassius auratus. Gen. Comp. Endocrinol. 32:213-220.

Breton, B., B. Jalabert, R. Billard & C. Weil, 1971. Stimulation in vitro de la liberation d'hormone gonadotrope hypophysaire par un facteur hypothalamique chez la Carpe (Cyprinus carpio L.). C.R. Acad. Sci., Paris 273D:2591-2594.

Callard, G.V., 1982. Aromatase in the teleost brain and pituitary: role in hormone action. In: C.J.J. Richter (Ed): Proceedings of the International Symposium on Reproductive Physiology of Fish. Pudoc, Wageningen. (In press).

Chang, J.P., A.F. Cook & R.E. Peter, 1982. Influences of catecholamines on gonadotropin secretion in goldfish, Carassius auratus. Gen. Comp. Endocrinol. (In press).

Chang, J.P., & R.E. Peter, 1982a. Actions of dopamine on gonadotropin release in goldfish, Carassius auratus. In: C.J.J. Richter (Ed.): Proceedings of the International Symposium on Reproductive Physiology of Fish. Pudoc, Wageningen. (In press).

Chang, J.P., & R.E. Peter, 1982b. Effects of dopamine on gonadotropin release in female goldfish, Carassius auratus. Neuroendocrinology. (In press).

Chang, J.P., & R.E. Peter, 1982c. Effects of pimozide and des-Gly^{10}, [D-Ala^{6}] luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration and ovulation in female goldfish, Carassius auratus. Gen. Comp. Endocrinol. (In press).

Crim, L.W., 1982. Control of gonadotropic hormone secretion (GTH) by the rainbow trout pituitary gland. Evidence of GTH inhibition by catecholamine and stimulation of GTH release by some other neuroregulatory factors. In: K. Lederis & D.S. Farner (Eds.): Neurosecretion. Plenum, New York. p. 446.

Crim, L.W. & D.M. Cluett, 1974. Elevation of plasma gonadotropin concentration in response to mammalian gonadotropin releasing hormone (GRH) treatment of the male brown trout as determined by radioimmunoassay. Endocr. Res. Comm. 1:101-110.

Crim, L.W., & D.M. Evans, 1979. Stimulation of pituitary gonadotropin by testosterone in juvenile rainbow trout (Salmo gairdneri). Gen. Comp. Endocrinol. 37:192-196.

Crim, L.W. & D.M. Evans, 1980. LH-RH-stimulated gonadotropin release from the rainbow trout pituitary gland: an in vitro assay for detection of teleost

gonadotropin releasing factor. Gen. Comp. Endocrinol. 40:283-290.

Crim, L.W., & D.M. Evans, 1982. Positive steroid feedback on gonadotropic hormone in juvenile rainbow trout: evidence of gonadotropin synthesis and release following testosterone administration via silastic capsules. In: C.J.J. Richter (Ed.): Proceedings of the International Symposium on Reproductive Physiology of Fish. Pudoc, Wageningen. (In press).

Crim, L.W., D.M. Evans, D.H. Coy, & A.V. Schally, 1981a. Control of gonadotropic hormone release in trout: influence of synthetic LH-RH and LH-RH analogues in vivo and in vitro. Life Sci. 28:129-135.

Crim, L.W., D.M. Evans, & B.H. Vickery, 1982. Manipulation of the seasonal reproductive cycle of the landlocked salmon (Salmo salar) with LHRH administration at various stages of gonadal development. Can. J. Fish. Aqua. Sci. (In press).

Crim, L.W., & R.E. Peter, 1978. The influence of testosterone implantation in the brain and pituitary on pituitary gonadotropin levels in Atlantic salmon parr. Ann. Biol. Anim. Biochim. Biophys. 18:689-694.

Crim, L.W., R.E. Peter & R. Billard, 1976. Stimulation of gonadotropin secretion by intraventricular injection of hypothalamic extracts in the goldfish, Carassius auratus. Gen. Comp. Endocrinol. 30:77-82.

Crim, L.W., R.E. Peter & R. Billard, 1981b. Onset of gonadotropic hormone accumulation in the immature trout pituitary gland in response to estrogen or aromatizable androgen steroid hormones. Gen. Comp. Endocrinol. 44:374-381.

Dodd, J.M., P.A.C. Stuart-Kregar, J.P. Sumpter, L.W. Crim & R.E. Peter, 1978. Premature sexual maturation in the Atlantic salmon (Salmo salar L.). In: P.J. Gaillard & H.H. Boer (Eds.): Comparative Endocrinology. Elsevier/North-Holland, Amsterdam. p. 101-104.

Donaldson, E.M., G.A. Hunter & H.M. Dye, 1981/1982. Induced ovulation in coho salmon (Oncorhynchus kisutch). II. Preliminary study of the use of LH-RH and two high potency LH-RH analogues. Aquaculture. 26:129-141.

Donaldson, E.M., G.A. Hunter, H.M. Dye & G. Van der Kraak, 1982. Induced ovulation in Pacific salmon using LH-RH analogs and salmon gonadotropin. In: B. Lofts (Ed.): Proceedings of the Ninth International Symposium on Comparative Endocrinology. University of Hong Kong Press, Hong Kong. (In press).

Dubois, M.P., R. Billard, B.Breton & R.E. Peter, 1979. Comparative distribution of somatostatin, LH-RH, neurophysin, and α-endorphin in the rainbow trout: an immunocytological study. Gen. Comp. Endocrinol. 37:220-232.

Goos, H.J.Th. & O. Murthanoglu, 1977. Localisation of gonadotropin releasing hormone (GRH) in the forebrain and neurohypophsis of the trout (Salmo gairdneri). Cell Tiss. Res. 181:163-168.

Hontela, A., 1982. Regulation and physiological significance of daily cycles in gonadotropin hormone levels in the female goldfish. Ph.D. Thesis, Department of Zoology, University of Alberta.

Hontela, A. & R.E. Peter, 1978. Daily cycles in serum gonadotropin levels in the goldfish: effects of photoperiod, temperature, and sexual condition. Can. J. Zool. 56:2430-2442.

Hontela, A., & R.E. Peter, 1980. Effects of pinealectomy, blinding, and sexual condition on serum gonadotropin levels in the goldfish. Gen. Comp. Endocrinol. 40:168-179.

Idler, D.R. & L.W. Crim, 1982. Gonadotropin releasing factor(s) (GtH-RF) from hypothalamus of winter flounder. In: B. Lofts (Ed.): Proceedings of the Ninth International Symposium on Comparative Endocrinology. University of Hong Kong Press, Hong Kong. (In press).

Jiang, R., S.Huang & W. Zhao, 1980. Changes of serum gonadotropin levels before and behind induced spawning in grass carp and silver carp. J. Fish. China. 4:129-133.

Kah, O., P. Chambolle, P. Dubourg & M.P. Dubois, 1982a. Distrubution of immunoreactive LH-RH in the brain of the goldfish. In: C.J.J. Richter (Ed.): Proceedings of the International Symposium on Reproductive Physiology of Fish. Pudoc, Wageningen. (In press).

Kah,O., R.E. Peter, P. Dubourg & H. Cook, 1982b. Effects of monosodium L-glutamate on pituitary innervation in goldfish, Carassius auratus. Gen. Comp. Endocrinol. (In press).

King, J.A. & R.P. Millar, 1979. Heterogeneity of vertebrate luteinizing-releasing hormone. Science. 206:67-69.

King, J.A. & R.P. Millar, 1980. Comparative aspects of luteinizing hormone-releasing hormone structure and function in vertebrate phylogeny. Endocrinology. 106:707-717.

Lin, H.-R., R.E. Peter, C.S. Nahorniak & O. Bres, 1982. Actions of the superactive analogue of Des-Gly^{10}[D-Ala^{6}]LRH ethylamide (LRH-A) on gonadotropin secretion in goldfish. In: B. Lofts (Ed.): Proceedings of the Ninth International Symposium on Comparative Endocrinology. University of Hong Kong Press, Hong Kong. (In press).

Münz, H., B. Claas, W.E. Stumpf & L. Jennes, 1982. Centrifugual innervation of the retina by luteinizing hormone releasing hormone (LHRH)-immunoreactive telencephalic neurons in teleostean fishes. Cell Tiss. Res. 222:313-323.

Münz, H., W.E. Stumpf & L. Jennes, 1981. LHRH systems in the brain of platyfish Brain Res. 221:1-13.

Nagahama, Y. & R.E. Peter, 1982. Effects of brain lesions on gonadotrop ultrastructure and serum gonadotropin levels in goldfish. Cell Tiss. Res. (In press).

Nozaki, M. & H. Kobayashi, 1979. Distribution of LHRH-like substance in the vertebrate brain as revealed by immunohistochemistry. Arch. Histol. Japan. 42:201-209.

Olivereau, M. & P. Chambolle, 1978. Ultrastructure des cellules gonadotropes de l'Anguille normale et après injection d'oestradiol. C.R. Acad. Sci. Paris. 287D:1409-1412.

Olivereau, M. & J. Olivereau, 1979a. Effect of estradiol-17β on the cytology of the liver, gonads and pituitary, and on plasma electrolytes in the female freshwater eel. Cell Tiss. Res. 199:431-454.

Olivereau, M. & J. Olivereau, 1979b. Estradiol-positive feedback on gonadotropic (GTH) cells in freshwater male silver eels. Gen. Comp. Endocrinol. 39:247-261.

Pan, C.-H., M.-Q. Feng, N.-C. Ling, S. Pao, W.-Q. Xu, G.-X. Xu & R.-C. Shen, 1979. Immunocytochemical studies on gonadotropin releasing hormone (GRH) secretory nucleus of the carp (Cyprinus carpio). Acta Biol. Exp. Sinica. 12:306-310.

Pan, X., D. Chen, Z. Zhao, Y. Zhu, X. Zhao, H. Lin, Z. Liu, Z. Liu & D. Lin, 1981. Studies on mechanism of action of luteinizing releasing hormone (LH-RH) in ovulation of fish. Scientia Sinica. 24: 982-988.

Peter, R.E., 1970. Hypothalamic control of thyroid gland activity and gonadal activity in the goldfish, Carassius auratus. Gen. Comp. Endocrinol. 14:334-356.

Peter, R.E., 1980. Serum gonadotropin levels in mature male goldfish in response to luteinizing hormone-releasing hormone (LH-RH) and des-Gly^{10}-[D-Ala^{6}]-LH-RH ethylamide. Can. J. Zool. 58:1100-1104.

Peter, R.E., 1982. Neuroendocrine control of reproduction in teleosts. Can. J. Fish. Aquatic Sci. 39:48-55.

Peter, R.E., J.P. Chang, A.F. Cook & C.S. Nahorniak, 1982. Neuroendocrine regulation of gonadotropin and growth hormone secretion in goldfish. In: B. Lofts (Ed.): Proceedings of the Ninth International Symposium on Comparative Endocrinology. University of Hong Kong Press, Hong Kong. (In press).

Peter, R.E. & L.W. Crim, 1978. Hypothalamic lesions of goldfish: effects on gonadal recrudescence and gonadotropin secretion. Ann. Biol. Anim. Bioch. Biophys. 18:819-823.

Peter, R.E., L.W. Crim, H.J. Th. Goos & J.W. Crim, 1978. Lesioning studies on the gravid female goldfish: neuroendocrine regulation of ovulation. Gen. Comp. Endocrinol. 35:391-401.

Peter, R.E., O. Kah, C.R. Paulencu, H. Cook & A.L. Kyle, 1980. Brain lesions and short-term endocrine effects of monosodium L-glutamate in goldfish, Carassius auratus. Cell Tiss. Res. 212:429-442.

Peter, R.E. & C.R. Paulencu, 1980. Involvement of the preoptic region in gonadotropin release-inhibition in goldfish, Carassius auratus. Neuroendocrinology. 31:133-141.

Pickford, G.E., W.R. Knight, J.N. Knight, R. Gallardo & B.I. Baker, 1981. Long-term effects of hypothalamic lesions on the pituitary and its target organs in the killifish Fundulus heteroclitus. I. Effects on the gonads, thyroid, and growth. J. Exp. Zool. 217:341-351.

Schreibman, M.P., L.R. Halpern, H.J. Th. Goos & H. Margolis-Kazan, 1979. Identification of luteinizing hormone-releasing hormone (LH-RH) in the brain and pituitary gland of a fish by immunocytochemistry. J. Exp. Zool. 210:153.159.

Schreibman, M.P., H. Margolis-Kazan & L. Halpern-Sebold, 1982. Immunoreactive gonadotropin and luteinizing hormone releasing hormone in the pituitary gland of neonatal platyfish. Gen. Comp. Endocrinol. (In press).

van den Hurk, R., 1982. Effects of steroids on gonadotropic (GTH) cells in the pituitary of rainbow trout, Salmo gairdneri, shortly after hatching. Cell Tiss. Res. 224:361-368.

Weil, C., R.Billard, B. Breton & B. Jalabert, 1978. Pituitary response to LH-RH at different stages of gametogenesis in the rainbow trout (Salmo gairdneri). Ann. Biol. Anim. Bioch. Biophys. 18:863-869.

AROMATASE IN THE TELEOST BRAIN AND PITUITARY: ROLE IN HORMONE ACTION

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Summary

For certain androgen-dependent behavioral and neuroendocrine responses, prior conversion to estrogen is essential. Aromatization of androgen to estrogen occurs in the brain of representatives of all major vertebrate groups, although activity is extraordinarily high in teleosts. Retention of estrogen in discretely dissected brain regions following androgen perfusion, corresponds to the neuroanatomic distribution of the enzyme. By contrast, no free estrogen is recovered from brain after perfusion with estrogen. An active conjugating system for estrogen present in the gill inactivates or excretes circulating estrogen. Thus, only estrogen synthesized in close proximity to central targets is biologically active. High levels of aromatase have also been identified in teleost pituitary. In an *in vitro* translocation system, testosterone, presumably via conversion to estradiol, competes with ^{3}H-estradiol for nuclear binding sites. We conclude that, in teleosts, neither the brain nor the pituitary is wholly dependent on circulating estrogen, assuming aromatizable substrate is available.
Keywords: brain, pituitary, aromatase, estrogen, teleost.

Introduction

A relationship between the gonads and breeding behavior in humans and domestic animals has been known since ancient times. Nevertheless, identification of the active principles and their mechanism and sites of action are relatively recent developments. It is now accepted that sex steroids acting on target cells in the brain and elsewhere in the central nervous system (CNS) trigger sex behavior in adults and permanently alter the 'maleness' or 'femaleness' of the CNS of fetal and neonatal animals. Via complex positive and negative feedback mechanisms, androgens and estrogens control hypophysiotrophic centers in the brain and, to some extent, affect the pituitary directly. Through these actions they are responsible for the rhythmic patterns of gonadal development characteristic of ovulatory and seasonal cycles. There is good evidence that the mechanism by which steroids interact with neuroendocrine tissues is the same as that in peripheral steroid targets, and includes, in sequence, binding to specific cytosolic receptors, translocation of this complex to nuclear acceptor sites, and, ultimately, alteration of genomic functions (McEwen et al., 1979).

One concept that is now outdated is that sex steroids are sex specific, namely, that androgens are male hormones whereas estrogens are female hormones. From the earliest behaviorists, it was known that androgens and estrogens often share common actions that are independent of gender. To illustrate, copulatory behavior in castrated rats may be activated either by testosterone or by estradiol. 'Paradoxical' effects of sex steroids have now been documented for every major vertebrate group. In addition, the gonads of both sexes are capable of synthesizing and secreting androgen and estrogen. The relative output of these two classes of steroid does not necessarily conform to expectations based on sex. In female rabbits, hamsters, and turtles, to cite three examples, circulating androgens are much greater than circulating estrogens and fluctuations are keyed to the ovulatory cycle. Similar examples may be found in this symposium.

Androgens and estrogens are, in fact, components of the same steroidogenic pathway. Testosterone and androstenedione are the immediate and obligatory precursors of estradiol and estrone, respectively. Conversion of androgen to estrogen (aromatization) is controlled by a P-450 linked enzyme complex termed aromatase. This enzyme is not, however, an exclusive property of the primary estrogen secreting organs (placenta, gonads) but exists also in a variety of peripheral non-glandular tissues (fat, muscle, bone). Of particular importance to the neuroendocrinologist was the identification in 1971 by Naftolin, Ryan, and colleagues of low levels of aromatase activity in the brain (Naftolin et al., 1975). Numerous physiological and behavioral studies in the last ten years support the idea that aromatization to estrogen in the brain itself is essential for the expression of certain central androgen actions; however, since separate binding mechanisms for androgen and estrogen are simultaneously present in the brain of both sexes, it is likely that androgens have biological actions in their own right. Indeed, complex behavioral responses appear to be a composite of several androgen- and estrogen-dependent components. Natural changes in brain aromatase in relation to age, gender, and reproductive cycles as well as substances which regulate aromatase in brain *in vivo* and *in vitro* have been reported (Naftolin et al., 1975; Callard, 1980; Callard et al., 1980b; 1981a; 1982). By contrast, little is known about aromatase in the pituitary.

The first biochemical identification of activity in this gland was reported from this laboratory and was the result of investigations in a teleost, the longhorn sculpin (Myoxocephalus octadecimspinosus) (Callard et al., 1981b). We review here aromatization in the neuroendocrine tissues of teleosts and speculate on the function of this enzyme in mediating androgen action.

Brain

As part of a phyletic survey, aromatase activity in the brain of certain non-mammals was found to be much higher than in common laboratory species (Callard et al., 1978). In the sculpin, for example, estrogen yields were ≈190 fmol/mg tissue, 1000-fold greater than that reported for adult rat and rabbit (0.1-1.0 fmol/mg) and higher than other species tested. To date, seven other teleost genera have been studied: Pseudopleuronectes, Opsanus, Coris, Serranus, Paracentropristis, Spicara, and Pagellus (Callard & Reinboth, in preparation). Although none have levels quite as high as the sculpin, it would appear that the potential for synthesizing substantial amounts of estrogen may be a general characteristic of the teleost brain. Notable omissions from this survey, however, are fresh-water species and representatives of less advanced orders. In contrast to teleosts, brain conversions are low in elasmobranchs (Squalus, Raja), only tentatively identified in Petromyzon, and undetectable in Myxine and Amphioxus (Callard et al., 1978; 1980a).

The idea that estrogen synthesis in neuroendocrine tissues occurs in close proximity to receptors is supported by the observation that, in mammals, activity is restricted to the hypothalamus, preoptic area, septum, and amygdala, structures which have high concentrations of estrogen-binding cells, and are known by other criteria to control reproduction and sex behavior. That estrogen formed locally is retained in the same brain regions was first demonstrated directly using the isolated, perfused Rhesus brain, a preparation which precludes aromatization in peripheral tissue (Naftolin et al., 1975). It seems paradoxical in the light of these early studies that, in the teleost, as in other non-mammals, aromatase is found outside the 'reproductive brain', in pallial, midbrain, and hindbrain areas which have little or no labelling in autoradiograms. Nevertheless, retention of authentic estrogen in discrete brain regions is consistent with their enzyme levels (Callard et al., 1981a; Fig. 1).

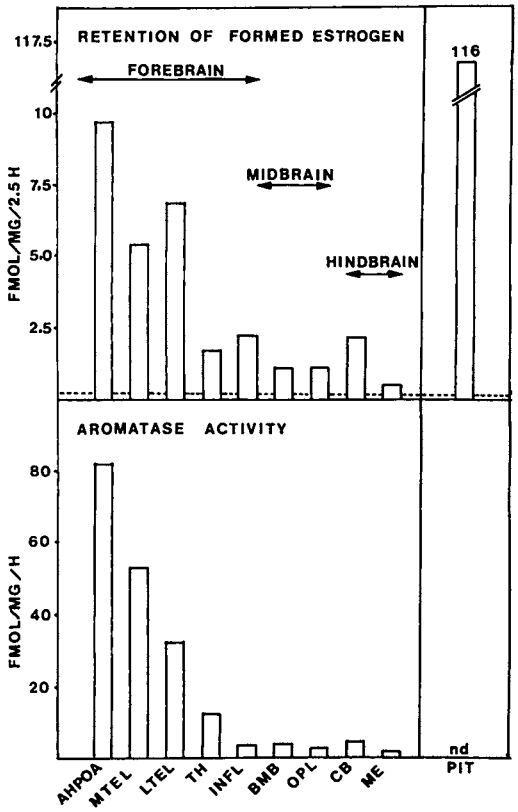


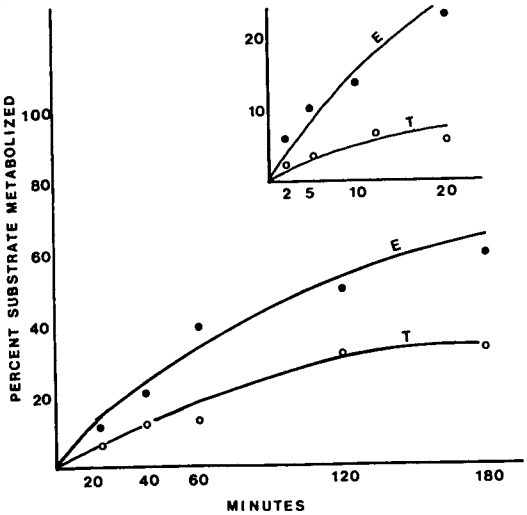
Figure 1. Comparison of aromatase activity as determined by homogenate assay (lower panel) and estrogen retention in the same regions after perfusion with ^{3}H-androgen (upper panel) (Myoxocephalus).

These results imply that estrogen formation in situ (aromatization) may be an alternative to conventional steroid receptors for achieving high local concentrations of active hormone (estrogen). Such a mechanism might be essential where estrogen exerts effects via non-genomic mechanisms (e.g. short-latency electrophysiological changes). Even in regions with conventional steroid receptors, responses may depend on local hormone concentrations in excess of those in plasma. In the sculpin, gonadal aromatase is low or undetectable. By contrast, teleost gonads are known to synthesize large amounts of androgen from C-21 precursors. Thus, the gonad of the fish may serve only as a source of aromatizable pro-hormone.

A final consideration is whether circulating estrogen is present in a form which has access to target sites within the brain. Shortly after birth in rodents, plasma estrogen is extensively bound to α-fetoprotein, although androgens are poor ligands for the same binder. Coincidentally, brain aromatase activity is relatively high. Presumably, estrogen that occupies receptor sites in the brain at this stage of development is derived solely from local synthesis (McEwen et al., 1979). We found recently an analogous system in sculpin. In order to test the distribution of circulating estrogen, the isolated teleost

head was perfused with ^{3}H-estradiol (Callard & Claiborne, in preparation). In contrast to results with androgen, no free estrogen was recovered from brain although small amounts of labeled steroid in polar fractions of the tissue extracts were judged to be estrogen conjugates. A large fraction of radioactivity in the spent perfusate was also found in the conjugate fraction. In order to identify the site of estrogen metabolism, tissues sampled from the sculpin head and trunk were incubated with radiolabeled substrate (Callard and Manz, in preparation). Activity could be ranked as follows: gill>> liver=kidney=gut>brain=ovary>muscle. Conversion in gill filaments was so extensive that 3 h after addition of substrate only 40% of the added radioactivity was ether-extractable (Fig. 2).

Figure 2. Conversion of ^{3}H-substrate (4 nM) to polar metabolites by gill filaments. Minced tissues (≈50 mg) were incubated at 22° for the time indicated. Steroids were separated into 'free' and 'polar' fractions by ether extraction.



Moreover, estrogen was metabolized twice as efficiently as testosterone. Mild acid hydrolysis (pH 5.0, 37° x 24 h) with or without Helix pomatia digestive juice (sulfatase/glucuronidase) indicated that a portion of the estrogen metabolites were sulfoconjugates; glucuronidase (sulfatase-free) was ineffective. In the sculpin, therefore, the gill reduces free estrogen in the circulation and, in this respect, resembles the mammalian kidney. Whether or not the gill also excretes conjugated steroids is not known. If so, conjugated steroids recently identified as teleost pheromones may enter the environment via this route. From experiments in Necturus testis, we know that conjugated estrogens are not acceptable ligands for receptor binding (Mak et al., submitted). Hence, in this teleost, only estrogen synthesized in close proximity to central targets is biologically active. An intriguing question is whether high brain aromatase levels in the sculpin are a cause or a consequence of estrogen inactivating pathways in peripheral tissues.

Pituitary

Until recently, pituitary tissues were believed to be aromatase-negative (Naftolin et al., 1975; McEwen et al., 1979). Using the same homogenate assay as for brain, no detectable estrogen was formed by the gland of rats, rabbits, monkeys, or the human fetus. Furthermore, following administration of ^{3}H-androgen, no estrogen was found in the hyophysis of rats and monkeys although brain regions known to have aromatase were positive. By contrast, when the isolated sculpin head was perfused with androgen, large amounts of authentic estrogen were recovered from the pituitary (Callard et al., 1981a; Fig. 1). We postulated that this estrogen was derived from the brain, since, in teleosts, the anterior and posterior lobes are not readily separable and both receive direct neural input from the hyopthalamus. To test this idea, the brain/pituitary complex was cultured intact; alternatively, the stalk was sectioned and the two tissues cultured separately (Callard et al., 1981b). Following addition of ^{3}H-androgen, radiolabeled estrogen products were isolated and identified. Not only was the pituitary capable of aromatizing androgen independently of the brain, but activity was even greater per unit weight than in adjacent preoptic/hypothalamic regions known to have high aromatase (3700 vs. 600 fmol/mg tissue, respectively). In a subsequent experiment, the pituitary was sectioned transversely into cephalic and caudal halves. In all cultures, the caudal half synthesized far more estrogen than the matched anterior half (2:1 to 8:1) (Callard et al., in preparation). Although pituitary cytology has not been examined in this fish, the data indicate that aromatase may be restricted to certain secretory cell types. Using similar explant culture techniques, we found that aromatase is not unique to the sculpin pituitary but can also be identified in other teleosts (see above; Callard & Reinboth, in preparation), in avian and reptilian adenohyophysis, and in transformed pituitary cell strains derived from rodents (Callard et al., submitted). In the latter studies, activity was present in somato/lactotropes but not in corticotropes or presumptive gonadotropes, supporting the idea that aromatase within the gland is not ubiquitous.

There is good evidence that estrogens or aromatizable androgens acting directly on the teleost pituitary have positive feedback effects. Moreover, an aromatase inhibitor (ATD) can block responses to aromatizable androgen (Crim et al., 1981). Although there is no biochemical evidence for estrogen receptors, the pituitary is heavily labeled in autoradio-

grams following treatment with ^{3}H-estradiol (Davis et al., 1977). This tissue, therefore, seemed to be advantageous for studying directly the relationship between estrogen synthesis, binding and translocation of estrogen formed *in situ*, and a quantifiable biological response. An *in vitro* translocation system was developed (Callard & Manz, in preparation; Table 1).

Table 1. Inhibition of uptake of ^{3}H-estradiol by pituitary cell nuclei (*Myoxocephalus*) (Callard & Manz, in preparation).

Radioinert competitors (200-fold excess)	% inhibition
Estradiol-17β	90.5
Progesterone	0
Testosterone	78.7
5α-dihydrotestosterone	54.4
ATD	0
ATD + testosterone	38.4

The quantity of ^{3}H-estradiol bound to cell nuclei from the teleost gland closely resembled that reported for mammalian pituitary *in vitro* (4.9 vs 7.8 fmol/10mg tissue, respectively) (Leavitt et al., 1969). Estradiol but not progesterone competed for nuclear binding sites, a good index of receptor specificity. Total displacement of radiolabeled estrogen was also obtained with a 200-fold excess of an aromatizable androgen; however, the non-aromatizable androgen, DHT, was partially effective. Since DHT and especially its 3β-reduced metabolite are known to bind to mammalian estrogen receptors, we also tested the effects of testosterone in the presence of an aromatase inhibitor. In amounts equimolar with substrate, ATD reduces estrogen yields by 50% (Callard et al., 1980b). Although ATD alone did not bind, it blocked the ability of testosterone to compete with radiolabeled ligand. Subsequent experiments showed that estrogen binding was slightly greater in the cephalic half of the gland; however, despite lower levels of aromatase in this region, testosterone completely displaced ^{3}H-estrogen in both anterior and posterior portions. Similar methods applied to brain failed to detect specific binding. Relative to pituitary, binding in brain is generally low. We conclude that this is a useful system for further examining quantitative relations between aromatization and the biological expression of steroid action.

Conclusions

These and other recent developments in endocrinology indicate clearly that Starling's definition of a hormone is not strictly valid. Not always is the active molecule present in the blood. In the case of the brain, pituitary, and many other androgen targets, conversion of circulating pro-hormone to one or more active metabolites at its site of action may be required. This added step in the events leading to biological activation provides an opportunity for regulation and serves, therefore, as a 'fine-tuning' mechanism for adjusting responses. At the same time it is a site of potential defects.

References

Callard, G.V., Z. Petro, & K.J. Ryan, 1978. Conversion of androgen to estrogen and other steroids in the vertebrate brain. Amer. Zool. 18:511-523.

Callard, G.V. 1980. Aromatization is cyclic-AMP dependent in cultured brain cells. Brain Research 204:461-464.

Callard, G.V., Z. Petro, & K.J. Ryan, 1980a. Aromatization and 5α-reduction in brain and non-neural tissues of a cyclostome, *Petromyzon marinus*. Gen. Comp. Endocrinol. 42:155-159.

Callard, G.V., Z. Petro, & K.J. Ryan, 1980b. Aromatization of androgen to estrogen by cultured turtle brain cells. Brain Research 202:117-130.

Callard, G.V., Z. Petro, K.J. Ryan, & J.B. Claiborne, 1981a. Estrogen synthesis *in vitro* and *in vivo* in the brain of a marine teleost (*Myoxocephalus*). Gen. Comp. Endocrinol. 43: 243-255.

Callard, G.V., Z. Petro, & K.J. Ryan, 1981b. Biochemical evidence for aromatization of androgen to estrogen in the pituitary. Gen. Comp. Endocrinol. 44:359-364.

Crim, L.W., R.E. Peter, & R. Billard, 1981. Onset of gonadotropic hormone accumulation in the immature trout pituitary gland in response to estrogen or aromatizable androgen. Gen. Comp. Endocrinol. 44:374-381.

Davis, R.E., J.I. Morrell, & D.W. Pfaff, 1977. Autoradiographic localization of sex-steroid concentrating cells in the brain of the teleost *Macropodus operculatis*. Gen. Comp. Endocrinol. 33:496-505.

Leavitt, W.W., J.P. Friend, & J.A. Robinson 1969. Estradiol-specific binding by pituitary nuclear fraction *in vitro*. Science 165:496-498.

McEwen, B.S., P.G. Davis, B. Parsons, & D.W. Pfaff, 1979. The brain as a target for steroid hormone action. Ann. Rev. Neurosci. 2:65-112.

Naftolin, F., K.J. Ryan, I.J. Davies, V.V. Reddy, F. Flores, Z. Petro, R.J. White, Y. Takaoka, & L. Wolin, 1975. The formation of estrogens by central neuroendocrine tissues. Rec. Prog. Horm. Res. 31:295-319.

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GONADOTROPIC CELLS IN THE PITUITARY OF TELEOSTS

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Summary

Immunocytochemical and histophysiological evidence points to globular basophils as the source of a glycoprotein gonadotropic hormone (GTH). This cell type may be situated in various parts of the adenohypophysis, but in many species is concentrated in the ventral region of the proximal pars distalis (PPD). It has irregular cisternae of the granular endoplasmic reticulum (GER), secretory granules of varying electron density, 200-500 nm in diameter, and larger, less electron dense globules. Among the contents of granules and globules are material reacting with anti-(β)-teleost-GTH, and acid phosphatase. Extrusion of GTH is accompanied by a loss of granules and globules and an increase in number and size of the GER cisternae. Cisternal cells and a variety of other cell types have been described as a separate type of gonadotrop. However, it seems that even if two morphological types of gonadotrops can be distinguished, both may be able to produce maturational as well as vitellogenic GTH. The physiological significance of secretory globules apart from granules is unknown. The production and extrusion of GTH is regulated by peripheral and central factors. Among the central stimuli are various types of neurosecretory fibres that in many teleosts penetrate the adenohypophysis and directly innervate glandular cells, including gonadotrops.
Keywords: gonadotropic cells, immunocytochemistry, histophysiology, neuroendocrine innervation.

Introduction

In most vertebrates, including the teleosts, the pituitary gland takes a central position in the endocrine system (review: Holmes & Ball, 1974). It consists of a neural component or neurohypophysis and a glandular part or adenohypophysis, derived from an ectodermal placode. The adenohypophysis secretes more than six different hormones, each of which is believed to be formed in a separate cell type.

The cell types can be recognized at the light microscopical level by their distribution and the stainability of the secretory granules, and at the electron microscopical level by the size and shape of the secretory granules and the form of the GER. Thus, in teleosts cells secreting GTH are situated in the pars distalis, often in the ventral part of the PPD, where they may form a solid ventral rim of cells, as for example in cyprinodonts. In other cases they are spread throughout the rostral pars distalis (RPD) and the PPD, as for example in salmonids and eels (reviews: van Oordt & Peute, 1982,1983).

Gonadotrops belong to the basophilic cell types. This means that the contents of their granules react with periodic acid and Shiff's reagent (PAS). They also stain with Alcian blue (AB) and other dyes for strongly acid groups. Like other basophils the GTH-cells have irregular, more or less dilated cisternae of the GER, and usually round secretory granules with contents of varying electron density. Among such basophils the gonadotrops are characterized by the relatively large diameter of their granules (200-500 nm), and especially by the presence of much larger, round or irregularly shaped globules, with more electron translucent contents.

Identification of GTH-cells

A modern approach to the question of identifying the gonadotrops is immunocytochemistry. The first to apply this method on teleost material were Mc Keown and van Overbeeke (1971) and Billard et al. (1971). Applying the double antibody fluorescence technique with anti-ovine-LH or anti-carp-GTH as first antibody, reactions were found in two types of basophils of the pars distalis, considered to be the gonadotrops and the thyrotrops. In view of the chemical relation between GTH and thyrotropic hormone (TSH), a reaction of thyrotrops with anti-GTH is no surprise. In that respect, Goos et al. (1976) have warned that when heterologous antibodies are being used, one has to be aware of unspecific reactions and of immunoreactive determinants, present in molecules of chemically related constituants of pituitary cells. As a result, other cell types than gonadotrops may react with anti-GTH.

A good example is the results of Peute et al. (1982) with the African catfish, Clarias lazera. Three cell types cross-reacted with anti-carp-GTH: large, strongly PAS-positive cells with granules and globules, forming the main cell type in the PPD; small, mainly AB-positive cells with fine granules, also in the PPD; and some erythrosinophilic cells in the pars intermedia. An anti-salmon-GTH and an antibody against the β-subunit of

carp-GTH reacted with constituants of the globular basophils only. Obviously, the antiserum against carp-GTH contained more and less specific antigen determinants than the other antibodies.

Other important points in immunocytochemical studies are the techniques of processing of the pituitaries and the dilution of the first antibody, applied in the double immunocytochemical technique. Leunissen et al. (1982) observed that in plastic embedded blocks of glutaraldehyde and paraformaldehyde fixed rainbow trout (Salmo gairdneri) pituitaries, depending on the concentration, anti-carp- and anti-salmon-GTH reacted either with constituants of all cell types, or did not react at all. In cryoultramicrotome sections of the same material only dilutions lower than 1 : 8000 of the two antigonadotropins and an antibody against the β-subunit of carp-GTH at a dilution of 1 : 1000 - 4000 selectively reacted with the contents of the granules and globules of the presumed gonadotrops.

These examples may suffice to show that immunocytochemical techniques need to be perfected, and one improvement might be the introduction of monoclonal antibodies to absolutely pure hormones. Even so, these techniques will never suffice to identify the cellular source of pituitary hormones, but will always have to be combined with histophysiological research. In fact, such a combination is general practice. For example, the work by Leunissen et al. (op.cit.) forms part of a series of studies on the gonadotropic cells in the rainbow trout, carried out by a group of comparative endocrinologists of the Utrecht University.

These studies include the work by van den Hurk (1982) on the development of the gonadotrops. In trout larvae of 45-100 days old, basophils can be found in the dorsal and in the ventral PPD. Both show an immunocytochemical reaction with anti-salmon- and anti-carp-GTH, but only the dorsal ones react with anti-human-β-TSH. So, it seems that the dorsal basophils are thyrotrops and the ventral cells gonadotrops. This is in accordance with the fact that the dorsally situated cells were seen to differentiate first, together with the thyroid follicles, and the ventral basophils somewhat later, along with the gonads. In addition, the development of the ventral cells can be enhanced by exogenous androgens. Gielen et al. (unpublished results) have shown that androgens act on the pituitary even after its heterotopic transplantation, and that this direct action leads to the production and storage of granules and globules. The release of GTH is minimal. Removal of the androgens results in a loss of secretory vesicles and an apparent dedifferentiation of the cells.

Maturation of the gonadotrops can also be obtained by injecting GTH, which stimulates the development of the gonads and in this way enhances storage of secretory material in the gonadotropic cells. (Gielen et al., 1982). Reciprocally, according to van Putten et al. (1981), removal of the gonads in adult rainbow trout leads to a strong extrusion of GTH and to a loss of the globules and most of the granules. These secretory vesicles are replaced by an increasing number of dilated cisternae of the GER. In extreme cases the cisternae coalesce to form a big vacuole. Similar changes have been noticed during the annual reproductive cycle by Peute et al. (1978), and strengthen the opinion that in the rainbow trout globular and cisternal basophils belong to one and the same type of gonadotropic cell. A second type of presumed gonadotrop could not be found. It is true that in the PPD of normal and gonadectomized rainbow trout van Putten et al. (1981) observed basophils differing from the globular cells by the absence of globules, and from the cisternal cells by the presence of numerous granules and by the small dimensions of the GER cisternae. This somewhat intermediate form tended to be concentrated in the rostro-dorsal PPD. In juvenile trout van Putten et al. (unpublished results) could stimulate these cells with goitrogens, and observed an immunocytochemical reaction within the secretory granules with anti-human-β-TSH. So, it seems that these cells are thyrotrops, not a second type of gonadotrop.

One or two types of GTH-cells

That does not mean that in the pituitary of the rainbow trout such a second type of gonadotrop does not exist. Olivereau (1978) and Olivereau and Nagahama (1982) described two types in pituitaries of mature trout. One is comparable to the globular type of gonadotrop, referred to by the members of the Utrecht group. It is abundant in the PPD, can be found in the RPD, and has secretory granules with PAS- and AB-positive contents that cross-react with anti-salmon-GTH. The other one does not react with the anti-GTH, has almost completely PAS- and AB-negative contents, and is mainly restricted to the dorsal glandular strands of the PPD. Cells of this second type predominated during vitellogenesis, appeared active mainly in spring, and showed hyperplasia and hypertrophy in three years old females with immature gonads.

This puts us in the middle of the controversy of one or two types of gonadotrops in the teleost pituitary. There is no problem about one of these cell types, which in most of the species, studied so far, seems to resemble the globular basophils of the rainbow trout. The difficulty comes in with the second type, and the reasons for that are first of all the fact that many authors did not succeed in finding a second type of GTH-cell, and secondly the lack of uniformity in

the description of such a cell type. For example: in some publications on the pituitary of salmonids the globular and cisternal gonadotrops are considered to represent two functionally different types of gonadotropic cells (e.g. Cook & van Overbeeke, 1972; Ueda & Hirashima, 1979; Ueda, 1980); in Sarotherodon mossambicus basophils in the dorsal PPD with relatively small secretory granules have been coined as the second gonadotropic cell type (Bern et al., 1974); in the stickleback (Gasterosteus aculeatus) cells with a relatively electron dense cytoplasm, situated among the ventral basophils in the PPD (Slijkhuis, 1978), and in the channel catfish (Ictalurus punctatus; Massoud et al., 1980) and the platyfish (Xiphophorus maculatus; Schreibman & Margolis-Kazan, 1979) cells in the pars intermedia have been described as the second type of gonadotropic cell.

Matters have become even more complicated by the recent results of Burton et al., (1981). The authors described two morphologically different types of basophils in the pars distalis of the winter flounder (Pseudopleuronectes americanus). During the spawning season both types showed immunofluorescence when treated with antibodies against flounder maturational GTH and flounder glycoprotein-TSH. However, one type reacted with anti-flounder vitellogenic GTH during the vitellogenic period only, whereas the other reacted with this antibody throughout the reproductive cycle. Thus, for the basophils in the pars distalis of the winter flounder the traditional axiom of "one morphological cell type, one hormone" is no longer applicable, and the physiological differences between the two types seems to change with the time of year. It may be good to keep this in mind when trying to elicit the number of gonadotropic cell types in other teleosts, and it may well be that the present controversy is largely due to differences in the definition of the concept "cell type".

Function of granules and globules

It has been argued that if teleost gonadotrops produce two hormones at the same time, the one may be stored in the granules and the other in the globules (Boddingius, 1975; Chinese scientists, 1978). However, for several species, including the rainbow trout (van Putten et al., 1981), intermediate stages between granules and globules have been described, and the presence of glycoprotein gonadotropin has been demonstrated in both types of secretory vesicles (Leunissen et al., 1982). This does not preclude the presence of a second GTH in the granules, the globules or both. At any rate, the proteolytic enzyme acid phosphatase has been demonstrated in the granules and to a lesser extent in the globules of the gonadotrops in the rainbow trout (van Oordt, 1979), suggesting the cleavage of a prohormone and the formation of one or more physiologically active polypeptides. It should, however, be kept in mind that granules and globules are not indispensable for gonadotropin secretion, for strongly activated gonadotrops continue to secrete GTH after the secretory vesicles have disappeared from the cytoplasm (e.g. van Putten et al., 1981). It seems that we will have to wait for a better understanding of the mode of GTH production before we will be able to elucidate the significance of the presence of two different types of secretory vesicles, i.e. granules and globules in the GTH-cells of teleosts and other vertebrates.

Innervation of GTH-cells

The production, storage and extrusion of GTH fluctuates during the annual reproductive cycle and depends on stimuli of central and peripheral origin. In bony fishes, as in almost all other vertebrates these stimuli may reach the GTH-cells via the circulatory system. However, in many teleosts neurosecretory stimuli may also pass along nerve fibres piercing the laminae that separate the neuro- from the adenohypophysis, and penetrating into the endocrine parenchyma of the pars distalis. (Reviews: Ball, 1981; van Oordt & Peute, 1983). These fibres directly contact the hormone producing cells, including the gonadotrops, and may even terminate with synapses on these cells. The majority of fibres near the gonadotrops and making synaptoid contact with the cells have so-called large granulated vesicles of 60-100 nm (type B). Others, seldom making synaptoid contact, have elimentary granules of 100-200 nm in diameter (type A). This double innervation, however, is not necessarily the same for all teleosts and for all cells at all times of the reproductive cycle. In eels (Knowles & Vollrath, 1966), the tench (Tinca tinca; Vollrath, 1967) and in salmonids (Fridberg & Ekengren, 1977) all neurosecretory fibres end in the neurohypophysis; in the roach (Rutilus rutilus; Ekengren et al., 1978) neuroendocrine type B fibres innervate gonadotrops adjacent to the neurohypophysis, but do not penetrate deeper layers of the pars distalis (Bøge et al., 1974), leaving the majority of the GTH-cells without neuroglandular connections. Moreover, synaptoid contacts were observed with vesicular, not with globular gonadotrops. Very significantly, Abraham (1974) suggested that when in the mullet (Mugil cephalus) type A fibres were not observed between the gonadotrops, this might be due to the fact that the fish were not caught during the spawning season. In like manner, Kaul and Vollrath (1974) described a gradual decrease in the amount of granules in type A and B fibres innervating GTH-cells in the goldfish (Carassius

auratus) during and shortly after the nuptial period, as well as an increase in the amount of granules in the type B fibres that innervate the gonadotrops, following an oestradiol treatment.

Thus it seems that a direct innervation is not indispensable for the neuroendocrine regulation of the gonadotropic cells, and that when such a direct innervation is present, it changes with the physiological condition of the fish. This is not the place to discuss the origin of the various types of neurosecretory fibres innervating the GTH-cells and the nature of the releasing or inhibiting substances that each of them produces (review: Peter, this volume), but it may be good to point to the direct innervation as providing an extra morphological tool in the study of the central control of the GTH-cells in many teleosts.

References

Abraham, M., 1974. Ultrastructure of the cell types and the neurosecretory innervation in the pituitary of Mugil cephalus L. from fresh water, the sea and hypersaline lagoon. II. The proximal pars distalis. Gen. Comp. Endocrinol. 24:121-132.

Båge, G., B. Ekengren, B. Fernholm & G. Fridberg, 1974. The pituitary gland of the roach, Leuciscus rutilus. II. The proximal pars distalis and its innervation. Acta Zool. (Stockh.). 55:191-204.

Ball, J.N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians and reptiles. Gen. Comp. Endocrinol. 44: 135-170.

Bern, H.A., R.S. Nishioka & Y. Nagahama, 1974. The relationship between nerve fibres and adenohypophysial cell types in the cichlid teleost Tilapia mossambica. Recherch. Biol. Contemp. Manfred Gabe Memorial Vol. p.179-194.

Billard, R., B. Breton & M.P. Dubois, 1971. Immunocytologie et histochimie des cellules gonadotropes et thyréotropes hypophysaires chez la carpe, Cyprinus carpio. C. R. Acad. Sc. Paris Série D 272:981-983.

Boddingius, J., 1975. The cell types of the adenohypophysis in the rainbow trout (Salmo irideus). A histological study. Holywell Press Ltd., Oxford.

Burton, M.P., D.R. Idler & T.B. Ng, 1981. The immunofluorescent localization of teleost gonadotropins and thyrotropins in flounder pituitary. Gen. Comp. Endocrinol. 43:135-147.

Chinese scientists, 1978. The role of LH-RH in induction of spawning in the grass carp (Ctenopharyngodon idellus). II. Ultrastructural study of pituitary gonadotrops. Scientia sinica 21:397-400.

Cook, H. & A.P. van Overbeeke, 1972. Ultrastructure of the pituitary gland (pars distalis) in sockey salmon (Oncorhynchus nerka) during gonad maturation. Z. Zellforsch. 130:338-350.

Ekengren, B., K. Lindahl & G. Fridberg, 1978. Immunocytology and innervation of the gonadotropic cells in the teleost fish Rutilus rutilus. Acta Zool. (Stockh.) 59:125-133.

Fridberg, G. & B. Ekengren, 1977. The vascularization and the neuroendocrine pathways of the pituitary gland of the Atlantic salmon, Salmo salar. Can. J. Zool. 55:1284-1296.

Gielen, J.Th., H.J.Th. Goos, J. Peute, R.A. van den Bosch & P.G.W.J. van Oordt, 1982. The brain-pituitary-gonad axis in the rainbow trout, Salmo gairdneri: gonadal hormones and the maturation of gonadotropic cells. Cell Tissue Res., in press.

Goos, H.J.Th., R. Seldenrijk & J. Peute, 1976. The gonadotropic cells in the pituitary of the black molly, Mollienisia latipinna, and other teleosts, identified by the immunofluorescence technique in normal and androgen-treated animals. Cell Tissue Res. 167:211-219.

Holmes, R.L. & J.N. Ball, 1974. The pituitary gland. A comparative account. Cambridge Univ. Press, London.

van den Hurk, R., 1982. Effects of steroids on gonadotropic (GTH)cells in the pituitary of rainbow trout, Salmo gairdneri, shortly after hatching. Cell Tissue Res. 224:361-368.

Kaul, S. & L. Vollrath, 1974. The goldfish pituitary. II. Innervation. Cell Tissue Res. 154:231-249.

Knowles, F. & L. Vollrath, 1966. Neurosecretory innervations of the pituitary of the eels Anguilla and Conger. II. The structure and innervation of the pars distalis at different stages of the life-cycle. Phil. Trans. Roy. Soc. London B250:329-342.

Leunissen, J.L.M., A.M. de Leeuw, J. Peute & H.J.Th. Goos, 1982. Immunocytochemistry of gonadotropic cells and identification of cell types in ultrathin cryosections of the pituitary of the rainbow trout, Salmo gairdneri. Cell Tissue Res., in press.

Massoud, A.A., B.A. Simio & K.B. Davis, 1980. Two gonadotropic cell-types in the pituitary of the channel catfish. Amer. Zool. 20:729.

McKeown, B.A. & A.P. van Overbeeke, 1971. Immunohistochemical identification of pituitary hormone producing cells in the sokeye salmon (Oncorhynchus nerka, Walbaum). Z. Zellforsch. 112:350-362.

Olivereau, M., 1978. Les cellules gonadotropes chez les salmonides. Ann. Biol. anim. Bioch. Biophys. 18:793-798.

Olivereau, M. & Y. Nagahama, 1982. Immunocytological study of eel and trout gonadotrophs. Gen. Comp. Endocrinol. 46:365-366.

van Oordt, P.G.W.J., 1979. A typology of the gnathostome adenohypophysis with some emphasis on its gonadotropic function. Basic Appl. Histochem. 23:187-202.

van Oordt, P.G.W.J. & J. Peute, 1982. Gonado-

tropic cells in the vertebrate pituitary. Proc. Ninth Intern. Symp. Comp. Endocrinol. Hong Kong, in press.
van Oordt, P.G.W.J. & J. Peute, 1983. The cellular origin of pituitary gonadotropins in teleosts. In: W.S. Hoar, D.J. Randall & E.M. Donaldson (Ed.): Fish Physiology, vol. IX. Reproduction. Academic Press, Inc., New York, in press.
Peute, J., H.J.Th. Goos, M.G.A. de Bruyn & P.G.W.J. van Oordt, 1978. Gonadotropic cells of the rainbow trout pituitary during the annual cycle. Ultrastructure and hormone content. Ann. Biol. anim. Bioch. Biophys. 18:905-910.
Peute, J., C. de Zoeten-Kamp & P.G.W.J. van Oordt, 1982. Immunocytochemical identification of GTH- and TSH-cells in the pituitary of the African catfish, Clarias lazera. Gen. Comp. Endocrinol. 46:366.
van Putten, L.J.A., J. Peute, P.G.W.J. van Oordt, H.J.Th. Goos & B. Breton, 1981. Glycoprotein gonadotropin in the plasma and its cellular origin in the adenohypophysis of sham-operated and ovariectomized rainbow trout, Salmo gairdneri. Cell Tissue Res. 218:439-448.
Schreibman, M.P. & H. Margolis-Kazan, 1979. The immunocytochemical localization of gonadotropin, its subunits, and thyrotropin in the teleost, Xiphophorus maculatus. Gen. Comp. Endocrinol. 39:467-474.
Slijkhuis, H., 1978. Ultrastructural evidence for two types of gonadotropic cells in the pituitary gland of the male threespined stickleback, Gasterosteus aculeatus. Gen. Comp. Endocrinol. 36:639-641.
Ueda, H., 1980. Changes of two types of pituitary gonadotrophs in white-spotted char, Salvelinus leucomaenis, during gonadal development. Bull. Fac. Fish., Hokkaido Univ. 31:1-15.
Ueda, H. & T. Hirashima, 1979. On two different types of putative gonadotrophs in the pituitary gland of the masu salmon, Oncorhynchus masou. Anot. Zool. Jap. 52:114-124.
Vollrath, L., 1967. Über die neurosekretorische Innervation der Adenohypophyse von Teleostiern, insbesondere Hippocampus cuda und Tinca tinca. Z. Zellforsch. 78:234-260.

HORMONAL CONTROL OF THE REPRODUCTIVE FUNCTION OF STURGEONS (CHONDROSTEI)

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Summary

Peculiarities of the hormonal regulation of gonads function of Chondrostei at different stages of the reproductive cycle in nature and at fish farms were studied.

Radioimmunoassay of gonadotropin (GTH), sex steroids and cortisol in blood serum and histological and ultrastructural analysis of pituitary and gonads were used.

In the sea period at the beginning of vitellogenesis the activation of gonadotropic (GTH) cells, the increase of pituitary GTH as well as elevation of 17β-estradiol and cortisol levels in the blood of stellate sturgeon were observed. The content of GTH and sex steroids at the beginning of the anadromous migration from sea to river had mean values, GTH cells rich in secretory granulations predominated in the pituitary.

Closer to the spawning period the number of devasted GTH cells increased. At the begining of spawning GTH is released from the pituitary, its concentration in blood sharply rising. After spawning exhausted cisternal cells predominated in the pituitary, many cells were destroyed, the content of GTH in the pituitary and in blood as well as sex steroids and cortisol levels were low both in male and female.

Injections of either different hypophyseal preparations or Aci-GTH at fish farms caused the rise of blood GTH. The highest level was observed 4-8 hours after administration, depending on the preparation being used. At the time of ovulation this level slightly decreased, but was still significantly higher than in the same fish before injection.

Intramuscular administration of synthetic LH-RH was shown to activate GTH cells and to increase GTH concentration in blood of female stellate sturgeon 30 minutes after injection. In one hour a drop of blood GTH occurred, which was followed by a more significant rise lasting 4-15 hours. Ovulation was obtained, which is retained 5-6 hours in comparison to ovulation induced by GTH.

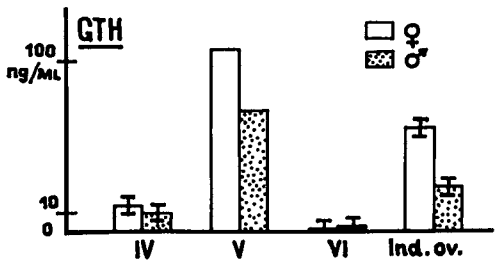

Fig. 1. GTH levels in the blood serum of Acipenser stellatus Pallas on various stages of the sexual cycle.
IY-before spawning, Y-spawning, YI-after spawning, Ind.ov.-induced ovulation.

LONG-TERM CHANGES IN PLASMA AND PITUITARY GTH AFTER CASTRATION OF RAINBOW TROUT AT AN IMMATURE STAGE

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In order to study hypothalamus-pituitary-gonad relationships during the establishment of puberty in rainbow trout, juvenile trout were castrated at an immature stage, and the GTH in the plasma and the pituitary was examined one year later when the controls were in full gametogenesis.

Material and methods

Six-month old male and female rainbow trout, weighing about 20 g, were castrated or sham-operated in June. We castrated the fish under binocular, totally castrating some and partially castrating others, leaving a very small part of the anterior part of the gonad. The fish were then stored in a raceway at the Gournay experimental trout farm. They were killed one year later. After the blood was sampled the plasma, obtained by centrifugation at 4°C, and the pituitaries, were frozen at -20°C until GTH (maturational) determination by RIA. The body and the gonads were weighed and a part of the gonad (when present) was fixed for histological examination.

Results (see Table)

Only one male in the sham-operated group was immature; all the others were in full spermatogenetic activity, showing some spermatozoa in the lobules. All the partially castrated males were in full spermatogenesis, the testis having reached the same size as in the sham-operated group (the GSI was similar), although its initial size had been strongly reduced by surgery. There was no regeneration of the sperm duct and the testis was entirely located in the anterior part of the body cavity. Body weight, although slightly lower in the castrated males, was not significantly different. Pituitary GTH was not significantly different between castrated and shams but plasma GTH, much higher ($P < 0.001$) in the completely castrated males than in the sham-operated group, was also higher in the partially castrated fish ($P < 0.05$). In all sham-operated females vitellogenesis had started. Some of the partially castrated ones were still immature while others showed signs of vitellogenesis. As compared to the sham-operated fish, the castrated had a higher plasma GTH level ($P < 0.001$) but the partial castrates did not. There was a significant difference ($P < 0.05$) in the pituitary GTH content between shams and castrates.

The results show that i) plasma GTH increases at the normal time of puberty in fish castrated at an immature stage. GTH level is higher than in the sham castrated in full gametogenesis suggesting a negative feed-back from the gonads; ii) the pituitary GTH is similar in the castrates as in shams at least for the males indicating that pituitary can build GTH in the absence of gonads. The pituitary gland can thus function normally (synthesis and release of GTH) without the gonads. This would suggest that, as in the higher vertebrates, puberty is controlled by the central nervous system (CNS). However, the plasma and pituitary GTH level and the response to castration are not as high as in older fish suggesting that this control of the CNS on the pituitary is not yet fully established. Finally these fish exhibited a considerable compensatory hypertrophic activity in their gonadal development. This demonstrates the ability of a few germ cells to divide actively and reinitiate a normal gametogenesis.

Table. GTH response to full or partial castration in juvenile rainbow trout. Values ± SD

Sex	Treatment	n	Body weight	GSI I	Plasma GTH ng/ml	Pitui. GTH ng/pit.
♂	Sham castration	10	673±160	1.14±1.1	3.79±2.61	18.86*11.6
	Full castration	6	574±112	——	16.46*** ±5.11	20.45±11.61
	Partial castration	13	682±143	1.39±0.89	5.93* ±2.34	——
♀	Sham castration	11	594±70	0.51±0.15	6.96±3.25	20.82±6.12
	Full castration	6	570±79	——	13.65*** ±2.16	12.35 ±6.28*
	Partial castration:					
	immature ♀	5	659±107	0.02*** ±0.01	9.67±4.69	15.15±6.54
	♀ in exog.vitell.	4	639±96	0.26* ±0.12	10.87±3.01	——

*P < 0.05; ***P < 0.001 compared to sham castrates by variance analysis

ACTIONS OF DOPAMINE ON GONADOTROPIN RELEASE IN GOLDFISH, *CARASSIUS AURATUS*.

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Intraperitoneal injection (ip) of drugs capable of blocking specific enzymes of catecholamine synthesis suggest that in goldfish, dopamine (DA) inhibits gonadotropin (GtH) release (Chang *et al.*, 1982). Further experiments demonstrated that ip injections of DA or its agonist, apomorphine (APO), decreased and pimozide (PIM), a DA antagonist, increased serum GtH levels in intact female goldfish. In the present experiments, the mechanism of DA inhibition of GtH release in female goldfish, acclimated to 16L:8D and 10–12°C, was further studied. In these experiments, DA, APO, and PIM, was dissolved or suspended in a vehicle of 0.7% NaCl with 0.1% $Na_2S_2O_5$.

A GtH release–inhibitory factor (GRIF) is present in the goldfish, and its influence can be abolished by lesions in the preoptic region, resulting in a prolonged massive spontaneous release of GtH (Peter & Paulencu, 1980; Peter, 1982). Serum GtH concentrations in preoptic lesioned goldfish remained elevated (200 to 400 ng/ml) for several days post–lesion compared to levels in sham operated animals (5 to 15 ng/ml). Compared to vehicle injected controls, ip injections of DA (10 & 100 ug/g) in preoptic lesioned fish on day 2 post–lesion significantly depressed the elevated serum GtH levels at 2 hours (h) post–injection. Both doses of DA reduced the elevated serum GtH levels by 40 to 50% ($P<0.02$). Similarly, ip injections of APO (20 ug/g) on day 1 post–lesion significantly reduced the lesion induced increase in serum GtH levels by 24 h post–injection. These results indicate that DA can act as a GRIF to inhibit spontaneous GtH release. However, intracranial (3rd ventricle) injections of DA did not alter serum GtH levels. This suggests that DA acts directly on the gonadotrophs to block the spontaneous GtH secretion.

Des Gly^{10}, [D–Ala^6] LHRH ethylamide (LHRH–A), dissolved in fish saline (PS), was effective in stimulating GtH release in goldfish when injected as two ip injections (0.1 ug/g), 12 h apart. The 1st LHRH–A injection is thought to potentiate the action of the 2nd injection (Peter, 1982). Ip injections of DA (10 & 100 ug/g) at 4 h after the 2nd LHRH–A injection significantly reduced the elevated serum GtH levels by 2 h after DA injection. The LHRH–A induced increase in serum GtH levels was also abolished when APO (10 & 20 ug/g) was injected simultaneously with the two LHRH–A injections. Administration of APO (20 ug/g, ip) with the 1st, but not the 2nd, LHRH–A injection was effective in abolishing the increase in serum GtH measured at 24 h after the 2nd LHRH–A injection (fig. 1). However, injection of APO with the 1st LHRH–A injection did not alter the LHRH–A induced increase in serum GtH levels measured at the time of the 2nd LHRH–A injection (fig. 1). These results indicate that DA can block the potentiating effects of multiple doses of the GtH releasing hormone (GnRH) on GtH release in goldfish, as well as decrease the GtH release–response.

Two injections of LHRH–A, 12 h apart, stimulated GtH release but did not significantly induce ovulation in gravid (sexually mature) female goldfish. Ip injections of PIM (10 ug/g) at the time of the 2nd LHRH–A injection potentiated the effects of LHRH–A on GtH release and increased the occurrence of induced ovulation (table I).

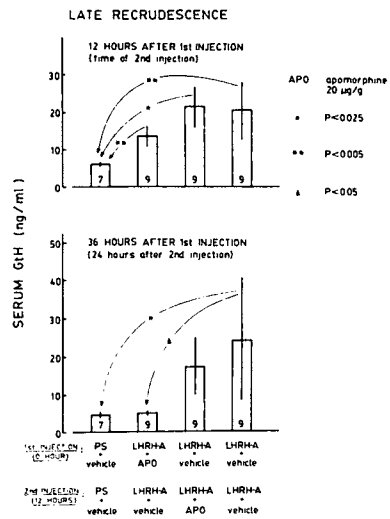

Fig. 1. Effects of APO, injected with the 1st or the 2nd LHRH–A injection, on serum GtH levels in female goldfish.

Table I. Effects of PIM on serum GtH levels and ovulation at times (h) after the 2nd LHRH–A injection. Values that are similar ($P>0.05$) are identified by similar superscripts.

treatment	n	GtH, ng/ml (24 h)	# ovulated (72 h)
PS+vehicle	8	7 ± 1^1	0^1
LHRH–A+PIM	9	294 ± 51^2	8^2
LHRH–A+vehicle	9	66 ± 19^3	2^1

In summary, DA has GRIF activity in the goldfish by actions directly on the gonadotrophs to inhibit the spontaneous release of GtH, and by blocking the actions of GnRH. The removal of the DA inhibition on GtH release may be an integral part of the mechanism regulating the preovulatory GtH surge and ovulation.

References

Chang, J.P., Cook, A.F., and Peter, R.E. 1982. Influences of catecholamines on gonadotropin secretion in goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* (in press).

Peter, R.E. 1982. Neuroendocrine control of reproduction in teleosts. *Can. J. Fish. Aquat. Sci.* 39: 48–55.

Peter, R.E., and Paulencu, C.R. 1980. Involvement of the preoptic region in gonadotropin release–inhibition in goldfish, *Carassius auratus*. *Neuroendocrinology*. 31: 133–141.

PRESENCE ET REPARTITION D'UN FACTEUR DE TYPE LHRH CHEZ L'ANGUILLE : ETUDE PAR DOSAGE RADIOIMMUNOLOGIQUE.

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avec la collaboration technique de N. Delerue-Le Belle et V. Lenoir(*).

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Summary : Eel brain contains a factor which produced a complete cross-reaction in a RIA for mammalian LHRH and which exhibited on Sephadex G25 a behavior similar to that of mammalian LHRH. This LHRH-like immunoreactive factor (IR LHRH) had a wider distribution in the eel brain than LHRH in mammals. Pituitary also contained a large amount of IR LHRH, at a concentration which was higher than in median hypothalamic lobe. Finally, plasma samples gave important cross reactions in the RIA. This RIA makes possible further physiological studies in the eel ; it can already be concluded that the lack of sexual development and the weak gonadotropic function of the eel are not due to a lack of IR LHRH.

Le cycle biologique de l'anguille européenne, *Anguilla anguilla*, se singularise par l'absence de développement sexuel tout au long de sa vie dans les cours d'eau, ceci étant dû à la faible activité de la fonction gonadotrope (Dufour et al., sous presse). Nous abordons ici l'étude du contrôle hypothalamique de la fonction gonadotrope par l'étude de la répartition d'un facteur immunoréactif de type LHRH (IR LHRH), au moyen d'un système de dosage radioimmunologique (RIA) préalablement mis au point pour la LHRH mammalienne (Kerdelhué et al., 1973, 1976).

Les échantillons sont prélevés sur des anguilles femelles de 200 à 350 g, aussitôt après décapitation, et congelés. Les tissus sont extraits dans HCl 1 N et les surnageants, après centrifugation, sont neutralisés et congelés jusqu'au dosage.

Les extraits de cerveau ou d'hypophyse d'anguille montrent une réaction croisée complète dans le RIA. Le cerveau entier contient 920 ± 135 pg IR LHRH dont la majorité (75 %) est présente dans l'ensemble du diencéphale et du mésencéphale, tandis que le télencéphale en contient 21 % et le cerveau postérieur 4 %, à la concentration respective de 25,0 ± 0,2, 7,8 ± 1,6 et 1,1 ± 0,3 pg/mg tissu frais. L'ensemble diencéphale et mésencéphale a été ensuite disséqué en trois parties, les lobes hypothalamiques (très développés chez les poissons), les lobes optiques et la partie intermédiaire (à la fois diencéphalique et mésencéphalique) qui contiennent respectivement 147 ± 10, 128 ± 58 et 350 ± 23 pg IR LHRH aux concentrations de 19 ± 1, 25 ± 2 et 30 ± 1 pg/mg. Quant aux lobes hypothalamiques, la majeure partie de l'IR LHRH est contenue dans le lobe médian (128 ± 20 pg) à une concentration de 34 ± 1 pg/mg, bien supérieure à celle des lobes latéraux (< 7 pg/mg). La présence d'IR LHRH n'a pas été détectée dans le sac vasculaire. L'hypophyse contient une grande quantité d'IR LHRH (304 ± 69 pg) à une concentration (112 ± 26 pg/mg) dépassant même celle du lobe médian de l'hypothalamus.

Dans les autres tissus étudiés, un début de réaction croisée est parfois observée. La concentration en IR LHRH y est < 0,02 pg/mg (moelle distale, foie, ovaire, muscle) ou est proche de cette valeur (tube digestif, peau).

Enfin, la présence d'IR LHRH a pu être détectée dans le plasma à des concentrations importantes (de 40 à plusieurs centaines pg/ml).

Une caractérisation partielle de l'IR LHRH cérébrale a été réalisée par filtration sur Séphadex G25 d'un extrait d'un pool de diencéphales et mésencéphales. L'activité IR LHRH est éluée en un pic de $K_D \simeq 0,76$, semblable à celui déterminé sur la même colonne pour la LHRH mammalienne.

En conclusion, le cerveau d'anguille contient un facteur IR LHRH plus largement réparti que chez les Mammifères, en accord avec les données immuno-histologiques obtenues chez les poissons, résultats suggérant que ce facteur peut avoir d'autres fonctions qu'hypophysiotrope (cf par ex. Münz et al., 1982, Kah et al., 1982). Dans l'hypophyse, l'IR LHRH est présente en quantité importante, ce qui est à rapprocher du fait que, chez les Téléostéens, l'éminence médiane est incorporée à l'hypophyse (revue de Ball, 1981). Le RIA mis en oeuvre permet donc de doser un facteur de type LHRH chez l'anguille et ouvre la voie à des études physiologiques. Il peut déjà être conclu que le manque de développement sexuel et la faible fonction gonadotrope de l'anguille ne peuvent pas être reliés à une absence d'IR LHRH.

Références

Ball, J., 1981. Gen. Comp. Endocrinol., 44, 135-170.

Dufour, S., N. Delerue-Le Belle, Y.A. Fontaine Gen. Comp. Endocrinol., sous presse.

Kah, O., P. Chambolle, P. Dubourg, M.P. Dubois, 1982. Abstract from this Symposium.

Kerdelhué, B., M. Jutisz, D. Gillessen, R. Studer, 1973. Biochem. Biophys. Acta, 297, 540-548.

Kerdelhué, B., S. Catin, C. Kordon, M. Jutisz, 1976. Endocrinology, 98, 1539-1549.

Münz, H., C. Class, W.E. Stumpf, L. Jennes, 1982. Cell. Tissue Res., 222, 313-323.

GONADOTROPIC HORMONE PRODUCTION IN ENZYMATICALLY DISPERSED PITUITARY CELLS OF THE TROUT, SALMO GAIRDNERI

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In order to study in vitro control of the activity of gonadotropic (GTH) cells in rainbow trout, pituitaries of juvenile fishes (ca. 1 yr.) are enzymatically dissociated according to the method of Fåhræus-van Ree et al.. The dispersed cells are kept in a suspension system for 72 hrs. or in primary monolayer cultures under different culture conditions for periods ranging up to 21 days. The cells are cultivated in control medium or in medium with either 17α-methyltestosterone (MT) or estradiol-17β (E_2) or with MT and dopamine (DA) in concentrations of 8.5×10^{-7}M and subsequently incubated for 3 hrs. in control medium or in medium with 8.5×10^{-7}M MT and/or synthetic LH-RH or with MT and/or 8.5×10^{-8}M des-Gly^{10}-|D-Ala^{6}|-LH-RH ethylamide (LH-RHa). GTH cells are identified by Alcian Blue-Periodic Acid Schiff-Orange G staining and double antibody immuno-enzyme cytochemical technique using anti-carpβGTH as first antibody. Percentages of immunoreactive cells among the isolated and cultivated cells are calculated. Secretory activity is estimated by measuring the GTH content in cell extracts and culture media by radioimmunoassay.

In both culture systems GTH cells show an accumulation of GTH (see table) and an increase in number of immunoreactive cells (fig. 2) after treatment with MT, indicating a stimulation of GTH synthesis by this steroid. E_2 seems to have the same effect. A synergetic stimulation of GTH synthesis is caused by MT and DA or by MT and a metabolite of DA. Autonomous GTH release is raised above that of the control medium level by these steroids. The effects of steroids are stronger with longer culture time. Primary monolayer culture gives a better result than the suspension system. Cells cultivated in steroid-enriched medium give a better monolayer (fig. 1) than those cultivated in control medium.

Treatment	72 hrs. suspension culture	
	cell extracts	medium
Untreated	0.20±0.04	0.31±0.02
Control	0.22±0.03	0.16±0.02
MT	0.68±0.03	0.36±0.02
MT + DA	1.09±0.16	0.48±0.03
	96 hrs. monolayer culture	
Untreated	0.56±0.03	0.69±0.12
Control	0.73±0.05	0.77±0.11
MT	3.03±0.64	1.30±0.23

Table of GTH content (AU) ± SD in extracts of untreated and treated cells (0.25×10^6) and culture media (0.5 ml).

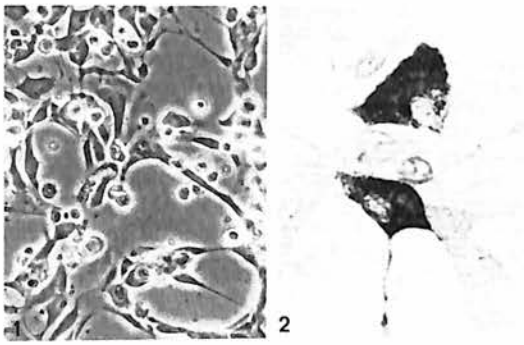

Fig. 1. Phase contrast micrograph of 1 week old primary monolayer culture in MT-enriched medium. X224. Fig. 2. Light micrograph of 3 week old culture in MT-enriched medium showing strong immunostaining of GTH cells with anti-carpβGTH. X560.

Subsequent treatment of the cells with MT for 3 hrs. causes stimulation of GTH synthesis and inhibition of GTH release. Subsequent treatment of the cells 18 hrs. after dissociation with LH-RH or LH-RHa for 3 hrs. does not affect GTH production, but stimulates GTH release into medium after priming of the cells with steroids for 72 hrs. GTH synthesis and release are both stimulated by simultaneous treatment of the cells with MT and hypophysiotropic substances after priming of the cells with steroids for 8 days.

These observations clearly show that the cells shortly after dissociation retain their ability to respond to steroids, but not to hypophysiotropic substances. After 72 hrs. the cells apparently recover and start to respond to LH-RH and its analogue.

Reference

Fåhraeus-van Ree, G.E., S.E.F.Guldenaar & J.Th.Gielen. Fine structure and function of isolated cells as revealed from pituitaries of immature rainbow trout, Salmo gairdneri, by means of a new enzymatic dispersion technique. Cell Tissue Res., in press.

DIRECT ACTION OF GONADAL STEROIDS ON THE MATURATION OF GONADOTROPIC CELLS IN THE RAINBOW TROUT, SALMO GAIRDNERI

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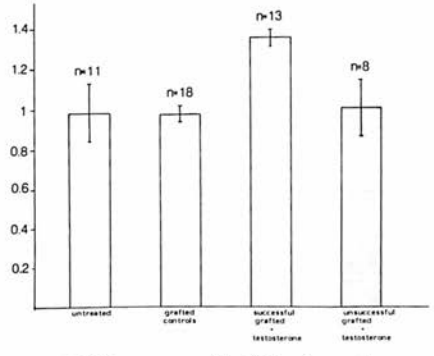
In juvenile trout it has been demonstrated that administration of gonadal steroids, e.g. testosterone leads to an accelerated maturation of the gonadotropic (GTH) cells. These maturational effects comprise synthesis and storage of GTH, accompanied by ultrastructural changes of the GTH-cells such as enlargement of the cell, nucleus and secretory granules, development of the Golgi apparatus and the appearance of globules. Similar signs of GTH-cell activation could also be achieved by injecting gonadotropin, and it has been proven that this effect is caused by endogenous gonadal hormones being synthesized as a result of the gonadotropic activity of the administered hormone (Gielen et al., Cell Tissue Res., 1982, 225, 45-57).

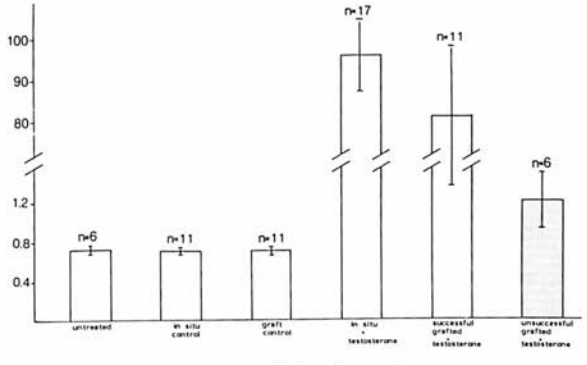
Do steroid hormones act on the GTH-cells directly or indirectly via affecting gonadotropin releasing hormone (GnRH)-centres in the brain ? To study the direct effect of steroids on the gonadotrops, the influence of testosterone on GTH-cells of ectopic implanted pituitary homografts was investigated. Pituitaries of male and female donor animals were transplanted into the caudal musculature of testosterone and cocoa butter treated host animals. Testosterone (1 µgr/gr b.w.) and cocoa butter treatment started 24 hours prior to the implantation of the pituitaries. GTH measurement by RIA and an EM study were carried out on the in situ and grafted pituitaries of the host animals. Plasma GTH levels were also analysed.

Testosterone treatment caused GTH-cell maturation of the in situ pituitaries as well as in the grafts. Accordingly, the GTH content of the in situ and the implanted pituitaries had increased in the testosterone treated animals.

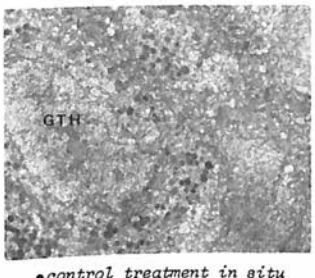
It can be concluded that testosterone acts directly on the maturation of the GTH-cells and that the brain is not necessarily involved in this process. Admittedly, one could argue that circulating GnRH was involved. However, in vitro studies using isolated pituitary cells confirm our conclusions (Fåhraeus-van Ree et al., this symposium).



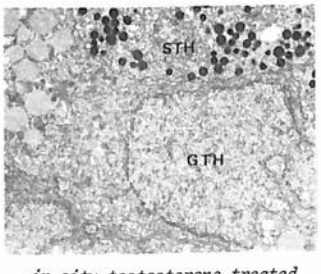
GTH(assay units/ml plasma)



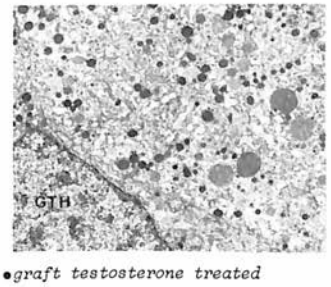
GTH(assay units/pituitary)



- *control treatment in situ*
- *control treatment graft*
- *untreated in situ*



- *in situ testosterone treated*



- *graft testosterone treated*

SEASONAL VARIATIONS IN SENSITIVITY TO GnRH OF JUVENILE RAINBOW TROUT, SALMO GAIRDNERI

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In juvenile trout the maturation of gonadotropic (GTH)-cells can be stimulated by gonadal steroids such as testosterone, both exogenous as well as endogenous secreted by the gonads as a result of GTH treatment (Gielen et al., 1982a). Testosterone acts directly on the gonadotrops (Gielen et al., 1982b).

As a consequence of the steroid hormone induced maturation of the GTH-cells, synthesis of gonadotropin is enhanced, but this is not accompanied by an increased release of GTH. Possible explanation for this observation might be that i) the GTH-cells are still not receptive for gonadotropin releasing hormone (GnRH) or ii) the GnRH-cells in the brain do not secrete their hormone or iii) the release is inhibited by the steroid.

To investigate the receptivity of the GTH-cells to GnRH testosterone pre-treated and non pre-treated juvenile, sexually immature trout were injected i.p. with a LH-RH analogue (des-Gly^{10}-|D-Ala^{6}|-LH-RH ethylamide). Testosterone was administered by a cocoa butter implantation (1 µgr/gr b.w.) 2 weeks prior to the LH-RHa treatment. GTH levels in the plasma and in pituitary extracts were analysed by RIA.

Two hours after a single injection of LH-RHa (50 ngr/gr b.w.) into non pre-treated and testosterone pre-treated animals the plasma GTH levels were increased in both groups (Fig. 1). Despite the release of gonadotropin the pituitary GTH content was not decreased, indicating that LH-RHa not only induced the release of GTH but also its synthesis.

Surprisingly, when LH-RHa (10 ngr/gr b.w.) was given at different times of the year the response was not always identical (Fig. 2). Fluctuations in response to LH-RHa and in basic GTH plasma levels (Fig. 3) might reflect a seasonal variation in the sensitivity to GnRH in juvenile trout.

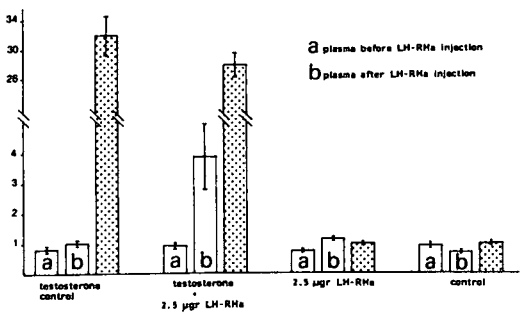

Fig.1. GTH level in plasma (open columns) and pituitary extracts after LH-RHa injection (assay units/ml plasma; a.u./pituitary).

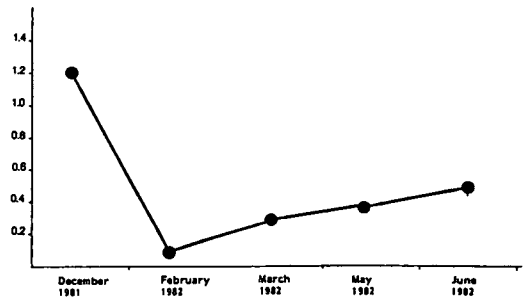

Fig.2. Increase of plasma GTH after injection of LH-RHa at different times of the year.

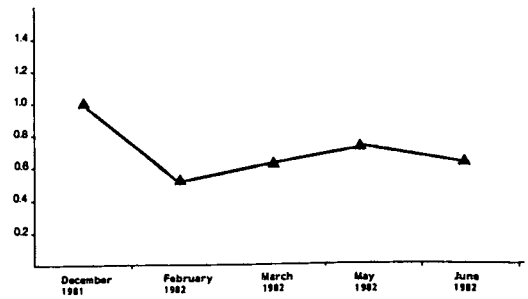

Fig.3. Basic plasma level of GTH in juvenile trout during part of the year (assay units/ml plasma).

References

Gielen J.Th., H.J.Th. Goos, J. Peute, R.A. van den Bosch, P.G.W.J. van Oordt, 1982a. The brain-pituitary-gonadal axis in the rainbow trout, Salmo gairnderi: Gonadal hormones and the maturation of gonadotropic cells. Cell Tissue Res., 225: 45-57.

Gielen J.Th., H.J.Th. Goos, Mariëlle G.A. de Mol Moncourt-de Bruyn, P.G.W.J. van Oordt, 1982b. Direct action of gonadal steroids on the maturation of gonadotropic cells in the rainbow trout, Salmo gairdneri. These proceedings.

DISTRIBUTION OF IMMUNOREACTIVE LH-RH IN THE BRAIN OF THE GOLDFISH

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Summary

Using an indirect immunofluorescence technique, LH-RH was detected in two hypothalamic centers : the ventrolateral NPP and the posterior NLT. Immunoreactive fibers were observed in many brain areas and in the proximal pars distalis of the pituitary.

Experiments based upon pituitary transplantations, brain lesioning studies and hypothalamic extracts injections have suggested that, in teleosts, the gonadotropic function of the pituitary is under hypothalamic stimulatory control. However the nature and the localization of the teleostean gonadotropin hormone-releasing hormone (Gn-RH) are not well established. Several authors attempted to localize LH-RH in the brain and pituitary of different species, however informations concerning the goldfish, which is one of the most studied teleost with regards to neuroendocrine control of reproduction, are lacking. We report here our observations on the immunocytochemical localization of LH-RH in the brain of the goldfish (Carassius auratus).

Males and females goldfish of the common variety were used in this study. Several fish were given colchicine intraperitoneally (10mg/100g body weight) 48 hours before decapitation. Paraffin or cryostat sections were processed for the indirect immunofluorescence technique using a primary antibody directed towards synthetic LH-RH. The specificity of the reaction was checked by ommission of one step of the reaction, use of other primary antisera or absorption of the antiserum with synthetic LH-RH.

No relationship was observed between the distribution of LH-RH and the sex or the reproductive cycle. Immunoreactive material was observed inconstantly in two groups of cell bodies of the hypothalamus. The first one was located in the ventrolateral parts of the nucleus preopticus periventricularis (NPP ; Peter and Gill, 1975) and consisted of bipolar neurons directed rostrocaudally. An other group of immunoreactive cell bodies was found in the posterior part of the nucleus lateralis tuberis (NLT ; Peter and Gill, 1975). These perikarya were less numerous and were observed only in two fishes. Immunoreactive fibers were widely distributed in the whole brain but were particularly numerous in several areas. On longitudinal sections, fibers directed rostrocaudally were observed in the ventral telencephalon and the olfactory tract. Whether they originated in the NPP is not sure. Fibers were also frequently observed in the area dorsalis and medialis of the telencephalon and along the wall of the ventricle. An important tract presumably originating from the cell bodies of the NPP travelled caudally to the NLT and possibly the pituitary where fibers were observed in the proximal pars distalis. Numerous fibers were found along the walls of the ventricle in the diencephalon. The habenular nucleus received a massive innervation as well as the optic tectum in the stratum album centrale and to a lesser extent the stratum griseum centrale and the stratum marginale. Immunoreactive fibers were also numerous at the level of the posterior commissure and in the lateral parts of the dorsal midbrain tegmentum. More posteriorly, on longitudinal sections, fibers were located in the nucleus diffusus lobi inferioris, the fasciculus longitudinalis medialis and the medulla oblongata. A few fibers were observed in the corpus of the cerebellum but none in the valvula.

Our results demonstrate that at least two LH-RH centers are located in the NPP and the posterior NLT. These findings are partly in agrement with previous reports on other teleosts (see reviews by Ball, 1981 ; Peter, 1982). The NLT is known as being involved in the regulation of the gonadotropic function of the pituitary : lesioning of the NLT pars posterior results in a blockage of gonadal recrudescence (Peter, 1982). More recently a gonadotropin inhibiting factor has been described in the NPP (Peter, 1982). Furthermore, both NPP and NLT are capable of concentrating labelled steroids (Kim et al. 1978).

References

Ball, J.N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians and reptiles. Gen. Comp. Endocr. 44 : 135-170.

Kim, J.A., W.E. Stumpf and M. Sar, 1978. Topography of estrogen target cells in the forebrain of goldfish, Carassius auratus. J. Comp. Neurol. 182 : 611-620.

Peter, R.E., 1982. Neuroendocrine control of reproduction in teleosts, Can. J. Fish Aquat. Sci. 39 : 48-55.

Peter, R.E. and V.E. Gill, 1975. A Stereotaxic atlas and technique for forebrain nuclei of the goldfish, Carassius auratus. J. Comp. Neurol., 159 : 69-102.

LOCALIZATION OF AROMATASE ACTIVITY IN THE BRAIN OF THE RAINBOW TROUT (SALMO GAIRDNERI)

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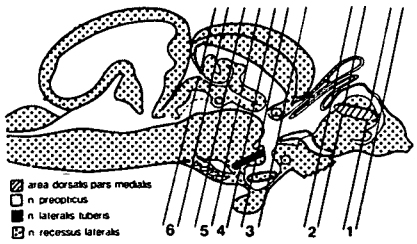
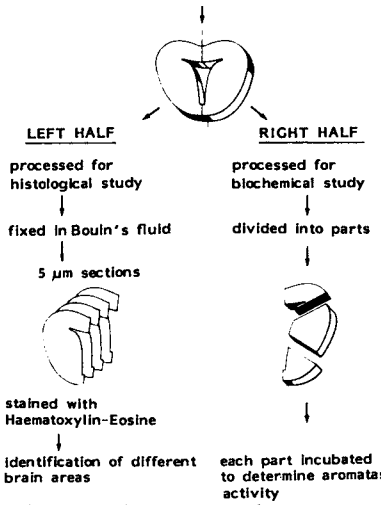
Aromatization (conversion of androgens into oestrogens) could be demonstrated in several parts of the brain (Lambert et al. 1982). In this study a method is presented for a more precize localization of the aromatase activity. It comprises a combination of a microanatomical and a biochemical appraoch.

Brains of adult trout were quickly frozen with CO_2. Transverse sections of 400 μm were cut at a temperature of -10^0C. The left half of these were processed for histological study to identify the different brain areas. The right halfs were cut into 2 or 3 pieces,

HISTOLOGICAL PROCEDURE

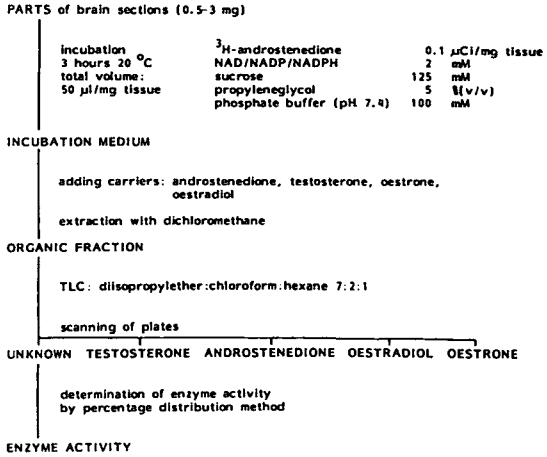


TRANSVERSE FROZEN SECTIONS (400μm)

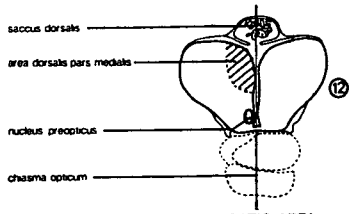


corresponding to distinct brain structures (i.e. hypothalamus, tectum opticum etc.). The aromatizing capacity was studied by incubating these pieces with ^{3}H-androstenedion and expressed as the amount of oestrone +oestradiol formed (fmol/mg tissue/min). Some of the preliminary results are shown as the figures in the O's

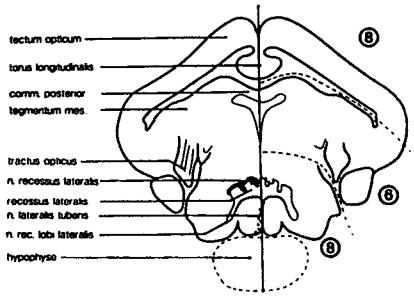
BIOCHEMICAL PROCEDURE



PRELIMINARY RESULTS



TELENCEPHALON AND PREOPTIC AREA



OPTIC TECTUM AND CAUDAL HYPOTHALAMUS

Reference:

J.G.D. Lambert and P.G.W.J. van Oordt: Cathechol-oestrogens in the brain of Salmo gairdneri. Gen. comp. Endocrinol.(46),p 401, (1982)

EFFECT OF SYNTHETIC LH-RH ON GTH CELLS OF E2-TREATED EELS

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Summary

Estradiol treatment induces the synthesis of gonadotropin (GTH) in the pituitary of Anguilla anguilla L. Injection of LHRH appears able to release GTH: exocytotic figures and lysis of some large globules and granules are observed. However, this response does not occur in all GTH cells which remain granulated.

Freshwater male silver eels have small GTH cells poorly differentiated containing abundant mitochondria, and rather few granules with a moderate electron density and an average diameter of 125 nm (80-200 nm) (Olivereau & Chambolle, 1978). Estradiol-17β (E2) treatment induces development and hyperplasia of GTH cells although a mitotic activity was not detected. A well developed Golgi complex, numerous secretory granules (200-500 nm diameter), dilated cisternae and some large globules (1.2-2.2 µm) are observed (Olivereau & Chambolle, 1979). As no macroscopic effect is discernible on the gonad, the release of the synthesized hormone remains questionable. Despite a careful study, exocytotic figures were not observed. Suppression of E2 treatment induces a gradual regression of GTH cells (Olivereau & Olivereau, 1979).

Twenty-two male silver eels were treated with E2 (10 or 13 injections, 125 µg/100 g, within 20 or 26 days respectively. Eight eels were subsequently injected with NaCl 0.6 % plus gelatin 1 % in the body cavity. Fourteen eels received LHRH in the same solvent (1 injection, or 2 injections 90 min apart, or 4 or 6 injections within 2 or 3 days respectively, 25 µg per injection). The eels were killed 1 h after the last injection. Pituitaries were fixed in 6.5 % glutaraldehyde solution in Sörensen buffer (pH 7.2) and post-fixed in 1 % osmium tetroxide. They were embedded in Epon and stained with uranyle acetate and lead citrate.

Solvent injections had no effect on the developed GTH cells. The response of these cells to LHRH is not uniform. Some areas of the proximal pars distalis were not clearly affected. Other islets of GTH cells appeared less granulated. Large vacuoles or dilated cisternae were more abundant at the periphery of the cells. Large globules were less numerous. Some globules showed an excentric dense core of a variable size, and a peripheral area of a low or very low electron density and a fine granular structure. A muriform structure was occasionally observed. In most cases, the membrane was no longer discernible. This process does not occur in all GTH cells after LHRH injections; it is rarely observed in E2-treated eels. In addition, several exocytotic figures were observed in LHRH-injected eels. The figures were located near the basal lamina, often facing a capillary. They occurred rarely in eels treated for one day, and were more abundant in those receiving 4 and 6 injections, whereas lytic processes in the globules were observed after a single injection. The Golgi area remained highly developed, and Golgi vesicles were abundant. These ultrastructural data suggest that some GTH is released after injection of synthetic LHRH although degranulated cells are not observed. These data agree with those of Crim & Evans reporting that LHRH increases plasma GTH levels in immature trouts previously treated with gonadal steroids.

References

Crim, L.W., & D.M. Evans, 1980. LHRH stimulated gonadotropin release from the rainbow trout pituitary gland: an in vitro assay for detection of teleost gonadotropin-releasing factor(s). Gen. Comp. Endocrinol. 40: 283-290.

Olivereau, M. & P. Chambolle, 1978. Ultrastructure des cellules gonadotropes de l'Anguille normale et après injection d'oestradiol. C. R. Acad. Sci. Paris, 287 D: 1409-1412.

Olivereau, M. & P. Chambolle, 1979. Ultrastructure of gonadotrophs in the eel following estradiol treatment. Proc. Indian Natl Sci. Acad. B 45: 478-482.

Olivereau, M. & J. Olivereau, 1979. Estradiol-positive feedback on gonadotropic (GTH) cells in freshwater male silver eels. Gen. Comp. Endocrinol. 39: 247-261.

THE FUNCTIONAL SIGNIFICANCE OF THE NUCLEUS OLFACTORETINALIS IN THE PLATYFISH, XIPHOPHORUS MACULATUS

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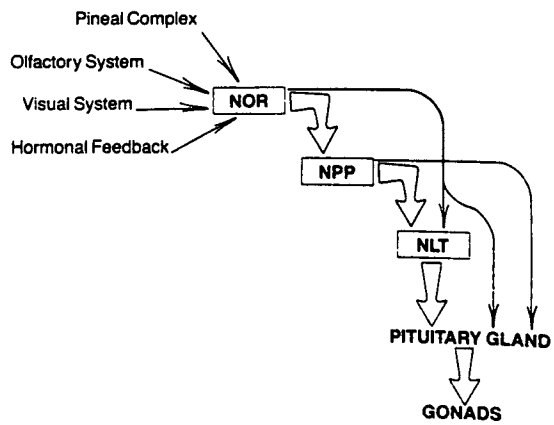
The nucleus olfactoretinalis (NOR) has been identified as an immunoreactive luteinizing hormone releasing hormone (ir-LHRH) containing center in the brain. Because it is located at the boundary between the ventral telencephalon and the olfactory lobe and sends projections to both the optic and olfactory nerves, it has been termed the nucleus olfactoretinalis telencephali (Münz et al., 1981). In our immunocytochemical (ICC) studies of sexually mature platyfish, ir-LHRH has also been localized in tracts between the NOR and the pineal complex (habenular nucleus) and between the NOR and the ir-LHRH containing neurons of the nucleus preopticus periventricularis (NPP) and the nucleus lateralis tuberis pars posterioris (NLT).

We have also found that during platyfish development the NOR is the first region in the brain in which ir-LHRH can be identified. It appears in sexually immature fish (stage 1) at 4 weeks of age in early maturing genotypes and at 9 weeks of age in late maturers. Ir-LHRH can first be demonstrated in neurons of the NPP as the process of sexual maturation is initiated (stage 2) and in the NLT one to two weeks later as pubertal development continues. The same sequence of development occurs in late maturers but with a more protracted time scale.

In an ICC study of hypophysectomized platyfish we found that there is a marked increase in the ir-LHRH content in perikarya and processes of the NOR but a depletion of ir-LHRH in the NPP and NLT. If hypophysectomized fish receive five 10ug injections of salmon gonadotropin on alternate days, there is an increase in the number of ir-LHRH containing fibers of the NPP and NLT but, although still above control levels, a significant decrease of ir-LHRH in the NOR. RIA analyses of whole brains confirm the decrease in LHRH content following hypophysectomy (mean $\pm$ S.E.: 649 $\pm$ 28 pg per brain compared to 446 $\pm$ 31 pg per brain) (Schreibman et al., 1982).

Based on our observations and because of its anatomical position, the NOR may play an important role in transmitting (translating) environmental cues to those centers in the brain that are involved in the development and maintenance of the reproductive system (see figure). The sequential appearance of ir-LHRH in brain nuclei beginning with the NOR and progressing in a caudal direction toward the pituitary gland, leads us to suspect that the NOR serves to initiate the activity in the other nuclei essential for the process of puberty to occur. We suspect, too, that the NOR is most active when gonadotropin levels are low, as for example in the immature fish and in the hypophysectomized adults that we have studied. (Supported by NIH-NIA (AG01938) and the City University of New York.

THE NOR: INTEGRATOR OF INTERNAL AND EXTERNAL SIGNALS FOR THE REGULATION OF REPRODUCTIVE SYSTEM MATURATION AND MAINTENANCE.



References

Münz, H., W.E. Stumpf, and L. Jennes, 1981. LHRH-systems in the brain of platyfish. Brain Res., 221:1-13.

Schreibman, M.P., L. Halpern-Sebold, M. Ferin, H. Margolis-Kazan and H.J.Th. Goos, 1982. The effect of hypophysectomy and gonadotropin administration on the distribution and quantity of LHRH in the brains of platyfish: A combined immunocytochemistry and radioimmunoassay study. Submitted to Brain Res.

SITE OF ACTION OF SOME AGRICULTURAL PESTICIDE IN THE HYPOTHALAMO-HYPOPHYSIAL-OVARIAN AXIS OF THE FRESHWATER CATFISH. HETEROPNEUSTES FOSSILIS (BLOCH)

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Deleterious effect of some agricultural pesticides on reproductive physiology of **Heteronopustes fossilis** is now well established (Singh & Singh, 1980a, b, 1981). These pesticides decrease gonadotrophin secretion and inhibit ovarian activity. The present study was undertaken to explore the action of four pesticides - Cythion, Paramar M50 (organophosphate), Aldrin and Hexadrin (organochlorine) at sublethal concentration in 3 sets of experiments on hypothalamo-hypophyseal-ovarian axis in **H. fossilis**.

Fish were exposed to sublethal concentration of pesticides for four weeks. In the first set of experiment effect of the above pesticides on ovarian 32p uptake in sham-hypophysectomized and hypophysectomized fish was studied. In the second experiment ovarian 32p uptake was measured in response to the injections of pituitary extract or blood serum collected from fish exposed to pesticides. And the third experiment was conducted to assess ovarian 32p uptake response after the intraperitoneal administration of hypothalamic extract pooled from pesticides exposed fish.

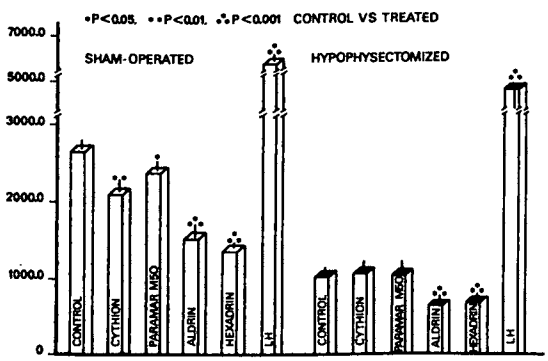

Fig. 1

All the four pesticides Cythion, Paramar M50, Aldrin and Hexadrin significantly reduced ($P<0.05$ to 0.001) the ovarian incorporation in sham-hypophysectomized fish. In hypophysectomized fish Cythion and Paramar M50 had no effect on 32p uptake of ovary but Aldrin and Hexadrin were potent enough to further reduce the 32p uptake by ovary ($P<0.001$, Fig. 1). This clearly indicate that these organochlorine compounds directly affect the ovary in **H. fossilis**. Gonadotrophic potency of the pituitary gland and the blood serum of the fish exposed to pesticides was substantially reduced ($P<0.001$) as reflected by decreased ovarian 32p uptake

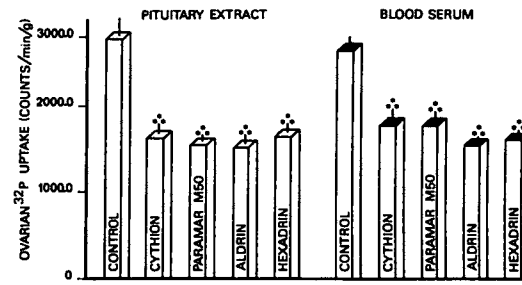

Fig. 2

in hypophysectomized recipients in comparison to the control (Fig. 2). Hypothalamic extract of pesticide exposed fish also showed significantly decreased amount of GnRH-like substance (Fig. 3). Thus these pesticides apparently inhibited the secretion of of GnRH-like substance which in turn reduced the gonadotrophin output followed by decreased ovarian activity involving hypothalamo-hypophysial-ovarian axis. However, Aldrin and Hexadrin belonging to organochlorine group of pesticides did also act directly on ovary bypassing hypothalamo-hypophysial sites in **H. fossilis**.

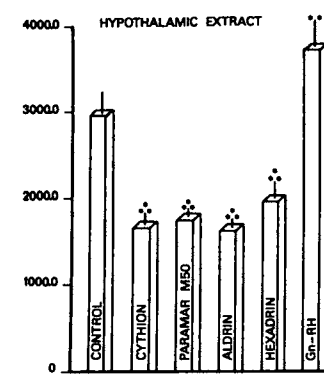

Fig. 3

Reference :

Singh, H. and T. P. Singh, 1980a. Effect of two pesticides on ovarian 32p uptake and gonadotrophin concentration during different phases of annual reproductive cycle in the freshwater catfish, Heteropneustes fossilis (Bloch). Environmental Res, 22 : 190-200.

Singh, H. and T. P. Singh, 1980b. Effects of two pesticides on testicular 32p uptake, gonadotrophic potency, lipid and cholesterol content of testis, liver and blood serum during spawning phase in in H. fossilis (Bloch). Endokrinologie, 76 : 288-296.

Singh, H. and T. P. Singh, 1981. Effect of Parathion and Aldrin on survival, ovarian 32p uptake and gonadotrophic potency in a freshwater catfish, Heteropneustes fossilis (Bloch). Endokrinologie, 77 : 173-178.

Acknowledgements :

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EXPRESSION OF H-Y ANTIGEN IN NON MAMMALIAN VERTEBRATES AND ITS RELATION TO SEX DIFFERENTIATION

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Summary

Among the factors involved in primary gonadal differentiation, a cell surface component, the H-Y antigen, has been proposed as the principal determining factor of mammalian testicular organogenesis. Since the H-Y antigen has been found in the heterogametic sex of species from all the classes of vertebrates, it has thus been assumed to play an important role in XY testicular or ZW ovarian differentiation. However in nonmammalian vertebrates, experimental findings suggest a role for both sex hormones and H-Y antigen in gonadal organogenesis, since the gonadal H-Y expression depends on a sex steroid control.
Keywords: H-Y antigen, gonadal sex differentiation, sex hormones, sex manipulation.

Introduction

H-Y antigen was first considered as a minor histocompatibility antigen specific of the male in mammals. It was originally discovered in the mouse, since male skin grafts on females were rejected while other graft combinations were tolerated in the highly inbred (syngeneic) C57BL/6 mouse strain (Eichwald & Silmser, 1955). This male-specific antigen was then termed 'H-Y' (Billingham & Silvers, 1960), 'H' as 'histocompatibility' and 'Y' as 'Y-chromosome', because the expression of this surface antigen was attributed to the Y-chromosome, being the main genomic difference between males and females of the same syngeneic strain.

Studies on the involvement of H-Y antigen in transplantation are numerous (review: Billingham and Hings, 1981). However, graft experiments are not the only way to study the H-Y antigen and a serological approach has been developed. H-Y antiserum is usually obtained by hyperimmunisation of female mice or rats with spleen cells from males of the same syngeneic strain. Even though the H-Y antigen is present on all male cells, only a few cell types are good targets for H-Y antibodies. Among them, the mouse epididymal spermatozoon is the most widely used in numerous serological assays (review: Koo,1981). H-Y typing is usually determined by serological absorption. This procedure uses the ability of the cells to be tested to remove H-Y antibodies from H-Y antiserum. The residual anti-H-Y activity of the recovered H-Y antiserum is then estimated in a direct serological assay using an H-Y positive target cell. However H-Y serology is far from being easy to handle, and numerous technical difficulties may led to conflicting results of H-Y serotyping. Because H-Y is a minor antigen, the main technical problem is the weakness of the H-Y antiserum. A solution to this problem is to use monoclonal H-Y antibodies.

In spite of these difficulties, since 1975 the H-Y antigen was suspected as being the mammalian testis determining factor, and, due to its phylogenetic conservation, to be involved in the differentiation of the heterogametic gonad in vertebrates (Wachtel et al., 1975a). In mammals at least, there is a strong body of evidence to suggest that H-Y might play a role in primary sex determination. Although this will not be discussed here in detail, it is important to know that, in mammals:

- testicular tissue is generally associated with the expression of H-Y antigen, whatever the sex genotype or the sex phenotype may be
- there is a gonad-specific receptor for H-Y antigen (in gonadal tissue of both sexes)
- H-Y antigen is presumed to control testis differentiation via its receptor
- dissociated ovarian cells reorganize into testicular-like structures in the presence of exogenous H-Y antigen (for review on mammals see: Haseltine & Ohno, 1981; Ohno,1979; Wachtel & Koo, 1981).

I Phylogenetic conservation of H-Y antigen and of its gonad-specific receptor

The homology of mouse H-Y antigen with a cross-reactive or identical plasma membrane component in other vertebrates has been demonstrated. Since direct cytotoxicity or binding assays cannot be used in nonmammalian vertebrates, H-Y typings are performed by serological absorption experiments using mouse or rat H-Y antiserum. Cells to be tested are incubated with H-Y antiserum. The residual anti-H-Y activity is then estimated on mammalian H-Y positive target-cells. A decrease of anti-H-Y activity indicates that the tested cells are H-Y antigen positive ($H\text{-}Y^+$), whereas no (or a few) loss of anti-H-Y activity indicates that the tested cells are H-Y antigen negative ($H\text{-}Y^-$). The serological assays that have been used for nonmammalian vertebrates are the following: cytotoxicity test on mouse sperm (Goldberg et al., 1971); cytotoxicity on rat epidermal cells (Scheid et al., 1972); protein A ro-

Table 1. H-Y antigen expression in fishes.

References	Species	H-Y antigen in	Tested tissues
Shalev et al. (1978)	Salmo gairdneri	female	blood
Müller & Wolf (1979)	Salmo gairdneri	?	gonads
	Salvelinus alpinus	?	gonads
	Rutilus rutilus	?	gonads
	Carassius auratus	?	gonads
	Barbus tetrazona	?	gonads
	Lebistes reticulatus	male	gonads
	Xiphophorus helleri	male	gonads
Pechan et al. (1979)	Xiphophorus maculatus	male (XY or YY)	brain
	Haplochromis burtoni	male	brain
	Orizias latipes	male (XY)	brain,liver
	Tilapia	male	
Shalev & Huebner(1980)	Lebistes reticulatus	male *	liver,heart,spleen,gonads
Wiberg (1982)	Anguilla anguilla	female (ZW) *	gonads
Zaborski & Bruslé	Serranus cabrilla	testicular part	blood,spleen,ovotestis

*Park & Grimm, 1981.

sette assay on mouse sperm (Koo & Goldberg, 1978); cytotoxicity on human lymphoblastoid cells (Fellous et al., 1978); immunobacterian rosette assay on mouse sperm (Zaborski, 1979a).

Using such techniques, antigenic determinants similar to H-Y have been found in birds (Wachtel et al., 1975a; Müller et al., 1980), reptiles (Zaborski et al., 1979; Engel et al., 1981), amphibians (Wachtel et al., 1975a; Zaborski, 1979b; Engel & Schmid, 1981), and fishes (see Table 1). Moreover, a strong correlation between the H-Y$^+$ sex and the heterogametic sex has been established (XY-male= H-Y$^+$; ZW-female=H-Y$^+$) at least within the birds and the amphibians. However, there are some intra-class exceptions, mostly in mammals, dependant on genetic anomalies. The use of detection of H-Y antigen as a tool for identification of an unknown heterogametic sex is not always possible because of technical difficulties in H-Y serology. It should be noted that, for reasons probably involving defects in cross-reactivity, the greatest problems are encountered in fishes. However, since the studies in fishes were generally performed on a preliminary way, further more conclusive studies are required. A strict homology between the H-Y$^+$sex and the heterogametic sex seems to be exagerated. However, the identification of the H-Y phenotype might be used as a substitute which reflects differences in sex alleles between male and female individuals, when sex chromosomes or the heterogametic sex are unknown.

Further studies have demonstrated the conservatism of the gonad-specific H-Y receptor. Wachtel et al. (1980) shown that soluble mammalian H-Y antigen and soluble avian H-Y antigen bound specifically to testicular cells from normal Xenopus males (in Xenopus, normal ZZ testes are H-Y$^-$ and normal ZW ovaries are H-Y$^+$). As in mammals, the homogametic gonad possess H-Y receptors, although normally H-Y$^-$. In a more functional perspective, Zenzes et al. (1980) cultivated dissociated testes from chickens in the presence of mammalian H-Y antigen and they observed that the testicular cells reorganized into ovarian-like structures (normal avian ovaries are H-Y$^+$).

The widespread phylogenetic representation of H-Y antigen as well as the phylogenetic conservatism of its gonads-specific receptor suggest that H-Y antigen could have an important sex-associated function in nonmammalian vertebrates.

II Manipulation of sex and of H-Y expression

Sex as well as H-Y manipulation by epigenetic factors is possible in nonmammalian vertebrates but not in mammals. All the observations that have been made so far in this respect are reviewed in this section.

Change of sex with change of H-Y expression

Sex hormone treatments in birds and amphibians. In female birds, sex dissymmetry of gonads is a specific feature. Only the left gonad differentiates into an ovary while the right one remains rudimentary. Sex dissymmetry occurs as well in feminized males. since male embryos treated with estradiol benzoate develop a left ovotestis while the right gonad remains testicular. Müller et al. (1979) have shown that such an ovotestis becomes H-Y$^+$like a control ovary (normal ZZ testes are H-Y$^-$). Similar results were obtained in quails: H-Y antigen was present

in ovotestes of male embryos feminized following diethylstilbestrol treatment, but H-Y was not induced in non-gonadal tissues such as spleen that remained H-Y$^-$ (Müller et al., 1980). Moreover, the quail ovotestis is only transiently feminized (as also in the chick) and reverts to a testis a few weeks after hatching. The H-Y study of the post-hatching period in treated males shown an evolution of H-Y expression: H-Y antigen disappeared from the reverting left ovotestis while the unfeminized fight testis remains H-Y$^-$ (Zaborski et al., 1981). In amphibians, complete and functional sex inversion can be achieved by steroid treatment of larvae. Wachtel et al. (1980) in the frog Xenopus laevis and Zaborski & Andrieux(1980) in the salamander Pleurodeles waltlii (both amphibians displaying a ZZ-male/ZW-female sex-determining mechanism) demonstrated that ZZ male larvae treated with estrogens were sex-inversed and subsequent adult 'neo-females' typed H-Y$^+$ in ovarian cells but remained H-Y$^-$ in non-gonadal cells, according to their homogametic sex genotype.

Temperature treatments in reptiles. In many species of turtle the sexual differentiation of the gonads is temperature dependant and complete sex-inversion can occur. In Emys orbicularis, egg incubation below 27.5°C results in 100% phenotypic males, while egg incubation above 29.5°C results in 100% phenotypic females. H-Y typing of blood cells in animals of each lot shown that some individuals were H-Y$^+$ and the others were H-Y$^-$. Since wild adult females are H-Y$^+$ (no heteromorphic sex chromosomes) H-Y$^+$ individuals were considered as genotypic females (and as sex-inversed in the phenotypic male lot), and H-Y$^-$ individuals were considered as genotypic males (and as sex-inversed in the phenotypic female lot). The H-Y typing of gonadal cells has shown that, whatever the blood H-Y phenotype, ovarian cells were H-Y$^+$ and testicular cells were H-Y$^-$ (Zaborski et al., 1982). Temperature dependant changes in sex hormone concentrations in the embryonic gonads could be involved.

Ovariectomy in birds. In the female chick, a left ovariectomy induces the transformation of the rudimentary right gonad into a testis. Studies of the H-Y antigen in such a model shown that it progressively disappeared in the initially H-Y$^+$ right gonad during the course of its masculinization, to reach a level of H-Y expression similar to that of H-Y$^-$ control testes. Non-gonadal cells of operated animals were not affected and remained H-Y$^+$ (Zaborski et al., 1980). Although the gonadal transformation occured without any added exogenous hormones, an important increase of testosterone and a decrease of estrogen production occured during the period of disappearance of H-Y antigen (Reyss-Brion et al., 1982).

Thus, sex hormone treatment or ovariectomy (which also influence the hormonal balance) both induce an inversion of H-Y phenotype on the gonadal cells. These experiments demonstrate that the H-Y structural gene(s) is present in both the heterogametic and the homogametic sex. The correlation between the presence of H-Y antigen and the presence of gonadal structures of the heterogametic sex is confirmed. However, these experiments do not permit one to discern whether the H-Y expression is the result of the treatment and the cause of the gonadal inversion or the result of the gonadal inversion.

Change of H-Y expression without change of sex

As sex steroids are apparently involved in the sexual differentiation of gonadal and H-Y phenotype, the question arose whether H-Y antigen expression might also be hormonally controlled in already differentiated gonads. The following experiments has been undertaken in adult amphibians belonging to either the ZZ-male/ZW-female or the XX-female/XY-male sex-determining system.

In Pleurodeles, when adult ZZ males were treated with estradiol-17beta, the testes were not feminized but became H-Y$^+$, and a drop of plasma testosterone was observed. Thus, the appearance of H-Y antigenis the direct consequence of the estrogen treatment and does not necessarily depend on the feminization of the gonads (Zaborski & Andrieux, 1980b). Though the estradiol treated testes were not inversed; the spermatogenesis was inhibited and the glandular tissue was atrophied. It is well known that the very same effects on testes are obtained when adult males are hypophysectomized. H-Y typing in such males in Pleurodeles shown that testicular cells became H-Y$^+$. Moreover, both plasma testosterone and estradiol levels were very low, suggesting that estradiol per se was not required to induce H-Y expression. This conclusion was confirmed in hypophysectomized female Pleurodeles: ovarian cells remained H-Y$^+$ as in normal ovaries although estradiol level as well as testosterone level were very low (Zaborski & Andrieux, 1980a).

Thus a repressor control exerted by androgens was suspected. However testosterone treatment of adult females failed to show any modification of the ovarian H-Y phenotype which remained H-Y$^+$, suggesting a constitutive expression of H-Y in ZW females, while it was inducible in ZZ males. To elucidate this specific problem, more precise and controlable in vitro experiments were undertaken with Pleurodeles ovaries. They shown that a three-week culture with dihydrotestosterone led to the inhibition of H-Y expression in ovarian tissues which be-

came H-Y⁻ (Penrad-Mobayed & Zaborski, 1982). Thus it is the absence (or a low level) of androgens rather than the presence of estrogens that controls the gonadal H-Y expression in a ZZ/ZW system.

Complementary experiments in the frog Rana ridibunda shown that estradiol treatment of XY adult males induced an inhibition of H-Y expression on initially H-Y⁺ testicular cells. It appears that the H-Y expression is repressed in ZW Pleurodeles ovary by an androgen, whereas the very same effect is induced by an estrogen in XY Rana testis.

Conclusion

Once the technical difficulties have been overcome, H-Y serology may be used as a tool in sex identification, since a stronger reactivity of the H-Y antiserum is observed in the heterogametic sex rather than in the homogametic sex. Associated with classical experiments, it also permits us to enhance our knowledge on the conditions of expression of H-Y antigen.

In nonmammalian vertebrates, the sex hormones appear to be the primary inducers of H-Y antigen. An inhibitory or repressor control by androgens in ZZ/ZW vertebrates and by estrogens in XX/XY vertebrates may also be proposed. However, since each sex produces both kind of sex hormones, the control involves a quantitative rather than a qualitative hormonal change. In all these respects, H-Y might play an intermediary role as a part of a chain of factors leading to morphogenetic events.

Indeed, the fact that the H-Y antigen might be involved in sex differentiation is an exciting suggestion. In this connection, what remains is to design convenient experiments of the 'cause and effect relationship' type using exogenous H-Y antigen and undifferentiated embryonic gonadal cells.

The role of H-Y antigen in the process of sex differentiation is far from being clear and it seems premature to adopt a definitive position regarding the present state of our knowledge on immunologic, genetic, hormonal and environmental sex factors.

References

Billingham, R.E. & I.M. Hings, 1981. The H-Y antigen and its role in natural transplantation. Hum. Genet. 58:9-17.

Billingham, R.E. & W.K. Silvers, 1960. Studies on tolerance of the Y chromosome antigen in mice. J. Immunol. 85:14-26.

Eichwald, E.J. & C.R. Silmser, 1955. Untitled communication. Transp. Bull. 2:148-149.

Engel, W., Klemme, B. & M. Schmid, 1981. H-Y antigen and sex-determination in turtles. Differentiation 20:152-156.

Engel, W. & M. Schmid, 1981. H-Y antigen as a tool for the determination of the heterogametic sex in amphibia. Cytogenet. Cell Genet. 30:130-136.

Fellous, M., E. Günther, R. Kemler, J. Wiels R. Berger, J.L. Guenet, H. Jakob & F. Jacob, 1978. Association of the H-Y male antigen with beta$_2$-microglobulin on human lymphoid and differentiated mouse teratocarcinoma cell lines. J. Exp. Med. 148: 58-70.

Goldberg, E.H., E.A. Boyse, D. Bennett, M. Scheid & E.A. Carswell, 1971. Serological demonstration of H-Y (male) antigen on mouse sperm. Nature 232:478-480.

Haseltine, F.P. & S. Ohno, 1981. Mechanisms of gonadal differentiation. Science 211: 1272-1278.

Koo, G.C., 1981. Serology of H-Y antigen. Hum. Genet. 58:18-20.

Koo, G.C. & C.L. Goldberg, 1978. A simplified technique for H-Y typing. J. Immunol. Methods 23:197-201.

Maurel, M.C. & P. Zaborski, 1982. Inhibition of H-Y antigen expression in the frog testis following estradiol treatment. Biol. Cell. 45:86.

Müller, U., A. Guichard, M. Reyss-Brion & D. Scheib, 1980. Induction of H-Y antigen in the gonads of male quail embryos by diethylstilbestrol. Differentiation 16: 129-133.

MUller, U. & U. Wolf, 1979. Cross-reactivity to mammalian anti-H-Y antiserum in teleostean fish. Differentiation 14:185-187.

Müller, U., M.T. Zenzes, U. Wolf, W. Engel & J.P. Weniger, 1979. Appearance of H-W (H-Y) antigen in the gonads of oestradiol sex-reversed male chicken embryos. Nature 280:142-144.

Ohno, S., 1979. Major sex-determining genes. Springer-Verlag, Berlin Heidelberg pp.140.

Park, E.H. & H. Grimm, 1981. Distribution of C-band heterochromatin in the ZW sex chromosomes of european and american eels (Anguillidae, Teleostomi). Cytogenet. Cell Genet. 31:167-174.

Pechan, P., S.S. Wachtel & R. Reinboth, 1979. H-Y antigen in the teleost. Differentiation 14:189-192.

Penrad-Mobayed, M. & P. Zaborski, 1982. Control of H-Y antigen expression in amphibian gonads: possible inhibitory role of androgens in the heterogametic ovary. Proc. XV Internat. Embryol. Conf. Strasbourg France p. 89.

Reyss-Brion, M., Th.M. Mignot & A. Guichard, 1982. Development of steroidogenesis in the right gonad of domestic fowl masculinized by left ovariectomy. Gen. Comp. Endocrinol. 46:68-74.

Scheid, M., E.A. Boyse, E.A. Carswell & L.J. Old, 1972. Serologically demonstrable alloantigens of mouse epidermal cells. J. Exp. Med. 135:938-955.

Shalev, A., I. Berczi & J.L. Hamerton, 1978.

Detection and cross-reaction of H-Y antigen by haemagglutination. J. Immunogenet. 5:303-312.
Shalev, A. & E. Huebner, 1980. Expression of H-Y antigen in the guppy (Lebistes reticulatus). Diefferentiation 16:81-83.
Wachtel, S.S., P. Bresler & S.S. Koide,1980. Does H-Y antigen induce the heterogametic ovary? Cell 20:859-864.
Wachtel, S.S. & G.C. Koo, 1981. H-Y antigen in gonadal differentiation. In C.R. Austin & R.G. Edwards (Ed.): Mechanisms of sex differentiation in animals and man. Academic Press, London. p. 255-299.
Wachtel, S.S., G.C. Koo & E.A. Boyse, 1975. Evolutionary conservation of H-Y (male) antigen. Nature 254:270-272.
Wachtel, S.S., S. Ohno, G.C. Koo & E.A.Boyse, 1975. Possible role for H-Y antigen in the primary determination of sex. Nature 257:235-236.
Wiberg, U., 1982. Serological cross-reactivity to rat anti-H-Y antiserum in the female european eel (Anguilla anguilla). Differentiation 21:206-208.
Zaborski, P., 1979a. Detection of H-Y antigen on mouse sperm by the use of Staphylococcus aureus. Transplantation 27:348-350.
Zaborski, P., 1979b. Sur la constance de l'expression de l'antigène H-Y chez le sexe hétérogamétique de quelques Amphibiens et sur la mise en évidence d'un dimorphisme sexuel de l'expression de cet antigène chez l'Amphibien Anoure Pelodytes punctatus D. C.R. Acad. Sci. Paris, D, 289:1153-1156.
Zaborski, P. & B. Andrieux, 1980a. H-Y antigen in sexual organogenesis of amphibians: recent studies on its expression in some experimental conditions. Proc. XIV Internat. Embryol. Conf. Patras Greece. p.114.
Zaborski, P. & B. Andrieux, 1980b. Hypothèse sur l'action inhibitrice de la testostérone dans l'expression de l'antigène H-Y chez un Triton. Arch. Anat. Microsc. Morphol. Exp. 69:333.
Zaborski, P. & S. Bruslé. H-Y antigen in a synchronous hermaphrodite teleost fish: restriction of its expression to the testicular part of the ovotestis of Serranus cabrilla L. Reprod. Nutr. Develop. (submitted).
Zaborski, P., M. Dorizzi & C. Pieau, 1979. Sur l'utilisation de sérum anti-H-Y de Souris pour la détermination du sexe génétique chez Emys orbicularis L. (Testidines Emydidae). C.R. Acad. Sci. Paris, D., 288:351-354.
Zaborski, P., M. Dorizzi & C. Pieau, 1982. H-Y antigen expression in temperature sex-reversed turtles (Emys orbicularis). Differentiation (to be published).
Zaborski, P.,A. Guichard & M. Reyss-Brion, 1980. H-Y antigen in ovariectomized chicks : disappearance of its expression during the transformation of the right gonad into a testis. Biol. Cell. 39:291-294.
Zaborski, P., A. Guichard & D. Scheib, 1981. Transient expression of H-Y antigen in quail ovotestis following early diethylstilbestrol (DES) treatment. Biol. Cell. 41:113-122.
Zenzes, M.T., E. Urban & U. Wolf, 1980. Mammalian cross-reactive H-Y antigen induces sex reversal in vitro in the avian testis. Differentiation 17:121-126.

TEMPERATURE AND STEROID EFFECTS ON GONADAL SEX DIFFERENTIATION IN RAINBOW TROUT

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Summary

Testicular homogenates of rainbow trout from D(ay) 100 after fertilization incubated in vitro in the presence of C14-labelled 11β-hydroxy androstenedione (11βAdione) are able to convert this steroid into 11-keto androstenedione (11-keto Adione), which is indicative of the presence of 11β-hydroxy steroid dehydrogenase (11β-HSD). Ovaries from D100 convert H3-labelled 17α-hydroxy progesterone (17αP4) into androstenedione, which indicates the presence of 17α, 20-desmolase. These and previous observations (van den Hurk et al., 1982a) show that just developing testes and ovaries possess the same enzyme systems, and testes in addition have those to form steroids with a functional group at the 11-position. Both ovaries and testes from D100 do not show 17β-HSD and aromatase activity and thus are unable to synthesize testosterone and oestrogens.

Administration of 11βAdione (60 µg/g) for 8 weeks from first feeding results in an all male trout population. Administration of a lower dose (6 µg/g) and of methyltestosterone (0.6 and 6 µg/g) also result in high proportions of males (94-99%). It is concluded that 11βAdione is an endogenous androgen in rainbow trout, which initiates the development of testes.

Contrary to its feminizing effect when suspended in aquariumwater, progesterone (P4) does not influence sex ratio when added to the food. Neither does exposure of 31 to 57 day old trout to high temperatures significantly alter the sex ratio.

Keywords: gonads, sex differentiation, steroids, temperature, Salmo gairdneri.

Introduction

In broods of rainbow trout, Salmo gairdneri, the sex ratio can be influenced by treatment with steroids (Johnstone et al., 1978; Okada et al., 1979; van den Hurk & Slof, 1981; van den Hurk et al., 1982b). This effect is dependent on type and dose of added steroid, time of start of treatment and method of steroid administration. In our experiments the sex ratio was shifted toward a male direction by treatment for 4 weeks with methyltestosterone or 11βAdione. The sex ratio was shifted toward a female direction by treatment with P4 suspended in aquarium water. The best results, 64-84% dominance of one sex were obtained when treatment was started between D43 and D57 after fertilization, a period that coincides with onset of gonadal sex differentiation. An earlier start of treatment with steroid, i.e. from hatching onward, inhibits normal development of gonads, often resulting in sterilization. Treatment from D57 onward does not affect the normal sex ratio. 8 weeks treatment with methyltestosterone and 11βAdione added to the food from the first day of feeding were carried out in order to investigate whether such long treatments will result in all male broods, and with P4 to study whether this steroid excerts a feminizing effect when added to the food.

Experiments in which gonadal homogenates are incubated in vitro with labelled steroid precursors demonstrate that undifferentiated gonads and just developing ovaries in rainbow trout possess enzyme systems which synthesize P4 and other C21-steroids, whereas just differentiating testes in addition can synthesize the androgens androstenedione and 11βAdione (van den Hurk et al., 1982a). These results and those from experiments in which fish were treated with steroids possibly indicate that the presence or absence of androgens at the start of gonadal sex differentiation determines gonadal sex. On account of its high androgenic potency in fish, we wondered whether 11-keto testosterone is synthesized in just differentiating testes. Therefore testicular tissue from D100 has been incubated in vitro in the presence of 11βAdione-C14 in order to determine a possible synthesis of other 11-steroids. Furthermore, homogenates of ovaria from D100 were incubated in vitro in the presence of 17αP4-H3, since it was not obvious from previous studies, whether just differentiating ovaries have 17α,20-desmolase for the synthesis of androstenedione.

Commercial production of monosex populations is very important in fish farming. Increasing the proportion of females results in a number of benifits in trout farming. Feminization can be achieved by steroid hormone administration, however, human consumption of steroid treated fish is not permitted. Cross fertilization of masculinized animals of female genotype with normal females produces all-female offsprings, but this is a time consuming and expensive method. In this respect, feminization with a biotic factor would be more attractive. Temperature may play an important role in determination of gonadal sex in teleosts (Bruslé & Bruslé, 1982). Possible influences of high temperature on gonadal sex differen-

tiation in rainbow trout were investigated.

Materials and methods

Animals

Fertilized eggs of rainbow trout, Salmo gairdneri, were incubated in aquaria at 11°C and 12 h 1:12h d. Hatching occurred at D28-29. Before and after treatment, fry were reared under the same conditions as the eggs. Fry were fed (Trouvit) 4% of their body weight daily.

Steroid and temperature treatments

200 fish were treated for 8 weeks from first feeding (= D47) until D105 with steroid added to the food or with control food. Preparations of the experimental diet has been described by van den Hurk (1982). Methyltestosterone was added to the food in concentrations of 0.6 and 6 µg/g, 11βAdione in 6 and 60 µg/g, and P4 in 60 and 600 µg/g. In each experiment the gonads of 100 randomly sampled fish were sexed at D120 or D150. Gonads were examined histologically (van den Hurk & Slof, 1981). Statistical analysis of sex ratio's was done with the chi-square test; Student's t-test for mean body weight, mean gonad weight and mean gonadosomatic index (GSI) was used. GSI was expressed in °/oo.

High temperature shocks were given at D31, D44 and D57 after fertilization, respectively. Fish were brought at the desired temperature level within 20 min. Subsequently, they remained at 25°C for 3h or at 28-29°C for 10 min. Another group of fish was kept from D43 to D57 at 22-23°C. After each experiment, temperature was gradually lowered again to 11°C.

In vitro experiments

34 testes from D100 fish were pooled and homogenized in 1 ml 0.1M phosphate buffer (pH 7.4) containing 0.25M sucrose at 0°C. 0.25 ml homogenate was incubated with 1 KBq 11βAdione-C14 (spec. act.: 2 GBq/mmol) dissolved in 24 µl propyleneglycol, 2mM each of NAD and NADPH and 0.15 ml phosphate buffer. Incubations were carried out at 20°C under continuous automatic shaking. After 3h enzyme reactions were terminated by adding 1 ml dichloromethane. 100 µg each of 11βAdione, 11-keto Adione, 11-keto testosterone and 11β-hydroxy testosterone were added to the incubation mixture before extraction with dichloromethane (3 x 10 ml). The extract was evaporated in vacuo and the residue was subjected to TLC in dichloromethane-methanol (97:3). After formylation and repeated TLC in dichloromethane-methanol, the 11-keto Adione fraction was further purified by recrystallization (see van den Hurk et al., 1982).

28 ovaries from D100 trout were pooled and homogenized in 1 ml 0.1M phosphate buffer (pH 7.4) containing 0.25M sucrose, 0.25 ml homogenate was then incubated with 37 KBq 17αP4-H3 (spec. act.: 396 GBq/mmol) dissolved in 25 µl propyleneglycol, 2mM each of NAD and NADPH and 0.15 ml phosphate buffer for 3h at 20°C. Before extraction, 100 µg each of 17αP4, androstenedione, testosterone, oestradiol-17β and oestrone were added as carrier steroids. Separation of steroids was carried out with TLC in benzene-ethylacetate (3:1). Androstenedione was further purified by crystallization to a constant specific activity.

Results

Steroid and temperature treatments

High percentages of males were observed after treating trout from D47 to D105 with 11βAdione and methyltestosterone (Table 1).

Table 1. Effects of androgens on sex ratio. Number of fish sexed = 100. Period of treatment = D47-105. a = $p < 0.0001$.

Treatment	Dose µg/g	%o	%o
11βAdione	6	94[a]	6
	60	100[a]	-
Methyltestosterone	0.6	99	1
	6	98	2
Control	-	47	53

The highest dose of 11βAdione used, even results in a 100% male population. Except for male fish treated with 6 µg/g 11βAdione, mean testis weight and GSI were strongly decreased in androgen treated trout (Table 2).

Treatment with P4 added to the food did not change a normal 1:1 distribution of males and females. Females treated with 600 µg/g P4 showed a significantly decreased ovarium weight (0.63±0.36 mg; $p = 0.0003$) and mean GSI (0.36±0.20; $p < 0.0001$) at D120 as compared to control values (1.41±0.42 mg and 1.09±0.25, respectively). Females treated with 60 µg/g P4 showed a significantly ($p = 0.01$) decreased mean GSI (0.76±0.25). Mortalities among groups of fish, exposed to steroid or control treatments, varied from 2-10%.

Neither high temperature shocks nor 2 weeks of enhanced temperature from D43 to D57 significantly changed the sex ratio. A shock of 25°C for 3h at D44 and D57 results in a tendency towards more females (59%) and males (61%), respectively. Mortality percentages varied from 10-25%. Mean gonad weight (1.59±0.62 mg; $p < 0.0001$) and mean GSI (0.66±0.47; $p < 0.005$) were significantly decreased at D150 in females kept for 2 weeks at 22-23°C as compared to control values (3.71±1.07 mg; 1.14±0.40).

In vitro experiments

After incubation of D100 testes with 11β Adione-C14, 11-keto Adione was identied as the only formed product. These young testes thus contain 11β-HSD, but lack 17β-HSD to synthesize 11β-hydroxy testosterone or 11-keto testosterone.

D100 ovaries possess 17α,20-desmolase activity, since 17αP4 appeared to be converted into androstenedione. D100 ovaries are not able to synthesize testosterone and oestrogens from 17αP4

Table 2. Effects of androgens on body weight, gonad weight and GSI. Period of treatment D47-105. Values are estimated at D150.

Compound	Dose (µg/g)	Mean body weight(g)	Mean gonad weight(mg)	Mean GSI (°/oo)
Females				
11βAdione	6	2.91±0.79	0.66±0.59[a]	0.25±0.27[a]
Control	-	3.39±0.76	3.71±1.07	1.14±0.40
Males				
11βAdione	6	4.29±1.94[c]	1.86±1.00	0.42±0.09
	60	3.53±0.92	0.69±0.29[b]	0.21±0.10[a]
MT	0.6	3.60±1.03	0.98±0.58	0.27±0.14[a]
	6	3.60±1.56	0.78±0.70[c]	0.20±0.11[a]
Control	-	2.79±0.82	1.46±0.69	0.50±0.18

a = $p < 0.0001$, b = $p < 0.005$, c = $p < 0.03$
MT = methyltestosterone.

Discussion

From previous (van den Hurk et al., 1982a) and present observations it appears that the main difference in steroidogenesis between just developing trout testes and ovaries from D100 is the potency of the testes to synthesize 11βAdione and 11-keto Adione through 11β-hydroxylase and 11β-HSD (Fig. 1). This may indicate that at the time these enzymes are present trout gonads differentiate into testes. 11-keto testosterone and 11β-hydroxy testosterone are not formed in D100 testes, which indicates that these androgens are not involved in gonadal sex differentiation in trout. Synthesis of these 11-steroids has previously been demonstrated in testes of one year old trout (Arai & Tamaoki, 1967).

The present observation that administration of 60 µg/g 11βAdione for 2 months from first feeding results in an all male trout population shows that this 11-steroid exerts a masculinizing effect on gonad differentiation. This finding, and results from in vitro studies, point to 11βAdione as an endogenous gonadal androgen in rainbow trout, which induces the onset of testis differentiation. A similar masculinizing effect was observed after addition of the artificial androgen methyltestosterone. Like the high dose (60 µg/g) of 11βAdione given, but contrary to the low dose (60 µg/g), administration of methyltestosterone (0.6 and 6 µg/g) results in a decrease of testis weight and GSI. Sterile gonads were not found in androgen treated fish. This is in contrast to the findings of Johnstone et al. (1978). Although these authors claimed all male broods after treating trout for 3 months with 3 µg/g methyltestosterone, 17% appeared to have sterile gonads. This might be due to the longer androgen treatment used. Okada et al. (1979) found 60, 80 and 88% males after feeding trout with 1, 5 or 10 µg/g methyltestosterone for 2 months from swim-up, respectively. The difference between the present results and those of Okada et al. is probably due to a difference in food preparation, since we used the method of Johnstone et al. (1978) in which steroid was incorporated into portions of defatted food.

From present and previous (van den Hurk et al., 1982a) in vitro experiments it appears that ovaries from D100 are unable to form oestrogens. This excludes oestrogens from a role as a "gyno-inducer" as suggested by Yamamoto (1969). Possibly it is the absence of enzymes to synthesize steroids with a functional group at the 11-position that enables ovarium development. P4 appeared to have a feminizing effect on the gonads, when added to aquarium water (van den Hurk & Slof, 1981). The relative high dose of P4 administered probably affects gonadal sex differentiation by a blockage of enzymes involved in the synthesis of androgens. Present investigations do not confirm a feminizing effect of P4 when added to the food. It does not appear to be any reason to use higher doses of P4 in future experiments, since the highest dose tested significantly decreased ovarium weight and GSI. P4 taken in by the alimentary tract is possibly more easily subjected to degradation by the liver than when taken up by the gills.

In teleosts, temperature may play an important role in determination of gonadal sex (Bruslé & Bruslé, 1982). From the few investigations reported so far it appears that a period of relatively high temperature may increase the amount of males or females, depending on the species investigated, whereas in some species a period of low temperature enhances the percentage of males. Recently, it has been found that a temporary rise in temperature from 30 to 39°C within 3h at D9 of development results in an all male brood from the African catfish, Clarias lazera (van den Hurk & Richter, unpubl.). In the present study, high temperature, either given as shocks or in a 2 weeks period did not affect the sex ratio in trout. Further studies have to be carried out to learn whether it is possible to influence sex in trout by keeping fish at high temperatures.

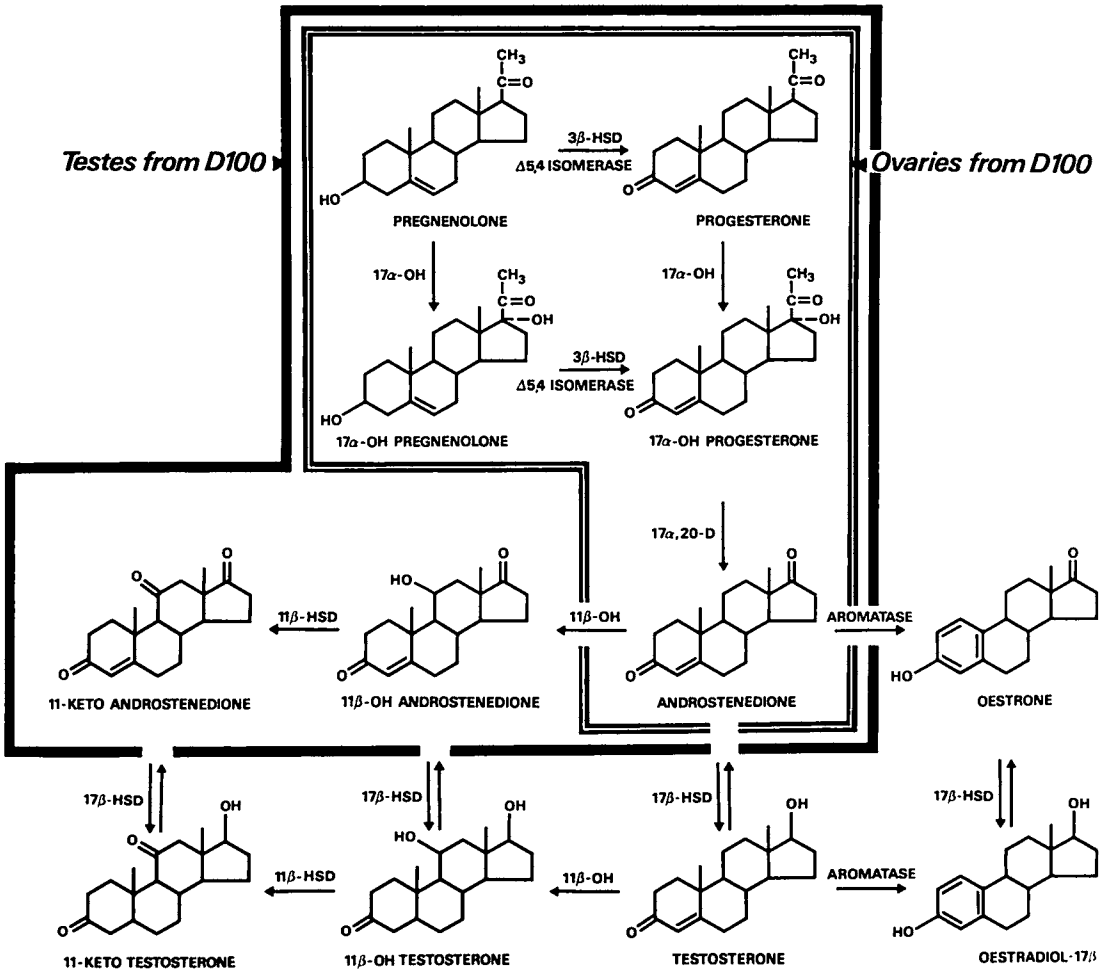

Fig. 1. Steroid biosynthesis in trout gonads from D100.
Enzyme abbreviations: OH = hydroxylase, D = desmolase, HSD = hydroxy steroid dehydrogenase.

References

Arai, R. & B. Tamaoki, 1967. Biosynthesis of 11β-hydroxy testosterone and 11-keto testosterone in the testis of rainbow trout. Can. J. Biochem. 45:1191-1195.

Bruslé, J. & S. Bruslé, 1982. La gonadogenese des poissons. Repr. Nutr. Dev. 22: in press.

Hurk, R. van den, 1982. Effects of steroids on gonadotropic (GTH) cells in the pituitary of rainbow trout, Salmo gairdneri, shortly after hatching. Cell Tiss. Res. 224:361-368.

Hurk, R. van den, J.G.D. Lambert & J. Peute, (1982a). Steroidogenesis in the gonads of rainbow trout fry, Salmo gairdneri, before and after the onset of gonadal sex differentiation. Repr. Nutr. Dev. 22:413-425.

Hurk, R. van den & G.A. Slof, 1981. A morphological and experimental study of gonadal sex differentiation in the rainbow trout. Cell Tiss. Res. 218:487-497.

Hurk, R. van den, G.A. Slof, J.G.D. Lambert, H.J.Th. Goos & P.G.W.J. van Oordt, 1982b. Effects of androgens on the development of the pituitary-gonadal axis in juvenile rainbow trout. Gen. Comp. End. 46: p. 394.

Johnstone, R., T.H. Simpson & A.F. Youngson, 1978. Sex reversal in salmonid culture. Aquaculture 13:115-134.

Okada, H., H. Matumoto & F. Yamazaki, 1979. Functional masculinization of genetic females in rainbow trout. Bull. Jpn. Soc. Sci. Fish. 45: 413-419.

Yamamoto, T., 1969. Sex differentiation, 117-175. In: Fish physiology, Vol. III. Acad. Press, New York & London.

PRACTICAL APPLICATION OF SEX MANIPULATION

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Abstract

Practical application of sex manipulation involves, induced maturation and ovulation, off season-spawning, mono sex culture, and production of sterile fish.

To induce final maturation and ovulation purified gonadotrophins seem to be effective alone or together with other hormones. Gonadotrophins also synchronize spawning time.

Off season spawning can be induced by photo or photothermal regulation, and in salmonids spawning have been obtained 90 days prior to and 220 days after normal spawning peak. This shows the possibilities to obtain fertilized eggs during most of the year, which allow the aquaculturist to develop an integrated processing marketing-program for fresh fish.

Mono sex culture are recognised as a solution to over-population caused by high fecundity. Mono sex culture also have advantage when one of the sexes have superior production traits. Mono sex culture can be produced by admimistration of sex hormone during the time gonads are not differentiated. A disadvantage with this method is that fish distined for human consumption have been fed hormones. This problem can be overcome by sex reversing the homogametic sex and then produce mono sex offspring. Crosses between related Tilapia species can in some cases produce all male population. Induced diploid gynogenesis can produce all female population.

Sterility can be produced by surgical castration, autoimmune castration, administration of high doses of sex steroids, hybridization between species and chromosomal manipulation.

Production of triploid fish by chromosomal manipulation appear to be a suitable technique in many species. However, the production traits in triploids have to be further investigated before the value of triploids in commercial fish farming can be evaluated.

Induced breeding

Induced breeding is essential for species which do not breed in captivity. It involves induction of final oocyte maturation and ovulation in females and induction of spermatogenesis in males (if necessary). Even when fish can breed in captivity and artificial incubation can be controlled, it is practiced to achieve better control of spawning time and fry production. For some species prespawning mortality can become evident. This is spesially a problem for salmonids during the holding period when brood stocks are taken from wild stocks. Induced maturation and ovulation can than reduce the prespawning mortality and thereby increase the egg takes. (Hunter et al. 1979).

The use of hormones to induce spawning has been widely practiced spesially in carps. Recent reviews include Chaudhuri (1976), Fontaine (1976) and Lam (1982). Today purified gonadotrophins are commercially available (Syndel Laboratories Ltd) and its effect to induce ovulation when given alone or together with other hormones are clearly shown in pasific salmon (Hunter et al. 1978, 1979, 1981, Donaldson et al. 1981, Donaldson et al. 1981 and Jalabert 1978, Atlantic salmon (Gjerde unpublished), rainbow trout (Gjerde unpublished) and Walleye (Lessman 1978)

Hormone injections also synchronize ovulation in large groups of animals which often are important when carrying out a selection program.

Disadvantages with hormone injection is extra laboure and stress caused by handling of fish. The commercially produced gonadotrophins is also relatively expensive, and it seems appropriated to look for

cheaper methods to induce ovulation and spawning.

Off season spawning

In many species off-season production of fertilized eggs would prove beneficial to aquaculturist and fisheries research. When producing pen-size fish schedual spawning would allow the fish farmer to develope an integrated processing marketing program for fresh fish. For research purpose, off-season production of fertilized eggs would give fish of standard weight or age several times during the year.

Off season-spawning can be induced in many species by regulation of photoperiod, photothermal treatment or photothermal treatment together with hormon administration. In salmonids regulation of photoperiod have shown to be effective for brood trout (Allison, 1951, Hazard and Eddy, 1951, Corson, 1955. Henderson, 1963, Carlson and Hale, 1973), coho salmon (MacQuarrie et al. 1978) pink salmon (MacQuarrie et al. 1979) and rainbow trout (Whitehead et al. 1978).

In coho salmon spawning has been obtained as much as 90 days prior to normal spawning (MacQuarrie et al. 1978), and in pink salmon spawning has been obtained 220 days after normal spawning (MacQuarrie el al. 1979).

This shows the possibilities to obtain fertilized eggs during most of the year in salmonids simply by regulation of photoperiod. However, the broodstock have to be kept in buildings were normal light can be excluded for one year minimum.

Photothermal treatment have produced precociously gravid catfish as early as April, and spawning was induced by administration of luteinizing hormone (LH), (Sundaraj and Vasal 1976). The normal spawning season was July-August. The process was repeated and the same set of fish spawned four times between April and July the same year and gave 2-6 times the number of eggs contained from untreated spawners. This means that higher numbers of eggs can be colledted from selected females, and will improve the selection intensity in a breeding scheme.

Mono sex culture

Mono sex culture has been recognized as the best solution to overpopulation caused by high fecundity, which is a main problem in Tilapia under almost any pond condition (Hickling, 1963). Mono sex culture also have advantages when one of the sexes have a better growth rate or a higher marketing value.

In fish, as in other animals, sex is genetically determinded, however, many species are not clearly differentiated into either sex at hatching. Sex reverse can be induced by administration of sex hormone at early stage of life. This has been reported for rainbow trout, Atlantic salmon, Coho salmon, Tilapia and goldfish (for review see Yamamoto, 1969, Schreck, 1974, Lam, 1982, Donaldson and Hunter, 1982). A disadvantage of technique is that all fish require treatment and fish destined for human consumption will have been fed hormons. When females are homogametic sex this problem can be overcome by producing fenotypic males from genetic females and mate them with normal females. This will give all female population without hormone treatment of the slaughtering fishes. The problem might be to distinguish between sex reversed genetic females (fenotypic males) and normal males.

In Tilapia mono sex male culture have been produced by crossing T. nilotica, T. aurea. (Pruginin et al. 1975).

Artificial gynogenesis have been induced in many species (Purdom, 1969, Stanly, 1976, Chourrout, 1980, Refstie et al. 1982) and will produce mono sex female populations when the female are the homogametic sex. However, the gynogenetic fish are higly inbreed, and will probably suffer from significant inbreeding depression (Aulstad and Kittelsen, 1981, Kincaid, 1976).

Sterile fish

Sterile fish have many advantages in fish farming. Problems with overpopulation caused by high fecundity and loss of growth and quality during maturation and spawning, can be avoided. For salmonids maturity also set an upper limit for size and slaughtering time.

Sterile fish can be produced by irradiation, chemosterilization, surgical techniques, immunological tech-

niques, hormone administration, hybridization and chromosome manipulation (for review see Stanly, 1978, and Donaldson and Hunter, 1982).

Irradiation claim placement of fish in water containing an isotope or injection of fish with an isotope and would not be acceptable unless the isotope had a very short half-life and could be used under controlled conditions. Chemosterilization also posess problems when the fish are used for human consumption and the effects are often temporary. These methods are therefore not likely to be practical procedures in fish farming.

Surgical castration is labor intensive and costly, and the fish can not be operated on until the gonads are of sufficient size. This ruling out its use in ocean ranching, and it is probably also too labor intensive for use in practical fish farming.

Little work has been conducted with immunological techniques in fish, and although some promising results are reported (Laird et al. 1978, 1981), more research is needed before this technique can be used in practical fish farming.

Hybridization between species often results in sterility in fish (Buss and Wright, 1957, Suzuki, 1974, MacCrimmon et al. 1974). Hybrids between salmonid species ordinary have low hatchability (for review see Chevassus, 1979). Refstie (1981) reared hybrids between four salmonid species together with their parent species in seawater and concluded that this hybrids was of no advantage in fish farming in seawater. Today it does not look as if production of sterile species hybrids have any advantages in practical fish farming.

Administration of relatively high doses of sex hormones at early stages of life, have produced sterile fish in many species and can easely be applied to fish farming conditions. However, when fish are destined for human consumptions, this method has the same disadvantages as using sex hormones to induce sex reverse.

Triploid fishes may be totally or partially sterile. Tripoloidy have been induced by heat shock in flatfish (Purdom, 1972) Salmonids (Chorrout, 1980, Thorgard and Jazwin, 1981, Holmefjord et al. 1982), Channel Catfish (Walters et al. 1981) and Carp (Ojima and Makino, 1978), and today techniques for production of high numbers of triploid salmonids by heat shock are available.

An alternaltive method to heat shock for production of triploid salmonids is productions of a tetraploid strain (Thorgaard et al. 1981, Refstie, 1981, Standly and Allen, 1979). Tetraploid fishes are expected to be fertile and will give triploid fish when back crossed to diploids.

Lincoln (1981) reports that fully motile spermatoza were produced from several triploid plaice. Thorgaard and Gall (1979) report that six triploid rainbow trout found in a full-sib family were normal in size and external appearance, and have undeveloped gonads.

More research is needed to study the degree of sterility, and production traits of triploids have to be investigated before their value in fish farming can be evaluated. However, the results so far are promising and production of sterile fish by chromosome manipulation have not the disadvantages as administration of sex hormones have, when the fish are destined for human consumption.

References

Allison, L.N. 1951. Delay of spawning in eastern brook trout by means of artificial prolonged light intervals. Prog. Fish-Cult., 13: 111-116.

Aulstad, D, and Kittelsen, A.,1971. Abnormal body curvatures of rainbow trout (Salmo gairdneri)inbred fry. J.Fish Res. Bd. Canada 28: 1918-1920.

Buss, K.W. and Wright, J.E. Jr.,1957. Appearance and fertility of trout hybrids. Trans. Am. Fish Soc. 87: 172-181.

Carlsson, A.R. and Hale, J.G. 1973. Early maturation of brook trout in the laboratory. Prog. Fish-Cult. 35: 150-153.

Chaudhuri, H., 1976. Use of hormones in induced spawning of carps. J. Fish.Res. Bd. Canada 33:940-947.

Chevassus, B. 1979. Hybridization in salmonids: results and perspectives. Aquaculture 17: 113-128.

Chourrout. D., 1980. Thermal induction of diploid gynogenesis and triploidy in the eggs of the rainbow trout (Salmo gairdneri Richardson). Reprod. Nutr. Dev. 20:727-733.

Corson, B.W., 1955. Four years progress in the use of artificially controlled light to induce early spawning of brook trout. Prog. Fish-Cult. 17:99-102.

Donaldson, E.M., Hunter, G.A. and Dye, H.M., 1981. Induced ovulation in coho salmon (Oncorhynchus kisutch). II. Preliminary study of the use of LH-RH and two high potency LH-RH analogues. Aquaculture 26:129-141.

Donaldson, E.M. and Hunter, G.A., 1982. Sex control in fish with particular reference to salmonids. Can. J.Fish Aquat. Sci. 39:99-110.

Fontaine, M., 1976. Hormones and the control of reproduction in aquaculture. J.Fish Res. Bd. Canada 33:922.939.

Hazard, T.P. and Eddy, R.E., 1951. Modification of the sexual cycle in brook trout (Salvelinus fontinalis) by control of light. Trans. Am. Fish Soc. 80:158-162.

Henderson, N.E., 1963. Influence of light and temperature on the reproductive cycle of the eastern brook trout, (Salvelinus fontinalis) (Mitchill). J.Fish Res. Bd. Canada 20: 859-897.

Hickling, C.F., 1963. The cultivation of Tilapia. Sci. Am. 208: 143-152.

Holmefjord, J., Refstie, T. and Høstmark, J., 1982. Preliminary results of induction of triploidy in salmonids using heat shocks. 33rd Annual Meeting of the EAAP, Commission of Animal Genetics, Leningrad 16-19 August 1982.

Hunter, G.A., Donaldson, E.M., Stone, E.T. and Dye, H.M., 1978. Induced ovulation of female chinook salmon (Oncorhynchus tshawytscha) at a production hatchery. Aquaculture 15: 99-112.

Hunter, G.A., Donaldson, E.M., Dye, H.M. and Peterson, K., 1979. A preliminary study of induced ovulation in coho salmon (Oncorhynchus kisutch) at Robertson Creek Salmon Hatchery. Fish Mar. Serv., Technical Report 899; 15 p.

Hunter, G.A., Donaldson, E.M. and Dye, H.M., 1981. Induced ovulation in coho salmon (Oncorhynchus kisutch). I. Further studies on the use of salmo pituitary preparations. Aquaculture 26: 117-127.

Jalabert, B., Goetz, F.W., Bretan, B. Foster, A. and Donaldson, E.M., 1978. Precocious induction of oocyte maturation on coho salmon (Oncorhynchus kisutch). J.Fish Res. Bd. Canada 35: 1423-1429.

Kincaid, H.L., 1976. Inbreeding in rainbow trout (Salmo gairdneri) J. Fish. Res. Bd. Canada 33: 2420-2426.

Laird, L.M., Ellis, M.A., Wilson, A.R. and Holliday, F.G.T., 1978. The development of gonadal salmon (Salmo salar L.) and a consideration of the possibility of inducing autoimmune destruction of the tesis. Ann. Biol. Anim. Biochem. Biophys. 18:1101-1106.

Laird, L.M., Wilson, A.R. and Holliday, F.G.T., 1981. Field trials of a method of induction of autoimmune gonad rejection in Atlantic salmon (Salmon salar L.) Reprod. Nutr. Dev. 20: 1781-1788.

Lam,T.J., 1982. Applications of endocrinology to fish culture. Can. J. Fish Aquat. Sci. 39: 111-137.

Lessman, C.A.,1978. Effects of gonadotrophin mixtures and two steroids on inducing ovulation in the Walleye. Prog. Fish-Cult. 40:3-5.

Lincoln, R.F., 1981. Sexual maturation in triploid male plaice (Pleuronectes platessa) and plaice x flounder (Platichthys flesus) hybrids. J. Fish. Biol. 19:415-426.

MacCrimmon, H.R., Stewart, J.E. and Brett, J.R. 1974. Aquaculture in Canada. The practice and promise. Bull. Fish. Res. Bd. Canada 188: 1-84.

MacQuarrie, D.W., Markert, J.R. and Vanstone, W.E., 1978. Photoperiod induced off-season spawning of coho salmon (Oncorhynchus kisutch). Ann. Biol. Anim. Biochem. Biophys. 18: 1051-1058.

MacQuarrie, D.W., Vanstone, W.E. and Markert, J.R., 1979. Photoperiod induced off-season spawning of pink salmon (Oncorhynchus gorbuscha) Aquaculture 18: 289-302.

Oijma, Y. and Makino, S. 1978. Triploidy induced by cold shock in fertilized eggs of the carp. Proc. Japan Acad. 54. Ser. B: 359-362.

Pruginin, Y. Rothbard, S. Wohlfarth, G., Halevy, A., Moav, R. and Hulata, G., 1975. All-male broods of Tilapia nilotica x T. aurea hybrids. Aquaculture 6:11-21.

Purdom, C.E. 1969. Radiation - induced gynogenesis in fish. Heredity, London 24: 431-444.

Purdom, C.E. 1972. Induced poliploidy in plaice (Pleuronectes platessa) and its hybrid with the flounder (Platichthys flesus). Heredity, London 29:11-24.

Refstie, T. 1981. Tetraploid rainbow trout produced by cytochalasin B. Aquaculture 25:51.58.

Restie, T., Stoss, J. and Donaldson, E.M., 1982. Production of all-female coho salmon (Oncorhynchus kisutch) by diploid gynogenesis using irradiated sperm and cold shock. Aquaculture (in press).

Shreck, C.B., 1974. Hormonal treatment and sex manipulation in fishes. P. 84-106. In C.B. Schreck (ed.) Control of sex in fishes. Extension Division, Virginia Polytechnic Inst. and State Univ. Blacksburg, V.A.

Stanley, J.G. 1976. Production of hybrid, androgenetic and gynogenetic grass carp and carp. Trans. Am. Fish Soc. 105: 10-16.

Stanley, J.G., 1978. Control of sex in fishes with special references to checking reproduction in grass carp. Proceedings of the International Grass Carp Conference, Gainesville, Florida, January 9-12, 1978: 42 pp.

Stanley, J.G. and Allen, S.K. jr. 1979. Polyploid mosaics induced by cytochalasin B in landlocked Atlantic salmon (Salmo salar). Trans. Am. Fish. Soc. 108: 462-466.

Sundararaj, B.I. and Vasal, S., 1976. Photoperiod and temperature control in the regulation of reproduction in the female catfish, (Heteropneustes fosilis) J.Fish Res. Bd. Canada, 33: 959-973.

Suzuki, R. 1974. Inter-crossings and backcrossing of F_1 hybrids among salmonid fishes. Bull. Freshw. Fish. Res. Lab. 24:11-32.

Thorgaard, G.H. and Gall, G.A.E. 1979. Adult triploids in a rainbow trout family. Genetics 93: 961-973.

Thorgaard, G.H., Jazwin, M.E. and Stier, A.R., 1981. Polyploidy induced by heat shock in rainbow trout. Trans. Am. Fish Soc. 110: 546-550.

Walters, W.R., Libey, G.S. and Chrisman, C.L., 1981. Induction of triploidy in channel catfish. Trans. Am. Fish Soc. 110-312.

Whitehead, C., Bromage, N.R. and Forster, J.R.M., 1978. Seasonal changes in reproductive function of the rainbow trout (Salmo gairdneri). J. Fish Biol.12:601-608.

Yamamoto, T., 1969. Sex differentiation. In W.S. Hoar and D.J. Randall (eds.). Fish Physiology, Vol 3: 117-175. Academic Press, New York, N.Y.

THE OCEAN RELEASE AND CONTRIBUTION TO THE FISHERY OF ALL-FEMALE AND STERILE GROUPS OF COHO SALMON (ONCORHYNCHUS KISUTCH)

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Introduction

The application of sex control techniques has the potential to maximize economic returns from the sea ranching of Pacific salmon. The production of all-female groups would increase the landed value of the catch by maximizing the proportion of fish containing roe. Sterilization provides a means of inhibiting the normal propensity for anadromous migration, thereby increasing the average size of the fish in the catch and extending the period that the fish are available to the fishery. Both sex control techniques eliminate precocious males. This study presents the results from the first ocean release of sterile and all-female groups of salmon.

Materials and Methods

Two groups of approximately 40,000 coho salmon were treated with either estradiol or methyltestosterone as eyed eggs, alevins and fry at the Capilano Salmon Hatchery. Treatment of the eyed eggs was conducted by immersion in water containing the steroids at 200 µg/l. Two treatments of 2 hr duration were applied 7 days apart. Alevins were treated similarily. Fry were fed for the first 90 days a diet containing either 5 mg/kg estradiol or 10 mg/kg methyltestosterone. Both groups were nose tagged and fin clipped at 6 months of age. Before release, a sample of 1,000 from each group were transferred to a seawater net pen to monitor gonadal morphology, survival and growth. Contribution to the fisheries was determined from nose tag recoveries and to the hatchery by fin clips.

Results

The estrogen and androgen treated groups contained > 97% female and sterile fish, respectively. Both groups survived at similar rates until maturation of the estradiol group. Sterile fish grew at a slower rate than female fish but unlike the females, continued to grow through the period of sexual maturation. As a result, at female maturity the carcass weights were similar in the two groups. Furthermore, the sterile fish did not display the flesh deterioration associated with sexual maturation.

To date, a total of 1007 tags have been recovered from the fishery. Both groups contributed to the commercial fisheries at similar rates, however, the sterile group contributed at a lower rate to the recreational fishery. In the fall of 1981, the all-female group returned to the hatchery as expected. Returning females from the treated group were similar in size and gonadosomatic index to untreated females. No sterile fish returned to the hatchery, however, a small number of fish which contained maturing single or partial testes did return.

Discussion

The results indicate that it is feasible on a production basis to produce essentially 100% female and sterile groups of coho salmon by steroid treatment in the early life stages. Further, fish treated in this manner maintain their altered gonadal phenotypes. The somewhat slower growth seen in sterile fish may be compensated for by continued growth and maintenance of high flesh quality in the sterile fish during and beyond the period of normal sexual maturation.

The tag data shows that both groups survived after release into the ocean and contributed significantly to the fishery. Interpretation of the differential contribution to the recreational fishery is not possible based on present data. The return to the hatchery of substantial numbers of phenotypically normal, exclusively female fish from the estradiol group demonstrates the feasibility of this treatment for enhancing the proportion of females in the commercial catch. To produce an all-female group suitable for broodstock purposes it would be necessary to use the alternate techniques described by Donaldson and Hunter (1982). Perhaps the most significant biological finding from this study is the demonstration that sterilization eliminates the propensity of the coho salmon to undergo its anadromous migration. The evaluation of the total contribution of the sterile fish to the fishery will await tag returns in subsequent years.

Reference

Donaldson, E.M. and G.A. Hunter, 1982. Sex control in fish with particular reference to salmonids. W.S. Hoar Symposium, Fish Reproduction: Behavior and Endocrinology. Can. J. Fish. Aquat. Sci., 39: 99-110.

THE PRODUCTION OF GENETICALLY ALL-FEMALE ATLANTIC SALMON STOCKS

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The attainment of sexual maturity is a major constraint on Atlantic salmon cultivation. In the fresh water rearing phase, precocious sexual maturity in males entering their second winter is associated with increased disease and mortalities. In the sea water rearing phase, maturity of adult fish marks the end of the period in which they may be usefully reared and often occurs at body weights which are undesirably or unacceptably low. In salmonids generally, male fish become mature at a younger age than do females. As a consequence of this, advantages might be expected to follow from growing only female stocks.

By treating fish shortly after first feeding with dietary 17β-oestradiol, it has proved possible to over-ride the normal expression of genetic sex in genotypically male fish in both salmon and rainbow trout (Johnstone et al., 1978). Groups of fish successfully treated in this way prove to be entirely female, with apparently normal gonads, and have the deferred maturation characteristics of the female type (Johnstone et al., unpublished results).

Oestrogen treatment is not always fully effective however, and normal male fish and fish with variable hermaphroditic gonads may occur. Such animals tend to retain the temporal pattern of sexual development typical of males (Johnstone et al., unpublished results).

An alternative method of producing all-female stocks exists. The normal genetic expression of sex in female rainbow trout may be overridden by exposure to dietary androgen resulting in the fish having gonads in which testicular elements predominate. In treated mixed-sex stocks, genetically female fish can be identified at maturity by their hermaphroditism or by the absence of patent sperm ducts. The genetic constitution of rainbow trout is such that when crossed with normal female fish, they yield all-female progeny (Johnstone, et al., 1979a; Bye and Lincoln, 1981). This technique has now been validated for Atlantic salmon.

In May 1979 a group of Atlantic salmon fry were fed a diet containing 17α-methyl-testosterone at 3 mg/kg diet for the first 60 days of feeding. Thereafter they were fed normally. At one year of age, a proportion of the stock (10%) became smolts and were transferred to sea water. As the remaining fish approached their second winter in fresh water (November 1981), they were classified as immature (80%) or precociously mature (20%) individuals (characterised by their darker colouration, more obvious parr marks and distended genital papillae). Mature fish were further categorised as those which gave milt by manual expression (14%) or as those which failed to do so (6%). Upon sacrifice and visual inspection of the gonads, the latter group was seen to contain animals with a range of testicular types varying from lobed testes lacking ducts to more normal testes blocked at the genital papilla.

Lobed testes from five individual animals were dissected out, chopped and placed in modified Cortland extender (1hr at 10°C) and the resulting milt solutions were used separately to fertilise the ova from several normal salmon females. Fertilisation rates were excellent and subsequent survivals to the eyed-egg stage were good (>95%). First feeding mortalities were satisfactory (<5%) in three out of the five groups, but were higher (20% and 41%) in the two other groups.

After eight weeks of feeding (July 1982), the gonads of 50 fish in each egg batch were inspected microscopically (x 25) following fixation in Bouins fluid. All animals examined were female. The sex ratio in a control group of 42 fish was 1.1 ♂ : 1.0 ♀. These results demonstrate that, as in rainbow trout, the Atlantic salmon female is homozygous for sex (XX).

The performance of the all-female progeny of precociously mature males will be compared, in future years, with the performance of all-females generated from sex-reversed male parents maturing relatively later in the rearing cycle.

References

Johnstone, R. et al., 1978. Aquaculture, 13, 115-134.

Johnstone, R. et al., 1979a. Aquaculture, 18, 13-19.

Bye, V. & Lincoln, R. 1981. Fish Farmer 4(8), 22-24.

MONOCLONAL ANTIBODIES IN THE STUDY OF GONADAL DIFFERENTIATION IN THE CARP (CYPRINUS carpio L.)

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Summary

Monoclonal antibodies (MA) have been raised against spermatozoa of carp, in order to study the appearance of specific antigens during gonado- and spermatogenesis in fish. After characterizing the antisera obtained, using immunohistochemical and immunological methods, eleven MA appeared to define cell membrane determinants specific for spermatogenic cells. These MA are expected to form a handsome tool in the study of cytodifferentiation during spermatogenesis in fish, and might be used to induce male sterility in carp.

Introduction

Studies on mammalian spermatogenic cells have shown that the membranes of spermatozoa, spermatids and spermatocytes contain determinants normally not present on somatic cells, whereas spermatogonia do not (Millette, 1979). In these studies it was concluded that the blood testis barrier formed by Sertoli cells provides an environment in which specific spermatogenic antigens may appear. These antigens are probably involved in cellular interactions and the process of cytodifferentiation in the mammalian testis.

MA derived from individual hybrid cell lines provide highly specific reagents, which can be used in the study of the membrane composition of individual cell types. Recently, Bechtol et al. (1979) and Gaunt (1982) succeeded in defining differentiation antigens of spermatogenesis in the mouse with MA.

In fish, little is known about the process of cytodifferentiation in the testis. However, it is likely that, here too, specific spermatogenic antigens are present, because Sertoli cells have been demonstrated in several species (Grier, 1981).

To obtain evidence of such antigens, MA have been raised against mature spermatozoa of carp.

Results and conclusions

Hybridomas were obtained from a fusion between cells of the mouse myeloma NS1 and splenocytes from Balb/c mice hyperimmunized with carp spermatozoa. The MA obtained were characterized in several tests as shown in table 1.

Table 1. Characteristics of the monoclonal antibodies specific for carp spermatozoa[1)]

MONOCLONAL ANTIBODIES	ELISA on SPERM	IMMUNOFLUORESCENCE smears of SPERM	IMMUNOFLUORESCENCE frozen sections TESTIS	HEAVY CHAIN TYPE 3)	AGGLUTINATION TITER 4)	COMPLEMENT CYTOTOXICITY LYSIS TITER 4)
WCS 1	-	-	+ 0[2)]	γ_{2a}	6	4
WCS 3	+	+	+ 0	γ_3	8	4
WCS 7	-	+	+ 0	μ	7	4
WCS 11	-	+	+ 0	μ,γ_{2a}	8	4
WCS 12	-	+	+ 0	μ,γ_{2a}	6	4
WCS 14	+	+	+ 0	γ_{2b}	6	4
WCS 16	-	+	+ -	-	6	4
WCS 17	+	+	+ 0	μ	5	2
WCS 27	-	+	+ 0	μ,γ_{2b}	5	5
WCS 28	+	+	+ 0	γ_3,γ_{2a}	11	3
WCS 29	+	+	+ 0	μ	11	3

1) before subcloning of the hybridomas
2) precursor cells
3) determined with anti-mouse Ig heavy chain antisera in an Ouchterlony double diffusion test
4) 2-log dilution

Eleven MA reacted with spermatozoa, and except for MA WCS 16, also with spermatogenic cells. However, none of the MA reacted with somatic tissue, i.e. gut, liver, brain, spleen and kidney as determined on frozen sections.

All MA belong to the mouse IgG- or IgM-class, and are able to agglutinate and, in the presence of complement, lyse mature sperm cells. This latter property suggests that the MA might be used to kill spermatogenic cells of carp in vivo, and so to induce male sterility.

References

Bechtol, K.B., S.C. Brown & P.H. Kennett, 1979. Recognition of differentiation antigens of spermatogenesis in the mouse by using antibodies from spleen cell-myeloma hybrids after syngeneic immunization. Proc. Natl. Acad. Sci. USA 76: 363-367.

Gaunt, S.J., 1982. A 28-K Dalton cell surfaca auto antigen of spermatogenesis: characterization using a monoclonal antibody. Dev. Biol. 89: 92-101.

Grier, H.J., 1981. Cellular organisation of the testis and spermatogenesis in fishes. Amer. Zool. 21: 345-357.

Millette, C.F., 1979. Cell surface antigens during mammalian spermatogenesis. Curr. Top. Dev. Biol. 13: 1-29.

FISH STERILISATION: THE AUTOIMMUNE APPROACH

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Recent work at Aberdeen University has shown that it is possible to induce an autoimmune reaction in Atlantic salmon (Salmo salar) leading to degeneration of either testis or ovary. This is proposed as a possible method for sterilising large batches of fish on farms. The processes involved in this response are now being investigated using rainbow trout (Salmo gairdneri) and Atlantic salmon as laboratory models. Clearly a cellular response is involved, as seen in histological studies (Laird et al., 1978, 1980), and it is important to know if a humoral (antibody) response also occurs. To this end sperm agglutination tests have been performed in the present study on serum from gonad injected trout.

Methods

Rainbow trout, weighing 35-40 g, were injected intraperitoneally with allogeneic gonad material (testis or ovary plus testis) emulsified in Freund's complete adjuvant (FCA). Each fish received 0.1 ml of a 50% gonad solution mixed with an equal volume of adjuvant. Fish were kept at the ambient temperature, 12-17°C, during the course of the experiment. Blood samples were taken 4, 6 and 8 weeks after immunisation. Sera were decomplemented at 50°C for 30 min. before use. Sera were serially diluted in Baker's buffer pH 8.1 (166 mM glucose, 34 mM NaCl, 17 mM Na_2HPO_4 and 1 mM KH_2PO_4) in microtitre plates. To each well was added 50 ul of fresh trout spermatozoa, adjusted to 4 x 10^7 cell/ml Baker's buffer. After 3 hours a sample was taken from each well and examined for sperm agglutination.

Results and discussion

Titres of 1:32 and 1:64 were reached as early as day 28 in the mixed gonad injected group, and the mean titres (± standard deviation) were 3.63 ± 1.92, 0.75 ± 0.50 and 3.25 ± 1.89 on days 28, 42 and 56 respectively. The mean titres for the testis injected group were 2.25 ± 1.16, 2.25 ± 1.26 and 2.25 ± 1.50. About 50% of the control FCA injected fish responded, compared to almost 100% of gonad injected fish. Mean titres were 1.50 ± 1.31 on day 28 and 3.00 ± 3.00 on day 56. A phosphate buffer injected group had significantly lower aglutination titres than all other groups at all times.

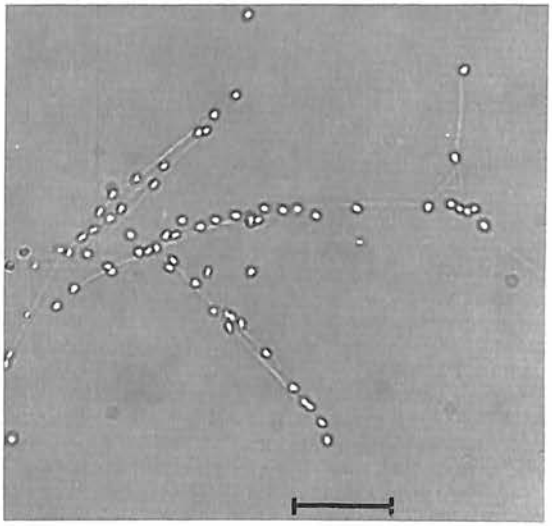

Fig. 1. Sperm agglutination seen after incubation with serum from a trout immunised 42 days previously with testis plus FCA. Bar = 20 µm.

This detection of sperm agglutinins is the first observation of possible auto-antibodies in fish. Agglutination occurred along the whole length of the sperm (Fig. 1). Indications that a humoral immune response is involved come from preliminary immunofluorescence studies using rabbit anti- trout immunoglubulin antiserum, showing the observed sperm agglutinins to be immunoglobulin sperm agglutinins. The detection of sperm agglutinins in the sera from some of the fish injected only FCA is in agreement with previous histological studies where an occasional fish was found to have a surface granuloma on the gonad. It now remains to be elucidated if the reason for this response is the route of injection, possibly allowing FCA to come into contact with the animals own gonad.

A major programme is now underway to evaluate this technique as a tool for the fish farming industry.

References

Laird, L.M., A.E. Ellis, A.R. Wilson & F.G.T. Holliday (1978). The development of the gonadal and immune systems in the Atlantic salmon (Salmo salar L.) and a consideration of the possiblity of inducing autoimmune destruction of the testis. Ann. Biol. anim. Bioch. Biophys. 18, 1101-1106.

Laird, L.M., A.R. Wilson & F.G.T. Holliday (1980). Field trials of a method of induction of autoimmune gonad rejection in Atlantic salmon (Salmo salar L.). Reprod. Nutr. Develop. 20, 1781-1788.

MONOSEX GRASS CARP PRODUCTION THROUGH BREEDING SEX-REVERSED BROODSTOCK

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Summary

Monosex grass carp provide a viable alternative aquatic plant control. Using a monosex population is not an infallible means of preventing reproduction as individuals are fertile, but it can be used in areas where grass carp have not been stocked. Further, this protocol should be applicable to other exotic species with similar reproductive biology.

Monosex grass carp can be obtained by induced gynogenetic fertilization (Stanley 1976). Female grass carp are presumed to be homogametic for sex determination (XX); therefore, all gynogenetic grass carp should be females. But the yield of viable offspring is insufficient to be practical.

To develop a program of monosexing that could meet large demands, we have studied functional sex reversal with methyltestosterone (MT). The sex-reversed fish are phenotypic males but genotypic females (XX) and when bred to normal females (XX), should produce only XX-progeny.

We have described the gonadal differentiation for grass carp and have developed an extended release capsule (Shelton & Jensen 1979). This capsule was implanted in the body cavity of grass carp of appropriate size and the treated fish were then stocked in ponds during the period of hormone release. Only female (XX) were treated, so at maturity, any males in the population would be considered to have been functionally sex reversed.

Two sizes of implant (5 & 12 mg) have been tested, both were effective but the smaller one permitted treating fish earlier in gonadal differentiation. We have had the greatest success using 80-110 mm, 10 to 16-week-old fish, then rapidly growing them to about 180-200 mm.

Fish were treated by this method in 1977, 1978, and 1979. During 1980, the first sex-reversed male matured and was used in the initial spawning test (Shelton 1982). About 160,000 eggs were fertilized with milt from the sex-reversed male (XX-"♂"); 380,000 eggs from the same female were fertilized with milt from a normal male (XY). There was no difference in development of the two groups; about 85,000 fry hatched in the experimental group; of about 500 that have been sexed; all were females. An additional 8,000-10,000 presumed monosex fish are being grown.

During 1981, 12 more sex-reversed males from the 1978 and 1979 year classes had motile spermatozoa, and several were used in a second spawning test. This year, 650,000 eggs were fertilized with milt from sex-reversed males. A total of about 250,000 fry were stocked in ponds for later examination.

Fish from the 1980 and 1981 year classes (presumed all-female) were treated with MT implants; later they will be examined as potential broodstock. Sex-reversed males may be available from the 1980 year class in 1982.

The mechanism of sex determination in fishes is unresolved, thus it is possible that some males may be produced in this breeding program. With this caveat, if no males are found in the presumed all-female populations, application of the approach can be considered. Relatively few males are required as the full reproductive potential of normal females is used.

References

Shelton, W. L., 1982. Production of reproductively limited grass carp for biological control of aquatic weeds-- Phase II. Off. Water Res. & Tech., USDI, Water Resour. Res. Inst. Bull. 45, Auburn Univ.

Shelton, W. L. & G. L. Jensen, 1979. Production of reproductively limited grass carp for biological control of aquatic weeds. Off. Water Res. & Tech., USDI, Water Resour. Res. Inst. Bull. 39, Auburn Univ.

Stanley, J. G., 1976. Production of hybrid, androgenic, and gynogenetic grass carp and carp. Trans. Am. Fish. Soc. 105:10-16.

Stanley, J. G. & A. E. Thomas, 1978. Absence of sex reversal in unisex grass carp fed methyltestosterone. In: R. O. Smitherman, W. L. Shelton, & J. H. Grover (Eds): Culture of exotic fishes symposium proceedings. Fish Cult. Sect., Am. Fish. Soc., Auburn AL. Pages 194-199.

PHEROMONES IN TELEOST FISH

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Summary

Pheromones control a number of social and sexual interactions in teleost fish and their interindividual integrative function can be regarded as an extension of the intercellular communication mode. Actually, ectocrine or pheromone-releasing cells represent the centrifugal component of the chemorecepto-signaling system which processes the chemical information flux through the organism. In this paper, the recent literature on fish pheromones is systematically reviewed and available data about their origin and chemistry are critically discussed. Integumentary peptides are suggested to mediate pheromonally conspecific recognition and aggregation whereas testicular or ovarian androgens in conjugated form might play the role of sex attractants in different species.
Key words: teleost, pheromone, behaviour, reproduction, growth.

Introduction

Teleost fish rely upon a complex multisensory communication system for their social and sexual interactions. The balance between physical (visual, auditory, hydrodynamic, tactile and bioelectric) vs. chemical signals (olfactory and gustatory) involved in intraspecific communication may vary in different species and even between sexes of the same species (Crapon de Caprona, 1982). This relates to both the transmission characteristics (speed, directionality and attenuation) of a given signalling output and the discriminatory potential of the corresponding sensory apparatus in the fish natural habitat.

For instance, although a number of teleosts can emit and perceive sounds of low frequency, predominantly below 1 KHz, their sonic exchanges may be hampered by high levels of ambient noise in the same frequency range originating from wave motion, friction by water currents, waterfalls, wind or from other soniferous species including man with his traffic and industry (Myrberg, 1978). Moreover, despite the fast and efficient propagation of acoustic energy in water, fishes can locate a sound source only in the near field, presumably by means of the lateral line (Tavolga, 1971).

Visual stimuli have the advantage of being immediate, directional and highly informative but their effective range is much shorter in water than in air depending upon turbidity and light quenching with depth. An even greater proximity or direct contact is needed for bioelectric (Hopkins, 1981), hydrodynamic (Tavolga, 1971) and tactile interactions.

The organs which, in fish, generate or detect physical signals are often highly sophisticated and specialized and their rapid, close-range mode of action contrasts with that characterizing the interchange of chemical messengers. In particular, odorous signals propagate slowly in the water, according to their speed of molecular diffusion and transport by currents, but their source can be traced by fish from very long distances, as during homing (Johnsen & Hasler, 1980), if they are associated with a differential rate of water flow allowing orientation by rheotaxis (Hara, 1971). In the absence of the directional clue inherent in a current, fish can still search along a concentration gradient by exploratory strategies that are more complex than true osmotropotaxis (Timms & Kleerekoper, 1972). Taste too can guide some fish to food by sensing chemical cues, even if released in still water (Hara, 1971). The use of gustatory receptors to reveal semiochemicals from conspecifics has been advocated for the response of the male of cave-dweller poeciliids to the female sex attractant (Parzefall, 1973) and in the case of the mouthing of the male's genital papilla by the female of Tilapia macrochir during courtship (Crapon de Caprona, 1976).

From an evolutionary viewpoint, chemoreception, with its extreme sensitivity and rather conservative traits, was probably perfected earlier than the perception of physical stimuli, being the dominant sense in the less organized aquatic invertebrates and unicellular organisms. Its role in intraspecific communication of metazoa can be regarded as either a specialized adjunct to its function in environmental scanning and interspecific recognition in prey-predator and host-parasite /symbiont relationships (Kittredge, 1974) or, rather, as the consequence of an interindividual extension of the intercellular communication mode which is chemically-mediated. This process must have occurred repeatedly and independently in different taxa when there was a selective advantage for a population to implement social integration or sexual coordination and it required the differentiation of environmentally diffusible chemical signals (pheromones) matched to that of specific receptors.

The pheromone concept in fish

Karlson and Lüscher(1959) proposed the term "pheromones" for "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process". They added also that pheromones, as chemical messengers, should be active in minute amounts and be relatively species-specific, only limited molecular overlapping between closely-related species being tolerated.

The requisite of a definite, stereotyped behavioural or developmental response in the recipient organism, as often observed in insects, is actually too restrictive to be adopted in the definition of pheromones in vertebrates. As argued by Mykytowycz (1979) in the case of mammals, their superior level of cerebral information processing makes the reaction to a given odour signal conditional on a set of manifold variables, such as previous experience, physiological condition, social status, ecological context etc. To a minor degree, this lack of strict automatism in the response to an olfactory stimulus is likely to occur also in teleosts, as discussed by Myrberg (1975) about parent discrimination of young in Cichlasoma nigrofasciatum.

The reverse possibility that a given response is triggered by a multi-component odorant mixture, as it is well documented in insects (Ritter, 1979), should also be kept in mind when hunting for fish pheromones. In this case, the species-specificity of the signal may be entrusted not only to molecules with different chemical structures (symbolic coding) but also to odorous blends with different ratios of the same ingredients (syntactic coding), as reported in lepidoptera (Ritter, 1979).

For the above criticism, we feel that the term "pheromones" is adequate to designate the intraspecific semiochemicals of fish only if it is given an acceptation more comprehensive then in the original definition.

The chemorecepto-signalling system

It must be pointed out that the pheromone-releasing or ectocrine cells (to be distinguished from esocrine cells whose secretions are essentially of metabolic significance) belong to the chemorecepto-signalling system which transduces and processes the chemical information flux through the organism.

By a drastically reductive approach, the body of a fish (and of any other animal as well) can be assimilated to a toroidal-like figure whose internal space is separated from the enclosed space by an inner interface and from the external space by an outer interface, as shown in Fig.1.

The enclosed space corresponds to all those spaces "inside" the body of a fish which open, or at least opened once, into the outer space, such as the cavities of the digestive

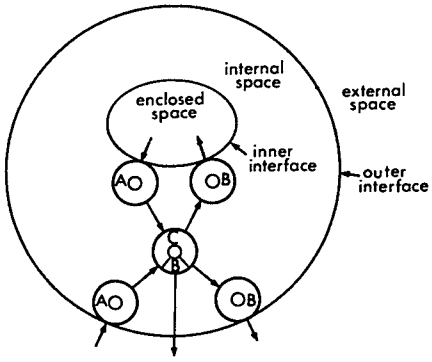

Fig.1. The chemorecepto-signalling system of a fish. The fish body is portrayed as a toroidal-like figure while chemorecepto-secretory cells are represented by two concentric rings. Signalling modes: A = centripetal; B = centrifugal; C = internal.

tract, the lumina of the gonoducts and of other secretory or excretory ducts, the ependimal canal, the encephalic ventricles etc. Both interfaces can be thought as selectively permeable to two bidirectional fluxes: one of energy-matter (to be channelled essentially into metabolism, growth, reproduction and reaction to "stress") and the other of chemical signals (to control the internal balance among the four energy-matter channels). To simplify, physical signals are not considered here.

The transfer of chemical signals across and between the two interfaces is not by diffusion or passive transport alone, but it is also relayed by cells of a specialized type, the chemorecepto-secretory cells. These cells are characterized by the faculty of secreting or stopping to secrete a chemical messenger after interacting with an incoming signalling molecule through a surface or cytoplasmic receptor. This function is requested for both information processing and transduction, i.e. to change the chemical vector.

According to wherefrom the first signal is received and whereto the second is directed, cellular relays can operate in three ways: centripetally, internally and centrifugally (Fig.1).

In the centripetal mode, the cell accepts the signal from the external or enclosed spaces and directs its own messenger into the internal space. The chemosensory cells such as olfactory, gustatory, vomero-nasal and paraneuronal cells of the open type (Fujita et al., 1980) work in this manner.

In the internal mode, cells receive and transmit signals only within the internal space. It is typical of nervous, neurosecretory, endocrine and paraneuronal cells of the closed type (Fujita et al., 1980).

In the centrifugal mode cells receive signals from the internal space and secrete semiochemicals into the external and enclosed

spaces. It is exhibited by ectocrine cells. However, a signal released into an enclosed space which communicates no longer with the outside (as in the case of the CSF-contacting neuron system around the encephalic ventricles (Franzoni & Fasolo, 1982)) cannot be regarded as pheromonal by definition. This situation resembles that of an animal smelling its own odour and it is classifiable as autoectocrine communication.

It should be emphasized that the above model refers to signalling modes and not to actual cell types. In the intricacy of feedback circuits, a given cell may, in fact, function in more than one way. For instance, a chemosensory cell is switching to the internal mode when it modulates its transmission in response to hormones. In male goldfish, it has been shown that the sensitivity to odours of the olfactory epithelium is androgen-dependent (Yamazaki, 1979).

Further, when an endocrine or neurosecretory cell succeeds to send its secretion into the external space to act as a pheromone, its role becomes ectocrine. Interestingly, the fish pheromones for which we have more hints about their chemical identity seem to be produced just by endocrine (Colombo et al.,1980) and neurosecretory cells (Richards, 1974) as discussed below.

Discrimination by means of pheromones

An impressive number of intraspecific interactions have been shown or suggested to be under pheromonal regulation in fish. First of all, teleosts use chemical cues to make important discriminations such as the recognition of one's species (Oshima et al., 1969; Höglund & Astrand, 1973; Bloom & Perlmutter, 1977), race or population (Nordeng, 1971,1977; Døving et al., 1974), brood (Myrberg, 1975; McKaye & Barlow, 1976), individual conspecifics (Todd et al., 1967; Richards, 1974), their social status (Todd et al., 1967), life stage (Miles, 1968), sex (Tavolga, 1956; Gandolfi, 1969; Crapon de Caprona, 1982) and sexual condition (Partridge et al., 1976; Crow & Liley, 1979; Honda, 1979, 1980a,b).

In some cases, this ability is dependent upon a previous exposure of the fish to the pheromone during a critical period (imprinting effect) (Myrberg, 1975; Crapon de Caprona, 1982). Recognition may trigger a quick change in behaviour (release effect). Alternatively, a pheromone may cause a slow physiological modification affecting later behaviour or even survival (priming effect). On the whole, pheromones have been reported to influence fish social behaviour (mostly through release effects), body growth and viability (mainly through priming effects) and reproduction (through both).

Valuable reviews on the topic of fish pheromones have been prepared by Bardach and Todd (1970), Hara (1971), Pfeiffer (1974), Crapon de Caprona (1976), Tavolga (1976), Barnett (1977) and Solomon (1977) and reference is made to them for a more extensive coverage of previous literature. Aim of this paper is to update the survey with a discussion on pheromone sources and chemistry and to present some unpublished data from our laboratory on the possible role of androgen conjugates in fish sexual communication.

Pheromonal control of social behaviour

Chemical communication has been documented or claimed to shape different fish social behaviours which may be broadly classified as hierarchical,associative (schooling), aggregative (homing) and alarmed (fright reaction).

Hierarchical behaviour

Research on the weight of olfaction in social hierarchy is limited to catfishes of the genus Ictalurus which are basically territorial and nocturnal animals with a keen sense of smell.

Yellow bullheads, I.natalis, have been found to use chemical cues not only to recognize individual conspecifics, but also to assess their relative dominant or submissive attitude in order to avoid undue challenges of social status (reviewed by Bardach and Todd, 1970; Solomon, 1977). However, when a dominant fish was returned to its territory after experiencing a losing encounter, it was immediately attacked by its formerly submissive neighbour, suggesting that stress had altered its odour.

As to the possible source of the pheromone(s), it was found that the integumentary slime, presumably uncontaminated from urine or fecal residues, was sufficient for donor identification, though less effectively than donor ambient water (Todd et al., 1967).

In an interesting study, Richards (1974) has shown that both urine and extracts of the urophysis provide information for discrimination of conspecifics in I.nebulosus.

Several and even unusual neuropeptides have been isolated from the fish urophysis (Bern, 1982). Nevertheless, it seems unrealistic to postulate that the urophysis contains a unique compound for each individual fish as suggested by Barnett (1981), especially if one considers the rather conservative nature of neuropeptide molecular evolution. A syntactic coding based on few compounds is more likely.

Receptors for neuropeptides are widespread in the body of vertebrates and thus it would not be surprising to find them also in the dendritic terminals of olfactory cells. Significantly peptides where reported to stimulate quite effectively the olfactory epithelium of the white catfish, I.catus (Suzuki & Tucker, 1971). An externalization for pheromonal communication of the internal neuropeptide signalling system is, therefore, conceivable.

However, if the hypothetical pheromonal neuropeptides are secreted by the urophysis,

they should overcome dilution in the vast external space without flooding receptors in the internal body volume. To comply with this restriction, the peptides might be either hormonally inactive or quickly removed from the circulation into the urine.

On the other hand, there are also hints to a completely different alternative: that the peptides are actually released from cells in the integument. In rainbow trout, typical olfactory bulbar responses were induced by skin mucus collected from conspecifics, as well as other teleosts, in such a way as to avoid contamination by alarm substances (Hara & MacDonald, 1976). Activity was found to be associated with heat-stable, non-volatile components of molecular weight between 500 and 1000 and was pH-dependent. Though not remarked by the authors, these properties seem to parallel those of small peptides.

Moreover, the conclusion by Richards (1974) that urine and urophysis were more effective for interindividual recognition than an integumentary extract prepared by homogenizing 1 cm^2 section of tissue in 12 ml of pond water is open to criticism. In fact, catfish were conditioned to manifest recognition by approach or flight reactions but, since a catfish skin homogenate must contain alarm substances (Pfeiffer, 1974), there was a strong bias towards flight responses, thus invalidating the assessment of recognition by means of integumentary signals.

The ability of fish to smell peptides (and aminoacids as well (Suzuki & Tucker, 1971)) is understandable if we consider that, in an aqueous medium odours correspond to water-soluble (i.e. polar) rather than volatile (often unpolar) compounds.

Whether fish contain pheromonal peptides in the skin remains to be demonstrated experimentally, but it is inspiring the fact that a number of interesting peptides have been revealed in the skin of amphibians (Erspamer et al., 1977) where, by the way, they might perform a similar function.

An advantage (for the fish) of the externalization not only of the receptors but also of the eventual peptide-releasing cells is that there is no need for special hormonally inactive peptides because, contrary to the urophysial model, there is no danger of hormonal unbalances.

Schooling and homing

It should be emphasized that the information conveyed by integumentary water-soluble odours from conspecifics may be used not only to stabilize hierarchical systems but also to support associative and aggregative behaviours such as schooling and homing, respectively.

A fish school is an integrated association of isospecific individuals which is stabilized cybernetically through visual, olfactory (especially at night) and hydrodynamic signals (Hemmings, 1966).

Wrede (1932) and Göz (1941) demonstrated that minnows, Phoxinus phoxinus, maintain cohesion because they are attracted by odours released from the skin mucus of school members. A similar observation was made by Kinosita (1975) in the marine catfish eel, Plotosus anguillaris, in which a dializable and heat-stable attractant is secreted from the body surface of schooling fish.

Considerable evidence exists that odour trails are utilized by anadromous salmonids for homestream selection (Johnsen & Hasler, 1980). Nordeng (1971, 1977) was the first to propose that orientation for homeward migration is actually provided by population-specific pheromones released from the skin mucus of resident juveniles and descending smolt.

Recordings from single cells in the olfactory bulb of the Artic char, Salmo alpinus, disclosed that fish could discriminate char populations from different areas on the basis of odorants contained in the integumentary slime (Døving et al., 1974). Since this is copiously secreted by the fish, it may be a major vehicle for impregnation of water with char odours. Differential responses of the olfactory bulb to homo- and heterospecific skin mucus were observed also in the rainbow trout, Salmo gairdneri (Hara & McDonald,1976), as discussed above.

Recently, Stabell and Selset (1980) have warned to avoid carefully contamination by intestinal juice in the collection of skin mucus for olfaction studies in fish. In fact, intestinal residues contain bile acid derivatives which are powerful odorants in teleosts (Selset, 1980), though they are not likely to contain peptides or aminoacids.

It is of interest that elvers of the catadromous species, Anguilla rostrata, are drawn upstream by a heat-stable, non-volatile substance emitted by the adults (Miles, 1968), and not by the young as in salmonids. Atema et al.(1973) reported that alewives (Alosa pseudoharengus) were attracted to their homestream by compounds of molecular weight less than 1000 Dalton which were not free aminoacids.

The possibility that fish are guided to their homestream also by non-pheromonal chemical cues washed out from soil (enviromones) or plants (allomones) cannot be presently dismissed. However, the demonstration that coho salmon imprinted to morpholine at the time of smolting were able to return as adults to the sites scented with it, in the absence of coho salmon upstream, (Johnsen & Hasler, 1980) is weakened by the fact that morpholine can act as a non-specific attractant for salmonids (Mazeaud, 1981).

Alarm substances

A class of integumentary pheromones, distinct from those presumably involved in hierarchical, associative and aggregative behaviours,

is represented by the so-called alarm substances of Ostariophysi and Gonorhynchiformes (cf. Pfeiffer, 1974).

These pheromones are stored in specialized epidermal elements, the club cells, which release them not by active secretion but upon breakage caused by mechanical skin injuries. Their intimidatory effect helps nearby conspecifics to escape further attacks by a predator but it is scarcely activated in case of cannibalism (Verheijen & Reuter, 1969).

Testosterone has been shown to reduce the number of club cells in male fathead minnows, Pimephales promelas, so they can rub their back to clean a spawning site without freeing alarm substance that would frighten away interested females (Smith, 1973). Malyukina et al. (1977) have also reported a decrease of alarm pheromone in the skins of spawning male and female minnows, P.phoxinus, and a depression of the fright reaction in starved or unhealthy recipients.

Alarm substances are apparently heat-labile and water-soluble and have been claimed to be either pterinoic compounds with UV autofluorescence (Pfeiffer & Lemke, 1973) or aminosugars without UV absorption (Kasumyan & Lebedeva, 1979). Whatever their nature, they represent a fascinating example of the evolution of an altruistic character, as discussed by Solomon (1977).

Pheromonal control of growth and viability

Convincing evidence exists that fish can respond to homospecific semiochemicals not only behaviourally but also physiologically (priming effects). In this kind of communication, the pheromonal influence penetrates deeply into the chemorecepto-signalling system of the recipient.

An interesting case is that in which chemical cues facilitates (beneficial conditioning) or inhibit (crowding factors) the growth and even the viability of conspecifics (cf. Solomon, 1977). It has been reported that fish live better in water where other fish had lived and that such a conditioning can make them more resistant to stress (Mc Cauley, 1968).

Conversely, when crowded, fish liberate in the medium species-specific inhibitors which stunt or shorten the life-span of smaller or weaker members of the group (Yu & Perlmutter, 1970). Crowding has been shown to promote the release of heart-rate depressants in the gold fish (Francis et al., 1974) and of immuno-suppressive factors in the blue gourami,Trichogaster tricopterus, thus increasing vulnerability by infective agents (Perlmutter et al., 1973).

Since larger animals produce more crowding factor, whereas smaller ones are more sensitive to it, in an overcrowded group of young fish those which are initially smaller will stop to grow and would eventually die (Rose & Rose, 1965). Significantly, fish schooling together in nature tend to be of similar size (Keenleyside, 1955) thus equalizing the adverse effects of crowding.

Besides, it is conceivable that, in nature, the main function of crowding factors is just to prevent crowding by favouring fish dispersal, their action becoming more dramatic only under abnormally high population densities. For this reason, research on crowding factors is likely to be of great importance for the practice of fish culture where productivity increases with the increase of stock densities.

Preliminary fractionation studies do not support the suggestion that a crowding factor and an aggregating pheromone might be actually the same substance acting in opposite ways at different concentrations (Solomon, 1977). The crowding factors of different fishes were extracted with organic solvents from the water where they had been secreted and thus appear to be lipids (Yu & Perlmutter, 1970; Pfuderer et al., 1974), whereas aggregating pheromones were found to be water-soluble (see above). Moreover, population density would be optimized more effectively by a feed-back mechanism based on two different signals for fish dispersal and aggregation then by one based on the level of a single signal only.

Pheromonal control of reproduction

Teleosts, as other animal groups, have amply exploited chemical communication for both the control of fertility and the coordination of sexual and epigamic behaviours.

Pheromonal control of fertility

Since reproduction increases population density, its depression by crowding factors appears to be a more radical and less wasteful mechanism of autoregulation than the stunting or killing of already born relatives.

Swingle (1953) found that, if uncrowded fish about to spawn were exposed to the holding water of a dense group, their spawning was prevented. Yu and Perlmutter (1970) quote the thesis work by Greene (1964) who extracted a species-specific spawning inhibiting factor from goldfish water with chloroform. Rose (1959) discovered that, in the live-bearing guppy, Lebistes reticulatus, the number of young per female was inversely proportional to the number of females per tank but was increased by replacing most of the water daily.

It is really unfortunate that these original findings did not arouse sufficient interest to stimulate further investigation. Density-dependent inhibitors of fecundity would be obviously detrimental in the rearing of fish for breeding, especially if the latter are genetically selected.

It should be noted that fertility may be depressed not only by crowding but also by isolation. Females of Pterophyllum scalare, which had been isolated chemically from the male for two months, were found to spawn at lower rates

with respect to non-deprived controls (Chien, 1974).

Sexual behaviour

Much more information is available on the role of chemical signals in fish sexual behaviour. The following interactions have been reported to be mediated by pheromones:
a) attraction of the male by the female in Poecilia reticulata (Gandolfi, 1969), Ictalurus punctatus (Timms & Kleerekoper, 1972) and Brachydanio rerio (Bloom & Perlmutter, 1978; van den Hurk et al., 1982);
b) attraction of the male by the ovulated or responsive female only, with stimulation of male courtship in Bathygobius soporator (Tavolga, 1956), Colisa lalia, C.labiosa (Rossi, 1969), Astyanax mexicanus (Wilkens, 1972), Carassius auratus (Partridge et al., 1976), Poecilia mexicana (Parzefall, 1973), Poecilia reticulata (Crow & Liley, 1979), Poecilia chica (Hilton Brett & Grosse, 1982) Hypomesus olidus (Okada et al., 1979), Plecoglossus altivelis (Honda, 1979), Salmo gairdneri (Honda, 1980a), Misgurnus anguillicaudatus (Honda, 1980b) and Haplochromis burtoni (Crapon de Caprona, 1980);
c) increase of male to male interactions induced by the female in Colisa lalia, C.labiosa (Rossi, 1969), Mollienesia latipinna (Thiessen & Sturdivant, 1977) and Poecilia chica (Hilton Brett & Grosse, 1982);
d) attraction of the female by the male in Blennius pavo (Laumen et al., 1974), Brachidanio rerio (Bloom and Perlmutter, 1977) and Gobius jozo (Colombo et al.,1980);
e) intrasexual attraction in Hypsoblennius robustus, H.jenkinsi, H.gentilis (Losey, 1979), Salmo gairdneri (Newcombe & Hartman, 1973), Macropodus opercularis (Davis & Pilotte, 1975) and Brachydanio rerio (Bloom & Perlmutter, 1977).

Parental behaviour

Chemical information is important also in epigamic behaviour of fish as exemplified by the parent-young interactions of cichlids. In Hemichromis bimaculatus, brooding behaviour and selective recognition of young by the parents are initiated by chemical emanations from the wiggling fry (Myrberg, 1966). Also in Cichlasoma nigrofasciatum (Myrberg, 1975) and C.citrinellum (McKaye & Barlow, 1976), parents learn to distinguish their brood by its odour.

Conversely, young cichlids can recognize conspecific adults, but not their parents, through urinary cues (Barnett, 1981), their detection of parentalness being essentially visual (Myrberg, 1975).

Studies on cichlids have provided consistent evidence of the importance of early experience (imprinting) in the development of appropriate responses to pheromones. In male Haplochromis burtoni, the first breeding experience seems to be essential in determining the subsequent preference for the odour of the isospecific gravid female (Crapon de Caprona, 1982), just as the first broodcare experience is decisive for the subsequent olfactory discrimination of young by cichlid parents (Myrberg, 1975).

Sources of sex pheromones

Several authors, who have demonstrated the occurrence of sex attractants in teleosts have also tried to point out their source and to give hints about their chemistry. A general consensus exists about the gonadal origin of fish sex pheromones, with the possible exception of Blennius pavo in which the male attractant is produced by specialized appendices of the anal fin spines (Laumen et al., 1974). Significantly Malyukina et al. (1974) reported that even microsmatic fish react to gonadal odours.

In the females of goby (Tavolga, 1956), goldfish (Partridge et al., 1976), ayu (Honda, 1979), trout (Honda, 1980a) and loach (Honda, 1980b) an ovarian pheromone eliciting male courtship has been shown to accumulate in the ovarian fluid chiefly or exclusively just after ovulation and to be released with it in the surrounding medium through the ovipore. Sexual attractants and stimulants were revealed in the ovaries of pond smelt (Okada et al., 1979), guppy (Crow & Liley, 1979) and zebrafish (van den Hurk et al., 1982). A testicular sex pheromone has been postulated also in the black goby (Colombo et al., 1980).

There are indications, however, that female fish may utilize also urine, besides ovarian fluid, as a pheromonal vehicle. Although females of Poecilia reticulata become maximally attractive to males during the period of receptivity which follows parturition, when the proximal end of their oviduct is open (Crow & Liley, 1979), the holding water of adult virgin females, whose oviduct is closed, still attracts males (Gandolfi, 1969; Crow & Liley, 1979; our own unpublished observations).

Similarly, even though male goldfish prefer the smell of recently ovulated over that of unovulated females (Partridge et al., 1976), yet they are easily attracted by the holding water of females in late vitellogenic condition when the ovary contains very little fluid (unpublished).

A more decisive experiment was recently made by us in Gobius jozo. Urine, free from ovarian contaminants, was obtained from both gravid (i.e. ovulated but unspawned) and post-vitellogenic females by tying up the urogenital papilla and collecting the urine (usually 0.1 ml) with a 1-ml syringe directly from the swollen urinary bladder 10-12 hr afterwards. It was then diluted 1:50 with saline. Ovarian fluid,free from urinary contaminants, was obtained by removing an ovary, slitting it open, shaking it in a watch glass containing 5 ml of fish saline for 5 min and pipetting off

the supernatant (cf. Tavolga, 1956).

Behavioural tests were performed on sexually active males isolated in 50-l aquaria which were provided with gravel on the bottom, a halved plastic tube as a shelter and running seawater. Behaviour was monitored for 10 min after the addition to the aquarium water of 1 ml of saline alone and for 10 min after the addition of 1 ml of either urine solution or ovarian washing.

Urine was much more effective than ovarian washing in triggering courtship and fluids obtained from gravid females were more effective than those of postvitellogenic ones. Male urine was uneffective (unpublished). This result contrasts with the observation by Tavolga (1956) that urine was inactive in another gobiid fish.

This variability imposes to assay only ovarian fluid uncontaminated from possible urinary attractants when trying to locate the source of a sex pheromone. The usual procedures of stripping, squeezing or even running a finger lightly along the fish sides do not seem to be safe enough.

The difference between ovarian fluid and urine as pheromonal vehicles is not a trivial one. Firstly, owing to peripheral metabolism and binding to plasma proteins, an ovarian hormone is not likely to be also a urinary pheromone whereas this possibility exists in the case of the ovarian fluid. Moreover since a systemic catabolite of an ovarian hormone is just a fraction of it, only an inactive ovarian product, sinthesized in parallel to hormones and scarcely transformed peripherally, is expected to reach a urinary concentration adequate for long-range transmission. However, a simple extra-ovarian molecular finishing (e.g. conjugation) may be required if the ovarian product is actually a propheromone.

Secondly, a urinary pheromone must be of low molecular weight, whereas such a restriction does not apply in the case of the ovarian fluid.

Thirdly, intraovarian secretion should allow the accumulation of a greater pheromonal charge than urinary excretion owing to lower clearance rate (especially in freshwater) and greater fluid storage capacity. Presently, it is unclear how fish exploit these vehicles to deliver sex pheromones, especially because available information about the chemistry of the latter is still scanty and contradictory.

Chemistry of sex pheromones

Disparate molecular identities have been proposed for female sex pheromones in fish such as estrogen in Lebistes (= Poecilia) reticulatus (Amouriq, 1965), a ether-soluble substance in Carassius auratus (Partridge et al., 1976), water-ether soluble basic substances in Plecoglossus altivelis (Honda, 1979), Salmo gairdneri (Honda, 1980a) and Misgurnus anguillicaudatus (Honda, 1980b), a protein in Hypomesus olidus (Okada et al., 1979), cholesterol esters (Algranati, 1979) and steroid glucuronides (van den Hurk et al., 1982) in Brachydanio rerio.

The latter finding is in keeping with previous reports from our laboratory about a testicular steroid pheromone in Gobius jozo (Colombo et al., 1979, 1980). In this species, the male bears a prominent mesorchial gland composed of Leydig cells which synthesize mainly 5β-reduced androgen conjugates, particularly etiocholanolone glucuronide (E-G), together with small amounts of non-conjugatable 11-oxygenated androgens, namely 11β-hydroxyandrostenedione and adrenosterone. While the latter compounds have probably a hormonal function, E-G was found to attract gravid females when tested at low concentration ($2 \cdot 10^{-6}$ M at the stimulus source) in a four-choice system. Unovulated females were unresponsive (Colombo et al., 1980).

Gravid females were also attracted by dilute male urine (1:600 dilution at the stimulus source) delivered into one arm of a glass Y-maze in a constant flow of seawater which was equal to that of the contralateral arm (76 % correct responses, n = 17, against 50 % in control runs, n = 42).

The pheromonal system of G.jozo is very interesting because it is characterized by:
a) undertaking of the centrifugal signalling mode by endocrine elements associated with their overdevelopment to cope with signal attenuation in the external space;
b) pheromonal androgens prevailing over but different from hormonal androgens to prevent flooding of hormonal androgen receptors;
c) pheromonal molecular form suitable for both urinary excretion and environmental diffusion as steroid conjugates are water-soluble;
d) female responsivity to the male pheromone induced by ovulation.

Most striking the pheromonal system of the boar displays just the same features, the only difference being that the testicular 5α-androst-16-enes, which induce the mating stance in the estrous sow, are volatile and released in the air with the breath (Gennings et al., 1977; cf. Colombo et al., 1980 for discussion).

We have also extended our study to species, such as Carassius auratus and Poecilia reticulata, in which it is the male that looks for the female for spawning (and not the other way round as in G.jozo) and should then plays the role of the receiver in the pheromonal interaction.

Male goldfish were tested in a two-choice system in pairs because single males are attracted not only by the odour of females but also by that of males. Pellets of fish food were given to fish immediately before the test to calm them down and to impregnate the water of the system with aspecific odours. As shown in Fig.2, male goldfish were attracted by E-G whereas females did not react. Males responded also to ovarian fluid (possibly contaminated with urine) but not to charcoal-tre-

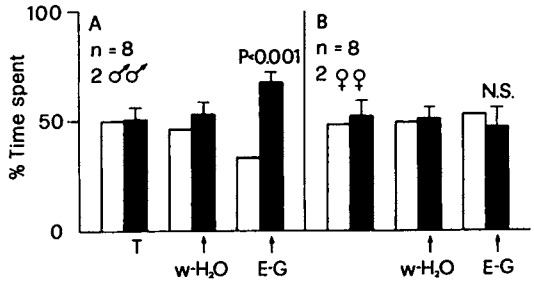

Fig.2. % Time spent by one of two male (A) or female (B) goldfish in the two compartments of a 150-l tank (halved by a panel ending at 6.5 cm from the bottom) for 30 min before any input (T) and during addition to one compartment (arrow) of 5 ml/min for 30 min of warm water alone (w-H_2O; 4°C above the tank water t°C) or etiocholanolone glucuronide (E-G) at a concentration of 1µg/ml in warm water. Student's t-test on matched pairs (with respect to w-H_2O).

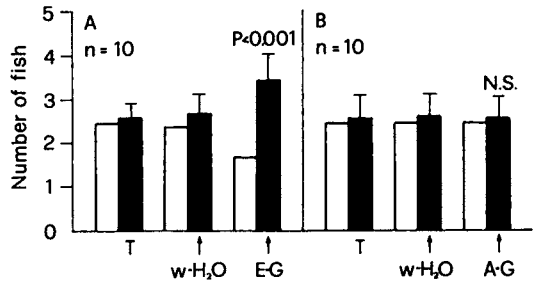

Fig.3. Number of fish (checked every 30 sec) among five male guppies visiting the two compartments of a 35-l tank (halved by a panel ending at 11.5 cm from the bottom) for 30 min before any input (T) and during addition to one compartment (arrow) of 4.2 ml/min for 30 min of warm water alone (w-H_2O), etiocholanolone glucuronide (E-G) (A) or androsterone glucuronide (A-G) (B) at a concentration of 0.6 µg/ml in warm water. Student's t-test as above.

ated ovarian fluid, testosterone, estradiol-17β and its 3-glucuronide.

In a similar test, also male guppies displayed a preference for E-G but not for its epimer, androsterone glucuronide (Fig.3), while females did not respond to both. These experiments, which will be reported in detail elsewhere demonstrate that the response to E-G is specific with respect to sex and chirality but not to species.

Presently, we have not yet established whether 5β-androgen conjugates are secreted by the ovary or excreted with the urine in the females of goldfish and guppy. However, Lambert and van den Hurk (this symposium) have revealed a high conversion rate of androstenedione to steroid glucuronides, mainly testosterone glucuronide, by the ovary of the African catfish, Claria lazera, just after ovulation. Significantly, high yields of testosterone glucuronide were also obtained after incubation of pregnenolone and progesterone with ovarian tissue from the Indian catfish, Heteropneustes fossilis (Colombo and Sundararaj, unpublished). Also the finding by Kime (this symposium) that the testicular glucuronide/free steroid ratio is highest in trout at spermiation, when environmental temperature is at its minimum, can be rationalized in terms of pheromone production.

The conclusion that not only the internal space but also the external space around fish may be sexualized by gonadal steroids supports the concept that the evolution of the bioregulatory function of sex steroids in vertebrates is aimed at the progressive elongation of the communication channel connecting the steroid-secreting with the steroid-responsive sites, as discussed by Colombo et al. (1980). According to this model, the ectocrine role of gonadal steroids originated from their endocrine function which, in its turn, emerged from their paracrine or intragonadal activity. In the female for instance, we may suppose that it was the estrogen in excess to that needed for granulosa mitogenesis and differentiation (Richards, 1975) that was utilized for the endocrine control of extra-ovarian targets, just as the hormonal progestogens were derived from the ovarian overflow of the maturational steroids involved in oocyte differentiation (i.e. resumption of meiosis) (cf. Colombo et al., 1980). At this point, we would like to suggest that pheromonal androgen conjugates represents an alternative utilization of the androgen intermediates left over by the decline of aromatization (van Bohemen & Lambert, 1981) and, possibly, 11β-hydroxylation in some male fish, occurring at the completion of gamete maturation.

References

Algranati, F.D., 1979. Role of sex attractant pheromone(s) on spawning aggregation in the zebra fish, Brachydanio rerio, and the isolation and partial purification of the attractants (abstract). 2nd Conf. Ethol. Behav. Ecol. Fishes, October 19-22, Illinois State Univ. Bloomington.

Amouriq, L., 1965. Origine de la substance dynamogène émise par Lebistes reticulatus femelle (Poisson Poeciilidae, Cyprinodontiforme). C. R. Acad. Sc. Paris, 260:2334-2335.

Atema, J., S. Jacobson, J. Todd & D. Boylan, 1973. The importance of chemical signals in stimulating behaviour of marine organisms, effects of altered environmental chemistry on animal communication. In: G. E. Glass (Ed.): Bioassay Techniques and Environmental Chemistry. Ann Arbor Science Publishers, Michigan. p. 177-197.

Bardach, J. E. & J. H. Todd, 1970. Chemical communication in fish. In: J. W. Johnston, D. G. Moulton & A. Turk (Ed.): Communication

by Chemical signals. Appleton-Century-Crofts, New York. p. 205.
Barnett,C., 1977. Aspects of chemical communication with special reference to fish. Biosci. Commun., 3:331-392.
Barnett,C., 1981. The role of urine in parent-offspring communication in a cichlid fish. Z. Tierpsychol., 55:173-182.
Bern,H.A., 1982. The caudal neurosecretory system of fishes: the beginning of "urophysiology". Gen. Comp. Endocrinol., 46:349-350.
Bloom,H. & A. Perlmutter, 1977. A sexual aggregating pheromone system in the zebrafish, Brachydanio rerio (Hamilton-Buchanan). J. Exp. Zool., 199:215-226.
Bloom,H. D. & A. Perlmutter, 1978. Possible pheromone mediated isolation in two species of cyprinid fish of the genus, Brachydanio. J. Fish Biol., 13:47-50.
Bohemen,van Ch. G. & J. G. D. Lambert, 1981. Estrogen synthesis in relation to estrone, estradiol and vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol., 45:105-114.
Chien, A. K., 1974. Reproduction behaviour of the angelfish, Pterophyllum scalare (Pisces: Cichlidae): Influence of male stimuli upon the spawning rate of females. Anim. Behav., 21:457-463.
Colombo, L., P. C. Belvedere & A. Marconato, 1979. Biochemical and functional aspects of gonadal biosynthesis of steroid hormones in teleost fish. Proc. Indian Natl. Sci. Acad., B45:226-234.
Colombo, L., A. Marconato, P. C. Belvedere & C. Friso, 1980. Endocrinology of teleost reproduction: a testicular steroid pheromone in the black goby, Gobius jozo L.. Boll. Zool., 47:355-364.
Crapon de Caprona, M-D., 1976. Chemical stimuli and reproduction in fish. Conclusion. Experientia, 32:1098-1100.
Crapon de Caprona, M-D., 1980. Olfactory communication in a cichlid fish, Haplochromis burtoni. Z. Tierpsychol., 52:113-134.
Crapon de Caprona, M-D., 1982. The influence of early experience on preferences for optical and chemical cues produced by both sexes in the cichlid fish Haplochromis burtoni (Astatotilapia burtoni, Greenwood, 1979). Z. Tierpsychol., 58:329-361.
Crow, R. T. & N. R. Liley, 1979. A sexual pheromone in the guppy, Poecilia reticulata (Peters). Can. J. Zool., 57:184-188.
Davis, R. E. & N. J. Pilotte, 1975. Attraction to conspecific and nonconspecific chemical stimuli in male and female Macropodus opercularis (Anabantoidei). Behav. Biol., 13: 191-196.
Døving, K.B., H. Nordeng & B. Oakley, 1974. Single unit discrimination of fish odours released by char (Salmo alpinus L.) populations. Comp. Biochem. Physiol., 47A:1051-1063.
Erspamer, V., G. Falconieri Erspamer & G. Linari, 1977. Occurrence of tachykinins (Physalemin or Substance P-like peptides) in the amphibians skin and their action on smooth muscle preparations. In: U.S. von Euler & B. Pernow (Ed.): "Substance P". Raven Press, New York.
Francis, A.A., F. Smith & P. Pfuderer, 1974. A heart-rate bioassay for crowding factors in goldfish. Prog. Fish. Cult., 36:196-200.
Franzoni, M.F. & A. Fasolo, 1982. Ultrastructural analysis of the posterior hypotalamus of salamander. J. Submicrosc. Cytol., 14: 355-368.
Fujita, T., S. Kobayashi, R. Yui & T. Iwanaga, 1980. Evolution of neurons and paraneurons. In: S. Ishii, T. Hirano & M. Wada (Ed.): Hormones, Adaptation and Evolution. Springer-Verlag, Berlin. p. 35-43.
Gandolfi, G., 1969. A chemical sex attractant in the guppy Poecilia reticulata Peters (Pisces, Poeciliidae). Monitore Zool. Ital., 3:89-98.
Gennings, J.N., D.B. Gower & L.H. Bannister, 1977. Studies on the receptors to 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol in sow nasal mucosa. Biochim. Biophys. Acta, 496:547-556.
Göz, H., 1941. Über den Art-und Individualgeruch bei Fischen. Z. vergl. Physiol., 29:1-45.
Hara, T.J., 1971. Chemoreception. In: W.S. Hoar & D.J. Randall (Ed.): Fish Physiology. Academic Press, New York. p. 79-120.
Hara, T.J. & S. MacDonald, 1976. Olfactory responses to skin mucous substances in rainbow trout Salmo gairdneri. Comp. Biochem. Physiol., 54A:41-44.
Hemmings, C.C., 1966. Olfaction and vision in fish schooling. J. Exp. Zool., 45:449-464.
Hilton Brett, B.L. & D.J. Grosse, 1982. A reproductive pheromone in the Mexican poeciilid fish, Poecilia chica. Copeia, 1982:219-223.
Höglund, L.B. & M. Åstrand, 1973. Preferences among juvenile char (Salvelinus alpinus L.) to intraspecific odours and water currents studied with the fluviarium technique. Inst. Freshwater Res. Drottningholm.,Report No 53: 21-30.
Honda, H., 1979. Female sex pheromone of the ayu, Plecoglossus altivelis, involved in courtship behaviour. Bull. Jap. Soc. Scien. Fish., 45:1375-1380.
Honda, H., 1980a. Female sex pheromone of rainbow trout, Salmo gairdneri, involved in courtship behaviour. Bull. Jap. Soc. Scien. Fish., 46:1109-1112.
Honda, H., 1980b. Female sex pheromone of the loach, Misgurnus anguillicaudatus,involved in courtship behaviour. Bull. Jap. Soc. Scien. Fish., 46:1223-1225.
Hopkins, C.D., 1981. On the diversity of electric signals in a community of mormyrid electric fish in West Africa. Amer. Zool., 21: 211-222.
Hurk, van den R., L.A.'t Hart, J.G.D. Lambert & P.G.W.J. van Oordt, 1982. On the regula-

tion of sexual behaviour of male zebrafish, Brachydanio rerio. Gen. Comp. Endocrinol., 46:403.
Johnsen, P.B. & A.D. Hasler, 1980. The use of chemical cues in the upstream migration of coho salmon, Oncorhynchus kisutch Walbaum. J. Fish Biol., 17:67-73.
Karlson, P. & M. Lüscher, 1959. "Pheromones": a new term for a class of biologically active substances. Nature, 183:55-56.
Kasumyan, A.O.& N.E. Lebedeva, 1979. New data on the nature of the alarm pheromone in cyprinids (Phoxinus phoxinus). Vopr. Ikhtiol., 19:890-895 (in Russian).
Keenleyside, M.H.A., 1955. Some aspects of the schooling behaviour of fish. Behaviour, 8:183-248.
Kinosita, H., 1975. Schooling behaviour of marine catfish eel (Plotosus anguillaris). In Modern Biological Sciences, vol.9, Iwanami, Tokyo.
Kittredge, J.S., 1974. Comparative biochemistry: marine biochemistry. In: R.N. Mariscal (Ed.): Experimental Marine Biology. Academic Press, New York. p. 225-267.
Laumen, J., U. Pern & V. Blüm, 1974. Investigations on the function and hormonal regulation of the anal appendices in Blennius pavo (Risso). J. Exp. Zool., 190:47-56.
Losey, G.S., 1969. Sexual pheromones in some fish of the genus Hypsoblennius Gill. Science, 163:181-183.
Malyukina, G.A., G.V. Devitsyna & E.A. Marusov, 1974. Communication on the basis of chemoreception in fishes. Zh. Obshch. Biol., 35:70-79 (in Russian).
Malyukina, G.A., A.O. Kasumyan, E.A. Marusov & N.I. Pashchenko, 1977. The alarm pheromone and its significance in fish behaviour. Zh. Obshch. Biol., 38:123-132 (in Russian).
Mazeaud, F., 1981. Morpholine, a nonspecific attractant for salmonids. Aquaculture, 26: 189-191.
McCauley, R.W., 1968. Suggested physiological interaction among rainbow trout fingerlings undergoing thermal stress. J. Fish. Res. Bd. Canada, 25:1983-1986.
Mc Kaye, K.R. & G.W. Barlow, 1976. Chemical recognition of young by the Midas cichlid, Cichlasoma citrinellum. Copeia, 1976:276-282.
Miles, S.G., 1968. Rheotaxis of elvers of the American eel (Anguilla rostrata) in the laboratory to water from different streams in Nova Scotia. J. Fish. Res. Bd. Canada, 25: 1591-1602.
Mykytowycz, R., 1975. Some difficulties in the study of the function and composition of semiochemicals in mammals, particularly wild rabbit, Oryctolagus cuniculus. In: F.J. Ritter (Ed.): Odour Communication in Animals. Elsevier/North Holland Biomedical Press, Amsterdam. p. 105-115.
Myrberg, A.A., 1966. Parental recognition of young in cichlid fishes. Anim. Behav., 14: 565-571.
Myrberg, A.A., 1975. The role of chemical and visual stimuli in the preferential discrimination of young by the cichlid fish Cichlasoma nigrofasciatum (Günther). Z. Tierpsychol., 37:274-279.
Myrberg, A.A., 1978. Ocean noise and the behaviour of marine animals: relationships and implications. In: J.L. Fletcher & R.G. Busnel (Ed.): Effects of Noise on Wildlife. Academic Press, New York. p. 169-208.
Newcombe, C. & G. Hartman, 1973. Some chemical signals in the spawning behaviour of rainbow trout (Salmo gairdneri). J. Fish. Res. Bd. Canada, 30:995-997.
Nordeng, H., 1971. Is the local orientation of anadromous fishes determined by pheromones?. Nature, 233:411-413.
Nordeng, H., 1977. A pheromone hypothesis for homeward migration in anadromous salmonids. Oikos, 28:155-159.
Okada, H., D.K. Sakai & K-i Sugiwaka, 1979. Chemical stimulus on the reproductive behaviour of the pond smelt. Sc. Reports Hokkaido Fish Hatch., 33:89-99.
Oshima, K., W.E. Hahn & A. Gorbman, 1969. Olfactory discrimination of natural waters by salmon. J. Fish. Res. Bd. Canada, 26:2111-2121.
Partridge, B.L., N.R. Liley & N.E. Stacey, 1976. The role of pheromones in the sexual behaviour of the goldfish. Anim. Behav., 24: 291-299.
Parzefall, J., 1973. Attraction and sexual cycle of poeciliids. In: J.H. Schröder (Ed.): Genetics and Mutagenesis of Fish. Springer-Verlag, Berlin. p.357-406.
Perlmutter, A., D.A. Sarot, M-L Yu, R.J. Filazzola & R.J. Seeley, 1973. The effect of crowding on the immune response of the blue gourami, Trichogaster trichopterus, to infectious pancreatic necrosis (IPN) virus. Life Sci., 13:363-375.
Pfeiffer, W., 1974. Pheromones in fish and amphibia. In: M.C. Birch (Ed.): Pheromones. North Holland/American Elsevier, Amsterdam. p. 269-296.
Pfeiffer, W. & J. Lemke, 1973. Untersuchungen zur Isolierung und Identifizierung des Schreckstoffes aus der Haut der Elritze, Phoxinus phoxinus (L.) (Cyprinidae, Ostariophysi, Pisces). J. Comp. Physiol., 82:407-410.
Pfuderer, P., P. Williams & A.A. Francis, 1974. Partial purification of the crowding factor from Carassius auratus and Cyprinus carpio. J. Exp. Zool., 187:375-382.
Richards, I.S., 1974. Caudal neurosecretory system: possible role in pheromone production. J. Exp. Zool., 187:405-408.
Richards, J.S., 1975. Estradiol receptor content in rat granulosa cells during follicular development:modification by estradiol and gonadotropins. Endocrinology, 97:1174-1184.
Ritter, F.J., 1979. Chemical Ecology: odour communication in animals: general introduction and overview. In: F.J. Ritter (Ed.): Chemical Ecology: Odour Communication in Animals. Elsevier/North Holland Biomedical

Press. p. 1-15.
Rose, S.M., 1959. Failure of survival of slowly growing members of a population. Science, 129:1026.
Rose, S.M. & F.C. Rose, 1965. The control of growth and reproduction in freshwater organisms by specific products. Mitt. int. Verein. theor. angew. Limnol., 13:21-35.
Rossi, A.C., 1969. Chemical signals and nest-building in two species of Colisa (Pisces, Anabantidae). Monitore Zool. Ital., 3:225-237.
Selset, R., 1980. Chemical methods for fractionation of odorants produced by char smolts and tentative suggestions for pheromone origins. Acta Physiol. Scand., 108:97-104.
Smith, R.I.F., 1973. Testosterone eliminates alarm substance in male fathead minnows. Can. J. Zool., 51:875-876.
Solomon, D.J., 1977. A reviw of chemical communication in freshwater fish. J. Fish Biol. 11:363-376.
Stabell, O.B. & R.Selset, 1980. Comparison of mucus collecting methods in fish olfaction. Acta Physiol. Scand., 108:91-96.
Suzuki, N. & D. Tucker, 1971. Amino acids as olfactory stimuli in freshwater catfish, Ictalurus catus (Linn.). Comp. Biochem. Physiol., 40A:399-404.
Swingle, H.S., 1953. A repressive factor controlling reproduction of fishes. Proc. Eight Pacif. Sci. Congr.,IIIA:865-870.
Tavolga, W.N., 1956. Visual, chemical and sound stimuli as cues in the sex discriminatory behavior of the gobiid fish Bathygobius soporator. Zoologica N.Y., 41:49-65.
Tavolga, W.N., 1971. Sound production and detection. In: W.S. Hoar & D.J. Randall (Ed.): Fish Physiology. Academic Press, New York. Vol.V:135-205.
Tavolga, W.N., 1976. Chemical stimuli in reproductive behaviour in fish:communication. Experientia, 32:1093-1095.
Thiessen, D.D. & S.K. Sturdivant, 1977. Female pheromone in the black molly fish (Mollienesia latipinna): a possible metabolic correlate. J. Chem. Ecol., 3:207-218.
Timms, A.M. & H. Kleerekoper, 1972. The locomotory responses of male Ictalurus punctatus, the channel fish, to a pheromone released by the ripe female of the species. Trans. Am. Fish. Soc., 101:302-310.
Todd, J.H., J. Atema & J.E. Bardach, 1967. Chemical communication in social behaviour of a fish, the yellow bullhead (Ictalurus natalis). Science, 158:672-673.
Verheijen, F.J. & J.H. Reuter, 1969. The effect of alarm substance on predation among cyprinids. Anim. Behav., 17:551-554.
Wilkens, H., 1972. Über preadaptation fur das Hohlenleben, untersucht am Laiverhalten ober-und unterrirdischer Population des Astyanax mexicanus (Pisces). Zool. Anz., 188: 1-11.
Wrede, W.L., 1932. Versuche über der Artduft der Elritzen. Z. vergl. Physiol., 17:510-519.
Yamazaki, F. & K. Watanabe, 1979. The role of steroid hormones in sex recognition during spawning behavior of the goldfish Carassius auratus. Proc. Indian Natl. Sci. Acad. Part B Biol. Sci., 45:505-511.
Yu, M-L & A. Perlmutter, 1970. Growth inhibiting factors in the zebrafish, Brachydanio rerio and the blue gourami, Trichogaster trichopterus. Growth, 34:153-175.

THE CONTROL OF GONADAL ANDROGEN BIOSYNTHESIS IN FISH

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Summary

The effect of temperature on reproduction in four species of fish, Salmo gairdneri, Carassius auratus, Sarotherodon mossambicus and Scyliorhinus caniculus is discussed. Incubations of testes with endogenous and exogenous precursors indicate that 1) utilisation of exogenous substrate increases with temperature, 2) competitive formation of 'inactive' steroids at high temperature limits 'active' steroid formation to the normal reproductive temperature, and 3) gonadotropin stimulation of steroidogenesis is temperature dependent. Seasonal studies indicate that factors other than temperature may also affect glucuronide synthesis and their possible nature is discussed.
Keywords: reproduction, fish, temperature, androgens, glucuronides, testis.

Introduction

One of the major factors influencing reproductive development in many species of fish is temperature, and this has often been considered to act via a hypothalamic thermoreceptor and thence release of pituitary gonadotropin to stimulate gonadal steroid production. Recently we made the suggestion (Kime, 1979) that temperature could also act directly on the steroidogenic enzymes of the testis to give maximal stimulation of steroid production at temperatures which were environmentally most suited for gonadal development. To test this hypothesis we incubated testes from a number of species at a range of temperatures and examined the effect of temperature on the nature and the yield of steroids produced from both endogenous and exogenous precursors. Some of these results have been reported in detail elsewhere (Kime, 1979; 1980; Kime & Hews, 1982; Kime & Hyder, 1982), but we consider it is now opportune to compare the results obtained in several different species and to discuss whether there is a general mechanism by which environmental temperature may act directly on the gonad to regulate reproductive development in fish.

Materials and Methods

Fish were obtained from commercial sources, and at the time of incubation were sexually mature and usually spermiating. Small pieces of testis (50-100 mg) were incubated in 1 ml Krebs-Ringer bicarbonate medium in the absence of cofactors. For incubations with radioactive precursors 1-2 µCi of tritiated steroid was added to the medium. When endogenous precursors were used, a preincubation was carried out for three hours, after which the medium was replaced by fresh medium containing salmon pituitary extract. After three hours the incubation medium was extracted, separated into free and conjugated fractions and chromatographed to separate individual steroids. Radioactive products were identified and quantitated by scintillation counting; non-radioactive products were quantitated by radioimmunoassay. Full details of these procedures are described in other publications from our laboratory.

Results and discussion

Investigations have so far been carried out on four species of fish: the rainbow trout (Salmo gairdneri), the goldfish (Carassius auratus), the tilapia (Sarotherodon mossambicus) and the dogfish (Scyliorhinus caniculus), and in each case major changes in the steroid profile were found to occur over the temperature range examined.

In the trout and goldfish, two distinct patterns were apparent when testis tissue was incubated with radioactive testosterone over a range of temperatures. Firstly, the two compounds which might be considered as the characteristic teleost androgens, 11-ketotestosterone and 11β-hydroxytestosterone rose from very low levels at 1° to a plateau corresponding very broadly to the temperature range over which reproductive development occurs and then at temperatures approaching the lethal limit for the species they declined again to low values (Figure 1a). Utilisation of substrate, however increased steadily with temperature (Figure 1b) and this increase is accounted for by the second distinct pattern found for formation of steroid glucuronides (Figure 1c). In both species there is a steady increase in formation of testosterone glucuronide with increase in temperature which is in contrast to the plateau found with the 11-oxygenated androgens. Since formation of the two products, glucuronides and 11-oxygenated androgens are in competition we have suggested that the preferential formation of glucuronide

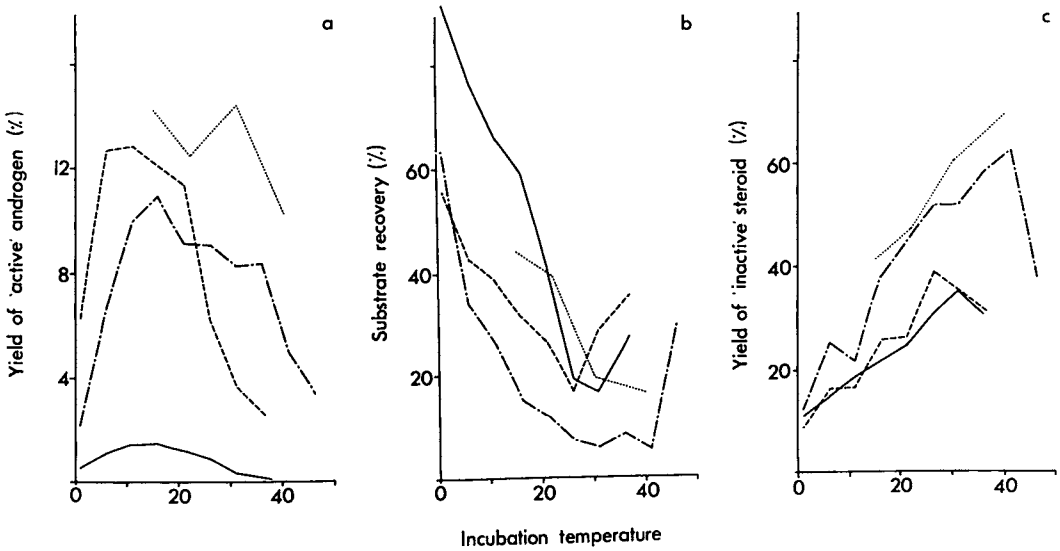

Figure 1. The effect of temperature on the metabolism of tritiated precursors in vitro for four species of fish. (- - - trout, ·-·-· goldfish, —— dogfish, ... tilapia)

at higher temperatures may be a protective mechanism to inhibit androgen synthesis and hence reproductive development at temperatures which may be too high for successful reproduction.

In an elasmobranch fish, the spotted dogfish Scyliorhinus caniculus, a very similar mechanism appears to be operative. When testis tissue of this species was incubated with radioactive pregnenolone, yields of the elasmobranch androgen, testosterone, showed a broad peak in yield around 11-16°. Again there was a steady increase in utilisation of substrate which was accounted for by the increasing yield of an unidentifiable but extremely polar steroid (Fig.1). The balance between the active androgen and this unusual, and probably inactive, steroid bears a remarkable resemblance to the pattern found in the trout and goldfish.

The fourth species which we have examined, the tilapia Sarotherodon mossambicus, again fitted this general pattern (Figure 1), but with a rather different variation. Utilisation of testosterone substrate again increased with temperature, but the yields of the 11-oxygenated androgens were fairly constant over the temperature range chosen (15-40°) although 11-ketotestosterone did show a small but significant drop in yield from 30-40°.Once again we found that the glucuronides increased with temperature, but in addition we found formation of substantial quantities of 5β-androstane-3α, 17β-diol which followed a similar but less steep pattern to that of the glucuronides. Although we had measured total glucuronide, we have shown that this fraction consists predominantly of the glucuronides of the 5β-diol and testosterone. The formation of 5β-androstane glucuronides is of particular interest since Colombo et al. (1982) have shown these compounds to have pheromonal activity in some species. Since pheromones must be species specific it is likely that sensitivity to these compounds has been developed in some species to indicate the presence of a potential mate but that the primary purpose of these 'inactive' metabolites is in some way connected with regulation of reproductive cycles.

In mammals, both glucuronides and 5β-reduced steroids are considered to be primarily hepatic metabolites serving no function other than deactivation and excretion. It is therefore very surprising to find them produced in such large quantities by fish testes. This fact and the differential effect of temperature on the rate of synthesis of the active and inactive steroids (Figure 2) suggests that these metabolites may play some protective role in the reproductive endocrinology of species such as fish in which environmental and body temperature vary over a considerable range. We can thus summarise the findings of our in vitro experiments with radioactive precursors in a general form applicable to all four species: 1) the metabolism of precursor to products increases with increase in temperature, 2) optimum formation of active androgen corresponds very broadly to the temperature for reproductive development of the species and decreases above and below this range, and 3) formation of an inactive metabolite increases steadily with increase in temperature and at high

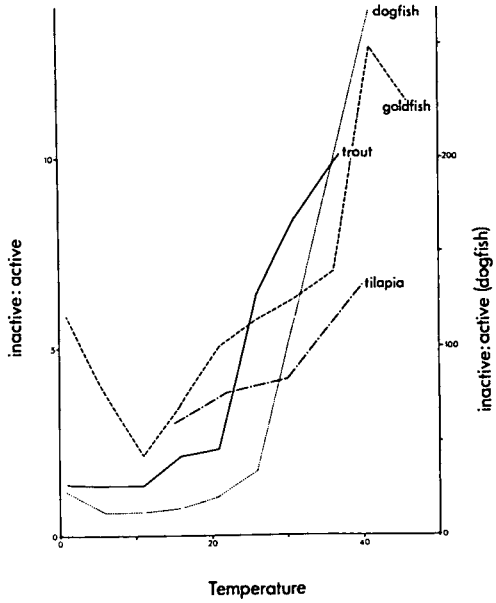

Figure 2. The effect of temperature on the ratio of inactive: active steroid in vitro.

temperatures is the predominant metabolite, these results are consistent with 1) Competitive inhibition of formation of the biologically active steroids at too high a temperature for successful reproduction, and 2) that in the absence of this competitive steroid, androgens would be formed optimally at around the lethal temperature for the species.

The experiments that we have so far described have involved the use of radioactive precursors, but formation of steroids from endogenous precursors might be expected to show the effect of temperature at earlier stages of steroidogenesis and might be nearer to the situation in vivo. Preliminary experiments with radioisotopes are, however, essential for the identification of the steroids formed so that suitable assays can be devised when we examine endogenous products. We have so far only examined formation of endogenous steroids in one species, Sarotherodon mossambicus. The effect of temperature was found to be rather different to that observed with exogenous precursors and all steroids showed a maximum yield at 22° with a steady drop to 40° (Figure 3). Glucuronides also peaked at this temperature but their relative yields increased at higher temperatures at which they were comparable to free steroid formation. Unfortunately no antiserum is available for measurement of the 5β-diol and its glucuronide which we would have expected to follow a similar rise with temperature. Although this experiment confirms the change in proportions of steroids with temperature and the increase in glucuronide at higher temperature, it differs from the experiments with exogenous steroid in the much more defined peak in total steroid formation. This indicates that temperature may play an even more important role in one of the early stages of steroidogenesis, possibly at the stage of gonadotropin stimulated cleavage of the cholesterol side chain. Figure 3 clearly shows that for the same amount of gonadotropin stimulation, yields of total unconjugated androgens are four times greater at 22° than at 40° (9.6 times if only testosterone is considered). In vitro incubations, even with the use of endogenous precursors, may not be a true representation of the situation in vivo but preliminary results with three species, Salmo gairdneri, Cyprinus carpio and Sarotherodon mossambicus indicate that the relative proportion of glucuronide is greater in fish held at higher temperatures than in those at the lower temperature, thus confirming the in vitro results (Kime & Manning, Unpublished observations).

Our studies have thus shown that the direct effect of temperature on the testis may affect both the relative proportions of the steroids formed and the total amount of steroid produced per unit of gonadotropin. It also follows that the relative proportions of gonadotropin and androgen in plasma will vary considerably with temperature. Temp-

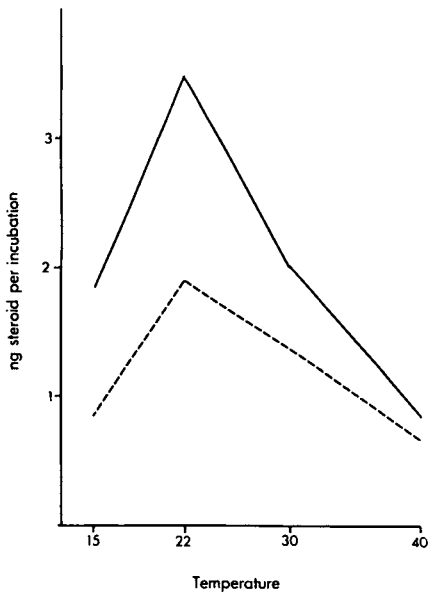

Figure 3. The effect of temperature on free and conjugated steroid formation from endogenous precursors in testes of Sarotherodon mossambicus (—— free,--- glucuronide).

erature may also affect other endocrine processes such as hepatic catabolism (Kime & Saksena, 1980), feedback of gonadal steroids on the pituitary, steroid action on target tissue (Dasmahapatra et al., 1981), receptor and protein binding, and hormone synthesis by the hypothalamus, pituitary, adrenal, thyroid and pineal. What has often been referred to as the 'hypothalamic thermoreceptor' may in fact be a temperature dependent enzyme system. The effect of temperature on reproduction in fish will be a reflection of all of these individual factors.

To determine whether glucuronides played a role in seasonal changes in reproductive activity we have measured plasma levels of both free and conjugated androgens during the seasonal cycle of the brown trout Salmo trutta (Kime & Manning, 1982). We found that plasma levels of glucuronides increased rapidly as spawning approached and, after spermiation, levels of these conjugates could exceed those of the free steroid. Since glucuronides are excreted approximately ten times more rapidly than the free steroid (Kellie & Smith, 1957), this indicates gonadal secretion to be of major importance. This increase in glucuronide secretion, however corresponds to a seasonal fall in environmental temperature and thus appears to contradict the findings both in vitro and in fish held at different temperatures. It would therefore appear likely that other factors apart from temperature, may well also regulate glucuronide synthesis and that glucuronides may play some role in the normal reproductive functioning in fish. In a recent extensive review, Dutton (1980) has described a large number of factors which have been shown to act directly on the glucuronyl transferase complex, but unfortunately the majority of this work is concerned with mammalian liver tissue and its validity to teleost testis may be questioned. Some of these factors which may be worth considering, however, are the effect of sex and adrenal steroids, phospholipids, unsaturated fatty acids, and some metal ions. Since concentrations of some of these factors have been shown to increase in teleost plasma during sexual maturation, it is possible that in some species they may play a role in regulating the balance of gonadal steroids, either by affecting the enzyme activity or its synthesis. The increase in glucuronide formation with sexual maturation is of particular interest with respect to its possible pheromonal role in some species (Colombo et al., 1982).

A fuller understanding of how these factors may affect gonadal steroidogenesis in fish and their relationship to the reproductive development and maturation of the species may lead to methods for the artificial manipulation of reproductive cycles in aquaculture. With the wide diversity of habitats and breeding conditions in fish it is likely that the relative importance of these different factors will vary considerably between species.

Acknowledgement

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References

Colombo, L., A. Marconato, P. Colombo Belvedere & S. Raineri, 1982. Pheromones in teleost fish. This volume.

Dasmahapatra, A.K., A.K. Ray & A.K. Medda, 1981. Temperature dependent action of estrogen in Singi fish (Heteropneustes fossilis Bloch). Endokrinologie 78: 107-110.

Dutton, G.J., 1980. Glucuronidation of drugs and other compounds. CRC Press, Boca Raton, Florida.

Kellie, A.E. & E.R. Smith, 1957. Renal clearance of 17-oxo steroid conjugates formed in human peripheral plasma. Biochem.J. 66: 490-495.

Kime, D.E., 1979. The effect of temperature on testicular steroidogenic enzymes of the rainbow trout, Salmo gairdneri. Gen.Comp. Endocrinol. 39: 290-296.

Kime, D.E., 1980. Androgen biosynthesis by testes of the goldfish (Carassius auratus) in vitro - the effect of temperature on the formation of steroid glucuronides. Gen.Comp.Endocrinol. 41: 164-172.

Kime.E., & E.A.Hews, 1982. The effect of temperature on steroid biosynthesis by testes of the dogfish, Scyliorhinus caniculum. Comp.Biochem.Physiol.71B: 675-679.

Kime,D.E., & M.Hyder, 1982. The effect of temperature and gonadotropin on testicular steroidogenesis in Sarotherodon (Tilapia) mossambicus in vitro. Gen.Comp.Endocrinol. In Press.

Kime,D.E., & N.J. Manning, 1982. Seasonal changes in free and conjugated androgens in the brown trout, Salmo trutta. Gen.Comp. Endocrinol. 46: In Press

Kime,D.E., & D.N. Saksena, 1980. The effect of temperature on the hepatic catabolism of testosterone in the rainbow trout (Salmo gairdneri) and the goldfish (Carassius auratus). Gen.Comp.Endocrinol. 42: 228-234.

STEROIDOGENESIS IN THE OVARIES OF THE AFRICAN CATFISH, CLARIAS LAZERA, BEFORE AND AFTER AN HCG INDUCED OVULATION

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The african catfish, Clarias lazera lends itself admirably to fish culture on account of its hardiness, its omnivorous character and its ability to live in poorly oxygenated water (Richter, 1976). Under natural conditions this fish shows an annual reproductive cycle, which culminates in a spawning once a year. In fish culture, it is desired to have young brood throughout the year. At the department of Fish Culture and Inland Fisheries of the Agriculture University, Wageningen, The Netherlands, catfish were cultured, which are able to develop vitellogenic oocytes at any desired time. Under these laboratory conditions, maturation and ovulation have to be induced by a treatment with gonadotropins, such as human chorionic gonadotropin (HCG) or carp pituitary suspensions (Eding et al., 1982). What happens in the ovary after such a treatment is unknown. Therefore, we studied with enzymecytochemical and biochemical methods the steroidogenesis in the ovaries before and after an induced ovulation by HCG.

To induce ovulation adult female catfish with ripe ovaries, aged 10-12 months were treated once with 4 I.U. of HCG per gram of fish. Sixteen hours after HCG injection all the animals have ovulated. At 0, 6, 10, 16, 28, 36, 48 and 72 hours after HCG injections respectively two animals were anaesthetized and the ovaries were removed and prepared for histological (Richter & van den Hurk, 1982), enzymecytochemical (van den Hurk & Richter, 1980) and biochemical (Lambert & van Bohemen, 1979) studies.

Morphological and enzymecytochemical results

The ovaries of control animals contain large postvitellogenic follicles. These follicles have completed their vitellogenesis and have reached a diameter of 1000-1200 µm. The nucleus is located centrally. The granulosa cells of these follicles constitute a thickened ring of cylindrical cells around the region of the future animal pole, while the cells at the opposite side are small and cubic. For tracing the site of steroid synthesis the cytochemical localization of the enzyme 3β-hydroxysteroid dehydrogenase is used. 3β-HSD activity is located only in special theca cells (stroma cells) around vitellogenic and postvitellogenic oocytes. The granulosa cells, although well-developed, give negative results for this enzyme. This indicates that, preceding oocyte maturation the ovarian steroidogenic potency is restricted to the special theca cells.

6 Hours after HCG. The first sign of maturation becomes visible. The nucleus or germinal vesicle has migrated to the micropyle, which is situated near the center of the future animal pole. A few hours later germinal vesicle breakdown takes place and the cylindrical cells of the granulosa begin to secrete an attachment disk, while at the same time the granulosa cells become smaller. No change in 3β-HSD activity can be observed.

10 Hours after HCG. The formation of the attachment disk has been completed and the granulosa now consists of nearly flat cells (fig. 1a). From this moment onwards a change in localization of 3β-HSD can be observed. Besides the special theca cells, the granulosa cells of the maturing follicles show a positive 3β-HSD activity (fig. 1b).

16 Hours after HCG. Many mature oocytes have been evacuated from their follicular envelopes towards the lumen of the ovary, but oviposition has not taken place. So a mass of ovulated ova is found in the ovarian cavities, and numerous postovulatory follicles can be observed in the ovarian wall. The 3β-HSD activity remains in the granulosa cells but these cells now belong to the postovulatory follicles.

28, 36, 48 and 72 Hours after HCG. During these periods the postovulatory follicles are still present in the ovary but they do not show a constant 3β-HSD activity. From ovulation onwards the 3β-HSD activity decreases slowly and 48 hours after HCG, about 30 hours after ovulation, activity can no longer be observed in the granulosa cells of the postovulatory follicles. The special theca cells, however, are still active.

In vitro incubations with ^{3}H-pregnenolone

The steroid synthesizing capacity of the ovaries of catfish before and after ovulation has been studied by incubating ovarian homogenates with tritiated pregnenolone to determine Δ^5 and Δ^4 pathway steroids and in particular the so-called oocyte maturation inducing steroids: deoxycorticosterone and 17α-hydroxy,20β-dihydroprogesterone. The bioconversion to water soluble compounds i.e. testosteroneglucuronide has also been studied, and it appeared that from ovulation

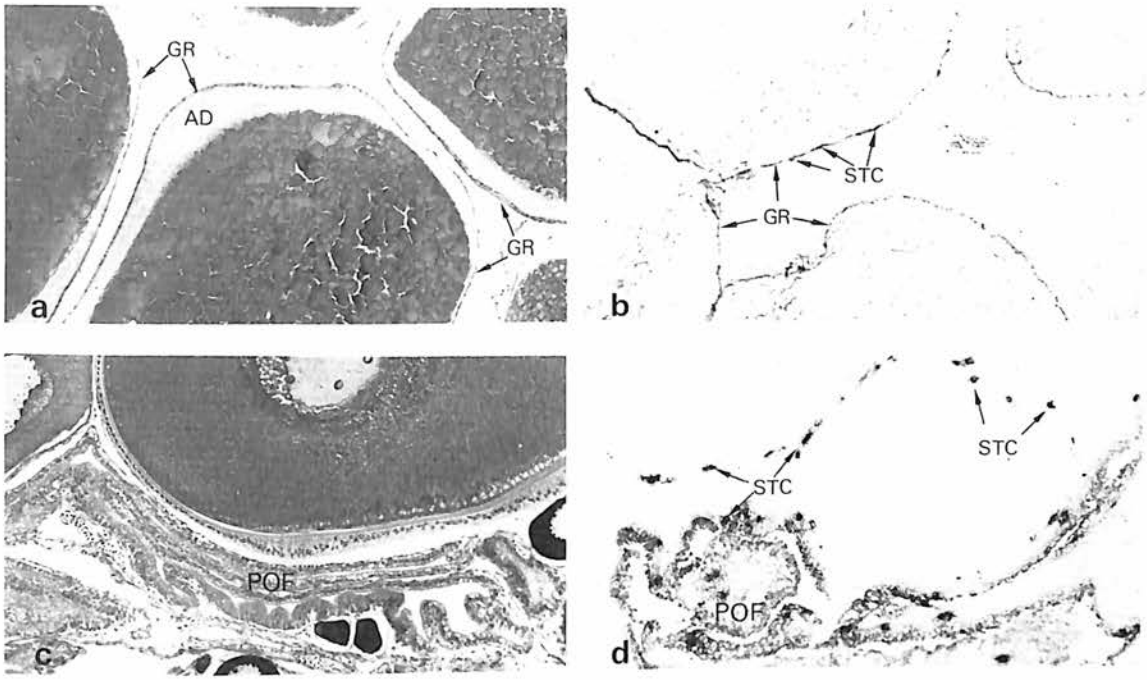

Fig. 1. 3β-HSD activity in the ovary of Clarias lazera. a. Ovary 10 hours after HCG injection; maturating follicles with a well-developed attachment disk (AD). (56x). b. Ovary 10 hours after HCG injection; 3β-HSD activity in granulosa cells (GR) and special theca cells (STC). (56x). c. Ovary 16 hours after HCG injection; postovulatory follicles (POF). (185x). d. Ovary 16 hours after HCG injection; 3β-HSD activity in granulosa cells of the postovulatory follicles and in special theca cells. (185x).

onwards the radioactivity was rather high in the remaining water fractions after organic extraction.

After incubation the steroids were isolated and purified, using thin layer techniques, derivative formation and recrystallization to constant specific activity. The quantitative data, the percentages of yield, were obtained a) by calculation on the basis of precursor initial radioactivity and corrected for procedural losses by a GC recovery determination of the added carriers, and b) by calculation of the percentage distribution of the radioactivity which is represented by the areas of the peaks on the radioscans of the thin layer plates.

The results of the pregnenolone incubation are summarized in fig. 2. In all the incubations carried out pregnenolone has been largely metabolized, as only a little of this precursor is left. The conversion to progesterone is nearly zero. This may be due to a rapid conversion to other products or to a lack of pregnenolone specific 3β-HSD. A lack of 3β-HSD is the most plausible explanation as incubations for shorter time periods never show a larger yield of progesterone. The biosynthetic pathway must therefore go via 17α-hydroxypregnenolone. This is confirmed by the results of incubations with shorter time periods.

HCG treatment effects a shift in synthesis. In control animals the yield of dehydroepiandrosterone is high, while the yield of 17α-hydroxyprogesterone is rather low at that moment. But by 6 hours after HCG treatment a complete shift can be observed. This indicates that gonadotropin stimulates the 17α-hydroxypregnenolone specific 3β-HSD and/or inhibits the 17α-hydroxypregnenolone specific 17α,20-C21 desmolase.

Deoxycorticosterone, a hormone that can induce maturation in catfish in vivo, is not formed. Therefore the maturation effect of deoxycorticosterone is pharmacological and not physiological. However, it seems to be that 17α-hydroxy,20β-dihydroprogesterone is the maturational factor as an increase in synthesis of this steroid can be observed just before ovulation.

The ovulation process can also be correlated with a change in androgen metabolism. The decrease of testosterone may be caused by a decrease in 17β-HSD activity as androstenedione accumulates, and by a conversion of testosterone to its glucuronide.

A comparison of the enzymecytochemical results with the biochemical data offers the possibility to get more information about the steroid synthesizing potency of each of the 3β-HSD positive structures. The special theca

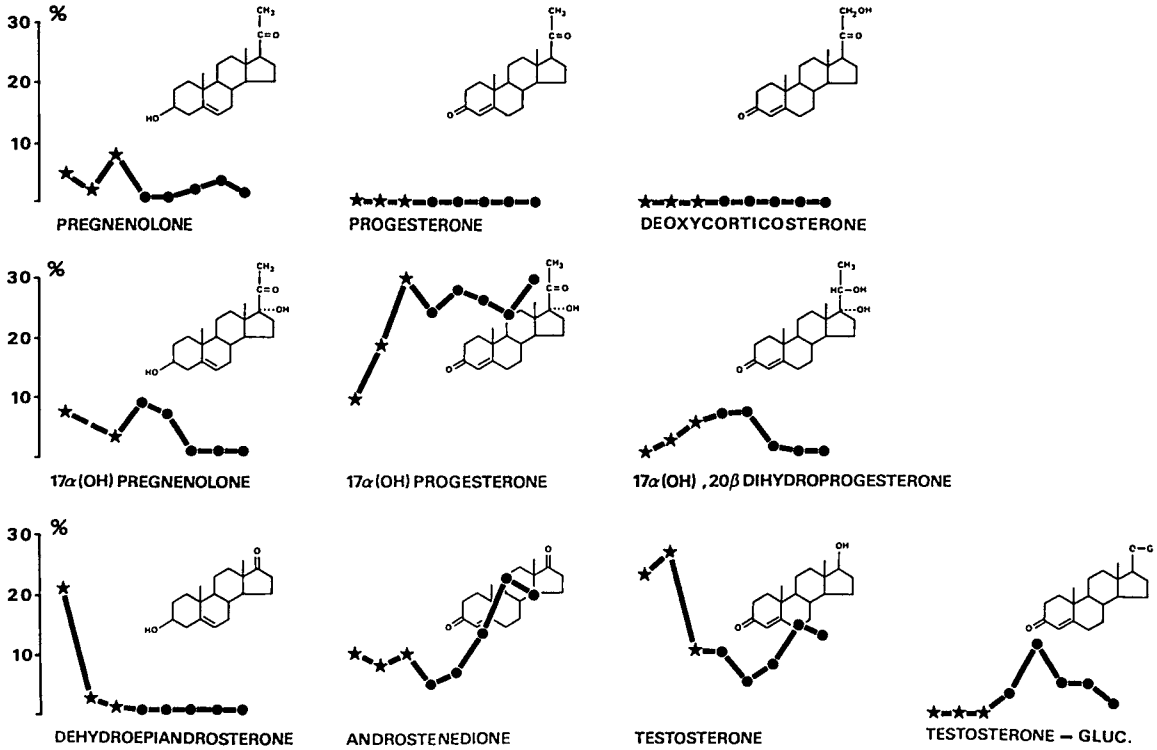

Fig. 2. Bioconversions in ovaries of Clarias lazera 0, 6, 10, 16, 28, 36, 48 and 72 hours after HCG injection. Percentages of yield of steroids isolated from incubations of ovarian homogenates with pregnenolone-7α-^3H. ★ before ovulation; ● after ovulation.

cells are present in control animals as well as in animals treated with gonadotropins, and they are always 3β-HSD positive. This may indicate that these cells are responsible for the synthesis of those steroids which are synthesized throughout the annual cycle (androgens). However, the granulosa cells around the oocytes, as well as in the postovulatory follicles are involved in steroid synthesis only during special physiological processes for instance during maturation with 17α-hydroxy,20β-dihydroprogesterone formation, and during vitellogenesis with an estrogen synthesis (van Bohemen & Lambert, 1981). The 3β-HSD activity in the granulosa cells of the postovulatory follicles should be considered as a residual activity of the granulosa cells before ovulation. The formation of testosterone glucuronide after ovulation can be correlated with the presence of postovulatory follicles but not with the 3β-HSD activity in these follicles. So it might be possible that the enzyme glucuronosyl transferase is located in the granulosa cells of these postovulatory follicles. The significance of these glucuronides is unknown. They may be of importance in a biological inactivation of steroids as suggested by Kime (1980) or they may be functioning as sex pheromones (van den Hurk et al., 1982; Colombo et al., 1982).

Identification of 17α-hydroxy,20β-dihydroprogesterone in plasma by GC-MS.

17α-Hydroxy,20β-dihydroprogesterone can be synthesized by the ovary just before ovulation. With preliminary experiments we have demonstrated the presence of this steroid in the plasma of the african catfish. These experiments were carried out with a gaschromatographical-mass spectrometrical method. 17α-Hydroxy,20β-dihydroprogesterone is derivatized to its methoxime-di TMS compound. From the electron impact (70 eV) fragmentation spectrum it appeared that the molecular ion peak is small but that the ions 388 and 298 are characteristic. Ion 388 is the total molecule without C20-C21 side chain (fig. 3). Following both these ions by selected ion monitoring after capillary gaschromatography of the extracted steroids from the plasma of catfish 28 hours after HCG injection it appeared that both ions were present and coincide with the retention time of the standard 17α-hydroxy,20β-dihydroprogesterone (MO-di TMS) (fig. 4). These ions could not be detected in plasma of control animals.

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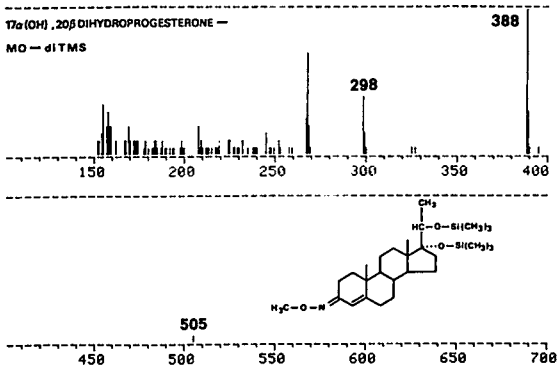

Fig. 3. Mass spectrum (EI) of 17α-hydroxy, 20β-dihydroprogesterone-methoxime-di TMS.

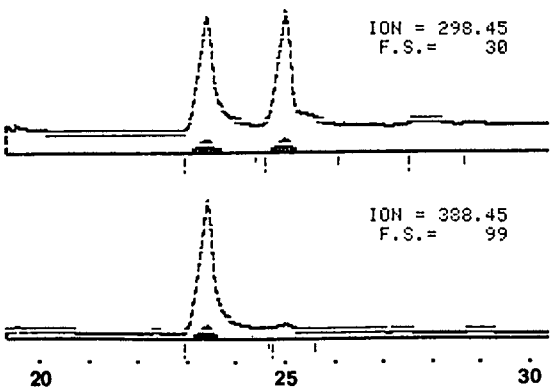

Fig. 4. Selected ion monitoring profiles at m/e 388 and 298 as recorded with a HP 5992B. GC-MS during SIM analysis of the organic fraction of plasma (Clarias lazera) after methoxime and TMS derivatization.

their competent technical assistence, Dr. C.J.J. Richter (Department of Fish Culture and Inland Fisheries, Agriculture University, Wageningen, The Netherlands) for the supply of catfish. HCG was a gift from Intervet International B.V., Boxmeer, The Netherlands.

References

van Bohemen, Ch.G. & J.G.D. Lambert, 1981. Estrogen synthesis in relation to estrone, estradiol, and vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 45: 105-114.

Colombo, L., A. Marconato, P. Colombo-Belvedere & S, Raineri, 1982. Pheromones in teleost fish. This symposium.

Eding, E.H., J.A.L. Janssen, G.H.J. Kleine Staarman & C.J.J. Richter, 1982. Effects of human chorionic gonadotropin (HCG) on maturation and ovulation of oocytes in the ovary of the african catfish Clarias lazera (C. & V.). This symposium.

van den Hurk, R., L.A. 't Hart, J.G.D. Lambert & P.G.W.J. van Oordt, 1982. On the regulation of sexual behaviour of male zebrafish, Brachydanio rerio. Gen. Comp. Endocrinol. 46: 403.

van den Hurk, R. & C.J.J. Richter, 1980. Histochemical evidence for granulosa steroids in follicle maturation in the african catfish, Clarias lazera. Cell Tissue Res. 211: 345-348.

Kime, D.E., 1980. Androgen biosynthesis by testis of the goldfish, Carassius auratus, in vitro: The effect of temperature on the formation of steroid glucuronides. Gen. Comp. Endocrinol. 41: 164-172.

Lambert, J.G.D. & Ch.G. van Bohemen, 1979. Steroidogenesis in the ovary of the rainbow trout, Salmo gairdneri, during the reproductive cycle. Proc. Indian. natn. Sci. Acad. B45: 414-420.

Richter, C.J.J., 1976. The african catfish, Clarias lazera (C. & V.), a new possibility for fish culture in tropical regions ? In: Aspects of fish culture and fish breeding. Ed. E.H. Huisman. Miscellaneous papers 13, Landbouwhogeschool, Wageningen, The Netherlands.

Richter, C.J.J. & R. van den Hurk, 1982. Effects of 11-desoxycorticosterone-acetate and carp pituitary suspension on follicle maturation in the ovaries of the african catfish, Clarias lazera (C. & V.). Aquaculture 28, in press.

PLASMA LEVELS OF SEX STEROIDS IN RELATION TO OVULATION AND SPERMIATION IN RAINBOW TROUT (SALMO GAIRDNERI)

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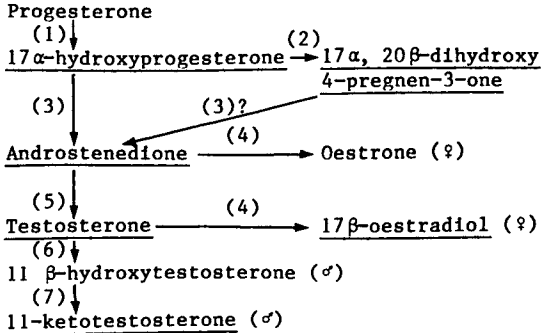
Summary

The physiological events associated with both ovulation and spermiation in rainbow trout involve a fall in plasma levels of androgens and a concomitant rise in plasma levels of progestagens. One possible explanation is that gonadotrophin blocks androgen biosynthesis in the gonads by inhibiting C21 → C19 desmolase, thereby causing the build-up of 17α-hydroxyprogesterone and its 20β-hydroxylated derivative, 17α, 20β-dihydroxy-4-pregnen-3-one. In the male rainbow trout, the highest levels of 17α, 20β-dihydroxy-4-pregnen-3-one have been found in spermiating fish. We propose that a major function of this steroid in the male is the control of the K^+ composition of seminal fluid.

Keywords: Salmo gairdneri; spermiation; ovulation; sex steroids; 17α, 20β-dihydroxy-4-pregnen-3-one.

Introduction

This paper describes the changes in plasma levels of sex steroids that take place around the time of ovulation and spermiation in rainbow trout. The steroids we have investigated (shown underlined) form a part of the Δ^4 biosynthetic pathway:



Most of the enzymes involved in this pathway have been demonstrated, by in vitro techniques, in ovaries (Sire & Depeche, 1981) and testes (Arai & Tamaoki, 1967; Depeche & Sire, 1982) of rainbow trout, and are (as indicated in the diagram): (1) 17α-hydroxylase, (2), 20β-hydroxysteroid dehydrogenase, (3) C21 → C19 desmolase, (4) aromatase, (5) 17β-hydroxysteroid dehydrogenase, (6) 11β-hydroxylase, (7) 11β-hydroxysteroid dehydrogenase. It is assumed in this study that the relative activity of these enzymes is the major influence on plasma levels of sex steroids. Gonadal formation of steroid glucuronides (Kime, this volume) and possible differential rates of release and metabolism of steroids have not been investigated.

One observation made in this study was that spermiating male fish had substantially elevated levels of 17α, 20β-dihydroxy-4-pregnen-3-one. This steroid has been implicated in the control of oocyte maturation in female rainbow trout. We suggest a function for it in the male.

Methods

Females

Details of the experimental work on female rainbow trout have been reported previously (Scott et al., 1982), or are awaiting publication (Scott et al., in press).

Males

A group of 16 male rainbow trout (18-months old) were kept in circular tanks supplied with river water. All milt was stripped out and collected at two-week intervals. Blood samples were taken every four weeks. Measurements were made of volume of milt, spermatocrit value, and sodium and potassium ion concentrations in seminal fluid. Sperm density and total sperm count were estimated from the spermatocrit.

Experimental

Three groups of spermiating male rainbow trout (c. 1000 g weight) were injected intraperitoneally with 3.0, 0.3 or 0 mg of 17α, 20β-dihydroxy-4-pregnen-3-one dissolved in 500 µl of a polypropylene glycol, 0.9% saline, dimethylsulphoxide mixture (50, 45, 5 v/v/v). The experiment ran from 11-19 January 1982. Milt was collected every day, and blood samples every two days.

All steroids were determined by radioimmunoassays carried out directly on heated, diluted plasmas (see Scott et al., 1982).

Results

Ovulation

(a) Ovulation and the events immediately preceding it (i.e. oocyte maturation) were associated with a fall in testosterone, androstenedione and 17β-oestradiol levels, and an abrupt rise in 17α-hydroxyprogesterone and 17α, 20β-dihydroxy-4-pregnen-3-one levels (Fig. 1).

(b) Injection of chum salmon pituitary powder extracts into mature female rainbow trout raised plasma levels of 17α, 20β-dihydroxy-4-pregnen-3-one and lowered plasma levels of testosterone (Fig. 2).

Spermiation

(a) Spermiation (the period when sperm could be expressed manually from male fish) lasted for 6-7 months in the particular strain of rainbow trout we studied (Fig. 3). The highest androgen levels coincided with the beginning of this period (November). 17α, 20β-dihydroxy-4-pregnen-3-one levels, however, peaked several months later (March) when sperm release was at its maximum. 17α-hydroxyprogesterone and 17β-oestradiol levels (not shown) did not vary significantly. Androgen levels, which started to rise again in June/July and accelerated sharply in September were correlated with the gonadosomatic index, and with the proportion of spermatocytes in the testis, but were not correlated with any of the milt parameters. 17α, 20β-dihydroxy-4-pregnen-3-one levels correlated with volume of milt ($r = 0.45$), total sperm count ($r = 0.43$), K^+ content ($r = 0.48$) and K^+/Na^+ ratio ($r = 0.52$) in seminal fluid (all $P < 0.001$, $n = 67$; steroid levels log. transformed)

(b) Injections of 17α, 20β-dihydroxy-4-pregnen-3-one into spermiating fish significantly raised the K^+/Na^+ ratio of the seminal fluid (Fig. 4) but did not affect the volume of milt or total sperm count. A linear relationship exists between the concentrations of K^+ and Na^+ in seminal fluid. The regression equation for calculating potassium ion levels (calculated from the data collected on days 0, 2, 4 and 6 of the experiment) was (0.240 x sodium ion concentration) - 0.973. The correlation coefficient was 0.83 ($n = 84$), and the intercept was not significantly different from zero. This latter fact means that observed-expected K^+ concentrations (as shown in Fig. 4) and K^+/Na^+ ratios (referred to above) are equivalent measurements.

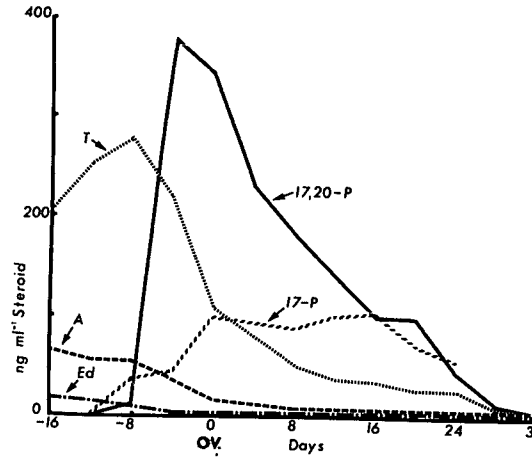


Fig. 1. Changes in plasma steroid levels of female rainbow trout around the time of ovulation.

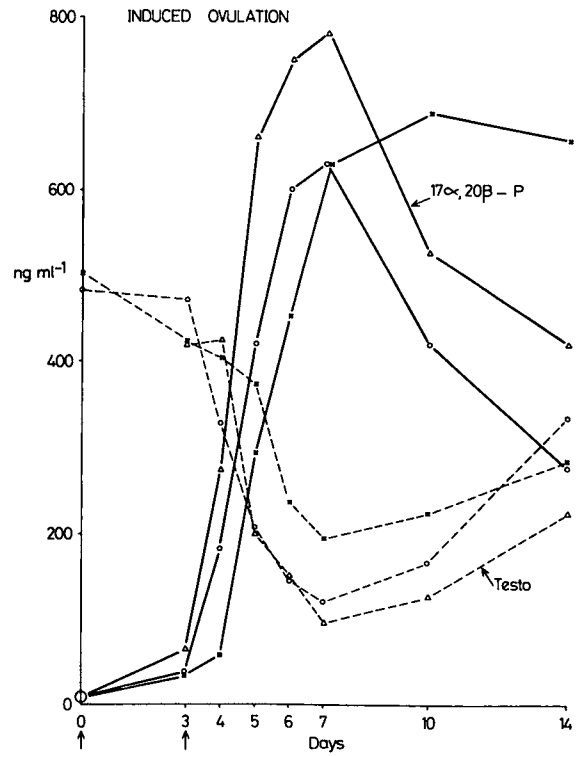


Fig. 2. Response of plasma levels of 17α, 20β-dihydroxy-4-pregnen-3-one and testosterone to intraperitoneal injections of salmon pituitary extract in three fish. Ovulation occurred between days 7 and 10. Arrows indicate injection days. 1st injection, 1.5 mg powder kg^{-1}; 2nd injection, 6 mg powder kg^{-1} body weight.

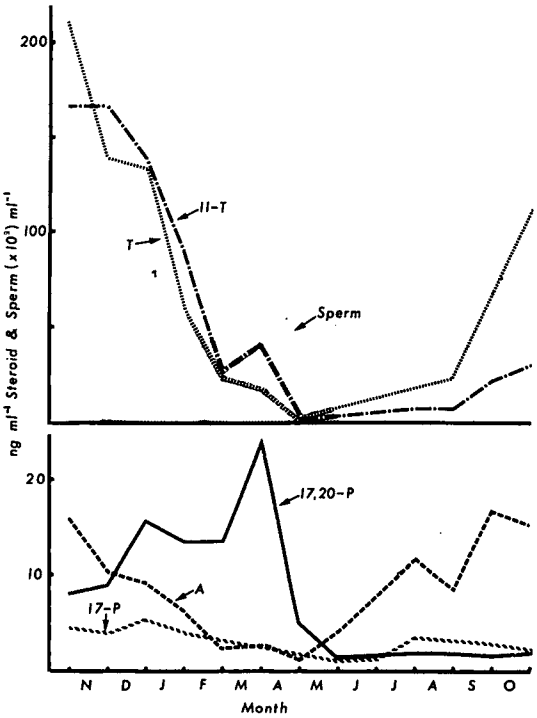


Fig. 3. Annual variations in plasma steroid levels and total sperm production in male rainbow trout.

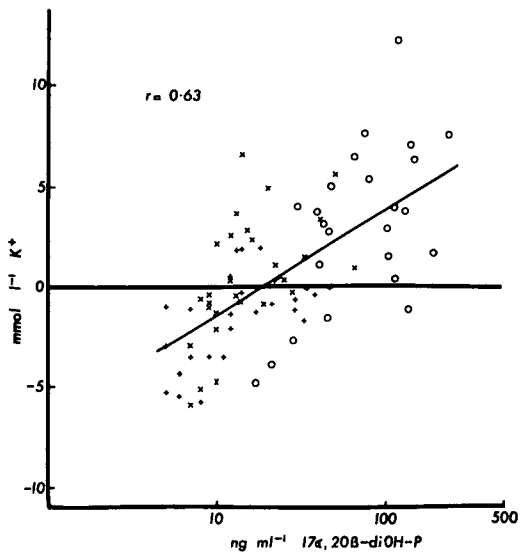


Fig. 4. Plot of observed-expected potassium concentrations in seminal fluids against levels of 17α, 20β-dihydroxy-4-pregnen-3-one in plasmas of male rainbow trout injected intraperitoneally with 3.0 mg (o), 0.3 mg (×) and 0 mg (+) of steroid. Includes data from days 2, 4 and 6 after injection.

Discussion

Our discussion will be limited to the two topics which, we feel, are of most interest: (1) the fall in plasma androgen levels at spermiation and ovulation, and (2) the role of 17α, 20β-dihydroxy-4-pregnen-3-one in males.

(1) We have demonstrated that oocyte maturation/ovulation, whether natural or induced, and spermiation are associated with a fall in plasma androgen levels. There are several possible explanations. One is that gonadotrophin directly inhibits C21 → C19 desmolase. This would readily explain why levels of one or both of the progestagens rise at virtually the same time as the androgen levels drop. Implicit in this proposed mechanism is the presence, prior to oocyte maturation and spermiation, of the branch route to androgen synthesis: 17α-hydroxyprogesterone→17α, 20β-dihydroxy-4-pregnen-3-one→androstenedione. There is some evidence that this does exist. Depeche & Sire (1982) have reported that 20β-hydroxysteroid dehydrogenase is present in trout testes at all stages of the reproductive cycle. Since 17α, 20β-dihydroxy-4-pregnen-3-one can be found in plasma only at spermiation, it is possible that during the rest of the reproductive cycle it is being efficiently converted to androstenedione.

However, it has been demonstrated in the rat that 17α, 20β-dihydroxy-4-pregnen-3-one cannot be subjected to side chain cleavage; in fact, this steroid has been shown to be a powerful inhibitor of desmolase activity (Inano et al., 1969). If this were also true in the rainbow trout, then there is another simple explanation for the observed changes in hormone levels: gonadotrophin stimulates 20β-hydroxysteroid dehydrogenase activity, which causes 17α, 20β-dihydroxy-4-pregnen-3-one levels to rise; this steroid inhibits C21 → C19 desmolase activity and causes the androgen levels to drop. One or other of the two mechanisms (or perhaps a combination of both) seem the most likely explanation for the grosser changes that take place in steroid levels.

(2) 17α, 20β-dihydroxy-4-pregnen-3-one was isolated from the plasma of male and female Pacific salmon over twenty years ago (see Schmidt & Idler, 1962). There is now much evidence to suggest that it is the mediator of gonadotrophin-induced oocyte maturation in female salmonids (see Scott et al., 1982 for references). There has been no suggestion as to its function in males. Schmidt & Idler (1962) reported levels of 80 ng ml^{-1} in pooled plasmas of mature male Oncorhynchus nerka. Campbell et al. (1980) failed to find any in male rainbow trout (< 9 ng ml^{-1}) but they undoubtedly sampled their fish too early in the season (December), before levels had risen above the limit of detection of their assay. We

have found levels as high as 60 ng ml^{-1} in some rainbow trout males. We have also clearly demonstrated that elevated levels of 17α, 20β-dihydroxy-4-pregnen-3-one are associated with spermiation. Since, by this stage, all meiotic and mitotic divisions have been completed and most of the spermatozoa are fully formed, a role for this steroid in the control of some aspect of spermatogenesis (as suggested by Depeche & Sire, 1982) would seem unlikely. Our initial thoughts were that 17α, 20β-dihydroxy-4-pregnen-3-one might control testicular hydration (cf. the hydration of oocytes that takes place concurrently with meiotic maturation), and that injection of this steroid would increase the total volume of the milt. However, although there was a significant correlation between plasma steroid levels and sperm volume, injections of 17α, 20β-dihydroxy-4-pregnen-3-one had no influence on this particular parameter.

Our interest in factors controlling the activation of spermatozoa (Baynes et al., 1981), however, led us to the discovery of another, much closer, association - between 17α, 20β-dihydroxy-4-pregnen-3-one levels and the potassium concentration of seminal fluid.

Seminal fluids of many vertebrates, among them the rainbow trout, have an unusually high potassium content (5-10 times that of blood plasma). It is known that, in rainbow trout testes, potassium ions play an important role in the inhibition of spermatozoan motility (see Baynes et al., 1981). It would not be unreasonable, in view of what is known of steroid-mediated ionic transport systems in other tissues, to expect a cation with such an important function to be under the control of a testicular steroid. Cortisol, desoxycorticosterone and aldosterone directly control the transport of K^+ and Na^+ across membranes of organs such as the kidney and gills. They are all, like 17α, 20β-dihydroxy-4-pregnen-3-one, C21 steroids. If the mechanism we suggest exists in the testis, and is in any way comparable, then we should expect to find specific receptors for 17α, 20β-dihydroxy-4-pregnen-3-one and also a Na^+/K^+-activated ATPase within the walls of the vas deferentia. This remains to be established.

Abbreviations used on figures:

17-P: 17α-hydroxyprogesterone
17,20-P: (17α 20β-P): 17α, 20β-dihydroxy-4-pregnen-3-one
A: Androstenedione
T: (Testo): Testosterone
Ed: Oestradiol
11-T: 11-ketotestosterone

References

Arai, R. & B. Tamaoki, 1967. Steroid biosynthesis in vitro by testes of rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 8:305-313.

Baynes, S. M., A. P. Scott & A. P. Dawson, 1981. Rainbow trout, Salmo gairdneri, spermatozoa: effects of cations and pH on motility. J. Fish Biol. 19:259-267.

Campbell, C. M., A. Fostier, B. Jalabert & B. Truscott, 1980. Identification and quantification of steroids in the serum of rainbow trout during spermiation and oocyte maturation. J. Endocrinol. 85: 371-378.

Depeche, J. & O. Sire, 1982. In vitro metabolism of progesterone and 17α-hydroxyprogesterone in the testis of the rainbow trout, Salmo gairdneri, at different stages of spermatogenesis. Reprod. Nutr. Dévelop. 22:427-438.

Inano, H., H. Nakano, M. Shikita & B. Tamaoki, 1967. The influence of various factors upon testicular enzymes related to steroidogenesis. Biochim. Biophys. Acta 137:540-548.

Schmidt, P. J. & D. R. Idler, 1962. Steroid hormones in the plasma of salmon at various states of maturation. Gen. Comp. Endocrinol. 2:204-214.

Scott, A. P., E. L. Sheldrick & A. P. F. Flint, 1982. Measurement of 17α, 20β-dihydroxy-4-pregnen-3-one in plasma of trout (Salmo gairdneri): seasonal changes and response to salmon pituitary extract. Gen. Comp. Endocrinol. 46:444-451.

Scott, A. P., J. P. Sumpter, and P. A. Hardiman. Hormone changes during ovulation in the rainbow trout. Gen. comp. Endocrinol. (in press).

Sire, O. & J. Depeche, 1981. In vitro effect of a fish gonadotrophin on aromatase and 17β-hydroxysteroid dehydrogenase activities in the ovary of the rainbow trout (Salmo gairdneri). Reprod. Nutr. Dévelop. 21:715-726.

STEROIDS IN PLASMA OF THE FEMALE RAINBOW TROUT BEFORE AND AFTER OVULATION BY NCI-GCMS

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In connection with the study of the hormonal regulation of reproduction in teleosts, research has been started to identify and quantify steroids in plasma of female rainbow trout, about three days before, and one and four weeks after ovulation. A new advanced method is used to determine steroids in plasma at a low level and for isolation and purification ion-pair systems are introduced. Identification and quantification takes place by capillary gaschromatography-negative chemical ionization-mass-spectrometry. To obtain full expression of the negative chemical ionization process, steroids with hydroxyl groups are derivitized to their heptafluorobutiryl (HFB) derivatives and steroids with keto groups to their o-pentafluorobenzyloxime (OPFB) derivatives. These compounds are synthetized to introduce electrophilic functions in the steroid molecules. When steroids contain both, hydroxyl and keto groups also OPFB-TMS derivatives were prepared.

An outline of the method

Extraction of steroids out of plasma

PLASMA (0.3 - 1.5 ml)
Adding: Internal Standards
- 2-Methoxyestradiol 2.0 ng
- 11β Hydroxyetiocholanolone 10-60 ng
$BaCl_2$ 10% (0.1 ml)(Precipitation of sulfates and phosphates)
Acetone (0.3 ml) and 4 N HCl (3 drops) (Precipitation of proteins)
H_2O up to a final volume of 3.0 ml.

Chloroform-Ethylacetate-Acetone 7.0 ml (70:28:2)
Extraction of the mixture for 2 min. on a Vibramix.
Centrifugation (10 min.,3000 rpm)

CLEAN-UP OF ORGANIC PHASE
Washing with 1) H_2O,2) NH_4OH (pH=8.3),3) H_2O
Drying by filtration
Evaporation by N_2(O_2-free) 50°C.
RESIDUE I

Isolation by ion-pair extraction

RESIDUE I dissolved in 2.0 ml Toluene (-20°C)
Adding: Tetramethylammoniumhydroxide (TMAOH) (20% in methanol) 60 ul, - 20°C.
Centrifugation (10 min., 3000 rpm)
TMAOH-FRACTION. . . . . TOLUENE-FRACTION

TMAOH-FRACTION (Phenolic steroids; Bile acids)
Adding: N-Tricine (10% in H_2O)(0.5 ml, 0°C)
Toluene-Diethylether 1:2 (2,1 ml)
Centrifugation (10 min.,3000 rpm)
Organic phase is washed with 0.2 N HCl (0.5 ml)
Adding: 2.0 Toluene

Drying by filtration
Evaporation under N_2(O_2-free) 50°C.
RESIDUE II

TOLUENE-FRACTION (Non-phenolic steroids)
Washing with 0.2 N HCl (0.5 ml)
Drying by filtration
Evaporation under N_2(O_2-free) 50°C.
RESIDUE III

Derivatization

KETO-FUNCTIONS to oxims by FLOROX-Reagent (O-Pentafluorobenzylhydroxylamine HCl/Pyridine)

HYDROXYL/PHENOLIC-FUNCTIONS to HFB-esters by Heptafluorobutirylimidazole or to TMS-ethers by N-Trimethylsilylimidazole

RESIDUE II

KETO-FUNCTIONS to oxims by MOX-Reagent (2% solution Methoxyamine HCl in Pyridine)

HYDROXYL/PHENOLIC-FUNCTIONS to TMS-ethers by N-Methyl-N-Trimethylsilyl-Trifluoroacetamide

Purification of the derivatives by liquid/liquid-extraction; Hexane-Acetonitrile 10:1 (2.0 ml)

Identification and quantification of the derivatives

Cap. Gaschromatography-Negative Chemical Ionization Mass Spectrometry
Column: Sp 2100 Fused silica, wide bore, 12.5 m
Programme temp. range: 160°-260°C.
Programme rate: 2.0°C/min.
Time 1 = 2.0 min., Carrier: Helium, flow 1.5 ml/min.
Transferline temp.: 275°C.
Source temp.: 200°C.; Analyser temp.: 185°C.
Reagent Gas: Methane
Source pressure : 0.5 Torr; 1.0 x 10^{-4} Torr.
Electron energy: 120 EV

TOTAL ION MONITORING; sc.w. 400-1000 M/z
EM 2600 Volt
QUANTIFICATION by SELECTED ION MONOTORING using Hp 100 Ion-software programme Hp 5985 GC/MS/DS;
EM 2800 Volt
Internal Standards: - 2-Methoxyestradiol
- 11β Hydroxyetiocholanolone

Identification

The more concentrated steroids in the plasma were identified by comparing the obtained spectra with the spectra of standards at the expected GC retention times (fig. 1). The identity of the other steroids was determined by confirmation of selected ions out of a total ion monitoring at distinct retention times.

In first instance attention is paid to the

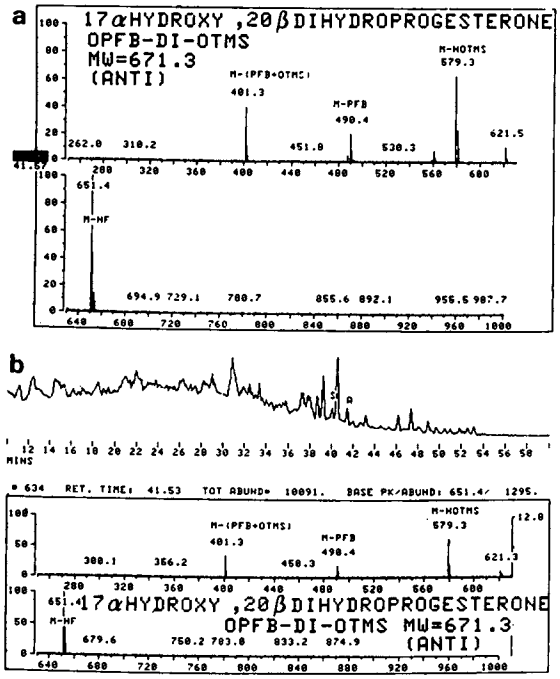

Fig. 1. a. Mass spectrum (NCI) of the standard; b. Mass spectrum out of plasma.

presence of androgens and oestrogens and their intermediates. A second group of steroids are the so-called maturation inducing steroids. A third group are some metabolites of androstenedione and testosterone.

Quantification

To quantify the steroids the abundance of selected ions i.e. the most characteristic ions and in most cases M-HF (M-20) were compared with the abundance of selected ions of the internal standards. These selected ions were taken out of a total ion monitoring. The results are summarized in Table 1.

Acknowledgement

These results were obtained with the aid of a HP 5985B-GC/MS/DS instrument kindly put at our disposal by: Applied Mass spectrometrical and Toxicological Laboratory (AMTOL B.V.), Kleverparkweg 13rd, 2023 CA Haarlem, The Netherlands.

Table 1. Levels of steroids (ng/ml) in plasma of Salmo gairdneri three days before (1), one week (2) and four weeks (3) after ovulation.

	1	2	3		1	2	3
Estrone	15.9	3.0	2.0	17α Hydroxy,20β -dihydroprogesterone	4.9	126.8	9.7
Estradiol	1.2	0.8	1.0	17α Hydroxy,20α -dihydroprogesterone	–	–	–
Estriol	–	–	–	Deoxycorticosterone	23.0	40.1	26.0
2-Methoxyestrone	1.9	1.7	1.8				
2-Methoxyestradiol	–	–	–	11α Hydroxyprogesterone	42.5	15.6	31.0
16-Ketoestradiol	–	–	–	Corticosterone	23.9	33.6	37.7
				20β Dihydrocortisone	–	–	–
Pregnenolone	5.2	17.7	10.0				
17α Hydroxypregnenolone	15.2	12.8	4.3	5β Pregnane 3α ,20α -diol	–	–	–
Dehydroepiandrosterone	3.5	30.7	15.5	5α Pregnane 3β ,17α ,20β -triol	–	–	–
Androst-4-ene 3β ,17β diol	–	1.8	1.6	5α Androstanedione	118.5	37.3	50.3
Progesterone	6.6	42.2	16.2	5α Androstane 3β ,17β -diol	+	+	+
17α Hydroxyprogesterone	49.4	245.7	29.0	5β Androstane 3α ,17β -diol	–	–	–
Androstenedione	148.3	66.3	28.4	Androsterone	–	–	–
Testosterone	167.3	25.9	17.5	Etiocholanolone	4.8	57.2	42.0
11β Hydroxyandrostenedione	+	+	+	11β Hydroxyetiocholanolone	–	–	–
11-Ketoandrostenedione	42.3	71.4	30.3	11-Ketoetiocholanolone	13.7	60.6	24.4
11β Hydroxytestosterone	13.3	5.1	3.6	5α Dihydrotestosterone	90.5	49.6	41.8
11-Ketotestosterone	36.6	10.3	6.2	5β Dihydrotestosterone	27.6	11.9	12.0

TOPOGRAPHICAL DISTRIBUTION OF STEROIDOGENIC ENZYMES IN RELATION TO SPERMATOGENESIS IN THE TESTIS OF SQUALUS ACANTHIAS.

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The process of spermatogenesis in vertebrates is, structurally and functionally, an extremely complex phenomena. Although the morphogenesis of spermatozoa is by now well understood, the functional changes which occur during the various stages of germ cell development are still largely unknown. Thus despite the fact that it is well established that spermatogenesis is androgen-dependent, it is still uncertain what role steroids play during the maturation of germ cells. It is essential, therefore, to not only determine, qualitatively and quantitatively the steroidal micro-environment associated with specific germ cell stages, but to also identify the cells responsible for steroid production. It is, however, technically very difficult to analyze the steroidogenic potential of the various germ cell stages in the testes of most laboratory animals. In these species the differentiation of germ cells is not synchronized in any one testicular region and since all stages of germ cell development are present simultaneously any changes in steroidogenesis associated with these stages would be hard to detect. To alleviate this problem we selected an animal model, the shark Squalus acanthias, where discrete germ cell stages are distinctly separated within the testis. This spatial segregation of the differentiating germ cells can actually be visualized in thick transverse sections of the testis, with the aid of a dissecting microscope. Using the gross criteria of opacity, color and relationship to the epigonal tissue the testis sections were separated into 3 zones. The composition of the seminiferous lobules present in these isolated regions, observed by light microscopy, is shown in Table I.

Table I

Morphological components of cysts

Zone	
I	Spermatogonia
II	Spermatocytes. Some cysts contain round spermatids
III	Mixture of cysts containing spermatids and spermatozoa

The steroidogenic potential of these zones was also investigated by incubating microsomes with various radioactive substrates. The topographical distribution of steroidogenic enzymes associated with each of the zones is summarized in Table II. As germ cell controls, free sperm stripped from the vas deferens were analyzed for the same enzymes.

Table II

Steroidogenic activities[a]

	17α hydroxylase (pmol/min/mg)	C-17,C-20 lyase (pmol/min/mg)	aromatase (fmol/min/mg)
Zone I	5.84 ±0.81	2.90 ±1.33	2.93 ±0.50
II	4.26 ±0.61	6.89 ±1.10	7.10 ±0.40
III	10.76 ±2.05	24.18 ±5.61	2.83 ±0.40
Free sperm	2.35[b]	-0-[b]	-0-[b]

[a]Product yield per mg microsomal protein (means ± sem) for separate microsomal preparations from 3 animals.

[b]Product yield per mg homogenate protein for a single pool of free sperm.

Light microscopic observations indicated that the interlobular tissue appeared poorly developed. Thus Leydig cells, if present, would be undifferentiated but could possibly synthesize small but important quantities of steroids. It is more probable, however, that the seminiferous lobules are responsible for the majority of the androgen and estrogen synthesizing activity of the shark testis. This study indicates, therefore, that the steroidogenic activity of the shark testis may vary, depending upon the stage of germ cell development.

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PLASMA STEROID PROFILE DURING THE SPAWNING SEASON OF PIKE

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Summary

It is well known that female pike do not spawn in captivity, even if captured after completion of vitellogenesis. Ovarian atresia of pike can be successfully prevented by several hormonal treatments which have been found effective in stimulating spawning in captivity (De Montalembert et al, 1978). However, the reproductive physiology of pike is largely unknown. The aim of this work was to investigate plasma profiles of sex-steroid hormones in connection with gonadal maturation of female pike during the reproductive cycle.

Plasma progesterone, testosterone and 17 β-oestradiol were assayed by high specific RIA methods in 22 female pike captured from lake Trasimeno shortly before and during the natural spawning season. Blood samples, obtained by branchial artery puncture on the capture of each pike, were centrifuged within 1 - 2 hours and the plasma stored at -20 °C until processing. The pike were subdivided into three groups depending on the stage of gonadal maturation according to histological findings: a post-vitellogenic group with the germinal vesicle (GV) located in a peripheral position (GV stage: 11 fish); a preovulatory group in which the GV was not visible and the ova were clear (GVBD stage or GV breakdown: 2 fish) and an ovulatory group (spawning stage: 9 fish).

Plasma levels of progesterone averaged 0.4 ± 0.2 ng/ml (Mean ± SD) without any significant change during the three gonadal maturation stages investigated.
Plasma testosterone levels were similar in both groups during the preovulatory stage resulting 2.6 ± 0.9 ng/ml and 2.6 ± 0.7 ng/ml respectively in the GV and GVBD stages, but significantly ($P < 0.01$) fell to 0.2 ± 0.2 ng/ml at spawning.
Plasma oestradiol concentration averaged 5.7 ± 2.3 ng/ml in pike having oocytes with the GV in peripheral position, was significantly ($P < 0.05$) lower in the GVBD stage group being 2.0 ± 0.3 ng/ml, then dropped ($P < 0.001$) to 0.3 ± 0.2 ng/ml, in the spawning pike.

RIA methodology is particularly suitable for endocrinological investigations in fish because its high specificity and sensitivity allow independent assays of steroid hormones on plasma samples as small as 0.1 to 0.2 ng/ml.

Even if progesterone is a significant precursor in the ovarian steroid biosynthesis of pike, its role in gonadal maturation is not remarkable, as ovulation causes no change in its peripheral plasma profile.

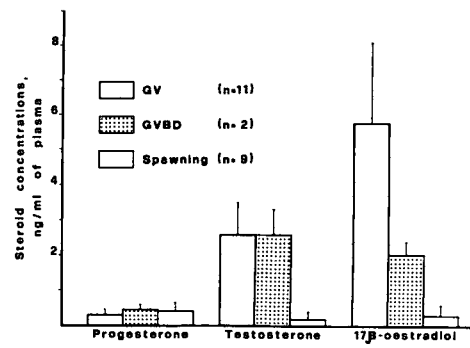

fig. 1. Sex-steroid hormonal profiles during ovarian maturation and spawning in pike.

Testosterone is an important precursor to C-18 steroid biosynthesis in the ovary and is synthetized and released only in the preovulatory phases. However, its physiological role in female pike reproduction is still uncertain. 17 β-oestradiol has a stimulating effect in vitellogenesis but a probable inhibitory effect on the resumption of meiosis and ovulation as demonstrated by its constantly decreasing plasma level during the last stages of the reproductive cycle.
The decreasing oestradiol profile, in contrast to the constant level of testosterone, suggests that in the preovulatory stages the aromatizing enzymes are partially inactivated. Ovulation is associated with a significant drop in both androgen and oestrogen plasma levels suggesting that the biosynthetic steroid pathway is blocked between progesterone and testosterone.

References

De Montalembert G., B. Jalabert, C. Bry, 1978 Precocious induction of maturation and ovulation in northern pike (Esox lucius). Ann. Biol. anim. Bioch. Biophys. 18(4):969-975.

SEASONAL VARIATIONS OF PLASMA ANDROGENS AND GONAD HISTOLOGY IN MALE SPOTTED SEATROUT, CYNOSCION NEBULOSUS (FAMILY: SCIAENIDAE).

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Several members of the perciform family Sciaenidae are important recreational and commercial fishes in coastal waters of the southeastern U.S.A. In addition, two sciaenids, spotted seatrout (Cynoscion nebulosus) and red drum (Sciaenops ocellatus), have been proposed as species suitable for mariculture in Texas. However, the reproductive physiology of these species is largely unknown. The purpose of this initial study, therefore, was to determine both the histological and the endocrine changes which occur during gonadal maturation and spawning in natural populations of male spotted seatrout.

Spotted seatrout were caught with artificial lures from early April until the end of October 1981 and bled within two min of capture. This species has a prolonged spawning season in the subtropical waters of south Texas (April to October). Over 83% of the males collected between April and September (mean temp. 27.2 °C) were running ripe. In contrast all the fish obtained after the onset of colder weather in mid October were spent (mean temp. 20.4 °C).

Histological examination revealed the typical teleostean pattern of spermatogenesis in C. nebulosus. All stages of spermatogenesis (from spermatogonia to spermatozoa) were found. The testes of running ripe fish collected in April contained large numbers of primary and secondary spermatocytes, as well as spermatids and spermatozoa, whereas those of running ripe individuals collected in June contained mainly spermatids and spermatozoa. The lobules of running ripe fish obtained in September were filled primarily with spermatozoa and those of spent individuals collected at the end of October were empty and partially collapsed.

Testosterone (T), 11-ketotestosterone (11-KT) and 17α-hydroxy-20β-dihydroprogesterone (17α, 20β OHP) were measured by radioimmunoassay using antisera which did not cross-react significantly with other steroids. Alterations in the circulating levels of T and 11KT were observed during the reproductive season. Highest levels of 11KT were detected in fish blood collected during the peak spawning period in April (Fig. 1). During this early part of the spawning season mature fish had higher plasma 11KT concentrations then running ripe individuals. 11KT subsequently declined in both mature and running ripe spotted seatrout to less than 2 ng/ml in July.

Testosterone exhibited a similar pattern

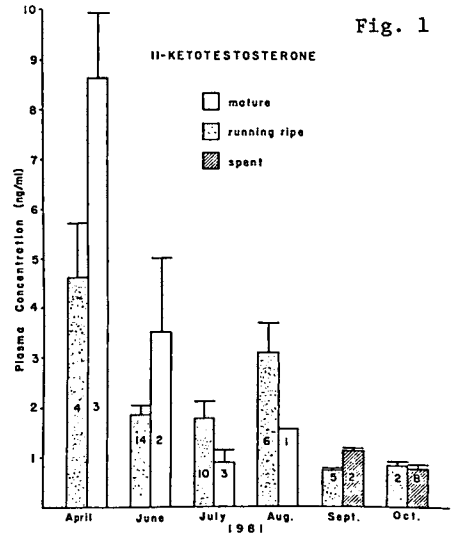

Fig. 1

of plasma fluctuations in male fish during the spawning season (Fig. 2). Maximum levels (2.4 ng/ml) were detected in April. Plasma T concentrations gradually fell during the summer and by September were only 0.2 ng/ml in running ripe fish. Prelimary data indicated that plasma levels of 17α, 20β OHP in male spotted seatrout were low (< 0.6 ng/ml) and did not fluctuate during the reproductive season.

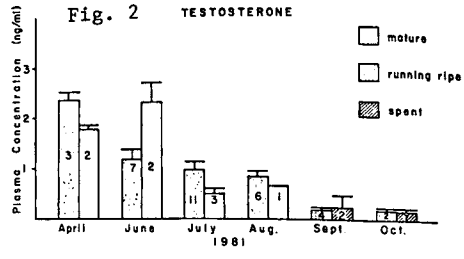

Fig. 2

Conclusions

These results suggest that high plasma concentrations of 11KT and T may be important in the spring for testes maturation and the early stages of spermatogenesis in spotted seatrout. However, in midsummer the final stages of spermatogenesis occur in the absence of high circulating androgen levels. High levels of 17α, 20β OHP may not be necessary for spermatogenesis to occur in spotted seatrout.

OVARIAN STEROID METABOLISM IN THE IMMATURE EUROPEAN EEL *(Anguilla anguilla)*.

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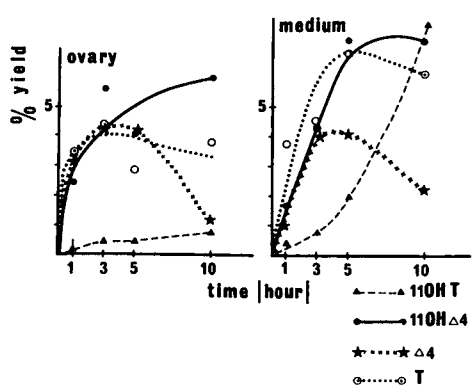
Summary

The steroid production pattern of the ovary in the immature silver eel is directed towards the synthesis of 11 oxoderivative androgens : no aromatisation of androgens has been found in the ovary ; the affinity of plasma binding proteins (PBP) for estradiol (E_2) and testosterone (T) is very high ; the metabolic clearance rate (MCR) of E_2 is 2.5 times lower than that of T, despite plasma level (PL) of E_2 (0.05, 0.5) lower than that of T (0.8, 3.5 ng/ml).

The European eel differentiates sexually in continental waters but supposedly spawns in the vicinity of the Sargasso sea. In normal freshwater female eels, oocytes are at the prophase stage of the first meiotic division. The gonadal development occurs only during their marine migration or when treated with fish gonadotropin hormone. Colombo & Colombo-Belvedere (1976) showed that the ovary of immature silver eel synthesizes androgens and 11-oxoderivatives. We performed a comparable kinetic study of ovarian synthesis and investigated sexual steroid metabolism.

Results - Incubation of (4-^{14}C) progesterone with slices of ovarian tissue from immature silver eel yielded androgens (androstenedione (Δ_4), T) and their 11-oxoderivatives ; 11- hydroxyandrostenedione (11 OH Δ_4), 11-hydroxytestosterone (11 OH T) and 11-ketotestosterone were the major transformation products. The yield-time curves of the characterized products are given in the figure above.



Yield-time curves of the characterized products of the ovary of the immature silver eel, incubated with (4-^{14}C) progesterone.

Other compounds are yet to be identified, some of them being probably 5-β reduced C 19 steroids. In our experimental conditions, no estrogens were found. - E_2 seemed to be present in the plasma but its level (0.05, 0.5) was lower than that of T (0.8, 3.5). The MCR of E_2 (0.57 ml/kg x h) was 2.5 times lower than that of T (1.48 ml/kg x h). - The affinity of PBP for E_2 and T is very high (97 % E_2 bound, 98 % T bound). - After intraveinous injection of a dose of labelled tracer, we observed that both hormones were mainly metabolized in the liver. T gave rise to a metabolite that appeared in the plasma, its concentration increasing as a function of time ; its chromatographic properties were different from that of E_2.

Discussion - In our experiments, yields of 11-oxoandrogens increased continuously with time while yields of Δ_4 and T decreased at the end of the incubation (Fig.). Since these last compounds are key intermediates, it can be supposed that their conversion into estrogens is reduced by the large production of 11-oxoderivatives. The occurence of an extraovarian aromatisation of T has not been shown. However, a local aromatisation in central nervous system or hypophysis which has been shown in some Teleosts (Callard et al., 1981) could occur in eel without modifying PL of E_2. Despite the protection of E_2 against peripheral catabolism (high affinity of PBP) the PL of this hormone is very low. This confirms our hypothesis that the ovarian production pathway is not directed towards the synthesis of estrogens. The large conversion into 11-oxoandrogens indicates that the ovarian production pattern is comparable with that generally admitted (rev. Ozon, 1972) for the testis of fishes.

References

Callard, G.V., T. Petro, K. Ryan, J.B. Clairbone, 1981. Estrogen synthesis in vitro and in vivo in the brain of a marine Teleost *(Myoxocephalus)*. Gen. and Comp. Endocrinol., 43, 243-255.

Colombo, L., P. Colombo-Belvedere, 1976. Steroid biosynthesis by the ovary of the European eel, at the silver stage. Gen. and Comp. Endocrinol., 28, 371-385.

Ozon, R., 1972. Androgens in Fishes, Amphibians, Reptiles and Birds. In "Steroids in non mammalian Vertebrates". D.R. Idler ed., Acad. Press, N.Y., 328-389.

PRESERVATION OF FISH GAMETES

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Summary

In this review a discussion of the biology of teleost sperm cells and the factors regulating their motility is followed by a description of techniques for the short-term preservation of teleost spermatozoa and ova. Subsequently, general aspects of the freezing and thawing of cells are discussed and studies on the cryopreservation of teleost spermatozoa are reviewed with particular emphasis on salmonids including pre-freezing effects, freezing, thawing, post-thawing and insemination. Cryopreservation techniques are described for salmonid spermatozoa which provide fertility rates close to those obtained with fresh spermatozoa. Factors which require further study include, 1) evaluation of gamete quality prior to preservation and also fertilization, 2) reduction of cell density of frozen-thawed spermatozoa required for successful fertilization, 3) extension of the post-thaw motility of cryopreserved spermatozoa.
Keywords: Oncorhynchus, Salmo, gametes, cryopreservation, spermatozoa, ova.

Introduction

Considerable progress has been made recently in the development of techniques for fish gamete preservation. This applies in particular to the cryopreservation of sperm cells. There are a number of reasons for this increased interest. The intensified cultivation and propagation of fish requires proper techniques for gamete handling which include intermediate storage. Here short-term preservation is indicated to facilitate artificial reproduction or increase its efficiency.

Cryopreservation as a storage-technique with practically no time limit, has a much greater application potential than short-term preservation techniques particularly with respect to fish genetics. Examples of massive losses of fish stocks due to water pollution, habitat destruction or overfishing are well known. Recent research in population genetics has clearly demonstrated that the above mentioned factors as well as measures such as artificial propagation of the indigenous fish stocks, transplants or selective fishery can alter gene frequencies considerably or lead to the complete loss of some genes (see Ryman,1981; Hynes et al. 1981; Ricker, 1981). These are justifications for the establishment of gene banks which preserve the genetic originality of populations and keep them available for re-introduction when general conditions for survival have improved.

In fish culture systems, selective breeding, as in other domestic animals, is essential for the improvement of production traits. A well known example is the Atlantic salmon industry in Norway. Cryopreservation of spermatozoa provides a useful tool to maximize the number of offspring from a fixed number of selected broodstock by time and location independent gamete availability.

The production of mono-sex all-female stocks has become feasible by using a so-called indirect feminization technique, i.e., sperm-cells from phenotypic males having a female genotype result in genetically all-female offspring (Hunter et al., 1982a,b). Cryopreservation of these sperm cells has the advantages of 1) identifying the genetic sex of the donor fish post mortem and 2) permitting the most efficient use of the milt.

A final example refers to some tropical species where catch of wild spawners often is an essential step for the collection of ripe gametes (Kuo, 1982). If sperm cells were constantly available, any limitation in the success of artificial reproduction would be reduced to the females.

In this paper, no reference will be made to the cryopreservation of ova and embryos since this subject is reviewed elsewhere in this publication by B. Harvey.

The biology of teleost sperm cells

Teleost spermatozoa all lack a head cap, the acrosome. Major morphological differences have been observed between spermatozoa from species with external or internal fertilization (Billard, 1970). The former category is characterized by primitive sperm cells. Heads are often

ovoid and the mid-piece is small, not visible with the resolution of light microscopy. It contains a few often fused mitochondria. The tail is relatively long (10-20 times the head length) and has most often the typical 9 + 2 arrangement of microtubules (see Baccetti et al., 1979). In viviparous fish, head and midpiece are elongated (Gardiner, 1978a). The midpiece can contain a substantial number of mitochondria as described in Poecilia reticulata. There is sufficient evidence to believe that these structural differences also reflect differences in the metabolic activity of these cells. Spermatozoa from oviparous fish show limited glycolytic activity and depend on oxidative metabolism (Mounib, 1967). Spermatozoa from viviparous species can convert exogenous sugars to lactic acid and use the energy to prolong the duration of motility (Gardiner, 1978b). However, one has to critically remark that experimental data from only a few species are available to form this generalized picture.

Teleost spermatozoa are immotile in the testis and often in the seminal plasma. The induction of motility can be related to the particular environmental conditions during spawning. In freshwater spawners sperm cells become activated by the hypotonicity of freshwater. In saltwater spawners, hypertonicity induces motility (Morisawa and Suzuki, 1980).

By far the most work has been done in salmonids. There is little doubt that K^+ from seminal plasma blocks motility. Dilution of K^+ or interaction with other ions removes this block (see Baynes et al., 1981).

The stimulation of motility in viviparous fish is less understood. Both a relative change between ions and the mechanical breakdown of spermatozeugma have been suggested as possible motility inducing mechanisms (Morisawa and Suzuki, 1980; Billard, 1978a).

Motility is limited to a period of seconds to minutes in freshwater spawners because of lysis (Billard, 1978a). Motility is considerably longer in saltwater spawners, 15 minutes in Atlantic cod (Davenport et al., 1981) or several days in herring (Yanagimachi, 1957). These examples show how well spermatozoa from saltwater species are adapted to hypertonic conditions possibly explaining their hardiness when applying cryopreservation procedures (vide infra).

Spermatozoan motility can be enhanced in some physiological solutions with activating properties. Alkaline pH has been reported repeatedly to intensify and prolong motility in various fresh and saltwater species (Petit et al., 1973; Hines & Yashouv, 1971; Billard, 1980). In salmonids, ovarian fluid and fish Ringer's are superior to water (Ginsburg, 1963).

Short-term preservation of spermatozoa

Spermatozoa which are immotile in seminal plasma are suitable for short-term preservation since no energy is required for locomotion. Therefore, diluents to reduce motility or to supply substrate are not necessary.

The most important factors determining the success of storage are:
- a reduction in temperature
- provision of gaseous exchange
- prevention of bacterial growth
- prevention of desiccation

Low temperatures just above freezing point have never been reported to cause any harm to fish spermatozoa. Thus storage around 0°C is most suitable.

The effect of various gases is known for salmonid spermatozoa. Storage under aerobic conditions, preferably under O_2 maintains the ability to become motile for 2 weeks. CO_2, which is commonly used in mammalian spermatozoa, kills the cells after a relatively brief exposure (Buyukhatipoglu & Holtz, 1978). This reflects the cell's dependence on aerobic metabolism.

For practical reasons a uniform availability of O_2 to all cells within a sample has to be ensured. Since O_2 has to diffuse at rather low temperatures into the sample, the critical diffusion distance is of importance. This was demonstrated in an experiment with rainbow trout sperm. When milt-height within the storage container was either 2.5 or 6.5 mm, motility could still be induced after 17 and 28 days of storage, respectively. Storage at 31 mm, in contrast, retained some motility to 4 days only. When repeating this experiment with 6 mm samples fertility was unchanged relative to controls at Day 39 (Stoss et al., in preparation).

As far as other species are concerned, requirements for gaseous exchange are less clear. There are indications that somewhat anaerobic storage conditions maintained motility relatively well, such as in white bass, Atlantic cod and milkfish (Clemens & Hill, 1969; Mounib et al., 1968; Pullin & Kuo, 1980).

Storage diluents have been used occasionally. Diluted sperm from Atlantic cod, grey mullet or channel catfish in

isotonic media retained the ability to become activated for 20, 23 and 63 days, respectively (Mounib et al., 1968; Chao et al., 1975; Guest et al., 1976). A clear advantage of diluted versus undiluted storage has however not been demonstrated.

Another mode of storage is by supercooling. Spermatozoa are kept at several degrees below 0°C and freezing of the suspension medium and cells is prevented by the presence of cryoprotectants. A few, quite promising attempts have been reported from salmonid spermatozoa. Modified Cortland's medium, which is based on the mineral content of blood plasma and seminal plasma K^+ content was used in combination with DMSO or ethylene glycol. In both Atlantic salmon and rainbow trout, cells stayed viable for at least 5 weeks. Fertility of cells was not tested beyond this period (Truscott et al., 1968; Sanchez-Rodriguez & Billard, 1978).

Short-term preservation of ova

Just as the prevention of motility is an important prerequisite for successful sperm storage, the prevention of activation is of importance for ova. This may be rather difficult in a number of species such as carps in which ova autoactivate after natural or artificial spawning (Yamamoto, 1961).

There is in general little available data on storage of ova and some results obtained in rainbow trout are presented in Fig. 1.

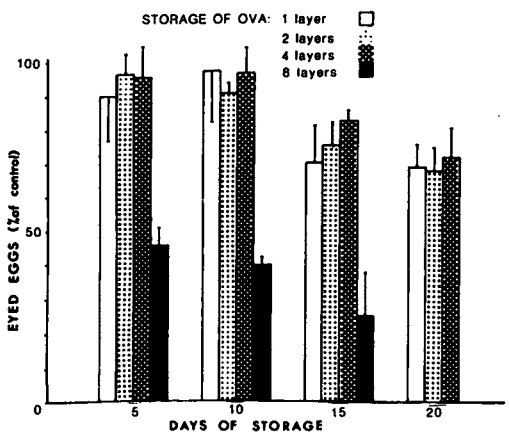

Fig. 1. Storage of rainbow trout ova in one or several layers. Air, 1°C, 125 IU penicillin and 125 ug streptomycin per g ova. X ± SD, n = 4 (Pueschel, 1979).

These data indicate that rainbow trout ova should be kept in not more than four layers (approx. 0.8 mm) under air at 1°C and protected by antibiotics. A previous comparison between various gases showed little difference between O_2 or air but demonstrated the unsuitability of N_2 or a mixture of O_2 (95%) and CO_2 (5%) (Pueschel, 1979). In this study 70% fertility was retained for 20 days (Fig. 3).

Cryopreservation of spermatozoa

1. General Aspects

In this section, events associated with the freezing and thawing of cells will be briefly reviewed. For more detailed information, papers by Meryman (1971a,b), Mazur (1977) and Farrant (1980) should be consulted. When talking about cryopreservation we usually mean storage of cells or tissue at -196°C, the temperature of liquid nitrogen (Fig. 2). This is far

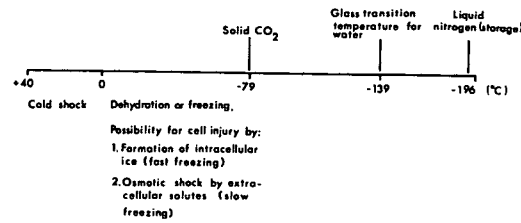

Fig. 2. Cell injury during freezing.

below the glass transition temperature for water, indicating that molecules are in a fixed state and motionless. Storage time is only limited by external influences such as background irradiation which theoretically limits the period of storage to somewhere between 200 - 32,000 years (see Ashwood-Smith, 1980; Whittingham, 1980).

Cell injury therefore is not related to the length of storage but to the transition of the cells from ambient temperatures at collection to approximately -80°C. It is in this range that 3 types of cell injury may occur (Fig. 2). The first one, referred to as cold shock, is caused by cooling at above freezing temperatures. This appears to be of little importance in fish spermatozoa, but may be more important in fish ova. Two types of injury are possible between approximately 0°C and -80°C being related either to the formation of intracellular ice or osmotic shock. This will be explained further.

At a few degrees below 0°C, the cell suspension medium freezes. The cell however, stays unfrozen since the ice crystals cannot grow through the cell membrane. Because the intracellular liquid water has a lower chemical potential than

the frozen extracellular water, intracellular water leaves the cell and freezes externally. This process of dehydration can virtually continue until the cell is dehydrated and thus has reached a suitable state for storage at low temperature. This process of dehydration is time-dependent.

When cooling proceeds too fast, the temperature at which the intracellular water freezes, will be reached before the cell is entirely dehydrated. Formation of significant amounts of intracellular ice is associated with the death of the cell.

During freezing, solutes such as salts concentrate severalfold in a small volume of remaining liquid. If freezing is carried out at a slow rate, the cells may become sufficiently dehydrated but the exposure to concentrated solutes may cause osmotic trauma. Further, there is a possibility that bound water would also be removed from the cells.

This model underlines the importance of the freezing rate. Optimum freezing rates are specific for a particular cell type. They depend on parameters such as the cell's permeability for water, the specific temperature coefficient, the amount of free water within a cell or the diffusion surface. Knowing these parameters, a mathematical model can be applied to optimize freezing rates (Mazur, 1963, 1977).

Under most circumstances, injury by osmotic shock and by intracellular ice, i.e., fast freezing and slow freezing injury overlap, allowing no survival. There are, however, examples where a small window for survival exists. Cryoprotectants open or widen this window by reducing primarily injury associated with slow freezing. The mechanisms involved are not completely understood but there is evidence that cryoprotectants do not alter the cell's susceptibility to slow freezing injury but that they alter the properties of the extracellular medium. Further, cryoprotectants lower the temperature for freezing of intracellular water. Consequently, freezing rates can not be dealt in isolation, but in relation to a specific cryoprotectant and its concentration.

Thawing is the reverse of the events just described. In the case of spermatozoa, fast thawing is always attempted. Usually, small amounts of intracellular ice are present in the cell (Leibo et al. 1978). If thawing proceeds slowly, this ice can recrystalize to larger crystals, which then can cause irreversible damage. By thawing quickly, recrystalization can be prevented.

Attempts to cryopreserve fish spermatozoa have primarily focussed on salmonid species and we are now able to achieve high fertility in these spermatozoa (Mounib, 1978; Stein & Bayrle, 1978; Buyukhatipoglu & Holtz, 1978; Bayrle, 1980; Erdahl & Graham, 1980; Stoss & Holtz, 1981a,b; Stoss & Refstie, 1982).

Success has also been achieved in the saltwater spawners Sparus auratus, Dicentrarchus labrax (Billard, 1978b), Gadus morhua (Mounib et al., 1968), Pleuronectes platessa (Pullin, 1972) and Clupea harengus (Blaxter, 1953). High fertility was reported in Labeo rohita (Withler, 1982) but results in other freshwater spawners are in general quite variable (see Sin 1974; Moczarski, 1976, 1977; De Montalembert et al., 1978; Bayrle, 1980; Withler, 1982).

In a number of species we therefore still stand at the beginning of adapting cryopreservation procedures. Experience obtained in salmonids has shown how important all steps of the entire cryopreservation procedure are to ensure good survival.

Table 1 lists the various phases of the cryopreservation procedure and variables within each phase which may influence post-thaw survival. The highly interactive nature of all input-variables involved was ponted out by Farrant (1980). Table 1 will serve as a guideline to discuss factors influencing post-thaw survival.

2. Pre-freezing effects

Differences in post-thaw fertility between different males have been reported several times (Ott & Horton, 1971a). Pooling of rainbow trout milt from several individuals improved the fertility drastically according to Legendre and Billard (1980) but no such effect was found in another study (Stoss & Holtz, in preparation). It has been suggested by the former authors that aging of sperm cells in the testis during the period of spermiation is one reason for differences between males. Similar effects have been found to occur in short-term preserved sea bass spermatozoa (Billard et al., 1977).

By collecting milt from coho salmon at intervals of 5 days from the beginning of spermiation, we could not find any differences related to either the stage of spermiation or the particular male (Stoss et al., in preparation). Since the spermiation period was limited to 3 weeks by the natural

Table 1. Variables affecting post-thaw fertility of cryopreserved spermatozoa.

Phase		Variables
Pre-freezing	-	Males selected
	-	Nature and concentration of cryoprotectant
	-	Equilibration time and temperature
	-	Interval cell collection-freezing
Freezing	-	Freezing rate
	-	Cryoprotectant
	-	Extender
	-	Dilution rate
Storage	-	Temperature
	-	Background irradiation
Thawing	-	Thawing rate
	-	Thawing solution
Post-thawing, insemination	-	Motility induction and duration
	-	Removal of cryoprotectant
	-	Incubation
	-	Cell density for insemination
	-	Females selected for fertilization

death of the fish, advanced stages in spermiation such as those occurring in rainbow trout, may never be reached by species of the genus Oncorhynchus.

Cryoprotectants may affect spermatozoa at above freezing temperatures. Such an influence has been observed when using glycerol in various salmonid species (Truscott et al., 1968; Ott & Horton, 1971a; Erdahl & Graham, 1980) or in the grouper Epinephelus tauvina (Withler & Lim, 1982). Sperm cells from the saltwater spawners Gadus morhua and Mugil cephalus were, however, not effected by glycerol (Mounib et al., 1968; Chao et al., 1975). A toxic effect has also been observed when using dimethylsulfoxide (DMSO) at concentrations between 6.8-12.5% in the final dilution and equilibrating trout sperm for 1 min or longer (Fig. 3). Data obtained in sockeye salmon (Oncorhynchus nerka), showed however, that DMSO at 6.8% was tolerated when it was added slowly to the cells.

Allowing no equilibration resulted in best fertility (Fig. 3). A similar finding has been made in various salmonid species (Ott & Horton, 1971b; Legendre & Billard, 1980; Bayrle, 1980) and in sea bream (Billard, 1978b) common carp (Moczarski, 1977) and channel catfish (Guest et al., 1976). It also supports the hypothesis that cryoprotectants do not have to permeate the cells in order to provide protection during freezing and thawing (Mazur & Miller, 1976).

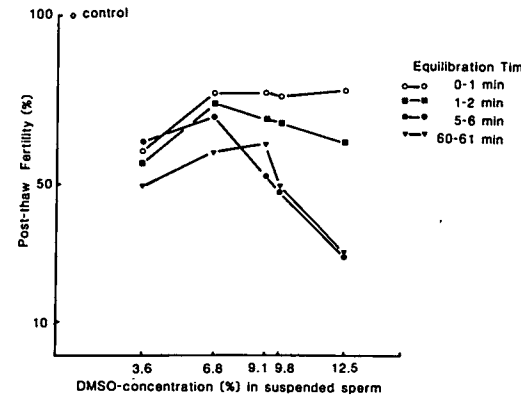

Fig. 3. DMSO-concentration and equilibration time in rainbow trout spermatozoa (X, n = 5, pooled SD = 5.1) (Stoss & Holtz, in preparation).

The importance of using fresh cells for cryopreservation has been shown in rainbow trout (Stoss & Holtz, in preparation). Short-term storage (0°C, air) of milt between collection and freezing for only one hour resulted in a decrease in post-thaw fertility when compared to 20 min of storage.

3. Freezing and thawing

Freezing rates in conjunction with the cryoprotectant have been investigated in spermatozoa from sea bass and seabream (Billard 1978b). Optimal conditions were achieved when freezing cells at 10°C/min and using between 5-15% DMSO in the diluent. Slower and higher rates were less suitable. In Atlantic cod a rate of 5°C/min was applied with good success whereas 1°C/min resulted in no survival (Mounib et al., 1968).

In a number of freshwater species, freezing has been carried out by pelleting suspended sperm onto dry ice (Nagase and Niwa, 1964). The transition from 0°C to -70°C lasts approximately 2 min resulting in a rate of 35°C/min. Since the rate decreases above -60°C, other estimates may be lower.

The freezing rate achieved by the pellet technique has been suitable for salmonid spermatozoa, (Stein & Bayrle, 1978; Buyukhatipoglu & Holtz, 1978; Legendre & Billard, 1980; Stoss & Refstie, 1982), Coregonus species (J. Piironen & H. Hyvarinen, personal communication) and the pike Esox lucius (De Montalembert et al., 1978).

The composition of the sperm extender has always been of concern and much work has been carried out to optimize the extender composition. Mineral concentrations have often been based on analysis of seminal plasma. The exceptional fertilization results obtained with a very unconventional extender in Atlantic salmon by Mounib in 1978 led to a comparison of extenders from the literature (Fig. 4).

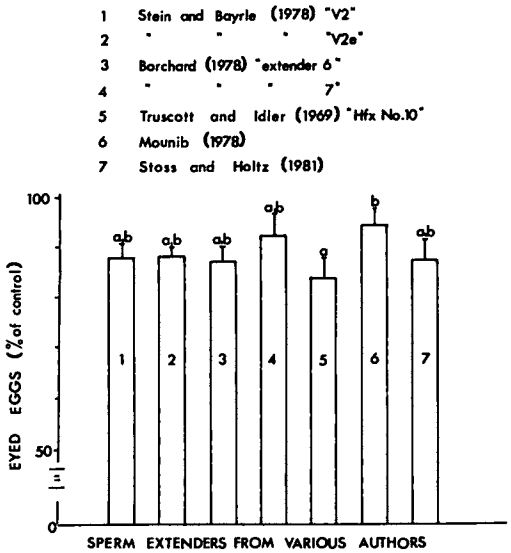

Fig. 4. Effect of extenders on post-thaw fertility in rainbow trout spermatozoa (x ± SD, n = 5) (Stoss & Holtz, in preparation).

In all cases the procedure of freezing and thawing described by Stoss & Holtz (1981a) was carried out. Clearly, little variability was caused by the various extender media. Only the results obtained with the extenders by Truscott & Idler (1969) and by Mounib (1978) were different from each other.

In pursuing the question about the optimal extender composition further, it was subsequently found that even an aqueous solution of DMSO provided cryoprotection and post-thaw fertilities of 44% and 67% were obtained in Atlantic salmon and rainbow trout spermatozoa (Stoss & Refstie, 1982; Stoss & Holtz, in preparation). An extender consisting of 300 mM glucose and DMSO (6.8% final concentration) has been used with good success in Salmo and Oncorhynchus species (Stoss & Refstie, 1982; Stoss et al., in preparation). The extender composition is relatively unimportant due to the fact that with the technique described sperm cells are exposed to the extender for only a few seconds. This is the case both prior to freezing and after thawing.

A relatively simple extender based on NaCl, $NaHCO_3$ and glycine was used successfully in Atlantic cod and plaice (Mounib et al., 1968; Pullin, 1972), while a mixture of diluted seawater and glycerol has produced good results in herring spermatozoa (Blaxter, 1953).

Various dilution rates of milt with extender have been tested. In salmonids, no differences were found when using rates of 1:1 to 1:19 (Truscott & Idler, 1969; Ott & Horton, 1971b; Buyukhatipoglu & Holtz, 1978; Bayrle, 1980). Legendre and Billard (1980), however, reported inferior post-thaw fertility results at dilution rates of 1:1 and 1:9 compared to 1:3. In pike, sea bass and sea bream spermatozoa a decrease in fertility was obtained when dilution rates exceeded 1:2 (De Montalembert et al., 1978; Billard, 1978b). Interpretation of these results is difficult. Assuming that the concentration of cryoprotectant was kept constant in the final dilution and that the different cell density was accounted for at insemination, little difference between dilution rates would be expected.

The effect of thawing rates has been investigated in pellet frozen spermatozoa from chum salmon (Stoss et al., in preparation). By adjusting the temperature of the thawing solution, rates between 140°C/min to 1500°C/min could be obtained without overheating the sperm cells. Post-thaw fertility almost identical to fresh sperm controls was obtained with the highest thawing rate; rates of 600 or 140°C/min were less suitable.

4. Post-thawing and insemination

After thawing, spermatozoa may show different motility characteristics than prior to freezing. Spontaneous motility induction has been observed (Stoss and Holtz, 1981a). The duration of motility can be reduced, as in grouper spermatozoa, from 1/2 hr in fresh cells to 30 sec in frozen-thawed cells (Withler and Lim, 1982).

Motility in fresh pink salmon (Oncorhynchus gorbuscha) spermatozoa was prolonged from 30 sec to 10 min when activated with an 120 mM $NaHCO_3$-solution to which IBMX (3-isobutyl-1-methylxanthin) had been added. An attempt to prolong motility with IBMX in freeze-thawed cells failed (Stoss et al., in preparation).

The rapid loss of motility is correlated

with a rapid decrease in post-thawing fertility. A delay of only 30 sec between thawing and insemination reduced fertility significantly in rainbow trout sperm (Fig. 5).

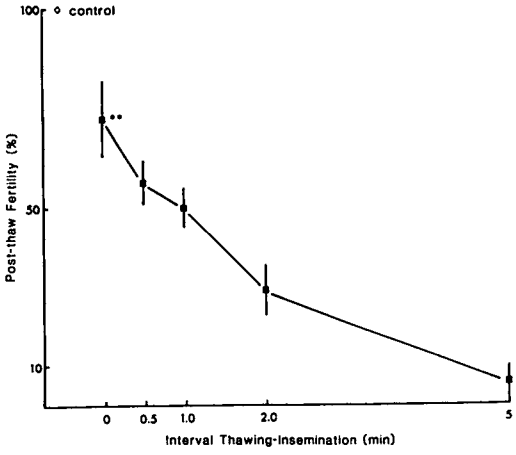

Fig. 5. Interval thawing-insemination in pellet-frozen rainbow trout spermatozoa (X ± SD, n = 7; Holtz & Stoss, 1981a).

In testing the effect of thawing frozen rainbow trout sperm pellets in various solutions, only the ones which induced motility (ovarian fluid, 120 mM $NaHCO_3$, 120 mM NaCl) resulted in appreciable success.

Not all cells may survive the cryopreservation procedure. Densities of frozen-thawed sperm cells have therefore to be relatively high to reach a plateau in fertilizing capacity. This is reached at a dilution factor of approximately 1:100 in rainbow trout spermatozoa (Legendre & Billard, 1980; Stoss & Holtz, 1981a). In fresh spermatozoa dilution rates between 10^{-3} to 10^{-4} still result in high fertility (Billard, 1981).

Conclusions

Relatively simple procedures can be applied to short-term preserve immotile spermatozoa and non-activated ova.

Cryopreservation procedures have been refined for salmonid spermatozoa. These illustrate the importance of each step involved. A number of problems remain, relating to all species.

1) Gamete quality is still an unpredictable influence. Both aging of cells and nutritional factors can affect spermatozoa (Billard et al., 1977; see Scott & Baynes, this volume). The different capabilities of ova from individual females to become fertilized by cryopreserved spermatozoa still requires clarification.

2) High cell densities of frozen-thawed spermatozoa are still required to achieve fertility close to fresh sperm results. To assess further improvements, not only maximum fertility per se but also cell density at insemination should be reported.

3) Post-thaw motility can be rather brief which in turn limits the period of fertility. Enhancing motility may improve the consistency of fertilization results.

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References

Ashwood-Smith, M.J., 1980. Low temperature preservation of cells, tissues and organs. In: M.J. Ashwood-Smith and J. Farrant, (Ed.): Low Temperature Preservation in Medicine and Biology. Pitman Medical Ltd., Tunbridge Wells, Kent. p. 19-44.

Baynes, S.M., A.P., Scott & A.P. Dawson,1981. Rainbow trout Salmo gairdnerii Richardson, spermatozoa: effects of cations and pH on motility. J. Fish Biol. 19:259-267.

Bayrle, H., 1980. Untersuchungen zur Optimierung der Befruchtungsfahigkeit von gefrierkonserviertem Fischsperma. Dissertation, Tech. Univ. Munchen.

Billard, R., 1970. Ultrastructure comparee de spermatozoides de quelques poissons teleosteens. In: B. Baccetti, (Ed.): Comparative Spermatology. Academic Press, New York, London. p. 71-79.

Billard, R., 1978a. Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. Aquaculture 14:187-198.

Billard, R., 1978b. Some data on gametes preservation and artificial insemination in teleost fish. Actes Colloq. Centre National pour l'Exploitation des Oceans (CNEXO) 8, 59-73.

Billard, R., 1980. Reproduction and artificial insemination in teleost fish. Proc. 9th Intern. Congr. Anim. Reprod. and Artif. Insem., Madrid 1980, RT-H-3, 327-337.

Billard, R., 1981. Short-term preservation of sperm under oxygen atmosphere in

rainbow trout (Salmo gairdneri). Aquaculture 23:287-293.
Billard, R., J., Dupont, & G. Barnabe, 1977. Diminution de la motilite et la duree de conservation du sperme de Dicentrarchus labrax L. (poisson, teleosteen) pendant la periode de spermiation. Aquaculture 11:363-367.
Blaxter, J.H.S., 1953. Sperm storage and cross-fertilization of spring and autumn spawning herring. Nature (Lond.) 172:1189-1190.
Buyukhatipoglu, S., & W. Holtz, 1978. Preservation of trout sperm in liquid or frozen state. Aquaculture 14:49-56.
Chao, N.H., H.P., Chen & I.C. Liao, 1975.Study on cryogenic preservation of grey mullet sperm. Aquaculture 5:389-406.
Clemens, H.P. & L.G. Hill, 1969. The collection and short-term storage of milt of white bass. Prog. Fish-Cult. 31:26.
Davenport, J., S. Lonning & E. Kjorsvik, 1981. Osmotic and structural changes during early development of eggs and larvae of the cod, Gadus morhua L. J. Fish Biol. 19:317-331.
DeMontalembert, G., C. Bry & R. Billard, 1978. Control of reproduction in northern pike. Am. Fish. Soc. Spec. Publ. 11:217-225.
Erdahl, D.A. & E.F. Graham, 1980. Preservation of gametes of freshwater fish. Proc. 9th Intern. Congr. Anim. Reprod. Artif. Insem., Madrid 1980 RT-H-2, 317-326.
Farrant, J., 1980. General observations on cell preservation. In: M.J. Ashwood-Smith & J. Farrant (Ed.): Low Temperature Preservation in Medicine and Biology. Pitman Medical Ltd., Tunbridge Wells, Kent. p. 1-18.
Gardiner, D.M., 1978a. Fine structure of the spermatozoon of the viviparous teleost Cymatogaster aggregata. J. Fish Biol. 13: 435-438.
Gardiner, D.M., 1978b. Utilization of extracellular glucose by spermatozoa of two viviparous fishes. Comp. Biochem. Physiol. 59:165-168.
Ginsburg, A.S., 1963. Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. J. Embryol. Exp. Morphol. 11:13-33.
Guest, W.C., J.W. Avault & J.D. Roussel, 1976. Preservation of channel catfish sperm. Trans. Am. Fish. Soc. 105: 469-474.
Hines, R. & A. Yashouv, 1971. Some environmental factors influencing the activity of spermatozoa of Mugil capito Cuvier, a grey mullet. J. Fish Biol. 3: 123-127.
Hunter, G.A., E.M. Donaldson, F.W. Goetz & P.R. Edgell, 1982a. Production of all-female and sterile coho salmon, and experimental evidence for male heterogamety. Trans. Am. Fish. Soc. 111: 367-372.
Hunter, G.A., E.M. Donaldson, J. Stoss & I. Baker, 1982b. Production of monosex female groups of chinook salmon (Oncorhynchus tshawytscha) by the fertilization of normal ova with sperm from sex reversed females. Presented at Int. Symp. Genetics in Aquaculture, Galway, Ireland, March 29-April 3, 1982.
Hynes, J.D., E.H. Brown, J.H. Helle, N. Ryman & D.A. Webster, 1981. Guidelines for the culture of fish stocks for resource management. Can. J. Fish. Aquat. Sci. 38:1867-1876.
Kuo, C.M., 1982. Progress in artificial propagation of milkfish. ICLARM Newsletter 5, 8-10 (International Centre for Living Aquatic Resources Managment, MCC P.O. Box 1501, Makati, Metro Manila, Philippines).
Legendre, M. & R. Billard, 1980. Cryopreservation of rainbow trout sperm by deep-freezing. Reprod. Nutr. Dev. 20: 1859-1868.
Leibo, S.P., J.J. McGrath & E.G. Cravalho, 1978. Microscopic observations of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. Cryobiology 15:257-271.
Mazur, P., 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J. Gen. Physiol. 47:347-369.
Mazur, P., 1977. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiology 14:251-272.
Mazur, P. & R.H. Miller, 1976. Survival of frozen-thawed human erythrocytes as a function of the permeation of glycerol and sucrose. Cryobiology 13:523-536.
Meryman, H.T., 1971a. Cryoprotective agents. Cryobiology 8:173-183.
Meryman, H.T., 1971b. Osmotic stress as a mechanism of freezing injury. Cryobiology 8:489-500.
Moczarski, M., 1976. Cryobiological factors in grass carp preservation. Proc. 8th Intern. Congr. Anim. Reprod. Artif. Insem., Krakow 1976 4: 1030-1033.
Moczarski, M., 1977. Deep freezing of carp Cyprinus carpio L. sperm. Bull. Acad. Pol. Sci. Ser. Sci. Biol. 15: 187-190.
Morisawa, M. & K. Suzuki, 1980. Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. Science 10:1145-1147.
Mounib, M.S., 1967. Metabolism of pyruvate, acetate and glyoxylate by fish sperm. Comp. Biochem. Physiol. 20: 987-992.
Mounib, M.S., 1978. Cryogenic preservation of fish and mammalian spermatozoa. J. Reprod. Fertil. 53: 13-18.
Mounib, M.S., P.C. Hwang & D.R. Idler, 1968.

Cryogenic preservation of Atlantic cod (Gadus morhua) sperm. J. Fish. Res. Board Can. 25:2623-2632.

Nagase, H., 1964. Deep freezing bull semen in concentrated pellet form. I.Factors affecting survival of spermatozoa. Proc. 4th Intern. Congr. Anim. Reprod. Artif. Insemin., Trento 1964 3:410-415.

Ott, A.G. & H.F. Horton, 1971a. Fertilization of steelhead trout (Salmo gairdneri) eggs with cryo-preserved sperm. J. Fish. Res. Board Can. 28: 1915-1918.

Ott, A.G. & H.F. Horton, 1971b. Fertilization of chinook and coho salmon eggs with cryo-preserved sperm J. Fish. Res. Board Can. 28:745-748.

Petit, J., B. Jalabert, B. Chevassus & R.Billard, 1973. L'insemination artificielle de la truite (Salmo gairdneri Richardson). I. - Effets du taux de dilution, du pH et de la pression osmotique du dilueur sur la fecondation. Ann. Hydrobiol. 4:201-210.

Pueschel, H., 1979. Eibildung und Eikonservierung bei der Regenbogenforelle. M.Sc. Thesis, University of Goettingen, Institute for Animal Breeding and Genetics.

Pullin, R.S.V., 1972. The storage of plaice (Pleuronectes platessa) sperm at low temperatures. Aquaculture 1: 279-283.

Pullin, R.S.V. & C.M. Kuo, 1980. Developments in the breeding of cultured fishes. Paper presented at Symp. on Advances in Food-Producing Systems for Arid and Semi-Arid Lands, Kuwait City, 1980.

Ricker, W.E., 1981. Changes in the average size and average age of Pacific salmon. Can. J. Fish. Aquat. Sci. 38: 1636-1656.

Ryman, N., 1981. Fish Gene Pools. Ecol. Bull. NFR (Naturvetensk. Forskningsraadet) 34.

Sanchez-Rodriguez, M. & R. Billard, 1977. Conservation de la motilite et du pouvoir fecondant du sperme de truite arc-en-ciel maintenu a des temperatures voisines de 0°C. Bull. Fr. Piscic. 265: 143-152.

Sin, A.W., 1974. Preliminary results on cryogenic preservation of sperm of silver carp (Hypophthalmichthys molitrix) and bighead (Aristichthys nobilis). Hong Kong Fish. Bull. 4: 33-36.

Stein, H. & H. Bayrle, 1978. Cryopreservation of the sperm of some freshwater teleosts. Ann. Biol. anim. Bioch. Biophys. 18:1073-1076.

Stoss, J. & W. Holtz, 1981a. Cryopreservation of rainbow trout (Salmo gairdneri) sperm. I. Effect of thawing solution, sperm density and interval between thawing and insemination. Aquaculture 22:97-104.

Stoss, J. & W. Holtz, 1981b. Cryopreservation of rainbow trout (Salmo gairdneri) sperm. II. Effect of pH and presence of a buffer in the diluent. Aquaculture 25:217-222.

Stoss, J. & T. Refstie, 1982. Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. Aquaculture (in press).

Truscott, B. & D.R. Idler, 1969. An improved extender for freezing Atlantic salmon spermatozoa. J. Fish. Res. Board Can. 26:3254-3258.

Truscott, B., D.R. Idler, R.J. Hoyle & H.D. Freeman, 1968. Sub-zero preservation of Atlantic salmon sperm. J. Fish. Res. Board Can. 25: 363-372.

Whittingham, D.G. (1980). Principles of embryo preservation. In: M.J. Ashwood-Smith and J. Farrant (Ed.): Low Temperature Preservation in Medicine and Biology. Pitman Medical Ltd., Tunbridge Wells, Kent. p. 65-83.

Withler, F.C., 1982. Cryopreservation of spermatozoa of some freshwater fishes cultured in South and Southeast Asia. Aquaculture 26: 395-398.

Withler, F.C. & L.C. Lim, 1982. Preliminary observations of chilled and deep-frozen storage of grouper (Epinephelus tauvina) sperm. Aquaculture 27:389-392.

Yamamoto, T., 1961. Physiology of fertilization in fish eggs. Int. Rev. Physiol. 12:361-405.

Yanagimachi, R., 1957. Studies of fertilization in Clupea pallasii. Parts I-III. Zool. Mag. (Tokyo) 66:218-233.

CRYOBIOLOGY AND THE STORAGE OF TELEOST GAMETES

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Summary

The problem of gamete storage in teleosts, whether by chilling or freezing, is too often attacked empirically. This approach, though understandable given the practical nature of the problem, has produced a large and confused literature documenting a wealth of methods without unifying biological theme. Teleost embryos represent a particular cryobiological challenge; the few published attempts to freeze them make it clear that extrapolation of mammalian results, while basically effective for sperm preservation, will not work for embryos.

This paper reviews cryobiological principles as they apply to the chilling, supercooling and freezing of teleost spermatozoa, eggs and embryos. I discuss the problems - biological as well as technical - involved in freezing any cell type, including the role of cryoprotectant, formation of intracellular ice, and freezing and warming rates, and attempt to relate them to such peculiarities of teleost gametes and embryos as size, permeability, motility and activation.

Introduction

The impact of cryobiology on animal husbandry is undoubted, and it is not surprising that a good deal of effort has been put into the transfer of the resulting benefits to fish culture. Such efforts have not always been rewarded, and it is the purpose of this paper to suggest reasons for this failure.

Cryobiology can play a role in fish culture and research in three ways. First, the problem of non-coincident maturation of males and females can partly be overcome if spermatozoa or ova are held in storage. Artificial fertilization thus becomes more convenient, and physiologists and fish farmers alike can benefit from this approach. Second, programs of selective breeding can be undertaken. This includes not only the gradual stock improvement that is a greater or lesser concern to every commercial fish farmer, but also attempts, particularly in the Third World, to breed indigenous species. Third, cryobiology can play an important part in the conservation of germplasm. In this application, gametes from threatened species or strains are banked as a hedge against the dwindling genetic variability caused by environmental disturbances and extensive monoculture.

The success of cryobiology in fish reproduction has been limited in two ways. The first of these stems from attempts at wholesale transfer of mammalian techniques without a corresponding understanding of fundamental cryobiology; the second from physiological pecularities of fish gametes that place rather severe constraints on cryopreservation success. I shall therefore first review the fundamentals of cryobiology, then consider those aspects of the morphology and physiology of spermatozoa and ova that relate directly to application of these principles. Some of the discussion will reflect my own experience in the cryopreservation of sperm (and the attempted cryopreservation of ova and embryos) from warm-water and cold-water teleosts.

Cellular and Physical Consequences of Cooling

Several excellent recent reviews cover the fundamentals of cryobiology; the reader is referred in particular to those of Mazur (1981) and Ashwood-Smith (1980).

When a cell is cooled in an aqueous medium the temperature will generally fall below the freezing point of both cell and medium before freezing occurs; that is, both cell and medium will supercool. In most cases the cell will supercool farther than the surrounding medium so that, when ice first forms in the medium (usually slightly below $0^{o}C$), dissolved solutes become progressively concentrated outside the cell while the cell water remains unfrozen. The cell responds osmotically to the resulting solute imbalance by losing water to the surrounding medium.

If cooling is sufficiently slow, the cell will lose enough water to remain in osmotic equilibrium with the concentrating brine surrounding it. If cooling is rapid, however, the cell will equilibrate by freezing internally upon seeding by ice crystal puncture through the cell membrane. Under most conditions intracellular freezing is fatal. Avoidance of intracellular freezing by slow cooling is not, however, a panacea; lowering the temperature too slowly prolongs the hyperosmotic state of the medium to the point where high external salt concentrations cause irreparable membrane damage. Damage from intracellular freezing or extracellular salt concentrations is most likely to occur between about $-15^{o}C$ and $-50^{o}C$; beyond $-50^{o}C$, the temperature can be rapidly lowered to a convenient storage point, usually $-196^{o}C$, the temperature of liquid nitrogen. No thermally driven reactions occur at this temperature

and storage is essentially indefinite; in fact, storage lower than -130°C, the glass transition point of water, will likely be as good. Warming, of course, is equally hazardous, because the same physical processes must take place in reverse.

Fortunately, some of the problems attendant on the threat of intracellular freezing and solute injury can be minimized by the addition of cryoprotectants to the cells and medium before cooling. Cryoprotectants may or may not permeate the cell; permeating compounds include dimethylsulfoxide (DMSO) and glycerol, while those that presumably simply coat the cell surface include sugars and such high molecular weight polymers as polyvinylpyrollidone (PVP) and various dextrans. The dependence of protection on permeation is still a matter of cryobiological debate (Mazur 1970); one advantage of non-permeating cryoprotectants is their ease of removal upon thawing. The protective action of permeating cryoprotectants is presumed to be colligative.

Factors affecting the cryobiological survival of teleost gametes and embryos

Cell size, cooling rate, and water permeability

One of the most important theoretical developments in cryobiology has been the quantitative description of the dependence of cell survival on size, cooling rate, and water permeability. The presently accepted model, first proposed by Mazur in 1963, permits calculation of the intracellular water content as a function of temperature, so long as the cell's surface area, its permeability to water, and the dependence of water permeability on temperature are known. In practical terms the model has produced some useful rules of thumb, namely:

(a) the larger the cell (and the lower its surface area:volume ratio) the slower it must be cooled in order to have time to dehydrate sufficiently to prevent intracellular freezing.

(b) the lower the cell's permeability to water, the slower it must be cooled.

Neither of these rules place any severe constraints on the freezing of spermatozoa. Fish ova, on the other hand, seem cryobiologically disqualified on both counts. Even the smallest teleost ovum is more than one hundred times the diameter of the average spermatozoon, and the water permeability of the unfertilized ovum, if not low in an absolute sense, is effectively minimal because of the enormous distances water molecules must travel in order to enter or leave the cell (for a discussion of the problem of effective water permeability in teleost eggs, see Loeffler and Løvtrup (1970) and Harvey and Chamberlain 1982). Although it is still impossible to arrive at a quantitative prediction of the optimal cooling rate for any teleost egg, experience in our laboratory has shown rates as low as $0.01^{\circ}C\ min^{-1}$ to be ineffective for rainbow trout eggs (Harvey and Ashwood-Smith 1982). Part of the blame for this failure can probably be laid to problems of cryoprotectant penetration (see below), but observation of ice formation within the ova makes it difficult to avoid the conclusion that dehydration is simply too slow.

Size is again a problem with the teleost embryo, although paradoxically less severe for relatively early stages of development. The gastrulating embryo, though roughly the same size as the unfertilized egg, is composed of many cells of a cryobiologically manageable size; it follows that, suitably protected at least some of these embryonic cells should survive freezing. We have shown this to be true using half-epiboly embryos from Brachydanio rerio: in embryos cooled at $0.05^{\circ}Cmin^{-1}$ to -196°C, individual cell survival is high (Harvey 1982b). Yet the practical importance of this result is minimal, for reasons to be detailed below.

In fact there has been almost no success in any attempts to freeze macroscopic bits of tissue. Rajotte and Mazur (1981) report resumption of biochemical function in fetal rat pancreases cooled to -196°C, but this tissue is sheetlike, and the distance covered by water during dehydration is not more than a few cell layers. Luyet and Gehenio (1954) have published accounts of cryopreservation of large tissue aggregates, such as embryonic chick hearts, by very rapid freezing, and have suggested that vitrification of cell water can permit some cell survival. We have only been able to preserve epithelia from B. rerio embryos using this method, although modifications such as made by James (1980) for cryopreservation of schistosomulae of Schistosoma mansoni may yet make ultra-rapid freezing feasible.

Cryoprotectants: kind, amount and permeation

There is a bewildering arsenal of cryoprotectants of known or imagined efficacy, and present theories to account for intracellular and extracellular cryoprotection are shaky enough that the list can only get longer. There is an understandable tendency for those attempting to cryopreserve a new cell type to follow precedent and choose one of the most popular cryoprotectants in mammalian systems, DMSO or glycerol. This is what most investigators have done for fish spermatozoa (see review by Scott and Baynes 1980), yet other compounds may be more effective. We compared methanol (when combined with milk powder as extracellular protectant) with DMSO and glycerol in the cryopreservation of Sarotherodon (Tilapia) mossambicus sperm, and found that, on a molar basis, protection was more than twice that of the more commonly

used compounds (Harvey 1982a). We also found strong, and unexpected, synergistic effects when methanol, DMSO, and glycerol were combined with milk powder or egg yolk, suggesting that various combinations be tried before a cryoprotectant mixture is chosen.

Spermatozoa are sufficiently small that cryoprotectant penetration is rapid, and no 'equilibration period' is required (Harvey 1982a). Penetration of the unfertilized ovum, however, is slow, and poses a cryobiological problem at least as great as the low cooling rate theoretically required. Trout eggs, for example, reach only 23% of the expected equilibrium value after incubation in methanol for 2 hours; DMSO and glycerol penetrate even more slowly (Harvey and Ashwood-Smith 1982). This low level of protection ensures that, even though cooling rates are very low indeed, survival is unlikely. The only way out of this dilemma is to force the cell to take up a sufficient amount of cryoprotectant by bathing it in a very high initial concentration, as was done by Rajotte and Mazur (1981) with fetal rat pancreas. This method does work, but again the size of the tissue is limiting: not only are high concentrations (> 2M) of most cryoprotectants likely to be toxic, but cells, once loaded with these compounds, become extraordinarily sensitive osmotically so that, upon thawing, removal of cryoprotectant by dilution must be so slow that toxic effects begin to show up. We have shown this with Brachydanio embryos protected with 25% glycerol and cooled slowly to -196°C: upon warming, the embryo shows little sign of damage and a high percentage of cells are alive. Yet removal of the cryoprotectant, even by very slow dialysis, is impossible (Harvey 1982b). Nevertheless, individual cells or small clumps of cells can be separated from the thawed embryo and remain viable after dilution, suggesting a potential method for the preservation of genetic information should techniques for nuclear transplantation be developed for teleosts.

Storage at relatively high subzero temperatures

Storage of teleost spermatozoa is a practical problem, and several investigators have attempted to use the most practical means at hand, namely the freezing cabinet of a laboratory refrigerator. Unfortunately, cryobiological theory states that at the temperature likely to be encountered (approx. -15°C) cells are only partially dehydrated and therefore bathe in a concentrated 'brine' of solutes that will damage the cell membrane ('solution effects'). We have confirmed this for spermatozoa of Brachydanio; no amount of augmented extracellular cryoprotectant seems able to prevent cell death within a few hours, and preservation of spermatozoa can only be done below about -79°C (the temperature of solid CO_2, at which dehydration will be complete) or between 0° and 5°C (see below).

Unfertilized ova, however, tolerate supercooling well, and techniques to store them between 0° and -5°C may prove very useful in alleviating problems of short-term conservation (1-2 months). Harvey and Ashwood-Smith (1982) and Stoss and Donaldson (1982) reported the ability of the protected salmonid egg to supercool as low as -16°C, and recent experiments in our laboratory have shown rainbow trout eggs to be fertile after a month's storage at -2°C. This is not a surprising result - the drop from normal ambient temperature is not great, and the major challenge appears to be providing an appropriate osmotic environment. The eggs of tropical fishes are less able to withstand cooling; we have never succeeded in cooling the unfertilized eggs of Brachydanio lower than 10°C before fertilization is severely affected. Storage at 5°C for more than 30 minutes results in complete infertility.

Motility, activation and oxygen requirements

Teleost gametes are wonderfully adapted for external fertilization and embryonic development in a variety of aquatic environments, yet several of the strategies adopted for survival pose real problems for the cryobiologist.

Motility in teleost spermatozoa is normally confined to a period of frenetic activity whose short duration is offset by the production of vast numbers of cells. These cells are thus not specifically adapted (as are mammalian sperm) for utilization of exogenous energy sources once motility has commenced, although they appear quite capable of so doing when provided with the opportunity. For cryopreservation, though, it is advantageous to keep the diluent as simple as possible, and in the absence of added energy sources it is important to ensure that spermatozoa are not activated before freezing starts. In many cases the osmotic pressure of the added cryoprotectant will be sufficient to inhibit motility; we have found powdered milk, itself an excellent extracellular cryoprotectant, to be an infallible inhibitor of motility. Some degree of motility may, incidentally, be triggered by the freeze-thaw process; Stoss (1982) has noted this phenomenon in salmonid sperm, as have we for Brachydanio; membrane damage and consequent depolarization may account for it.

Spermatozoan motility becomes a more severe problem when milt is to be stored above 0°C for several days or weeks (chilled storage); so little work has been done in this area that one cannot yet say whether motility should be blocked from the outset or whether spermatozoa should be supplied with complex exogenous energy sources throughout the storage period. These two approaches essentially represent a choice between undiluted

and diluted ("extended") storage. Our own experience suggests that few assumptions can safely be left untested: we had, for example, assumed that, in a tropical fish such as Sarotherodon or Brachydanio, motility would surely be limited by the very act of lowering the temperature to 0°C - a drop of 25-30 degrees from the normal ambient temperature for these cells. Yet observation of sperm suspensions with the cryomicroscope showed that roughly 50% of the spermatozoa were weakly motile at 0°, and that isolated cells were capable of sluggish tail movement as low as -9°C. Energy reserves are clearly being utilized at these low temperatures and, for storage to be successful, motility must either be inhibited completely or encouraged by providing a suitable energy source. Inhibition of motility can of course be achieved by storing undiluted milt, yet we have found the viability of undiluted Sarotherodon mossambicus milt to drop severely within several days, while that of sperm provided with exogenous nutrients from an egg-yolk diluent to last 3 or 4 times as long. Inhibition of spermatozoan motility by elevating K^+ concentration is well known (Sneed and Clemens 1956), although the effect is complicated by the presence of Ca^{2+} or Mg^{2+} ions (Scott and Baynes 1980). We found 25-50 mM K^+ to inhibit motility in Sarotherodon mossambicus sperm held in an egg-yolk-citrate diluent, but this inhibition lasted no more than a few hours.

Egg activation is another peculiar property that makes short-term or chilled preservation difficult. There is a large literature on this phenomenon in fishes (Yamamoto 1961, Kalman 1959, Potts and Rudy 1969) and further insights may well be gained from recent summaries of work on echinoderm ova and the role of ionic triggers in fertilization and activation (Epel 1980; Jaffe 1980). Years ago, Suzuki (1959) elegantly demonstrated that eggs of certain warm-water fishes became infertile as soon as elevation of the chorion severed the protoplasmic-micropylar connection. We have found mechanical activation and subsequent parthogenetic development to be a major stumbling block in storage of the unfertilized ova of B. rerio, and Withler (1980) has reported similar problems with ova of Thai carps and catfishes. Interestingly, activation poses no such problem in salmonids.

We are presently exploring ways in which activation can be limited. Ficoll, a high molecular weight polymer (m.w. 400,000) occasionally used as an extracellular cryoprotectant, delays activation in zebra fish ova at a concentration of 12%, and activation can be completely inhibited by immersing the eggs in silicone oil. Ca^{2+}-free solutions are ineffective in delaying activation. It is probably safe to say, however, that such methods only serve to limit a train of events begun by the act of forcibly expressing eggs from the female, and that a distinction must be made between activation as a result of osmotic, ionic or pH changes, and mechanical activation. The latter is much less under the control of the experimenter, and can likely only be prevented by scrupulous attention to the female's state of readiness.

References

1. Ashwood-Smith, M.J. 1980. Low temperature preservation of cells, tissues and organs. In: M.J. Ashwood-Smith and J. Farrant, Eds: Low temperature preservation in medicine and biology. Pitman Medical, Tunbridge Wells.
2. Epel, D. 1980. Ionic triggers in the fertilization of sea urchin eggs. Ann. N.Y. Acad. Sci. 339: 74-85.
3. Harvey, B. 1982a. Cryopreservation of Sarotherodon (Tilapia) mossambicus spermatozoa. In preparation.
4. Harvey, B. 1982b. Cooling of embryonic cells, isolated blastoderms and intact embryos of the zebra fish to -196°C. In preparation.
5. Harvey, B., and Ashwood-Smith, M.J. 1982. Cryoprotectant penetration and supercooling in the eggs of salmonid fishes. Cryobiology 19: 29-40.
6. Harvey, B., and B. Chamberlain, 1982. Water permeability in the developing embryo of the zebra fish Brachydanio rerio. Can. J. Zool. 60: 268-270.
7. Harvey, B., R.N. Kelley and M.J. Ashwood-Smith, 1982. Cryopreservation of zebra fish spermatozoa using methanol. Can. J. Zool. in press.
8. Jaffe, L. 1980. Calcium explosions as triggers of development. Ann. N.Y. Acad. Sci. 339: 86-101.
9. James, E.R. 1980. Cryopreservation of Schistosoma mansoni schistosomulae using 40% methanol and rapid cooling. Cryo-Lett. 1: 535-544.
10. Kalman, S.M. 1959. Sodium and water exchange in the trout egg. J. Cell. Comp. Physiol. 54: 115-162.
11. Loeffler, C. and S. Løvtrup. 1970. Water balance in the salmon egg. J. Exp. Biol. 52: 291-298.
12. Luyet, B.J. and P.M. Gehenio. 1954. Effect of the rewarming velocity on the survival of embryonic tissues frozen after treatment with ethylene glycol. Biodynamica 147: 213-223.
13. Mazur, P. 1981. Fundamental cryobiology and the preservation of organs by freezing. In: A.M. Karow and D.E. Pegg, Eds: Organ preservation for transplantation. Marcel Dekker, New York and Basel.
14. Mazur, P. 1970. Cryobiology: the freezing of biological systems. Science 168:939-949.
15. Mazur, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J. Gen. Physiol. 47:347-369.

16. Potts, W.T., and P.O. Rudy. 1967. Water balance in the eggs of the Atlantic salmon, Salmo salar. J. Exp. Biol. 50: 223-237.
17. Rajotte, R.V. and P. Mazur. 1981. Survival of frozen-thawed fetal rat pancreases as a function of the permeation of dimethyl sulfoxide and glycerol, warming rate, and fetal age. Cryobiology 18:17-32.
18. Scott, A.P. and S.M. Baynes, 1980. A review of the biology, handling and storage of salmonid spermatozoa. J. Fish. Biol. 17: 707-739.
19. Sneed, K.E. and H.P. Clemens. 1956. Survival of fish sperm after freezing and storage at low temperatures. Prog. Fish Cult. 18: 99-103.
20. Stoss, J. and E.M. Donaldson. 1982. Studies on cryopreservation of eggs from rainbow trout (Salmo gairdneri) and coho salmon (Oncorhynchus kisutch). Aquaculture in press.
21. Stoss, J. 1982. Personal communication.
22. Suzuki, R. 1959. Sperm activation and aggregation during fertilization in some fishes II. Effect of distilled water on the sperm-stimulating capacity and fertilizability of eggs. Embryologia 4:359-367.
23. Withler, F. 1980. Chilled and cryogenic storage of gametes of Thai carps and cat fishes. Can. Tech. Rep. Fish. Aq. Sci. No. 948.
24. Yamamoto, T. 1961. Physiology of fertilization in fishes. Intern. Rev. Cytol. 12: 361-405.

CRYOPRESERVATION OF RAINBOW TROUT SPERMATOZOA: VARIATION IN MEMBRANE COMPOSITION MAY INFLUENCE SPERMATOZOAN SURVIVAL

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Summary

After cryopreservation with a sucrose-based diluent, the spermatozoa of a sprat-fed group of rainbow trout gave a higher fertilization rate than spermatozoa of a group fed on a commercially-produced pellet diet. With egg yolk included in the diluent, however, fertilization was not significantly different. The results suggest that substances in egg yolk become associated with the spermatozoan membrane and that this association is influenced by membrane composition.

Introduction

Inconsistent results from cryopreservation experiments are generally accepted as inevitable. They do indicate, however, that more factors are influencing the experimental material than are being controlled or examined. Using factorial experimental design, we have examined the effects and interactions of three variables: egg yolk in the freezing diluent, sperm variability, and variation in the egg batches used to test fertilizations.

Methods

Sperm was obtained from two groups of rainbow trout (Salmo gairdneri), one fed on chopped sprat, the other on a pellet diet.

To simplify experimental design we used a constant freezing rate (30°C min^{-1}) and a uniform fertilization technique throughout. A sucrose-based freezing diluent was used with 0, 5, 10 or 20% fresh egg yolk added. Frozen sperm was tested by fertilizing batches of mixed eggs on two different occasions. The survival of the sperm was estimated by fertilization rate as a percentage of control fertilizations with excess fresh semen. The GLIM (Generalized Linear Interactive Modelling) system was used to analyse the data for main effects and interaction with the linear model:

$$P = \mu + F + D + E + F.D + F.E + D.E + F.D.E. + \Sigma_{ijk}$$

P is percentage fertilization,
μ is the population mean, Σ_{ijk}, the error,
F is the effect due to the feeding regime,
D is the effect due to freezing diluent,
E is the effect due to egg batch,
F.D, F.E, D.E and F.D.E. are the effects due to interaction of the factors.

Results and discussion

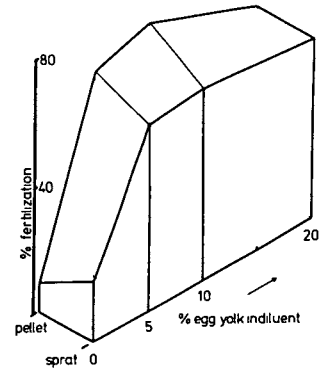

Figure. Mean fertilization rate as a percentage of the controls, with the sperm from the sprat and pellet-fed groups and the four levels of egg yolk in the diluent.

The fertilization rate for sperm from sprat-fed fish was significantly greater ($P < 0.05$) than for the pellet-fed group when no yolk was included in the diluent. There was no significant difference between the two groups of sperm, however, with egg yolk present at any of the three concentrations.

Although the fatty acid composition of the spermatozoa did differ between the two groups (most notably 20:5ω3 - 8% and 14% for sprat- and pellet-fed respectively) it is unlikely that the differences observed in the cryopreservation work can be attributed to this alone.

The improvement in survival of the sperm with egg yolk in the diluent is clear, but analysis of the data with GLIM shows significant interaction ($P < 0.01$) between feeding and diluent (F.D), and between diluent and egg batch (D.E). Such interaction confounds interpretation of main effects. The causes of these interactions are probably (1) that egg yolk in the diluent interferes with fertilization (we have other evidence for this) and (2) substances in egg yolk become associated with the membrane and confer a different degree of cryoprotection on the two types of sperm. Interaction is probably a major cause of unexplained variation in cryopreservation work.

CRYOPRESERVATION OF RAINBOW TROUT SPERM: EFFECT OF EQUILIBRATION

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Introduction

This study presents the results of three experiments carried out to evaluate the influence of equilibration time on fertilizing capacity of cryopreserved rainbow trout (Salmo gairdneri Richardson) sperm.

Methods

The sperm was collected by hand stripping and stored in glass tubes for each male separately. Before collecting the sperm the glass tubes were inserted into the same water in which the males were kept and therefore had the same temperature as the sperm.

The visually unobjectionable sperm samples were mixed.

For diluting the sperm the following extenders were used:

V2 e 750 mg NaCl, 38 mg KCl, 200 mg $NaHCO_3$, 100 mg Glukose, 100 cm^3 H_2O, 20 cm^3 egg yolk (used in experiment 2)

V2 f 750 mg NaCl, 200 mg $NaHCO_3$, 100 mg Glukose, 100 cm^3 H_2O, 20 cm^3 egg yolk (used in experiment 1 and 3)

For cryoprotection dimethyl sulfoxide (DMSO) was used. In experiment 1 and 2 10 cm^3 DMSO were added to 100 cm^3 of V2 e respectively V2 f. In experiment 3 9 cm^3 DMSO were added to 100 cm^3 of V2 f.

The temperature of the extenders was also adapted to that of the sperm in the way mentioned above.

The sperm was diluted with this solutions at a dilution rate (sperm : extender) of 1 : 3 (experiment 1 and 2) respectively 1 : 6 (experiment 3).

For freezing the pelleting technique of Nagase et al. (1964) was used.

Immediately after diluting respectively after equilibration of up to 55 minutes the samples were dropped with a syringe on carbon ice (-79°C) and frozen to pellets of 0,2 cm^3. The frozen pellets were stored in liquid nitrogen (-196°C).

For thawing, 3 of the pellets were put in 10 cm^3 of a thawing solution (1 g $NaHCO_3$ in 100 cm^3 H_2O) and immediately and strongly shaked. Just before the pellets were totaly thawed, the solution was poured over the eggs. For control egg samples were also inseminated with fresh sperm. On average 149 $\pm$ 45 ($\bar{x} \pm s$; n = 135) eggs were inseminated per replicate.

For the analysis of variance the percentages of the fertilization rates were transformed with the arcus-sinus-transformation. F-test and multiple range test (Duncan) were used to test the significance.

Results

With fresh sperm the following fertilization rates were achieved:

Experiment 1 96,7 % $\pm$ 0,6 ($\bar{x} \pm s$; n=2)

Experiment 2 96,8 % $\pm$ 2,6 ($\bar{x} \pm s$; n=4)

Experiment 3 82,9 % $\pm$ 15,8 ($\bar{x} \pm s$; n=4)

The fertilization rates obtained with cryopreserved sperm are presented in fig. 1 - 3.

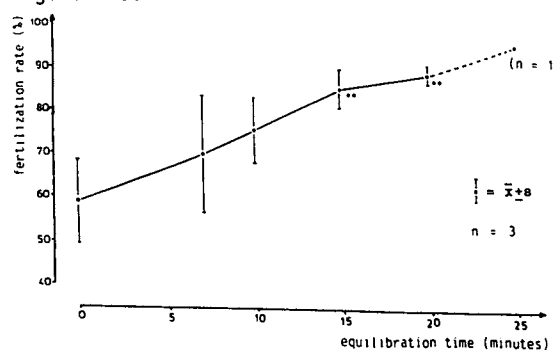

fig. 1. Experiment 1: Results of the fertilization tests with cryopreserved sperm

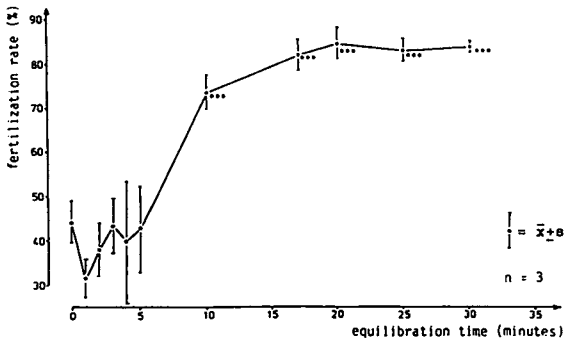

fig. 2. Experiment 2: Results of the fertilization tests with cryopreserved sperm

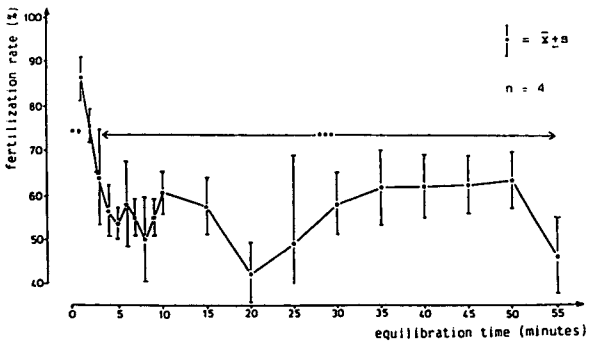

fig. 3. Experiment 3: Results of the fertilization tests with cryopreserved sperm

*** highly significant difference ($P < 0,001$) respectively

** significant difference ($P < 0,01$) in comparison with the results without equilibration.

Conclusion

Rainbow trout sperm was cryopreserved using the egg yolk containing extenders V2 e and V2 f (Bayrle 1980). Equilibration of the diluted sperm prior to freezing caused rising fertilization rates in two experiments and a falling fertilization rate in a third one. Sperm quality may be the reason of the different reactions on equilibration.

References

Bayrle, H., 1980. Untersuchungen zur Optimierung der Befruchtungsfähigkeit von gefrierkonserviertem Fischsperma. Dissertation TU München, Weihenstephan.

Nagase, H., T. Niwa, 1964. Deep freezing bull semen in concentrated pellet form., I. Factors affecting survival of spermatozoa. 5th. Int. Congr. Anim. Reprod. and A.I., Trento.

PHYSIOLOGICAL STUDY ON THE INITIATION OF SPERM MOTILITY IN CHUM SALMON, ONCORHYNCHUS KETA

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Introduction

Much evidence has been accumulated concerning the detailed mechanism of sperm motility in marine invertebrates and mammals. However little is known about sperm motility in teleosts. Recent work by Morisawa & Suzuki(1980) using fishes showed that sperm motility is inhibited in the male reproductive organ by potassium which is found at high concentration in the seminal plasma in chum salmon and a reduction of potassium concentration in the surrounding environment at spawning is the factor which initiates sperm motility. Morisawa & Okuno(1982) further demonstrated in the spermatozoa of another salmonid fish, rainbow trout, whose plasma membrane was removed by the detergent Triton X-100 that the release of suppression by potassium induces the increase of intraflagellar cyclic AMP and the synthesized cAMP convert the immotile axoneme(9+2 structure; motile apparatus in sperm flugellum) to a motile one, resulting in the initiation of sperm motility.

Here we demonstrated that cAMP is also the factor to trigger the initiation of axonemal movement i.e. sperm motility in another salmonid fish, chum salmon.

Methods and Results

The plasma membrane of chum salmon sperm, which were collected by inserting a pipette into the sperm duct, were removed by mixing on ice 1 volume of semen with 20 volumes of the extracting medium(0.15 M KCl, 0.5 mM $CaCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol(DTT), 2 mM Tris buffer at pH 8.2 and 0.04 % Triton X-100) for 30 seconds. These demembranated spermatozoa are immotile because they lack an energy supply(ATP) and the components essential for axonemal movement. We tried the reactivation of immotile axoneme by mixing at room temperature the demembranated spermatozoa with the reactivating medium containing 0.15 M KCl, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 2 mM EGTA, 1 mM DTT, 20 mM Tris buffer, pH 8.2 and 0.2 mM ATP in the absence or presence of 20 μM cAMP. Observations were made with dark-field microscopy.

When cAMP was absent from the reactivating medium, no axonemal movement occurred suggesting that an energy supply alone is not enough to induce axonemal movement. In contrast, when the demembranated sperm were suspended in reactivating medium containing ATP and cAMP, axonemal movement was observed. Axonemal movement occurred with a velocity of 55 μm/sec. These results suggest that the axoneme of chum salmon spermatozoa is functionally immotile and is converted to the motile state by exposure to cAMP.

Discussion

In the previous paper(Morisawa & Okuno,1982), we described in the rainbow trout that the reduction of potassium concentration surrounding spawned spermatozoa affects membrane-mediated processes in intact spermatozoa and then induces the synthesis of intraflagellar cAMP. The cAMP then converts the immotile axoneme to a motile state resulting in the initiation of sperm motility. In chum salmon sperm motility is also initiated by the reduction of potassium during natural spawning(Morisawa & Suzuki, 1980). Therefore an external signal, change in potassium concentration also induces an intraflagellar factor cAMP through some change of the plasma membrane. The synthesized cAMP then converts the axoneme from an immotile state to a motile one and the motile axoneme conveys motility to the spermatozoa.

In the reproductive physiology of fish, the mechanism involved in sperm motility have been not well known. We have shown here that fish spermatozoa are a useful material for investigating the mechanism of initiation of sperm motility. Further studies using fish spermatozoa may provide much valuable evidence for molecular processes of cell motility as well as for fish culture studies i.e. artificial insemination of fish.

References

Morisawa, M. & K. Suzuki, 1980. Osmolality and potassium ion: Their roles in initiation of sperm motility in teleosts. Science, 210: 1145-1147.

Morisawa, M. & M. Okuno, 1982. Cyclic AMP induces maturation of trout sperm axoneme to initiate motility. Nature, 295:703-704.

NEW APPROACHES FOR CRYOPRESERVATION OF SPERM OF GREY MULLET, Mugil cephalus

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Summary

Potency of milt extenders, sodium citrate, glucose, lactose, fructose, mullet serum, and Ott-Horton's extenders 129 and 164 supplemented with or without potassium chloride or sodium chloride was compared. Glucose and fructose were favorable and supplement of potassium chloride was beneficial for the motility of the mullet sperm.

Combination of freezing media and packing conditions of the diluted milt in PE straws provided a series of freezing rates ranged 1.5-876°C/min.

Extender and cryoprotectant employed in the most satisfactory group was a mixture of 80% diluted glucose solution (5%) and 20% DMSO. Milt was mixed with equal volume of the mixture and frozen at 29°C/min to -196°C. The best cryopreserved sperm recovered 90% motility, and provided 28.64% fertility (45.17% in control) and 62.60% hatching rate (100% in control).

Table: Osmotic pressure of various extenders used for the study and their effect on motility of grey mullet sperm

Extender	Concentration (mM)	Averaged osmotic pressure (mOsm/kg)	Best motility ☆ and duration
Sodium citrate	100	220	+++ 30"
	200	435	++ 1'17"
	300	630	++++ 1' 2"
	400	840	+++ 48"
	500	1150	++ 10"
Glucose	200	165	+++ 2' 3"
	400	330	++++ 1' 6"
	500	360	+++++ 2'14"
	600	660	+++ 1'16"
	1000	900	+++ 41"
Lactose	200	210	+++ 1'57"
	400	370	+++ 56"
	500	455	+++ 1' 9"
	600	560	+++++ 1'35"
	1000	570	+++++ 1'50"
Fructose	200	175	++ 10"
	400	365	++ 10"
	500	435	+++++ 2'46"
	600	530	++ 28"
	1000	910	++ 10"
Mullet serum		465	++++ 1'58"
▶129		378	++ 2'18"
▶164		428	+++ 10"
NaCl+KCl	100;100	383	+++ 1'11"
NaCl+Glucose	100;200	378	++++ 1'15"
KCl+Glucose	10;390	345	++++ 1'18"
KCl+Glucose	50;350	340	+++++ 1'17"
KCl+Glucose	100;200	370	+++++ 1' 5"
▶129 with KCl	15	290	++ 1'15"
▶129 with KCl	20	395	++ 1'35"
▶129 with KCl	25	487	++++ 30"
▶129 with KCl	40	690	+++++ 30"

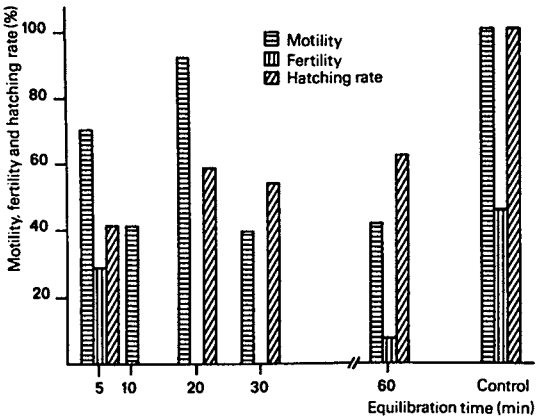
☆ +++++ very strong; ++++ strong; +++ moderate; ++ weak

Methods and Results

Extenders were solutions of salt, saccharide or mullet serum with HEPES buffer adjusted to a stable pH of 7.7. Organic compounds were included in the case of extenders 129 and 164 (Ott & Horton, 1971) and one of the ingredient KCl was concentrated for comparison as function of ions to influence sperm motility was a possible factor other than pH and osmotic pressure (Hines & Yashouv, 1971)

Extenders with cryoprotectant of better result were chosen in cryopreservation test at the ratio of 1:1 with milt. After equilibration, the mixture was dispensed into PE straws and frozen at different rates by using the freezing media of dryice, isopropanol with dryice, LN_2 vapor or LN_2 in combination with various packing of straws in canister of bamboo, plastic, PE, PB, cellulose thimbles, stainless, stainless wire, or being nake. Guest et al. (1976) indicated that extended equilibration appear best for channel catfish while minimal equilibration and rapid freezing are best for salmonids. It was found that for grey mullet equilibration within 1 h and mediate freezing rate between 21 and 119°C /min are favorable.

Motility reading and fertility evaluation of cryopreserved-thawed sperm of the best group showed a better result (Fig. 1) than previous study (Chao et al. 1975).



References

Chao, N.H., H.P. Chen & I.C. Liao, 1975. Aquaculture. 5: 389-406.

Guest, W.C., J.W. Avault, Jr. & J.D. Roussel, 1976. Trans. Am. Fish. Soc. 3: 469-474.

THE CHEMICAL COMPOSITION OF THE COMPANION FLUIDS OF THE GAMETES IN THE COMMON CARP (Cyprinus carpio)

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Introduction

A knowledge of the chemical composition of the ovarian fluid and the seminal plasma is necessary to a better understanding of the physiology of fish gametes as well as to a more practical goal, that of composing a diluent for artificial insemination or for the storage or deep-freezing of the gametes. The present paper describes the chemical composition of these companion fluids in the common carp.

Material and methods

The fish were reared in our experimental facilities at Jouy-en-Josas in recycled water (temperature: 13 to 20°C) under natural photoperiod. They were collected in June and given a treatment of carp crude pituitary extracts (3 mg/kg body weight for females and 1.5 mg/kg for males). After gamete collection, the seminal plasma was obtained by centrifugation (20 min at 3600 x g) and the ovarian fluid was taken by simply pouring off the excess fluid. Osmotic pressure (OP) and pH were measured immediately after collection and the remaining fluids were stored at -20° C until analysis. The fertility of the gametes of each male and female was estimated after insemination and 3-day incubation of the eggs at 20°C. Analysis included measurement of the ash after ashing at 350°C for 24 h and of the cations with flame photospectrometer Eppendorf. Total phosphorus and proteins were measured by colorimetry. Aminoacids were measured according the Menezo et al (1978) after 24-hour hydrolysis (HCl 6N).

Results

The composition of the seminal plasma of the common carp was characterized by a high content of potassium which was the major cation found. Contrary to trout, the K did not inhibit sperm motility and even seemed to potentiate it. The protein content of the seminal plasma was relatively low (1.2 g/l) but the concentration of amino acids and small peptides was very high (36 µM/ml). The overall composition of the seminal plasma was very different from that of the blood plasma. Highly significant correlations were found between total protein (TP) and phospholipids (PL) (0.8), P and PL (0.71), TP and P (0.94), but there was no correlation between the fertilizing ability of the spermatozoa (% of fertilization) and any of the measured compounds.

The inhibition of sperm motility in the male genital tract was due to the relatively high osmotic pressure of the seminal fluid (286 mOsmoles/kg). Motility initiated after dilution occured in a range of 15o-200 mOsmoles.

Table. Composition of ovarian fluid and seminal plasma in common carp. Values±SD; n = 10; (1) % of dry matter; (2) pooled samples

	Ovarian fluid	Seminal plasma
Water %	97	98.5
Organic matter (1)	73	58
Ash (1)	27	42
Na g/l	2.8±0,34	1.18 (2)
K g/l	0.48±0.2	1.7 (2)
Ca mg/l	105±3	28.5 (2)
Mg mg/l	15±6	6.5 (2)
P mg/l	113±60	33±20
PL mg/l	44±30	5.6±1
TP g/l	4.1±2.3	1.2±0.3
Amino acids µM/ml	10.6 (2)	36.7 (2)
OP mOsmoles/kg	305±12.9	286±10
pH	8.51±0.11	7.96±0.1
Fert. %	78±14	96±1.4

Except for K, which was 3 times higher in carp than salmonids or cod, the mineral composition of carp ovarian fluid was comparable to that of other fish species. Egg fertility was negatively correlated with K (-0.69). The P concentration was 4 times lower than in the blood plasma and the protein content was 10 times lower. Highly significant correlations were found between TP and PL (0.97), Ca and P (0.93), K and Mg (0.93) and organic matter and PL (0.97). The osmotic pressure was surprisingly high (305 mOsmoles), being even more elevated than in the seminal plasma so that, contrary to the case in trout, the spermatozoa did not move when directly added to the eggs. When artificial insemination was carried out in trout with ovarian fluid, fertilization occurred as soon as the gametes were mixed, while in carp it occurred only after a diluent had been added.

VITELLOGENESIS IN FISHES

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Summary

Egg yolk lipid and protein components are discussed along with considerations of their origins. In recent years, plasma vitellogenin has been confirmed as the precursor of the major yolk proteins. Several vitellogenins and vitellogenin derivatives have been characterized in some detail. Endogenous synthesis of some yolk components is possible although the qualitative and quantitative importance of endogenous vitellogenesis remains to be determined. Recent studies of estrogen induction of vitellogenesis have considered the role of estrone as well as estradiol, primary and secondary responses to estrogen and induction of vitellogenin mRNA. Annual cycles of sex steroids are discussed as are implications of the measurement of vitellogenin.
Keywords: vitellogenesis, lipovitellin, phosvitin, estrogen, yolk lipids.

Introduction

The dramatic growth in the ovaries of many fishes, from about 1% or less of body weight up to 20% or more, that occurs in the months prior to spawning is largely due to the accumulation by the oocytes of nutrient reserves for the embryos, the yolk. The synthesis of yolk materials, termed vitellogenesis, is believed to occur both within the oocyte (endogenous vitellogenesis) and external to the oocyte (exogenous vitellogenesis). In general, endogenous vitellogenesis is considered to preceed exogenous vitellogenesis but there is evidence for overlap of these phases. It may indeed be more accurate to consider the two types of vitellogenesis solely as processes rather than discreet temporal phases.

The major development in recent years in the study of vitellogenesis has been the demonstration in a variety of species of the presence of the female-specific plasma lipophosphoprotein complex, vitellogenin, which has been confirmed as precursor of the major yolk proteins. It is, however, important to recall that, unlike the case in Xenopus, precise quantification of the proportion of yolk materials derived directly from vitellogenin has not been performed in fish (Wallace & Selman 1981). As discussed below, there is evidence for a number of potential sources of fish yolk in addition to vitellogenin.

Traditionally, yolk material has been classified by the histochemical reactions of the various oocyte cytoplasmic inclusions as carbohydrate, lipid and protein yolk (Raven 1961). This review concentrates on the nature and origin of yolk lipids and proteins. Vitellogenin and its ovarian derivatives are discussed in some detail. Several other ovarian components are considered as are selected aspects of sex steroid hormone actions related to vitellogenesis and implications of the measurement of vitellogenin. The functions of gonadotropins in processes related to vitellogenesis are discussed elsewhere in this symposium (Idler 1982). Oocyte synthetic activity occurring prior to vitellogenesis has been recently reviewed (Guraya 1979; Bruslé 1980; Wallace & Selman 1981) as has the synthesis of "yolk vesicles" (Wallace & Selman 1981). Readers are encouraged to consult these works. It is, however, worth reiterating Wallace & Selman's (1981) point that "yolk vesicles" are likely precursors of cortical alveoli which are extruded at fertilization and hence should not be considered yolk. Raven (1961) previously pointed out that "carbohydrate yolk" in fishes is considered synonymous with "yolk vesicles."

Yolk material and its origins

(i) Lipids

"Lipid yolk" accumulation commences prior to that of protein yolk (Raven 1961) and its appearance can be considered to mark the start of endogenous vitellogenesis (Shackley & King 1977). Since the major yolk protein precursor, vitellogenin, and its major derivative, lipovitellin, contain lipid, it should be remembered that the classical term "lipid yolk" is normally reserved for lipid inclusions observed in anatomical studies to be distinct from vitellogenin-derived protein yolk granules. In some cases, however, like that of the gourami discussed below, substantial lipid is accumulated in the ovary although critical details of its origin are unknown. For this reason, I have considered yolk lipids from a variety of sources, including vitellogenin, in this section.

Lipid bodies appear initially in the perinuclear cytoplasm of the oocyte. They profiferate during the development and migration of Balbiani's body and occur frequently in association with that body

(Droller & Roth 1966; Beams & Kessel 1973; Chopra 1958; Guraya 1963, 1965; Nayyar 19 64). In early vitellogenesis, the lipid bodies consist largely of phospholipids (Chopra 1958; Guraya 1963, 1965; Nayyar 1964; Shackley & King 1977). A general classification of the early lipid inclusions as true yolk is of questionable accuracy as they have been observed to disappear in some species prior to, or during, subsequent exogenous vitellogenesis (Chopra 1958; Guraya 1963, 1965) but may persist in others (Shackley & King 1977). It is also noteworthy that the endogenous origin of lipid bodies has been ascribed solely on the basis of anatomical criteria. Detailed analysis of oocyte cytoplasmic lipids and a direct demonstration of the capacity of oocytes to synthesize them remain to be performed. In a recent study of rainbow trout, Salmo gairdneri, ovarian lipogenesis in vitro, it was found that radioactive acetate incorporated into lipids was recovered primarily in the polar lipid fraction of ovaries in early stages of recrudescence (Wiegand & Idler 1982). Whether the labelled polar lipids in this study correspond to oocyte lipid body phospholipids identified by others histochemically, or to structural polar lipids, or both, remains to be determined.

Lipid inclusions described in more advanced oocytes during after exogenous vitellogenesis has commenced consist primarily of triglycerides (Chopra 1958; Guraya 1965; Nakagawa & Tsuchiyer 1969 cited by Leger et al 1981). This pattern of shift in composition of oocyte lipid inclusions is common to a wide variety of vertebrates and invertebrates (Nath 1960; Raven 1961). The capacity of trout ovaries to synthesize triglycerides at a time when vitellogenin uptake is occurring suggests an endogenous source for some of this yolk (Wiegand & Idler 1982). An exogenous contribution to the triglyceride pool from plasma lipoproteins including vitellogenin is also likely (Leger et al 1981).

Vitellogenin is an important source of ovarian lipids and is likely the major source of polar lipids. Total lipid has been found to comprise 21.5% of S. gairdneri vitellogenin (Campbell & Idler 1980) and 21-22% of goldfish, Carassius auratus vitellogenin (Hori et al 1979). S. gairdneri vitellogenin, separated from other serum lipoproteins by centrifugation, has been found to have a distinct fatty acid complement from that of the other lipoproteins, (Leger et al 1981). Another preparation of S. gairdneri vitellogenin has been found to contain 280 ug polar lipids and 60 ug triglyceride per mg protein (Wiegand & Idler 1982). Approximately 87% of C. auratus vitellogenin lipid is phospholipid (Hori et al 1979). Based on yolk granule analysis, total lipid has also been estimated to comprise approximately 18% of the dry weight of lesser spotted dogfish, Scyliorhinus canicula vitellogenin (Craik 1978a). In general, fish vitellogenins have been found to have much higher lipid contents than that reported in Xenopus (Redshaw & Follett 1971). Of the ovarian derivatives of vitellogenin, only lipovitellin has been found to contain lipid, with contents ranging from 15-29% (Ando 1965; Jared & Wallace 1968; Plack et al 1971; Campbell & Idler 1980). Using a conversion factor of 25X lipid phosphorus determinations, the phospholipid content is estimated at 58% of lipovitellin lipid in coho salmon, Oncorhynchus kisutch (Markert & Vanstone 1971) and 66% in cod, Gadus morhua (Plack et al 1971). Vitellogenin and its derivatives are considered more extensively below.

An interesting series of papers has considered the accumulation of wax ester in the ovary of the opaline gourami, Trichogaster cosby. Wax ester is the major ovarian lipid in this fish, comprising about 37% of ovarian weight (Sand et al 1969). Wax ester is the major ovarian lipid in several other teleosts but comprises a lower proportion of total ovarian mass (Iyengar & Schlenk 1967; Kaitaranta & Ackman 1981). The source of the extremely high wax ester content in the ovaries of T. cosby is interesting in the overall view of teleost vitellogenesis. Of particular interest is the accumulation of the alcohol portion of wax esters.

In T. cosby, Sand et al (1969, 1971) found that ovarian wax ester alcohols can be synthesized in vivo from dietary acetate or derived from dietary carbon chains. The observation that de novo synthesis of the ovarian wax ester components can occur in vivo, although the site is unknown (Sand et al 1969), coupled with the observation that the ovary of at least one teleost species can synthesize fatty alcohols (Wiegand & Idler 1982), suggests that some ovarian wax ester components may be synthesized endogenously. The major potential exogenous source of ovarian alcohols is plasma fatty acid which is reduced to alcohol after uptake by the ovary. Since feeding experiments revealed that labelled fatty acid was transported primarily as triglyceride (Sand et al 1973; Rahn et al 1973), it is possible that either T. cosby vitellogenin is very unusual in terms of both its total and neutral lipid content, or other lipoproteins besides vitellogenin are involved the transport of lipid to the developing ovary. This species would appear to offer unique opportunities for the comparative study of vitellogenesis.

(ii) Vitellogenin, ovarian derivatives of vitellogenin and other ovarian proteins.

Vitellogenins have been demonstrated in a wide variety of teleost (for reviews, see Wallace 1978; Wallace & Selman 1981; also Campbell & Idler 1976, 1980; Le Menn 1979; Hara et al 1980; Nath & Sundararaj 1981) and

in several non-teleost fishes (Pickering 1976; Craik 1978a; Yu et al 1980, 1981). The hepatic origin of vitellogenin has been confirmed (Plack & Frazer 1971; Campbell & Idler 1976; de Vlaming et al 1977a; Le Menn & Lamy 1977; Larsen 1978; Yu et al 1980; Idler & Campbell 1980; Sundararaj & Nath 1981). In a number of the above studies, immunological relationships between vitellogenin and egg yolk proteins and induction of vitellogenin synthesis by estrogen in both sexes has been demonstrated.

Vitellogenin and their egg yolk derivatives have been characterized in some detail in a number of teleost species. Molecular weight determinations by gel filtration (Emmersen & Petersen 1976; Hara & Hirai 1978; Campbell & Idler 1980; Hara et al 1980) and electrophoresis (Hori et al 1979; de Vlaming et al 1980; Campbell & Idler 1980) have yielded values between 326,000 and 600,000 for the various intact molecules. Molecular weights of delipidated polypeptide monomers determined by electrophoresis in the precence of sodium dodecyl sulfate (SDS) range from 140,000 to 220,000 (Hara & Hirai 1978; Hori et al 1979; de Vlaming et al 1980; Roach & Davies 1980). Caution has been advised in the use of gel filtration for determination of vitellogenin molecular weights (Hori et al 1979; de Vlaming et al 1980), although close agreement between results using gel filtration and gradient gel electrophoresis has been found with S. gairdneri vitellogenin (Campbell & Idler 1980). Using native gel electrophoresis, two groups of investigators have found monomeric and dimeric forms of C. auratus vitellogenin along with a third, comparatively minor, component of intermediate molecular weight (Hori et al 1979; de Vlaming et al 1980). De Vlaming et al (1980) further found multiple molecular weight forms of delipidated C. auratus vitellogenin monomer.

Alanine, glutamic acid and leucine have been found to be the most abundant amino acids in S. gairdneri (Hara & Hirai 1978 Campbell & Idler 1980), C. auratus (de Vlaming et al 1980) and Japanese eel, Anguilla japonica (Hara et al 1980) vitellogenins. In contrast to the Xenopus vitellogenin (Redshaw & Follett 1971; de Vlaming et al 1980), the teleost vitellogenins were poor in serine. Other chemical analyses of teleost vitellogenins have revealed lower calcium and protein phosphorus concentrations than those found in Xenopus vitellogenin (Hori et al 1979; Campbell & Idler 1980; de Vlaming et al 1980; Hara et al 1980). It was previously pointed out (de Vlaming et al 1980) that concurrent low levels of protein phosphorus and serine in teleost vitellogenins are consistent, since the serine hydroxyl group is the principal site of phosphoprotein phosphorylation. Both carbohydrates and iron have been identified as components of teleost vitellogenins by histochemical methods (Hara & Hirai 1978; Hori et al 1979; Hara et al 1980) and the iron binding capacity of S. gairdneri, chum salmon, Oncorhynchus keta and A. japonica vitellogenins has been demonstrated using immunological and autoradiographic techniques (Hara 1976; Hara et al 1980).

When vitellogenin is taken up by oocytes in Xenopus, it is proteolytically cleaved to form the yolk proteins lipovitellin and phosvitin (Wallace 1978). While these processes have not been directly demonstrated in teleosts, there is substantial evidence that they do occur.

Gonadotropin-sensitive and selective uptake of S. gairdneri oocytes has been demonstrated in vitro (Campbell 1978; Campbell & Jalabert 1979). Autoradiography of oocytes from sheepshead minnow, Cyprinodon variegates, which had been injected 24 hrs. earlier with tritiated Xenopus vitellogenin revealed grain deposition over yolk spheres and fusing yolk masses, suggesting incorporation of the heterologous vitellogenin into yolk (Selman & Wallace 1982). Ng & Idler (1980) injected female winter flounder, Pseudopleuronectes americanus, with labelled serum phosphoprotein isolated from estrogen-treated males and demonstrated gonadotropin-sensitive uptake of label into yolk. The clearance from the plasma of phosphoprotein (an index of vitellogenin) and its appearance in yolk granules has been studied in the elasmobranch, S. canicula (Craik 1978b). A number of ultrastructural studies have shown incorporation of material taken up by pinocytosis into yolk spheres (for review, see Wallace & Selman 1981). Immunological relationships have been demonstrated between vitellogenins and various egg yolk proteins in a number of species (above) and close similarities in amino acid composition between vitellogenin and the major yolk protein, lipovitellin, have also been noted (Hara & Hirai 1978; Campbell & Idler 1980).

Lipovitellin molecular weights have been found to be lower than those of their vitellogenin precursors in several studies in which both were determined (Hara & Hirai 1978; Campbell & Idler 1980; Roach & Davies 1980; de Vlaming et al 1980) but not in A. japonica (Hara et al 1980). These latter authors suggested that eel vitellogenin may be incorporated into yolk without modification. Hori et al (1979) made a similar suggestion regarding C. auratus vitellogenin but this has been disputed by de Vlaming et al (1980) who isolated lipovitellins and phosvitins from C. auratus indicating that typical proteolytic metabolism of vitellogenin occurs upon uptake by the oocyte in this species. De Vlaming et al (1980) further found two molecular weight classes of lipovitellin subunits, each of which consisted of more than one polypeptide. The presence of high and low molecular weight subunits has been indicated using a 2-

mercaptoethanol-reduced preparation of S. gairdneri lipovitellin (Hara & Hirai 1978).

Low or undetectable levels of protein phosphorus have been reported for a number of teleost lipovitellins (Jared & Wallace 1968; Markert & Vanstone 1971; Plack et al 1971; Campbell & Idler 1980). Lipovitellin lipids are discussed in the preceeding section.

The phosphoprotein derivatives of vitellogenin, the phosvitins, have received more extensive study. These proteins may be isolated by redissolving a yolk dilution precipitate in saline and precipitating lipovitellin (and β-component) with ammonium sulfate (Wallace et al 1966; Markert & Vanstone 1971). The phosvitin can then be further purified by chromatography. Other procedures have been employed involving a series of extractions, precipitions and dialyses followed by chromatography (Mano & Lipman 1966) or the initial partition of egg proteins with water and phenol followed by ethanol precipitation of phosphoproteins from the aqueous phase and subsequent chromatography (Mano & Yoshida 1969; Inoue et al 1971).

The phosvitin content of fish ovaries appears to be quite variable. Jared & Wallace (1968) found very little phosvitin in the ovaries of several marine teleosts and suggested that developing eggs could obtain their required phosphate from the environment. In support of this, there is little phosvitin in the eggs of G. morhua (Plack et al 1971) or Pacific herring, Clupea pallasii (Inoue et al 1971; Inoue & Iwasaki 1980) although a high phosphoprotein concentration has been found in flounder ovaries (Mano & Lipmann 1966). Phosvitin has been found to comprise 3% of ovarian yolk in S. gairdneri (Campbell & Idler 1980).

Molecular weights of teleost phosvitins have been found to be extremely variable, and descrepancies exist between preparations from the same species. For example, molecular weights of 19,400 (Mano & Yoshida 1969) and 24,000 (Wallace et al 1966) have been determined for S. gairdneri phosvitin by sedimentation analysis. These values are comparable to phosvitin molecular weights in other salmonids, as determined by sedimentation analysis (Schmidt et al 1965; Mano & Yoshida 1969) or gel filtration (Markert & Vanstone 1971). However, Campbell & Idler (1980), using a procedure for isolation of S. gairdneri phosvitin similar to that of Wallace et al (1966), determined a molecular weight of 45,000 by gel filtration. In addition to differences in technique, part of such discrepancies could be due to interactions of subunits. Molecular weights ranging from 14,000 to 16,000 were determined by sedimentation alalysis for three phosvitins, of varying degrees of phosphorylation, isolated from ling (Mano & Lipmann 1966). About 30% of one fraction consisted of a species with molecular weight 37,000-40,000, suggesting the presence of a dimer (Mano & Lipmann 1966). Two molecular forms of C. auratus phosvitin, 7,600 and 14,500 were separated by gel filtration (de Vlaming et al 1980) and a low molecular weight phosvitin, 4,200 by sedimentation analysis, has been demonstrated in C. pallasii (Inoue et al 1971). Wiley & Wallace (1981) provided a detailed examination of molecular weight determinations of Xenopus yolk proteins with particular emphasis on the difficulties in determining molecular weights of highly phosphorylated proteins. These authors recommended the use of SDS-gradient gel electrophoresis in such cases.

In general, amino acid analyses of teleost phosvitins have revealed a predominance of serine. Proportions of serine in the preparations noted above range from 42% in S. gairdneri (Campbell & Idler 1980) to 75% in C. pallasii (Inoue et al 1971). An exception is the group of phosphoproteins isolated from shad in which serine content ranged from 9% to 22% (Mano & Lipmann 1966). The general observation of Mano & Lipmann (1966) that phosvitins are poor in histidine, sulfur-containing and aromatic amono acids has been verified in subsequent investigations (Inoue et al 1971; Table 3, Campbell & Idler 1980). The ester linkage of phosphate to serine has been studied in C. pallasii (Inoue et al 1971) and multiple forms of several teleost phosvitins with varying degrees of phosphorylation have been demonstrated (Mano & Lipmann 1966). The phosvitins of O. kisutch (Markert & Vanstone 1971) and S. gairdneri (Campbell & Idler 1980) were found to have no detectable levels of lipid.

Another yolk protein that may be derived from vitellogenin is the β^1 component. Jared & Wallace (1968) chromatographed yolk protein extracts of a variety of teleosts on TEAE-cellulose. In the case of the S. gairdneri extract, 2 distinct components, α and β, were detected by absorbance at 280mm and a third, γ, had low absorbance but a high content of protein phosphorus. The α and γ components, lipovitellin and phosvitin, were precipitated by dilution leaving the β component in solution. It was proposed that this protein, which contained neither lipid nor phosphorus was a serum protein (Jared & Wallace 1968). In contrast, Markert & Vanstone (1968) recovered a similar protein from several Oncorhynchus species that did precipitate with lipovitellin and phosvitin on dilution and coprecipitated with lipovitellin during ammonium acetate fractionation. This protein, designated β^1-component, was further studied in O. kisutch and found to have a molecular weight of 30,000 and to contain no lipid or phosphorus (Markert & Vanstone 1971). Its presence in serum from maturing females and estrogenized prepuberal fish was demonstrated by immunodiffusion. An immunological relationship between β^1-component and vitello-

genin has been demonstrated in S. gairdneri suggesting that β^1-component is a part of the vitellogenin complex (Campbell & Idler 1980).

The possibility of an endogenous contribution to yolk protein should not be ignored. In Brachydanio rerio, large yolk crystals of exogenous origin have been observed to be enclosed in an amorphous matrix that appears to be synthesized within the oocyte (Ulrich 1969). In a series of studies on fish gonadotropins (see Idler & Ng 1979), hormonal effects on ovarian metabolism have been investigated by measuring uptake of H_3PO_4 and H-leucine in vivo. It was consistently found that more isotope was incorporated into ovarian proteins which were insoluble in trichloroacetic acid, than could be accounted for by uptake of labelled vitellogenin. It remains to be investigated whether the additional labelled protein is true yolk and whether it is of endogenous or exogenous origin.

In addition to the major egg yolk constituents discussed above, some other materials have been studied whose origin is not known to be vitellogenin and may represent products of endogenous vitellogenesis or exogenous material of a non-vitellogenin source.

Sialoglycoproteins have been isolated from the eggs of S. irideus (Inoue & Iwasaki 1978) and C. pallasii (Inoue & Iwasaki 1980). The S. irideus sialoglycoprotein has a sialic acid content of 59% and a total amino acid content of 21%. It is about one quarter as abundant as phosvitin. A family of sialoglycoproteins was found in C. pallasii eggs with sialic acid contents of 5-6.5% and total amino acid contents 40-44%. They are about 10 times as abundant as phosvitin. Neutral and N-acetyl-amino sugars comprised the remainder of the preparations. Although the function of these sialoglycoproteins is unknown, it is worth noting that both the cortical alveoli (Wallace & Selman 1981) and the chorion (Tesoriero 1977, 1978) contain glycoprotein and at least one report exists of the detection of sialoglycoproteins in teleost cortical alveoli by histochemical means (Shackley & King 1977). Whether or not the sialoglycoproteins isolated by Inoue and collaborators are components of either of these structures or are true yolk remains to be determined.

There are a few reports indicating the possibility of uptake of proteins other than vitellogenin from the blood. S. gairdneri oocytes will sequester non-vitellogenin serum proteins in vitro (Campbell & Jalabert 1979). Uptake of these proteins is not sensitive to gonadotropin and occurs at a much lower rate than uptake of vitellogenin. In the ayu, Plecoglossus altevelis, one cross reaction between egg extract and antiserum to maturing female plasma is eliminated by absorbing the antiserum with male plasma (band C, Fig. 7 of Aida et al 1973). This suggests the presence in the egg of a protein common to both males and females and hence not stictly a vitellogenin. A similar protein common to male, non-vitellogenic female and vitellogenin female hagfish, (Eptatretus stouti) plasma has been found using an antiserum to egg yolk (Yu et al 1980).

Sex steroids and vitellogenesis

The induction of vitellogenin synthesis by estrogens has been demonstrated in a wide variety of teleosts (for review, see Wallace & Selman 1981; also Nath & Sundararaj 1981; Campbell & Idler 1976, 1980; Le Menn 1979; Hara et al 1980; Idler & Campbell 1980). Most studies have entailed the use of a single estrogen, normally estradiol. As is the case in Xenopus (Redshaw et al 1969), treatment with estradiol has been found to be more potent in the induction of vitellogenin than treatment with estrone in S. gairdneri (van Bohemen et al 1982a). However, higher plasma levels of estrone than estradiol during early phases of exogenous vitellogenesis in S. gairdneri (van Bohemen & Lambert 1981) and the observation that pretreatment with estrone can potentiate the subsequent vitellogenic response to estradiol, suggest that estrone may play a role in priming the liver for estradiol stimulation (van Bohemen et al 1982b). Estradiol was found to be more potent in inducing vitellogenin synthesis in hypophysectomized catfish Heteropneustes fossilis than either estrone or estriol (Sundararaj & Nath 1981). The relative potencies of estrone and estriol varied with dose in this latter study. Conversely, in ovariectomized, Tilapia aurea, estriol was found to be the most potent of the three estrogens in increasing plasma calcium and total protein levels (Terkatin-Shimony & Yaron 1978).

The response of plasma phosphoprotein, an index of vitellogenin, to three successive injections of estradiol has been studied in H. fossilis (Sundararaj & Nath 1981). Maximal phosphoprotein levels were recorded 2 days after each injection. Sucessive injections were administered after plasma phosphoprotein had returned to basal levels and each produced a higher maximum than its predecessor. Females gave a stronger response than males. This study supports the hypothesis that initial exposure to estrogen can prime the liver for subsequent stimulation. The primary and secondary responses of Xenopus to estrogen treatment have been examined in some detail (for review, see Tata & Smith 1979), but similar extensive work remains to be done in fishes.

The induction of vitellogenin in fish by estrogen has recently received attention at the nucleic acid level. Vitellogenin mRNA has been isolated from liver of estrogen-treated bullhead, Americanus nubulosa, and its cell-free translation product confirmed

as vitellogenin monomer by electrophoretic and immunological criteria (Roach & Davies 1980). Vitellogenin mRNA has also been isolated from estrogen-treated S. gairdneri and its cell-free translation product confirmed (Chen et al 1982).

High doses of androgen induce vitellogenin synthesis in Gobius niger (Le Menn 1979) and C. auratus (Hori et al 1979). Binding studies indicate that the androgen action is mediated by the estrogen receptor (Le Menn et al 1980), and the androgen effect is considered pharmaciological rather than physiological.

The relation of ovarian cycles to those of plasma sex steroid levels have been studied in a number of teleosts (for review, see Crim 1982; also Crim & Idler 1978; Billard et al 1978; Dindo & MacGregor 1981; van Bohemen & Lambert 1981; MacGregor et al 1981). As pointed out previously (Crim 1982), sex steroid levels tend to increase with ovarian development and decline after spawning. An interesting finding in the plaice, Pleuronectes platessa (Wingfield & Grimm 1977), S. trutta fario (Billard et al 1978), O. kisutch (Jalabert et al 1978), and S. gairdneri (Whitehead et al 1978; van Bohemen & Lambert 1981) is the decline in plasma estrogen levels which commences a month or more prior to spawning and while the ovary is still growing. This occurs in concert with a declining rate of estrogen synthesis in the ovary (van Bohemen & Lambert 1981) and may permit gonadotropin secretion to rise to ovulatory levels by reducing feedback inhibition. The decline also reduces a potential inhibition of gonadotropin-induced maturation (Jalabert 1975) and minimal levels may indeed signal maturation (Fostier et al 1978). Pertinent to the study of vitellogenesis is the observation that maximal serum estradiol levels preceed maximal serum phosphoprotein phosphorus levels by 3 months in S. gairdneri (Whitehead et al 1978). The persistence and magnification of the physiological effect of estrogen long after reduction of the level of stimulus is consistent with experimental findings in Xenopus (Dolpin et al 1971) and S. gairdneri (Elliot et al 1979).

Estrogen induction of vitellogenin synthesis has also been reported in cylostomes (Pickering 1976; Yu et al 1981). Estradiol has been reported in the plasma of the hagfish E. stouti (Matty et al 1976), but, to my knowledge, seasonal variations of plasma estrogens in cyclostomes have not been reported.

Vitellogenesis in the elasmobranch S. canicula has received detailed examination. Unlike the commonly studied temperate zone teleost fishes, oviposition may occur through most or all of the year with peak activity in winter (for review, see Sumpter & Dodd 1979). Therefore, although significant variation in gonadosomatic indices may occur during the year (Craik 1978a; Sumpter & Dodd 1979), the amplitude of the fluctuations is much less than that observed in teleosts like the salmonids. A reflection of the spreading out of reproductive effort is the observation that plasma vitellogenin levels are stable throughout most of the year and are much lower than those found in other oviparous vertebrates (Craik 1978a). In addition, the magnitude of the vitellogenic response to estrogen in lower than in other vertebrates (Craik 1978c). An annual cycle of plasma estradiol has been reported in this species with maximal levels in winter (Sumpter & Dodd 1979).

In addition to inducing the synthesis of vitellogenin, there is evidence that sex steroids have other roles related to the process of vitellogenesis. Estrogens, with estradiol being the most potent, are believed to have direct protective effects on follicles as evidenced by their ability to delay atresia induced by hypophysectomy (Sundararaj & Goswami 1968; Anand & Sundararaj 1974). In addition to vitellogenin, estrogen can also increase plasma levels of very low density lipoprotein which is an important carrier of triglyceride (Skinner & Rogie 1978). Estrogen mobilization of lipid into the plasma in excess of that contained in vitellogenin has been suggested in the elasmobranch S. canicula (Craik 1978c) and estrogen-induced increases in plasma phospholipid levels in C. auratus were found to be independent from those of protein phosphorus, an index of vitellogenin (Bailey 1957). It is also noteworthy that responses of plasma or serum levels of calcium (Woodhead 1969; Elliot et al 1980) and neutral lipids (Wiegand & Peter 1980a) to estrogen have been found to vary with season, reproductive state and temperature. Estradiol treatment has also been found to influence enzyme activities to favour glycolysis over gluconeogenesis in Platichthys flesus (Sand et al 1980). This is similar to changes observed in nature at the start of vitellogenesis. Similarly, estrogen-stimulated depletion of liver glycogen has also been reported (de Vlaming et al 1977a, b).

Annual cycles of plasma testosterone that coincide with the ovarian cycle have been noted in several teleosts (Campbell et al 1976; Wingfield & Grimm 1977; Scott et al 1980) and an elasmobranch (Sumpter & Dodd 1980) although their physiological role has not been determined. Experimental evidence suggests that testosterone may have a role in seasonal stimulation of gonadotropin production (Crim & Peter 1978; Crim & Evans 1979), as a precursor of brain and pituitary estrogens (Callard et al 1981a, b) and, pertinent to vitellogenesis, in the mobilization of free fatty acids (Wiegand & Peter 1980b).

Assay of Vitellogenin

The development of assay systems for vitellogenin may prove valuable for management of fish for which visual sex determination is difficult or impossible during early reproductive development. The ability to assess the proportion of females undergoing vitellogenesis in a population can allow estimation of the reproductive potential for a given season. On the basis of a tag and recapture study, it was predicted that Atlantic salmon, Salmo salar with plasma vitellogenin levels greater than 250 µg/ml, as determined by radioimmunoassay, would spawn within the year (Idler et al 1981). Idler et al (1979) reported plasma vitellogenin levels of 907 µg/ml in S. salar that were 4-5 months away from spawning. Taken together, these data suggest that female spawners of the year may be identifiable even earlier. An immunoagglutination system based on an antibody raised to S. gairdneri vitellogenin has permitted the identification of females during April and May in a population of S. trutta that spawn in December (Le Bail & Breton 1981). Their system also allows identification of female S. salar at similar early stages of vitellogenesis. Another method of vitellogenin detection, determination of serum phosphoprotein phosphorus, would appear to allow sexing of S. gairdneri about 4-5 months prior to spawning (Whitehead et al 1978). The presence of low levels of phosphoprotein phosphorus in male serum precludes earlier sex determination by this method. An advantage of an immunoassay system is sensitivity. For example, a S. gairdneri vitellogenin radioimmunoassay is sensitive to about 2-5 ng vitellogenin (Fig. 4, Campbell & Idler 1980) and the S. salar radioimmunoassay is sensitive to about 0.1 ng of yolk protein (likely β^1-component), representing about 4.7 ng of vitellogenin (Idler et al 1979). In contrast, an electrophoretic assay for S. gairdneri vitellogenin is sensitive to 30 ng of material (van Bohemen et al 1981). An immunoelectrophoresis system has been used to demonstrate a relation between plasma vitellogenin levels and gonadosomatic index between October and January for spring spawning pike Esox lucuis (Goedemakers & Verboom 1974).

Conclusions

The function of vitellogenin as the precursor of the major yolk products is well established. The qualitiative and quantitative importance of other sources of egg yolk such as endogenous synthesis or plasma sources other than vitellogenin is not well known. The derivation of yolk from other sources in addition to typical vitellogenins may be of special importance in those fish which have a high neutral lipid content in the ovary such as T. cosby. An interesting development, especially in light of similar findings in Xenopus (Wiley & Wallace 1981), is the finding of multiple forms of vitellogenin polypeptide monomer and its derivatives in C. auratus (de Vlaming et al 1980). Whether or not this phenomenon is widespread remains to be determined. Similarly, the demonstration of primary and secondary responses to estrogen in H. fossilis (Sundararaj & Nath 1981) merits comparative study. Further work will doubtless address the possibility of multiple vitellogenin genes in fish and compare molecular aspects of primary and secondary responses to estrogen with the Xenopus model.

With several basic aspects of vitellogenesis now well understood, further study should attempt to integrate vitellogenesis with other aspects of physiology such as the deposition and mobilization of nutrient reserves. This is of special interest with regards to those fish that fast during ovarian recrudescence. The role of thyroid hormones in vitellogenesis is not well understood although experimental evidence (Lewis & Dodd 1974) indicates a requirement. Finally, considering the great diversity of fish species, it should not be surprising if future comparative research uncovers unique strategies in the physiology of vitellogenesis in species faced with unique demands.

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References

Aida, K., P.-V. Ngan & T. Hibiya, 1973. Physiological studies on gonadal maturation of fishes-I. Sexual difference in composition of plasma protein of ayu in relation to gonadal maturation. Bull. Jap. Soc. Sci. Fish. 39: 1091-1106.

Anand, T.C. & B.I. Sundararaj, 1974. Ovarian maintenance in the hypophysectomized catfish, Heteropneustes fossilis (Bloch), with mammalian hypophyseal and placental hormones, and gonadal and adrenocortical steroids. Gen. Comp. Endocrinol. 22: 154-168.

Ando, T., 1965. Ultracentrifugal analysis of yolk proteins in rainbow trout egg and their changes during development. Can. J. Biochem. 43: 373-379.

Bailey, R.E., 1957. The effect of estradiol on serum calcium, phosphorus, and protein of goldfish. J. exp. Zool. 136: 455-469.

Beams, H.W. & R.G. Kessel, 1973. Oocyte structure and early vitellogenesis in the trout, Salmo gairdneri. Am. J. Anat. 136:

105-122.
Billard, R., B. Breton, A. Fostier, B. Jalabert & C. Weil, 1978. Endocrine control of the teleost reproductive cycle and its relation to external factors: salmonid and cyprinid models. In: P.J. Gaillard & H.H. Boer (Ed.): Comp. Endocrinol. Elsevier/ North-Holland, Amsterdam. 37-48.
van Bohemen, C.G. & J.G.D. Lambert, 1981. Estrogen synthesis in relation to estrone, estradiol, and vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 45: 105-114.
van Bohemen, C.G., J.G.D. Lambert & J. Peute, 1981. Annual changes in plasma and liver in relation to vitellogenesis in the female rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 44: 94-107.
van Bohemen, C.G., J.G.D. Lambert, H.J.Th. Goos & P.G.W.J. van Oordt, 1982a. Estrone and estradiol participation during exogenous vitellogenesis in the female rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 46: 81-92.
van Bohemen, C.G., J.G.D. Lambert, P.G.W.J. van Oordt, 1982b. Vitellogenin induction by estradiol in estrone-primed rainbow trout, Salmo gairdneri. Gen. Comp. Endocrinol. 46: 136-139.
Bruslé, S., 1980. Fine structure of early previtellogenic oocytes in Mugil (Liza) aurattus. Risso, 1810 (Teleostei, Mugilidae). Cell. Tiss. Res. 207: 123-134.
Callard, G.V., Z. Petro, K.J. Ryan & J.B. Claiborne, 1981a. Estrogen synthesis in vitro and in vivo in the brain of a marine teleost (Myxocephalus). Gen. Comp. Endocrinol. 43: 243-255.
Callard, G.V., Z. Petro & K.J. Ryan, 1981b. Biochemical evidence for aromatization of androgen to estrogen in the pituitary. Gen. Comp. Endocrinol. 44: 359-364.
Campbell, C.M., 1978. In vitro stimulation of vitellogenin incorporation into trout oocytes by salmon pituitary extracts. Ann. Biol. anim. Bioch. Biophys. 18: 1013-1018.
Campbell, C.M. & D.R. Idler, 1976. Hormonal control of vitellogenesis in hypophysectomized winter flounder (Pseudopleuronectes americanus Walbaum). Gen. Comp. Endocrinol. 28: 143-150.
Campbell, C.M. & D.R. Idler, 1980. Characterization of an estradiol-induced protein from rainbow trout as vitellogenin by the cross reactivity to ovarian yolk fractions. Biol. Reprod. 22: 605-617.
Campbell, C.M. & B. Jalabert, 1979. Selective protein incorporation by vitellogenic Salmo gairdneri oocytes in vitro. Ann. Biol. anim. Bioch. Biophys. 19: 429-437.
Campbell, C.M., J.M. Walsh & D.R. Idler, 1976. Steroids in the plasma of the winter flounder (Pseudopleuronectes americanus Walbaum). Gen. Comp. Endocrinol. 29: 14-20.
Chen, T.T., L.B. Agellon, P.C. Reid & D.A. Howard, 1982. Estrogen control of gene expression in rainbow trout. Proc. Can. Fed. Biol. Sci. 25: 45. Abst. 169.
Chopra, H.C., 1958. A morphological and histochemical study of the oocytes of the fish Ophiocephalus punctatus, with particular reference to lipids. Quart. J. Microscop. Sci. 99: 149-158.
Craik, J.C.A., 1978a. Plasma levels of vitellogenin in the elasmobranch Scyliorhinus canicula L. (lesser spotted dogfish). Comp. Biochem. Physiol. 60B: 9-18.
Craik, J.C.A., 1978b. Kinetic studies of vitellogenin metabolism in the elasmobranch Scyliorhinus canicula L. Comp. Biochem. Physiol. 61A: 355-361.
Craik, J.C.A., 1978c. The effects of estrogen treatment on certain plasma constituents associated with vitellogenesis in the elasmobranch Scyliorhinus canicula L. Gen. Comp. Endocrinol. 35: 455-464.
Crim, L.W., 1982. Environmental modulation of annual and daily rhythms associated with reproduction in teleost fishes. Can. J. Fish. Aq. Sci. 39: 17-21.
Crim, L.W. & D.M. Evans, 1979. Stimulation of pituitary gonadotropin by testosterone in juvenile rainbow trout (Salmo gairdneri). Gen. Comp. Endocrinol. 37: 192-196.
Crim, L.W. & D.R. Idler, 1978. Plasma gonadotropin, estradiol, and vitellogenin and gonad phosvitin levels in relation to the seasonal reproductive cycles of female brown trout. Ann. Biol. anim. Bioch. Biophys. 18: 1001-1005.
Crim, L.W. & R.E. Peter, 1978. The influence of testosterone implantation in the brain and pituitary on pituitary gonadotropin levels in Atlantic salmon parr. Ann. Biol. anim. Bioch. Biophys. 18: 689-694.
de Vlaming, V.L., M.J. Vodicnik, G. Bauer, T. Murphy & D. Evans, 1977a. Estradiol-17β effects on lipid and carbohydrate metabolism and on the induction of a yolk precursor in the goldfish, Carassius auratus. Life Sci. 20: 1945-1952.
de Vlaming, V.L., J. Singh, G. Paquette & R. Vuchs, 1977b. In vivo and in vitro effects of oestradiol-17β on lipid metabolism in Notemigonus crysoleucas. J. Fish. Biol. 10: 273-285.
de Vlaming, V.L., H.S. Wiley, G. Delahunty & R.A. Wallace, 1980. Goldfish (Carassius auratus) vitellogenin: induction, isolation, properties and relationship to yolk proteins. Comp. Biochem. Physiol. 67B: 613-623.
Dindo, J.J. & R. MacGregor III, 1981. Annual cycle of serum gonadal steroids and serum lipids in striped mullet. Trans. Am. Fish. Soc. 110: 403-409.
Dolphin, P.J., A.Q. Ansari, C.B. Lazier, K.A. Munday & M. Akhtar, 1971. Studies on the induction and biocynthesis of vitellogenin, an oestrogen-induced glycolipophosphoprotein. Biochem. J. 124: 751-758.
Droller, M.J. & T.F. Roth, 1966. An electron microscope study of yolk formation during oogenesis in Lebistes reticulatus Guppyi.

J. Cell Biol. 28: 209-232.
Elliott, J.A.K., N.R. Bromage & C. Whitehead, 1979. Effects of oestradiol-17β on serum calcium and vitellogenin levels in rainbow trout. J. Endocr. 83: 54P-55P.
Elliott, J.A.K., N.R. Bromage & C. Whitehead, 1980. Oestradiol-17β and vitellogenesis in the rainbow trout. J. Endocr. 87: 65P-66P.
Emmersen, B.K. & I.M. Petersen, 1976. Natural occurrence, and experimental induction by estradiol-17-β, of a lipophosphoprotein (vitellogenin) in flounder (Platichtys flesus L.). Comp. Biochem. Physiol. 54B: 443-446.
Fostier, A., C. Weil, M. Terqui, B. Breton & B. Jalabert, 1978. Plasma estradiol-17β and gonadotropin during ovulation in rainbow trout (Salmo gairdneri R.). Ann. Biol. anim. Bioch. Biophys. 18: 929-936.
Goedmakers, A. & B.L. Verboom, 1974. Studies on the maturation and fecundity of the pike, Esox lucius Linnaeus, 1758. Aquaculture. 4: 3-12.
Guraya, S.S., 1963. Histochemical studies on the yolk-nucleus in fish oogenesis. Z. Zellforsch. Mikrosk. Anat. 60: 659-666.
Guraya, S.S., 1965. A comparative histochemical study of fish (Channa maruleus) and amphibian (Bufo stomaticus) oogenesis. Z. Zellforsch. Mikrosk. Anat. 65: 662-700.
Guraya, S.S., 1979. Recent advances in the morphology, cytochemistry and function of Balbiani's vitelline body in animal oocytes. Internat. Rev. Cytol. 59: 249-321.
Hara, A., 1976. Iron-binding activity of female-specific serum proteins of rainbow trout (Salmo gairdneri) and chum salmon (Oncorhynchus keta). Biochim. Biophys. Acta. 427: 549-557.
Hara, A. & H. Hirai, 1978. Comparative studies on immunochemical properties of female-specific serum protein and egg yolk proteins in rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. 59B: 339-343.
Hara, A., K. Yamauchi & H. Hirai, 1980. Studies on female-specific serum protein (vitellogenin) and egg yolk protein in Japanese eel (Anguilla japonica). Comp. Biochem. Physiol. 65B: 315-320.
Hori, S.H., T. Kodama & K. Tanahashi, 1979. Induction of vitellogenin synthesis in goldfish by massive doses of androgens. Gen. Comp. Endocrinol. 37: 306-320.
Idler, D.R., 1982. Some perspectives on fish gonadotropins. This symposium.
Idler, D.R. & C.M. Campbell, 1980. Gonadotropin stimulation of estrogen and yolk precursor synthesis in juvenile rainbow trout. Gen. Comp. Endocrinol. 41: 384-391.
Idler, D.R., S.J. Hwang & L.W. Crim, 1979. Quantification of vitellogenin in Atlantic salmon (Salmo salar) plasma by radioimmunoassay. J. Fish. Res. Bd. Can. 36: 574-578.
Idler, D.R., S.J. Hwang, L.W. Crim & D. Reddin, 1981. Determination of sexual maturation stages of Atlantic salmon (Salmo salar) captured at sea. Can. J. Fish. Aq. Sci. 38: 405-413.
Idler, D.R. & T.B. Ng, 1979. Studies on two types of gonadotropins from both salmon and carp pituitaries. Gen. Comp. Endocrinol 38: 421-440.
Inoue, S. & M. Iwasaki, 1978. Isolation of a novel glycoprotein from the eggs of rainbow trout: occurrence of disialosyl groups on all carbohydrate chains. Biochem. Biophys. Res. Comm. 83: 1018-1023.
Inoue, S. & M. Iwasaki, 1980. Sialoglycoproteins from the eggs of Pacific herring. Eur. J. Biochem. 111: 131-135.
Inoue, S., T. Kaneda-Hayashi, H. Sugiyama & T. Ando, 1971. Studies on phosphoproteins from fish eggs. I. Isolation and characterization of a phosphoprotein from the eggs of Pacific herring. J. Biochem. 69: 1003-1011.
Iyengar, R. & H. Schlenk, 1967. Wax esters of mullet (Mugil cephalus) roe oil. Biochemistry. 6: 396-402.
Jalabert, B., 1975. Modulation par différents stéroids non maturants de l'efficacité de la 17-α-hydroxy-20β-dihydroprogesterone on d'un extrait gonadotrope sur le maturation intrafolliculaire in vitro des ovocytes de la truite arc-en-ciel Salmo gairdnerii. C. R. Acad. Sci. Paris. Ser. D. 281: 811-814.
Jalabert, B., F.W. Goetz, B. Breton, A. Fostier & E.M. Donaldson, 1978. Precocious induction of oocyte maturation and ovulation in coho salmon, Oncorhynchus kisutch. J. Fish. Res. Bd. Can. 35: 1423-1429.
Jared, D.W. & R.A. Wallace, 1968. Comparative chromatography of the yolk proteins of teleosts. Comp. Biochem. Physiol. 24: 437-443.
Kaitaranta, J.K. & R.G. Ackman, 1981. Total lipids and lipid classes of fish roe. Comp. Biochem. Physiol. 69B: 725-729.
Larsen, L.O., 1978. Subtotal hepatectomy in intact or hypophysectomized river lampreys (Lampetra fluviatilis L.): Effects on regeneration,blood glucose regulation and vitellogenesis. Gen. Comp. Endocrinol. 35: 197-204.
Le Bail, P.Y. & B. Breton, 1981. Rapid determination of the sex of prepuberal salmonid fish by a technique of immunoagglutination. Aquaculture. 22: 367-375.
Leger, C., L. Fremont, D. Marion, I. Nassour & M.-F. Desfanges, 1981. Essential fatty acids in trout serum lipoproteins,vitellogenin and egg lipids. Lipids. 16: 593-600.
Le Menn, F., 1979. Induction de vitellogénine par l'oestradiol et par des androgènes chez un téléostéen marin: Gobius niger L. C.R. Acad. Sc. Paris. Ser. D. 289: 413-416.
Le Menn, F. & M.M. Lamy, 1977. Protéins vitellines sériques et hépatiques chez un poisson téléostéen, Gobius niger L., soumis a des traitements hormonaux. Bordeaux Médical. 10: 213-216.

Le Menn, F., H. Rochefort & M. Garcia, 1980. Effect of androgen mediated by the estrogen receptor of fish liver: vitellogenin accumulation. Steroids. 35: 315-328.
Lewis, M. & J.M. Dodd, 1974. Thyroid function and the ovary in the spotted dogfish Scyliorhinus caricula. J. Endocr. 63: 63P.
MacGregor, R. III, J.J. Dindo & J.H. Finucane, 1981. Changes in serum androgens and estrogens during spawning in bluefish, Pomatomus saltator, and king mackerel, Scomberomorus cavalla. Can. J. Zool. 59: 1749-1754.
Mano, Y. & F. Lipmann, 1966. Characteristics of phosphoproteins (phosvitins) from a variety of fish roes. J. Biol. Chem. 241: 3822-3833.
Mano, Y. & M. Yoshida, 1969. A novel composition of phosvitins from salmon and trout roe. J. Biochem. 66: 105-108.
Markert, J.R. & W.E. Vanstone, 1968. Egg proteins of pacific salmon, genus Oncorhynchus: proteins in the soluble fraction. Can. J. Biochem. 46: 1334-1337.
Markert, J.R. & W.E. Vanstone, 1971. Egg proteins of coho salmon (Oncorhynchus kisutch): chromatographic separation and molecular weights of the major proteins in the high density fraction and their presence in salmon plasma. J. Fish. Res. Bd. Can. 28: 1853-1856.
Matty, A.J., K. Tsureki, W.W. Dickhoff & A. Gorbman, 1976. Thyroid and gonadal function in hypophysectomized hagfish, Eptatretus stouti. Gen. Comp. Endocrinol. 30: 500-516.
Nath, P. & B.I. Sundararaj, 1981. Isolation and identification of female-specific serum lipophosphoprotein (vitellogenin) in the catfish, Heteropneustes fossilis (Bloch). Gen. Comp. Endocrinol. 43: 184-190.
Nath, V., 1960. Histochemistry of lipids in oogenesis. Int. Rev. Cytol. 9: 305-320.
Nayyar, R.P., 1964. The yolk nucleus of fish oocytes. Quart. J. Micr. Sci. 105: 353-358.
Ng, T.B. & D.R. Idler, 1980. Gonadotropin regulation of androgen production in flounder and salmonids. Gen. Comp. Endocrinol. 42: 25-38.
Pickering, A.D., 1976. Effects of gonadectomy, oestradiol and testosterone on the migrating river lamprey, Lampetra fluviatilis (L). Gen. Comp. Endocrinol. 28: 473-480.
Plack, P.A. & N.W. Fraser, 1971. Incorporation of L-[^{14}C] leucine into egg proteins by liver slices from cod. Biochem. J. 121: 857-862.
Plack, P.A., D.J. Pritchard & N.W. Fraser, 1971. Egg proteins in cod serum: natural occurrence and induction by injections of oestradiol 3-benzoate. Biochem. J. 121: 847-856.
Rahn, C.H., D.M. Sand & H. Schlenk, 1973. Wax esters in fish. Metabolism of dietary palmidyl palmitate in the gourami (Trichogaster cosby). J. Nutr. 103: 1441-1447.
Raven, C.P., 1961. Oogenesis: The storage of developmental information. Pergamon Press, New York.
Redshaw, M.R. & B.K. Follett, 1971. The crystalline yolk-platelet proteins and their soluble plasma precursor in an amphibian, Xenopus laevis. Biochem. J. 124: 759-766.
Redshaw, M.R., B.K. Follett & T.J. Nicholls, 1969. Comparative effects of the oestrogens and other steroid hormones on serum lipids and proteins in Xenopus laevis Daudin. J. Endocr. 43: 47-53.
Roach, A.H. & P.L. Davies, 1980. Catfish vitellogenin and its messenger RNA are smaller than their chicken and Xenopus counterparts. Biochem. Biophys. Acta. 610: 400-412.
Sand, D.M., J.L. Hehl & H. Schlenk, 1969. Biocynthesis of wax esters in fish. Reduction of fatty acids and oxidation of alcohols. Biochemistry. 8: 4851-4854.
Sand, D.M., J.L. Hehl & H. Schlenk, 1971. Biocynthesis of wax esters in fish. Metabolism of dietary alcohols. Biochemistry. 10: 2536-2541.
Sand, D.M., C.H. Rahn & H. Schlenk, 1973. Wax esters in fish. Absorption and metabolism of oleyl alcohol in the gourami (Trichogaster cosby). J. Nutr. 103: 600-607.
Sand, O., I.M. Petersen & B. Korsgaard-Emmersen, 1980. Changes in some carbohydrate metabolizing enzymes and glycogen in liver glucose and lipid in serum during vitellogenesis and after induction by estradiol-17-β in the flounder (Platichtys flesus L.) Comp. Biochem. Physiol. 65B: 327-332.
Schmidt, G., G. Bartsch, T. Kitagawa, K. Fujisawa, J. Knolle, J. Joseph, P. De Marco, M. Liss & R. Haschemeyer, 1965. Isolation of a phosphoprotein of high phosphorus content from the eggs of brown brook trout. Biochem. Biophys. Res. Comm. 18: 60-65.
Scott, A.P., V.J. Bye & S.M. Baynes, 1980. Seasonal variation in sex steroids of female rainbow trout (Salmo gairdneri Richardson). J. Fish Biol. 17: 587-592.
Selman, K. & R.A. Wallace, 1982. Oocyte growth in the sheepshead minnow: uptake of exogenous proteins by vitellogenic oocytes Tiss. Cell. (in press).
Shackley, S.E. & P.E. King, 1977. Oogenesis in a marine teleost, Blennius pholis L. Cell Tiss. Res. 181: 105-128.
Skinner, E.R. & A. Rogie, 1978. The isolation and partial characterization of the serum lipoproteins and apolipoproteins of the rainbow trout. Biochem. J. 173: 507-520.
Sumpter, J.P. & J.M. Dodd, 1979. The annual reproductive cycle of the female lesser spotted dogfish, Scyliorhinus canicula L., and its endocrine control. J. Fish. Biol. 15: 687-695.
Sundararaj, B.I. & S.V. Goswami, 1968. Ef-

fects of estrogen, progesterone, and testosterone on the pituitary and ovary of catfish, Heteropneustes fossilis (Bloch). J. Exp. Zool. 169: 211-228.
Sundararaj, B.I. & P. Nath, 1981. Steroid-induced synthesis of vitellogenin in the catfish, Heteropneustes fossilis (Bloch). Gen. Comp. Endocrinol. 43: 201-210.
Tata, J.R. & D.F. Smith, 1979. Vitellogenesis: A versatile model for hormonal regulation of gene expression. Rec. Prog. Hor. Res. 35: 47-95.
Terkatin-Shimony, A. & Z. Yaron, 1978. Estrogens and estrogenic effects in Tilapia aurea (Cichlidae, Teleostei). Ann. Biol. anim. Bioch. Biophys. 18: 1007-1012.
Tesoriero, J.V., 1977. Formation of the chorion (zona pellucida) in the teleost Oryzias latipes. II. Polysaccharide cytochemistry of early oogenesis. J. Histochem. Cytochem. 25: 1376-1380.
Tesoriero, J.V., 1978. Formation of the chorian (zona pellucida) in the teleost Oryzias latipes. III. Autoradiography of [^{3}H] proline incorporation. J. Ultrastruct. Res. 64: 315-326.
Ulrich, E., 1969. Étude des ultrastructures au cours de l'ovogenèse d'un poisson téléosteen, le danio, Brachydanio rerio (Hamilton-Buchanan). J. Microscopie. 8: 447-478.
Wallace, R.A., 1978. Oocyte growth in non-mammalian vertebrates. In: R.E. Jones (Ed.): The vertebrate ovary. Plenum Press, New York. p. 469-502.
Wallace, R.A., D.W. Jared & A.Z. Eisen, 1966. A general method for the isolation and purification of phosvitin from vertebrate eggs. Can. J. Biochem. 44: 1647-1655.
Wallace, R.A. & K. Selman, 1981. Cellular and dynamic aspects of oocyte growth in teleosts. Am. Zool. 21: 325-343.
Whitehead, C., N.R. Bromage & J.R.M. Forster, 1978. Seasonal changes in reproductive function of the rainbow trout (Salmo gairdneri). J. Fish. Biol. 12: 601-608.
Wiegand, M.D. & D.R. Idler, 1982. Synthesis of lipids by the rainbow trout (Salmo gairdneri) ovary in vitro. Can. J. Zool. (in press).
Wiegand, M.D. & R.E. Peter, 1980a. Effects of sex steroids in plasma lipids in the goldfish, Carassius auratus. Can. J. Zool. 58: 967-972.
Wiegand, M.D. & R.E. Peter, 1980b. Effects of testosterone, oestradiol-17β and fasting on plasma free fatty acids in the goldfish, Carassius auratus. Comp. Biochem. Physiol. 66A: 323-326.
Wiley, H.S. & R.A. Wallace, 1981. The structure of vitellogenin. Multiple vitellogenins in Xenopus laevis give rise to multiple forms of the yolk proteins. J. Biol. Chem. 256: 8626-8634.
Wingfield, J.C. & A.S. Grimm, 1977. Seasonal changes in plasma cortisol, testosterone and oestradiol-17β in the plaice, Pleuronectes platessa L. Gen. Comp. Endocrinol. 31: 1-11.
Woodhead, P.M.J., 1969. Influence of oestradiol 3-benzoate upon the plasma content of calcium and vitamin A aldehydes in the cod, Gadus morhua. J. mar. biol. Ass. U.K. 49: 939-944.
Yu, J.Y.-L., W.W. Dickhoff, Y. Inui & A. Gorbman, 1980. Sexual patterns of protein metabolism in liver and plasma of hagfish, Eptatretus stouti with special reference to vitellogenesis. Comp. Biochem. Physiol. 65B: 111-117.
Yu, J.Y.-L., W.W. Dickhoff, P. Swanson & A. Gorbman, 1981. Vitellogenesis and its hormonal regulation in the Pacific hagfish, Eptatretus stouti L. Gen. Comp. Endocrinol. 43: 492-502.

EFFECTS OF FEEDING LEVELS AND TEMPERATURE ON THE DEVELOPMENT OF THE GONAD IN THE AFRICAN CATFISH CLARIAS LAZERA (C & V)

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Summary

The effect was studied of two temperatures 25 and 30°C and three feeding levels on the development of the gonad of the African catfish Clarias lazera. For males, the gonado somatic index was more affected by temperature than by feeding level. The combination of high temperature and high feeding level had a negative effect on the male gonado somatic index. At 25°C, the proportion of suitable male spawners was the highest, irrespective of feeding level. For females the gonado somatic index was more affected by feeding level than by temperature. A low feeding level had a positive effect on the female gonado somatic index. The better fed females had a higher ultimate absolute fecundity than poorly fed fish. Proportion hatching of fertilized eggs was much higher in the females kept at 25°C, indicating that 30°C is above optimum for controlled reproduction. Egg quality did not alter with decreasing feeding levels. A temperature of 25°C and a feeding level giving the most efficient feed conversion is recommended for conditioning brood fish for breeding.

Introduction

The effects of feeding levels and temperature on the gonad of C. lazera was studied under N. European light periodicity (march-january) to develop an optimum procedure for conditioning brood fish for breeding. The relationship between the amount of food administered and reproductive output was estimated.

Characteristics studied included gonadal growth, maturation stage of testis and ovary, absolute and relative fecundity of females and proportion of fertilized eggs that developed into normal larvae. In this brief account statistical analysis is not presented.

Raising of fish

The fish were raised from egg to fingerling in troughs with recirculating water at 29°C. Under optimum feeding conditions, a mean weight of 40 g was reached in about 60 days. At that age, the fingerlings were distributed over fibre-glass tanks and fed on trout pellets (45% protein) added to the water from automatic feeders. The effect was studied of two temperatures 25 and 30°C and three feeding levels on the development of the gonad. Level 2 was defined as that giving the most efficient feed conversion and the other two levels were derived from it: Level 1 with a third as much and Level 3 with three times as much as Level 2. The following groups will be distinguished in this paper: Groups 1-3 and 4-6 were raised at a water temperature of 30 and 25°C, respectively. The two sets of groups received, in order of ascending group number, food corresponding to Level 1, 2 and 3 respectively. Every two weeks, feeding levels were adjusted according to the observed growth rates. Development of gonads in 10 males and 10 females per group was studied for 5 sampling periods at about 120, 160, 200, 240 and 300 days after hatching of the eggs.

Somatic and gonadic growth

For males and females, somatic growth (wet body weight) was more affected by feeding level than by temperature. At an age of 300 days the largest fish were found in Group 6, in which mean weights of males and females were 800 and 600 g, respectively (Fig. 1 and 2).

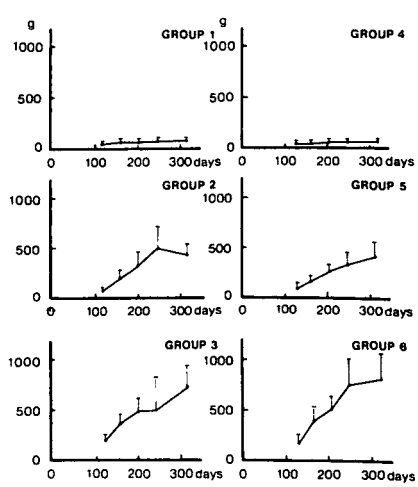

Fig.1. Growth of males (wet body weight) of *C. lazera* kept at 30 °C (left) and 25 °C (right) and various feeding levels.(For explanation see text.)

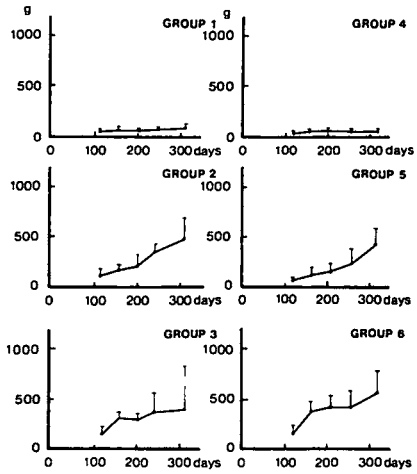

Fig. 2. Growth of females. For explanation see Fig. 1.

Testis growth was more affected by temperature than by feeding level. At the higher feeding levels, this effect was pronounced (Fig. 3).

Ovary growth was with the exception of the last sampling period more affected by temperature than by feeding level (Fig. 4).

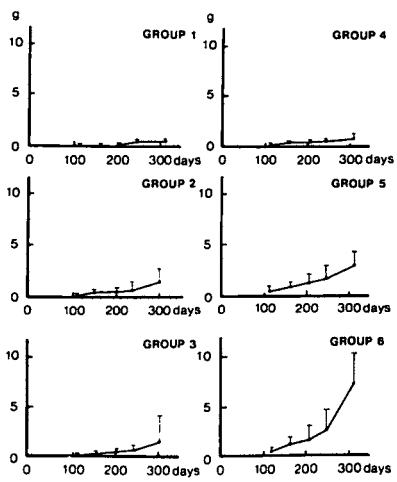

Fig. 3. Testis growth (wet weight). For explanation see Fig. 1.

Gonado somatic index

The gonado somatic index of a fish was calculated as follows:

$$GSI = \frac{\text{total gonad weight}}{\text{body weight - total gonad weight}} \times 100$$

For males the gonado somatic index was more affected by temperature than by feeding level. The combination of high temperature and high feeding level had a negative effect on the

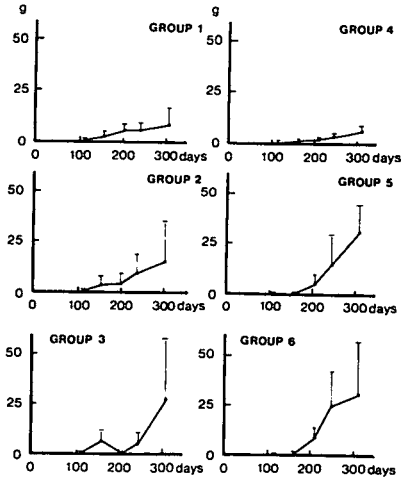

Fig. 4. Ovary growth. For explanation see Fig. 3.

gonado somatic index. For females the gonado somatic index, during most of the sampling periods, was affected by feeding level and not by temperature. A low feeding level had a positive effect on the female gonado somatic index.

Development of testis and ovary

The testis and ovary were examined histologically following autopsy of 10 males and 10 females per group in each sampling period. Central parts of the testis and ovary were fixed in calcium formaldehyde and embedded in paraffin wax. Sections 10 µm thick were stained with haemalumeosine.

C. lazera has a tubular testis which is characterized by the presence of spermatogenic cysts attached to seminiferous tubules. Testis development could be classified according to the following index: an undeveloped, spent or regressed testis; a developing testis with cysts mainly containing spermatogonia, spermatocytes, spermatids and occasionally spermatozoa; a ripe testis with cysts and tubules completely filled with spermatozoa. The developed (lateral) part of each testis was studied and scored according to the index.

The proportion of males with undeveloped and ripe testes was estimated (Fig. 5).

The highest proportion of suitable male spawners was encountered during the last four sampling periods at 25°C, irrespective of feeding level.

The ovary of C. lazera is a hollow sacklike structure consisting of an outer wall with lamellae penetrating the central lumen. The lamellae contain oogonia and oocytes in follicles at various stages of development: previtellogenic, vitellogenic, post-vitellogenic and atretic follicles (Richter and Van den Hurk, 1982). The proportion of females with

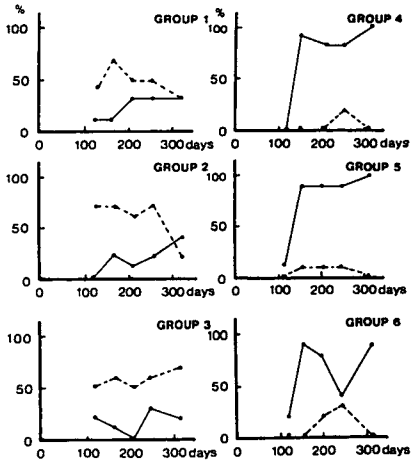

Fig. 5. Development of the testis. For explanation see Fig. 1.

●——● Proportion of males containing ripe spermatozoa (%)

●- - -● Proportion of males with undeveloped, spent or regressed testes (%)

predominantly post-vitellogenic oocytes (diameter > 1 mm) and with predominantly atretic oocytes in their ovaries was estimated (Fig. 6). A high temperature (30°C) resulted in an earlier onset of vitellogenesis at the lower feeding levels. The high proportion of females with atretic post-vitellogenic oocytes (Fig. 6, Groups 1-3) and the fluctuating proportion of females with "healthy" post-vitellogenic oocytes (Fig. 6, Group 3) indicated that complete regression of ovaries occurred at 30°C.

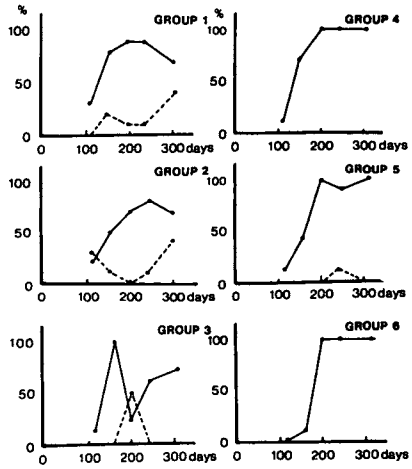

Fig. 6. Development of the ovary. For explanation see Fig. 5.

●——● Proportion of females containing ovaries with predominantly postvitellogenic oocytes (%)

●- - -● Proportion of females containing ovaries with predominantly atretic oocytes (%)

The ovaries of fish kept at 25°C contained few atretic oocytes. This agrees with our experience in recent years that adult C. lazera females can reproduce successfully throughout the year. The present histological examinations suggest a continuous slow "turnover" from vitellogenic through post-vitellogenic to atretic oocytes in the ovary of females kept at 25°C.

Absolute and relative fecundity of females

The total number of vitellogenic eggs per fish was estimated by counting the number of vitellogenic eggs (diameter > 0.35 mm) per 0.5 gram of ovary tissue. Absolute fecundity was defined as the total count of vitellogenic eggs at a given fish age. The number of fish containing vitellogenic eggs was not identical in all groups (Fig. 7).

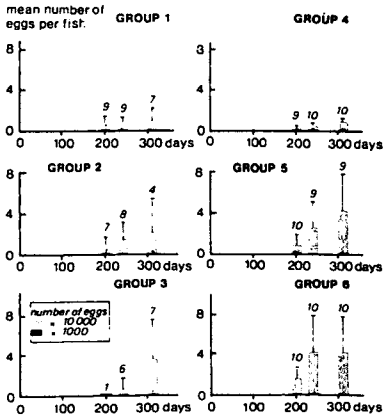

Fig. 7. Absolute fecundity of females (10 fish per group). The figures on top of the standard deviation bars refer to the number of fish containing vitellogenic eggs.

The absolute fecundity at all feeding levels reached a maximum on the last sampling day (300 days).

At that time the better fed females (Groups 3 and 6) had a higher absolute fecundity than the poorly fed ones (Groups 1 and 4).

The relative fecundity was defined as the total number of vitellogenic oocytes per gram body weight. These results and the number of fish containing vitellogenic eggs are presented in Fig. 8.

In both temperature groups the relative fecundity was negatively affected by increasing feeding level (compare Group 1 with 3 and Group 4 with 6).

Fertility of females

The egg quality of all groups was studied during the last three sampling periods. Ten females per group and per sampling period were reproduced by artificially induced breeding with HCG (Eding et al., poster)

After stripping, a sample of 200 eggs per brood fish was fertilized with milt from males of the brood stock of the hatchery and subsequently incubated in stagnant water at 30°C. The proportion of eggs hatched and the proportion of deformed larvae were calculated (Fig. 9), the difference being taken as an expression of female fertility, i.e. the proportion of fertilized eggs that develop into normal larvae.

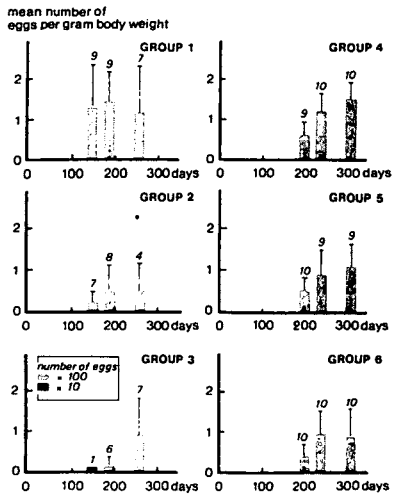

Fig. 8. Relative fecundity of females. For explanation see Fig. 7.

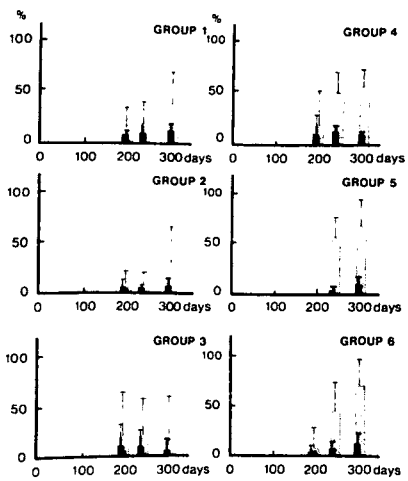

Fig. 9. Fertility of females. For explanation see Fig. 7.

- Proportion of eggs hatched
- Proportion of deformed larvae.

In all groups, the highest proportion hatched occurred in the last sampling period (300 days). The proportion of deformed larvae was fairly constant in all groups.

The proportion of eggs hatched was much higher in the groups kept at 25°C, indicating that 30°C is above optimum for controlled reproduction of C. lazera. Egg quality did not alter with decreasing feeding levels.

Discussion

The relationship between food supply and reproductive output has received relatively little experimental study (Wootton, 1979). Ration size was positively correlated with the percentage of yolk bearing oocytes per ovary but not with the atretic oocytes in the winter flounder Pseudopleuronectes americanus (Tyler & Dunn, 1976).

The present study on C. lazera has likewise shown that the better fed females were more fecund but has also established that egg quality was not impaired by low feeding levels.

The poorly fed females of C. lazera became mature at an unusually low body weight and had the highest relative fecundity. This means that food reduction in C. lazera leads to investment in the ovaries at the expense of somatic tissue. The three-spined stickleback (Gasterosteus aculeatus) has the same strategy (Wootton, 1979).

The present feeding experiments with C. lazera were carried out in the temperature range of its natural habitat. It is surprising that an increase of 5°C in temperature causes a marked disturbance in reproduction manifested by pronounced cyclic atresia of vitellogenic oocytes and regression of the testis. A temperature of 25°C and a feeding level giving the most efficient feed conversion is recommended for conditioning brood fish for breeding. Under these conditions hatchery raised C. lazera can be reproduced successfully throughout the year.

References

Richter, C.J.J. and R. van den Hurk, 1982. Effects of 11-desoxycorticosterone-acetate and carp pituitary suspension on follicle maturation in the ovaries of the African catfish, Clarias lazera (C & V). Aquaculture 29, 53-66.

Tyler, A.V. and R.S. Dunn, 1976. Ration, growth and measures of somatic and organ condition in relation to meal frequency in winter flounder, Pseudo pleuronectes americanus, with hypotheses regarding population homeostasis. J. Fish.Res.Bd.Can. 33: 63-75.

Wootton, R.J., 1979. Energy costs of egg production and environmental determinants of fecundity in teleost fishes. Symp.zool.Soc. Lond. (1979) No. 44, 133-159.

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THE INTER- AND INTRACELLULAR PASSAGE OF PROTEINS THROUGH THE OVARIAN FOLLICLE IN TELEOSTS

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Summary

Oocyte growth in teleosts primarily involves the sequestration and processing by the oocyte of a female-specific protein that is hepatically derived. The structure of vitellogenic follicles of two closely related euryhaline cyprinodonts, Fundulus heteroclitus and Cyprinodon variegatus, is described. The electron-dense tracer, horseradish peroxidase, and a larger heterologous protein, Xenopus laevis [^{3}H]vitellogenin, were used in this study to demonstrate the pathway by which exogenous proteins leave the maternal circulation, and pass through the follicle to yolk spheres within the growing oocyte. Proteins leave the perifollicular capillaries, and traverse the theca, the follicular epithelium, and the vitelline envelope. At the oocyte surface they are incorporated via micropinocytosis and translocated to enlarging yolk spheres. In contrast to studies on oocyte growth in teleosts which suggest that yolk is an autosynthetic product, this study substantiates the importance of heterosynthetic processes during oocyte growth.
Keywords: follicle, micropinocytosis, oocyte, teleost, vitellogenesis.

Introduction

Oocyte growth among nonmammalian vertebrates is associated with the accumulation of a hepatically derived yolk precursor protein, vitellogenin (VTG). This female-specific protein is the major phosphoprotein in female serum, is easily identified by labeling with [^{32}P]Pi, and can be induced in males by estrogen injection (Wallace and Selman, 1981). Subsequent to incorporation by the oocyte, VTG is cleaved into yolk proteins which accumulate in membrane-limited bodies ("extravesicular yolk").

Previous studies have indicated that oocyte growth in teleosts occurs primarily by the accumulation of both endogenously and exogenously synthesized yolk (e.g., Korfsmeier, 1966; te Heesen, 1977). Endogenous or "intravesicular" yolk resides within yolk vesicles which appear to be the precursors of cortical alveoli (Wallace & Selman, 1981). These structures release their contents to the perivitelline space at the time of fertilization and hence do not represent yolk in a strict sense. This consideration serves to focus on the mechanism of exogenous or "extravesicular" yolk formation as the major process contributing to formation of the relatively large teleost egg.

Several ultrastructural studies have described micropinocytotic activity at the surface of vitellogenic oocytes concomitant with the appearance of yolk bodies. However, only one previous study has described the incorporation of an extracellular tracer into yolk spheres in teleost oocytes (Wegmann & Götting, 1971). In the present study of vitellogenesis in two closely-related Cyprinodonts, the sheepshead minnow, Cyprinodon variegatus, and the killifish Fundulus heteroclitus, we a) present experimental evidence that growing oocytes incorporate exogenous proteins into developing yolk spheres and b) describe the pathway traversed by these proteins from the maternal circulation through the ovarian follicle into yolk spheres within the oocyte.

Materials and methods

Mature sheepshead minnows (C. variegatus) were collected in the vicinity of Crescent Beach, Florida, during their breeding season (April through September). Breeding killifish, F. heteroclitus, were collected in May and early June in the vicinity of Woods Hole, Massachusetts.

Pieces of ovary were placed in fish saline (FO solution; Wallace & Selman, 1978) and follicles were manually dissected, measured and rapidly placed in fixative for microscopy. The procedures used for specimen preparation and electron microscopy have been previously described (Wallace & Selman, 1979). Most tissues were embedded in Epon-Araldite although some were embedded in JB4 plastic (Polysciences) for light microscopy.

Horseradish peroxidase (HRP;Type II, Sigma) was dissolved in FO solution at a concentration of 30-40 mg/ml. Female C. variegatus, weighing approx. 2 g, were injected intraperitoneally with 6 mg HRP; F. heteroclitus females, weighing approx. 3 g, were injected with 9 mg HRP. At periodic intervals up to 12 hr, ovaries were removed and follicles were measured, routinely fixed, and washed for 2 hr. Follicles were sliced into halves or quarters, and were rinsed in 0.05 M phosphate buffer (pH 7.6) prior to incubation for 20 to 40 min at room temperature in DAB-reaction mixture (Graham & Karnovsky, 1966). Follicles from saline-injected fish were run as controls; in addition, follicles from HRP-injected fish were incubated in DAB-reaction mixture which

lacked H_2O_2. Following incubation, tissues were post-fixed and embedded. Thin sections were stained with lead solution prior to examination in the electron microscope; 1 µm sections were examined unstained in the light microscope.

[^{3}H]VTG was prepared from Xenopus laevis by injecting a female with 4 mg 17β-estradiol on day 0 and 5 mCi [^{3}H]leucine on day 7 (Wallace et al., 1972). [^{3}H]VTG was isolated from plasma (Wiley et al., 1979) on day 8 and had a specific activity of 0.5-1.3 x 10^3 cpm/µg protein. Fish were injected intraperitoneally with 1.25-2.7 mg [^{3}H]VTG and maintained for 24 hr, after which the ovaries were removed, washed in FO solution and follicles were prepared for microscopy. Slides dipped for light microscopic autoradiography were stored for 1-10 months at 4°C.

Results and discussion

The structure of vitellogenic follicles and the process of oocyte growth in both C. variegatus and F. heteroclitus are similar and will be treated together in this study. Fig. 1 shows oocytes in various size follicles in the ovary of C. variegatus. Oocytes within vitellogenic follicles are surrounded by several cellular and acellular layers (figs. 2,4). The oocyte is immediately invested by the acellular vitelline envelope (chorion) which is penetrated by cytoplasmic processes from both the oocyte and surrounding follicle cells. The follicular epithelium is comprised of a single layer of columnar cells which are periodically interrupted by large intercellular spaces. Within these spaces reside long microvillar processes from the oocyte and fibrillar appendages which extend from the outer surface of the vitelline envelope. A distinct basal lamina separates the follicle cells from the overlying theca. The thecal layer is composed of a scant connective tissue stroma and an extensive capillary network which supplies the entire follicle with maternal nutrients. A surface epithelium covers the entire follicle and consists of a single layer of squamous cells.

By the time vitellogenesis begins, the oocyte has already elaborated numerous lipid inclusions, yolk vesicles, and secondary lysosomes. Most characteristically, the oocyte begins to sequester material at its surface by micropinocytosis and yolk spheres form due to fusion of endocytotic vesicles and smooth-surfaced tubules. Yolk spheres migrate centripetally and fuse to form larger yolk bodies which subsequently coalesce to form a continuous yolk mass (fig. 2). As this occurs, yolk vesicles are displaced towards the periphery and become the definitive cortical alveoli.

In order to follow the route traversed by maternal protein during vitellogenesis, HRP and X. laevis [^{3}H]VTG were injected into females. HRP (MW 40,000) was used since it can be readily visualized by both light and electron microscopy. X. laevis [^{3}H]VTG (MW 465,00) has been chosen since its fate would mimic the fate of native VTG (Wallace et al., 1980). Females were sacrificed up to 24 hr after injection and vitellogenic follicles (C. variegatus: 0.4-0.7 mm; F. heteroclitus: 0.6-1.2 mm) were examined. Figs. 3-7 show the location of HRP within vitellogenic follicles. HRP reaction product is found within the lumen of the perifollicular capillaries, lining the lumenal surface of the endothelial cells and within intercellular spaces between adjacent endothelial cells (figs. 4,5). HRP reaction product is found amongst the stroma of the theca and within the basal lamina of the follicle cells. Materials must traverse the follicular epithelium and vitelline envelope to reach the oocyte surface. HRP reaction product is found within the intercellular spaces of the follicular epithelium (fig. 4) and within the pore canals of the vitelline envelope. These canals appear to provide a direct means of communication between the intercellular spaces of the follicular epithelium and the oocyte surface.

The surface of vitellogenic oocytes is projected into long microvilli which extend through the pore canals of the vitelline envelope and terminate deep within the intercellular channels of the follicular epithelium. Frequently, they are heavily stained and obscured by reaction product within the intercellular spaces of the follicular epithelium (fig. 4). The surface of vitellogenic oocytes displays extensive micropinocytotic activity (fig. 7). By 20 min after HRP injection (our shortest time interval), reaction product is found within endocytotic vesicles and smooth-surfaced tubules near the oocytes surface which appear to fuse to form peripheral yolk spheres (figs. 6,7). Yolk spheres containing HRP are readily visualized with the light microscope (fig. 3). Several hr after HRP injection, the disposition of HRP reaction product remains the same, except that larger yolk bodies residing deeper in the oocyte also contain HRP. Control follicles from uninjected fish show endogenous peroxidase activity only in erythrocytes within the capillaries; control follicles from HRP-injected fish were negative for HRP reaction product.

The distribution of [^{3}H]VTG within vitellogenic follicles was examined autoradiographically 24 hr after [^{3}H]VTG injection. Grains were found localized over peripheral yolk spheres and over fusing yolk masses (figs. 8,9).

We conclude from these studies that macromolecules leave the maternal circulation via an intercellular route. Upon reaching the follicular epithelium, they pass between, rather than through, adjacent follicle cells

en route to the oocyte. Circulating macromolecules, such as VTG and other serum proteins, are incorporated into the oocyte via micropinocytosis and translocated to yolk spheres in less than 20 min. These studies substantiate the importance of heterosynthetic processes during oocyte growth in teleosts.

References

Graham, K.C. & M.J. Karnovsky, 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291-302.

Korfsmeier, K.H., 1966. Zur Genese der Dottersystems in der Oocyte von Brachydanio rerio. Z. Zellforsch. Mikrosk. Anat. 71:283-296.

te Heesen, D., 1977. Immunologische Untersuchungen an exo- und endogenen Dotterproteinen von Brachydanio rerio (Teleostei, Cyprinidae) und verwandten Arten. Zool. Jb. Anat. Bd. 97:566-582.

Wallace, R.A. & K. Selman, 1978. Oogenesis in Fundulus heteroclitus. I. Preliminary observations on oocyte maturation in vivo and in vitro. Dev. Biol. 62:354-369.

Wallace, R.A. & K. Selman, 1979. Physiological aspects of oogenesis in two species of sticklebacks, Gasterosteus aculeatus L. and Apeltes quadricus (Mitchell). J. Fish Biol. 14:551-564.

Wallace, R.A. & K. Selman, 1981. Cellular and dynamic aspects of oocyte growth in teleosts. Am. Zool. 21:325-343.

Wallace, R.A., J.M. Nickol, T. Ho & D.W. Jared, 1972. Studies on amphibian yolk. X. The relative roles of autosynthetic and heterosynthetic processes during yolk protein assembly by isolated oocytes. Dev. Biol. 29:255-272.

Wallace, R.A., R.A. Deufel & Z. Misulovin, 1980. Protein incorporation by isolated amphibian oocytes. VI. Comparison of autologous and xenogeneic vitellogenins. Comp. Biochem. Physiol. 65B:151-155.

Wegmann, I. & K.J. Götting, 1971. Untersuchungen zur Dotterbildung in den Oocyten von Xiphophorus helleri (Heckel, 1948) (Teleostei, Poeciliidae). Z. Zellforsch. Mikrosk. Anat. 119:405-433.

Wiley, H.S., L. Opresko & R.A. Wallace, 1979. New methods for the purification of vertebrate vitellogenin. Anal. Biochem. 97:145-152.

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Fig. 1. The ovary of C. variegatus showing oocytes in various size follicles. a, primary growth phase; b, early yolk vesicle stage; c, late yolk vesicle stage; d, early vitellogenesis; e, late vitellogenesis. JB4-plastic, toluidine blue.

Fig. 2. A cross section through a vitellogenic follicle of C. variegatus showing the oocyte (o) and its surrounding vitelline envelope (v) and follicular epithelium (f). y, yolk; ca, cortical alveolus. Epon-Araldite, toluidine blue.

Fig. 3. Light micrograph of an unstained Epon-Araldite section of a follicle of C. variegatus from an animal sacrificed 20 min after HRP injection. HRP reaction product is seen within small spheres in the cortical ooplasm (o) and within the vitelline envelope (v). ca, cortical alveolus; y, unstained yolk sphere.

Fig. 4. Outer region of a follicle from F. heteroclitus which had been sacrificed 5 hr after HRP injection. Dense reaction product is seen within the extracellular components of the theca (Th), the basal lamina (B) overlying the follicular epithelium (F) and within the intercellular spaces (black arrows) between the follicle cells. The lumen (L) of a perifollicular capillary contains dense reaction product. SE, surface epithelium; V, vitelline envelope; white arrow, pore canal containing a microvillar process extending from the oocyte surface (O); white asterisk, fibrillar appendage of the vitelline envelope.

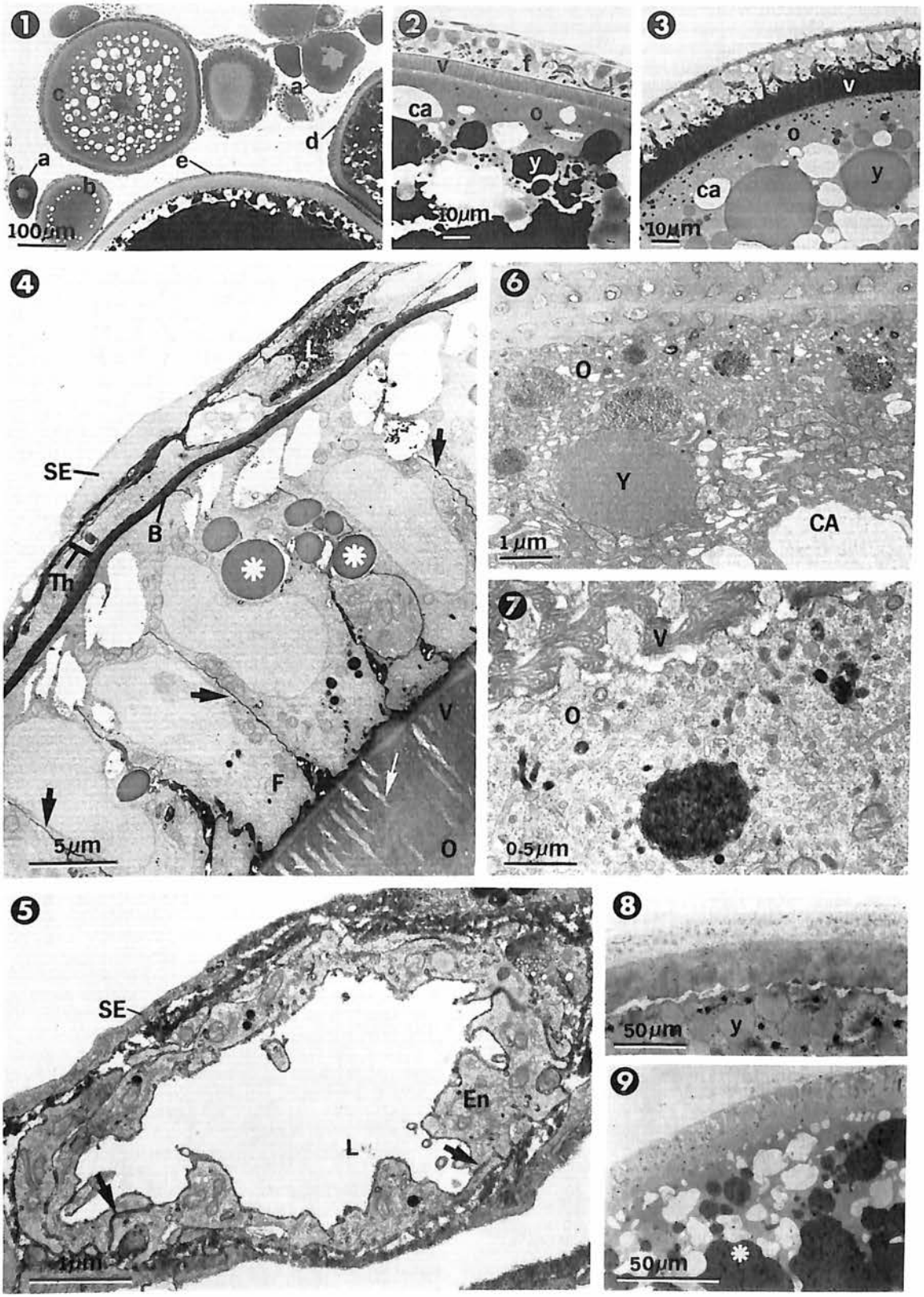
Fig. 5. A perifollicular capillary from C. variegatus, which was sacrificed 30 min after HRP injection. A thin layer of reaction product is found on the lumenal surface of the endothelial cells (En) and within the intercellular spaces of the endothelium (arrows). SE, surface epithelium; L, capillary lumen.

Fig. 6. Outer oocyte region from F. heteroclitus which was sacrificed 30 min after HRP injection. Reaction product is visible within small vesicles and tubules near the oocyte (O) surface and within peripheral yolk spheres. An unlabeled yolk sphere (Y) is closely associated with a labeled sphere. CA, cortical alveolus.

Fig. 7. Outer oocyte region from C. variegatus which was sacrificed 20 min after HRP injection. Reaction product is visible within tubules, small vesicles and a peripheral yolk sphere, which appears to have recently fused with reaction-containing vesicles. Unlabeled vesicles and tubules are also present. V, vitelline envelope; O, oocyte.

Fig. 8. Autoradiogram of a follicle from F. heteroclitus sacrificed 24 hr after injection of [^{3}H]VTG. Grains are concentrated as hot spots over small yolk spheres. y, unlabeled yolk spheres. Toluidine blue. (40 wk exposure).

Fig. 9. Autoradiogram of a follicle from C. variegatus, sacrificed 24 hr after injection of [^{3}H]VTG. Grains are concentrated over yolk spheres of varying sizes and over fusing yolk masses (white asterisk). Toluidine blue. (4 wk exposure).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BIOCHEMICAL COMPOSITION OF OVULES AND FECOND EGGS OF SEA BASS (Dicentrarchus Labrax), SOLE (Solea vulgaris) AND TURBOT (Scophthalmus maximus)

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Studies on the overall biochemical composition of fish eggs and ovules are of interest for reaching a better knowledge of different aspects of the reproduction biology and development of fish under artificial conditions. For instance, the viability of the eggs and larvae depends of different well known factors such as the characteristics of females (Blaxter, 1969) maturity of the eggs for artificial spawning and environmental conditions especially nutrition (Bagnenal, 1979; Fontaine et Ollivereau, 1962). But even when optimal conditions for egg development seem to be realized, no fertilization or embryological development occurs. A possible explanation for this phenomenon could be variations in the biochemical composition of ovules (Konovalov, 1980). Consequently, it is important to know the average content of their reserves involved in the fertilization processes of eggs and development of embryos. But at the time, more information is available about freshwater fish species (Ginzburg, 1972) than about marine fish species.

Hereafter our purpose is to present basic data concerning biochemical composition of three marine fish species actually involved in Aquaculture attempts: Turbot (Scophthalmus maximus), Sole (Solea vulgaris) and Sea Bass (Dicentrarchus labrax).

The analysis concern ovules at the end of oogenesis from wild fishes trawled or angled along Breton coasts, and naturally spawns (at morula stage) laid by captive spawners. Spawns are either naturally fertilized eggs or unfertilized ovules. Captive spawners are all fed on the same trash fish. Ovules are laid under natural light and temperature conditions ("natural spawning season") and artificial conditions (7 months shifted spawning season)- (Devauchelle, 1980).

Several 40 g batches of ovules or eggs were rinsed with distilled water and we measured their content in water (drying at 95°C), proteins (method of Kjedahl), ashes (by oxidization at 55°C), total lipids (method of Folch and Sloane, 1957), different classes of lipids (Beninger, 1982) fatty acids (Flanzy et al., 1976), cholesterol (method of Lieberman-Burchard, 1975), phospholipids and minerals.

All the results are presentages of ovules or eggs dry weight (Tables 1, 2, 3, Figures 1, 2, 3).

% DRY WEIGHT	TURBOT			SOLE			SEA BASS		
WATER	66,14 σ ±0,66	91,44 σ ±0,99	91,57 σ ±0,78	66,81 σ ±2,6	91,95 σ ±0,21	92,06 σ ±0,26	65,19 σ ±1,7	88,43 σ ±0,9	89,06 σ ±0,6
PROTEINS	74,71 σ ±1,55	69,94 σ ±9,9	62,86 σ ±1	73,77 σ ±0,92	62,33 σ ±4,5	67,78 σ ±3,1	63,88 σ ±2,4	54,18 σ ±9,8	52,64 σ ±2,6
LIPIDS	17,84 σ ±1,27	17,34 σ ±2,17	15,57 σ ±2,13	19,09 σ ±19,1	15,67 σ ±0,94	13,12 σ ±1,5	26,23 σ ±0,7	33,07 σ ±16	26,08 σ ±4,9
ASHES	4,16 σ ±0,16	10,58 σ ±6,1	11,30 σ ±6,6	5,62 σ ±0,5	8,11 σ ±1,5	9,82 σ ±3,9	4,19 σ ±0,3	6,19 σ ±0,5	6,59 σ ±0,4

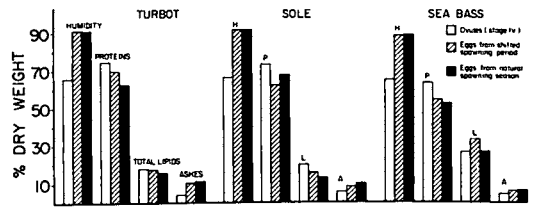

Table 1 and Figure 1: Global composition in fish ovules or eggs.

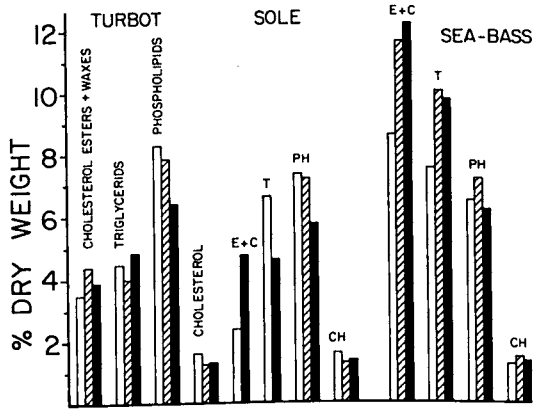

Figure 2: Lipids and different classes of lipids ovules and eggs.

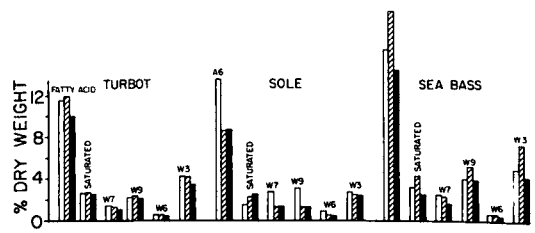

Figure 3: Fatty acids contents in fish ovules or eggs.

		% DRY WEIGHT			PPM DRY WEIGHT			
		CALCIUM (Ca)	PHOSPHORES (P)	MAGNESIUM (Mg)	ZINC (Zn)	COPPER (Cu)	IRON (Fe)	MANGANESE (Mn)
TURBOT		0.396 (σ = 0.071)	1.17 (σ = 0.03)	0.39 (σ = 0.03)	20 4 (σ = 14)	70.2 (σ = 23.9)	16.3 (σ = 3.2)	31 (σ = 0)
		1.8 (σ = 1.3)	0.59 (σ = 0.16)	1.78 (σ = 2.11)	19 5 (σ = 50.4)	67.1 (σ = 177.6)	56.6 (σ = 41.5)	0
		2.8 (σ = 1.6)	0.67 (σ = 0.41)	4.07 (σ = 0.64)	16 4 (σ = 50.3)	50 (σ = 111.8)	48.8 (σ = 27)	0
SOLE		0.316 (σ = 0.1)	1.15 (σ = 0.08)	0.72 (σ = 0.28)	96.8 (σ = 11.1)	0	44.2 (σ = 9.6)	0
		0.929 -	- -	1.02 -	- -	0	0	0
		0.854 (σ = 0.35)	1.11 (σ = 0.06)	0.99 (σ = 0.08)	88.3 (σ = 36.4)	0	94.3 (σ = 26)	0
SEA BASS		0.246 (σ = 0.09)	1.14 (σ = 0.05)	0.39 (σ = 0.05)	13 6 (σ = 8.15)	30.2 (σ = 15.6)	58.4 (σ = 7)	0
		1.115 (σ = 0.54)	0.68 (σ = 0.25)	0.83 (σ = 0.21)	138.4 (σ = 69.7)	17.8 (σ = 1.5)	63.25 (σ = 36)	0
		1.580 (σ = 0.74)	0.95 (σ = 0.02)	0.97 (σ = 0.16)	134 (σ = 22)	24.8 (σ = 7.2)	62.3 (σ = 22)	0

Table 2: Mineral contents in fish ovules or eggs.

		% DRY WEIGHT	10^{-5} DRY WEIGHT							
		TOTAL LIPIDIC PHOSPHORUS	LPC	SPH	PC	PS	PI	PE	DPG	AP
TURBOT										
		0.24 (σ = 0.031)	572 (σ = 60)	501 (σ = 132)	17 940 (σ = 2390)	189 (σ = 74)	1 463 (σ = 595)	2 390 (σ = 572)	324 (σ = 68)	437 (σ = 272)
		0.23 (σ = 0.03)	580 (σ = 84)	7 000 (σ = 2350)	17 700 (σ = 2720)	176 (σ = 49)	1 280 (σ = 672)	1 580 (σ = 822)	466 (σ = 132)	898 (σ = 608)
SOLE		0.27 (σ = 0.03)	658 (σ = 229)	1 371 (σ = 320)	19 890 (σ = 525)	246 (σ = 64)	558 (σ = 84)	3 670 (σ =1020)	632 (σ = 115)	134 (σ = 29)
		0.427 -	- -	3 766 -	28 900 -	589 -	3 177 -	5 218 -	611 -	422 -
		0.276 (σ = 0.002)	455 (σ = 11)	933 (σ = 142)	20 690 (σ = 275)	238 (σ = 51)	774 (σ = 107)	3 823 (σ = 752)	474 (σ = 51)	99 (σ = 27)
SEA BASS		0.245 (σ = 0.01)	2 158 (σ = 387)	664 (σ = 113)	18 390 (σ =1390)	326 (σ = 54)	260 (σ = 62)	2 529 (σ = 795)	115 (σ = 21)	132 (σ = 25)
		0.29 (σ = 0.02)	847 (σ = 277)	961 (σ = 264)	22 473 (σ =2157)	192 (σ = 80)	736 -	3 284 (σ = 623)	560 (σ = 165)	165 (σ = 74)
		0.22 (σ = 0.05)	396 (σ = 159	579 (σ = 20)	15 000 (σ = 319)	176 (σ = 38)	1 119 (σ = 437)	3 355 (σ = 362)	316 (σ = 129)	168 (σ = 27)

Table 3: Phosphatides in fish ovules or eggs.
LPC: Lysophosphatidylcholine; SPH: Sphingomyelin; PC: Phosphatidylcholine;
PS: Phosphatidyl Serine; PI: Phosphatidyl Inositol; PE: Phosphatidyl Ethanolamine
DPG: Diphosphatidyl Glyerol; AP: Phosphatidic acid

The main conclusions are presented below:
- Specific differences of biochemical composition of eggs and ovules appear, i.e. flatfish material contains less total lipids and fatty acids, but more proteins and minerals as compared to sea bass.

- The ovules from wild fish compared to the spawns of captive fish contain more proteins and lipids (especially cholesterol and phospholipids. On the other hand, their mineral content is lower.

- Shifted spawning season induces total lipids and mineral content decrease.

- Last of all, generally a higher total lipid content corresponds to very low viability rate of eggs.

Concerning the analyses of fatty acids (Fig. 3), we notice that saturated fatty acid rate is generally lower in ovules from wild fishes. On the other hand, poly unsaturated fatty acid seems to be more frequent in these ovules.

Mineral contents, and especially phosphorus content (Table 2) differ among different ovules or egg batches.

Phosphatides are given in Table 3 such as basic data.

All these results can be used in the future as indicators of the quality of the eggs, and consequently, indicators of the quality of larvae, as suggested by Nassour (1980)

working on Salmo gairdneri. On the other hand, it is well known that the composition of diets can influence the survival and the fecundity of spawners (Hilge, 1979; Wooton, 1973). So, when there is very little information for dietary requirements of spawners, the data about general composition of eggs could be used as a guideline for formulation of spawners feedstuffs as proposed by Ketola (1980) for amino acid patterns of diets.

References

Bagenal, T.B. (1969). The relationship between food supply and fecundity in brown trout Salmo trutta L. J. Fish. Biol., 1: 167-182.

Beninger, P.G. (1982). Etude biochimique comparé de deuz populations de bivalves Ruditapes decussatus et Ruditapes philippinarum. Thèse d'Etat: Université de Brest, 193 pp.

Blaxter, J.H.S. (1970). Development eggs and larvae. In: Fish physiology, Blaxter Ed. Academic Press. Inc., New York, 3: 178-271.

Devauchelle, N. (1980) Etude expérimentale sur la reproduction, les oeufs et les larves de bar Dicentrarchus labrax, daurade Sparus aurata, Mullet, Liza ramada, Rouget, Mullus surmuletus, Sole, Solea vulgaris, Turbot, Scophthalmus maximus. Thèse de 3è cycle UBO: 194 pp.

Flanzy, J., M. Bourbon, C. Léger and J. Pihet (1976). Application of carbawax 20 M as an open tubular liquid phase in analysis of nutritionally important fats and oils. J. chromatogr. Sci. 14: 17-24.

Folch, J.L. and S.G.H. Sloane (1957). A sample method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497-509.

Fontaine, M. et M. Olivereau (1962). Nutrition et sexualitè chez les poissons. Annales de la nutrition et de l'alimentation T 16: A 125-A 152.

Ginzburg, A.S. (1972) In: Fertilization in fishes and the problem of polyspermy. Ed. by B. Golek. Keter Press Jerusalem: 366 pp.

Hilge, V. (1979): Möglicher Einfluss verschiedener, Kombinierter protein und Fettgehalte des futters auf das Gonaden-Wachstum junger Spiefelkarpfen (Cyprinus carpio L.) Arch. Fischwiss, 29:(3):113-118.

Ketola, H.G. (1980). Amino acid requirements of fishes: A review. Proc. Cornell Nutr. Conf. Feed. Manuf. Nov. 1980: 43-48.

Konalov, Y.D. (1980). Viability of carps embryos and larvae as a fraction of the content of total protein and sulfhydryl groups in the gastrulation stage. Biology of Fishes: ISSN 0018-8166/79:65-68.

Nassour, I. (1980). Influence de la carence en acides gras essentiels sur les lipides de l'oeuf et du tissu adipeux chez la truite Arc-en-ciel (Salmo gairdneri). Mèmoire de DEZ.: 21 pp.

Wooton, R.J. (1973). The effect of size of food ration on egg production in the female three spined stickleback Gasterosteus aculeatus L.

STRUCTURAL STUDIES OF BONY FISH VITELLOGENIN

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Summary

Vitellogenin(VTG), originally named for the female-specific insect protein has been well characterized in avian and amphibian species as a serum protein specific for female, induible in animal by treatment with estrogen and an immediate precursor of egg yolk protein. It is also known that the VTG is a lipoglycophosphoprotein in nature and is a complex with lipovitellin and phosvitin (Bergink & Wallace, 1974, Christmann et al., 1977).

Here, we present identification, isolation and structural characterization of VTG of two bony fish, rainbow trout and Japanese eel. Schematic structural models of VTGs of the above two fish are proposed in comparison with those of Amphibia and Aves.

We purified a female-specific serum protein (FS) and two egg yolk proteins (E1 and E2) from serum and egg yolk of mature rainbow trout (Salmo gairdneri) by precipitation with low ionic strength buffers and gel filtration. The FS had iron-binding activity and was a lipoglycophosphoprotein. It was induced by administration of estradiol-17β in serum of both sexes. From these characteristics, the FS was defined to be a homologue of VTG of rainbow trout. The trout VTG had a m.w. of around 600,000, consisting of two identical polypeptides of 220-240,000 daltons associated by non-covalent forces and of some phospholipids and carbohydrates.

The egg yolk proteins, E1 and E2, of trout were precipitable together in a low ionic strength buffer as a E1-E2 complex. The m.w. of E1 and E2 were approximately 300,000 and 35,000, respectively. E1 was non-covalently bound dimer of two identical 130,000 components, which yet consisted of two subunits of 90,000 and 15,000 daltons associated by disulfide bonds in a molar ratio of 1 to 1. E2 was disulfide-linked dimer of 15,000 dalton subunit. Using antisera to VTG, E1 and E2, it was disclosed that the trout VTG contained both E1 and E2. This, together with the above m.w. analyses, indicated that the trout VTG monomer is composed of one E1 and one E2. This was further confirmed by comparison of the amino acid compositions among the three proteins (Table 1).

Eel VTG and egg yolk protein were also isolated from Japanese eel (Anguilla japonica). Structural analysis indicated that both the VTG and the egg yolk protein of eel have the same molecular form with the m.w. of 350,000, consisting of four identical polypeptides of 85,000 daltons. Schematic structural models of VTG from trout and eel, together with those of amphibian and avian VTG arranged from the structural data of Bergink and Christmann's groups are shown in Fig. 1. Structural similarity is apparent among VTGs presented, except for the eel VTG. This indicate that the phylogenetic divergence of VTG that occurred between Amphibia and Aves.

Table 1. Amino acid composition of E1, E2 and VTG of rainbow trout.

Amino acid (mol.wt.)	E1 (210,000)	E2 (30,000)	E1+E2	VTG/2 (240,000)
Asp	145	42	187	185
Thr	101	11	112	109
Ser	91	29	120	165
Glu	220	27	247	253
Pro	102	9	111	115
Gly	77	15	92	93
Ala	270	9	279	257
Cys/2	15	7	22	26
Val	146	21	167	156
Met	49	8	57	56
Ile	112	14	126	120
Leu	197	19	216	208
Tyr	55	11	66	66
Phe	88	5	93	89
His	42	6	48	47
Lys	119	29	148	156
Arg	84	9	93	100
Total	1913	271	2184	2201

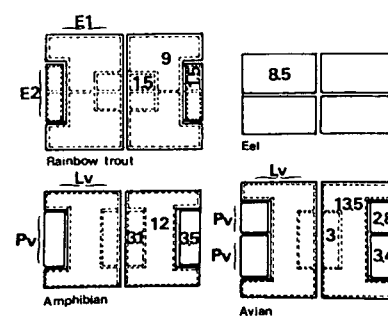

Fig. 1. Schematic models of various VTG molecules. Numbers in the scheme represent the m.w.($\times 10^4$). Lv:lipovitellin, P:phosvitin

References

Bergink E.W. and R.W. Wallace, 1974. Precursor-product relationship between amphibian vitellogenin and the yolk proteins, lipovitellin and phosvitin. J. Biol. Chem. 249, 2897-2903.

Christmann J.L., M.J. Grayson and R.C.C. Huang, 1977. Comparative study of hen yolk phosvitin and plasma vitellogenin. Biochemistry 16, 3250-3256.

OESTRADIOL-INDUCED FEMALE SPECIFIC PROTEINS IN THE PLASMA AND IN THE OOCYTES OF GASTEROSTEUS ACULEATUS F. TRACHURUS. AN IMMUNOLOGICAL APPROACH.

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Vitellogenesis was induced in non-sexually mature ♂♂ and ♀♀ three spined sticklebacks by injecting 6 times 0.66 µg oestradiol-3-benzoate/g fresh body weight. After polyacrylamide gradient electrophoresis four new bands appeared in the plasma of the treated fishes : two lipoprotein bands (fig. 1) and two bands with very high lipid concentration. These bands also appeared if only 1/10 of the above mentioned hormone concentration was injected, but the response was very weak if only 1/100 was injected. After bidimensional electrophoresis the strongest lipoprotein band (Vg 1) was split up into three different proteins (640,000 D, 550,000 D and 520,000 D).

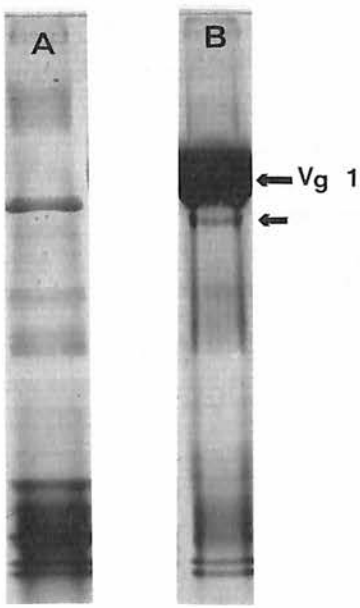

fig. 1 Electrophoretic pattern of plasma proteins from ♀♀ Gasterosteus aculeatus.
A. non vitellogenic control
B. oestradiol induced vitellogenic ♀

An injection with 1.32 µg or 13.2 µg medroxyprogesterone-acetate/g fresh body weight did not induce vitellogenesis 12 days after the injection. This confirmed the results of Hori et al. (1979) with goldfish. Antibodies prepared against Vg1 did not react with plasma of non-oestradiol treated ♀♀, but after electrophoretic separation positive results were obtained at the level of Vg 1 with plasma from fishes of both sexes showing oestradiol-induced vitellogenesis. Homogenates of ripe eggs showed, after agarose electrophoresis, two immunological cross reactions with the Ab : one with a lipovitellin component and one with an anodic migrating component (pH 8.8) which we believe represents phosvitin.

Tromp-Blom (1959) distinguished 6 stages in the development of the oocyte of the stickleback. In the non-oestradiol treated ♀♀ of this experiment, stage 1 to 4 were present. Stage 5 was only present in the oestradiol treated ♀♀ but stage 6 was lacking in both groups.
The stage 4 oocytes of oestradiol treated ♀♀ reacted positively with Ab against Vg1 at the level of the thecacells and the oolemma. This was not the case for the control fishes. Positively reacting 'vesicles' were never found in the cytoplasm of stage 4. Peripherally located 'vesicles' in the cytoplasm of stage 5 oocytes reacted positively with Ab against Vg1 (fig. 2). This indicated that during exogenous vitellogenesis only from stage 5 on proteins related with Vg 1 were accumulated in the oocytes. The proteins accumulated in the oocytes during the endogenous vitellogenesis were therefore immunologically different from Vg 1; this being the most concentrated vitellogenic lipoprotein présent in the plasma during exogenous vitellogenesis. Although chemically changed, as shown by electrophoresis, the components derived from this lipoprotein and present in the oocytes were still immunologically reactive with Ab against Vg 1.

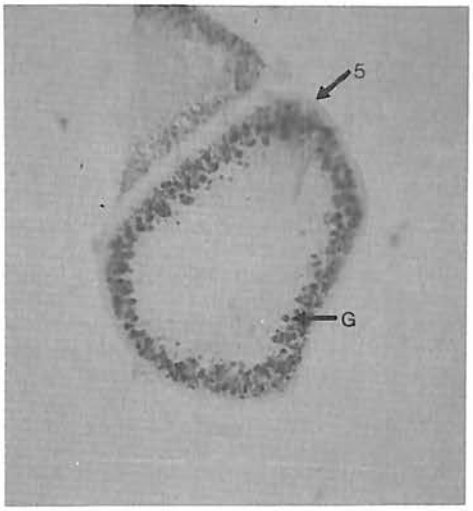

fig. 2 Oocyte (stage 5) of oestradiol injected Gasterosteus aculeatus treated with Ab against vitellogenin 1 and stained with PAP.

References

Hori, S.H., T. Kodoma & K. Tanahaski, 1979. Induction of vitellogenin synthesis in goldfish by massive doses of androgens. Gen. Comp. Endocrin. 37 : 306-320.

Tromp-Blom, N., 1959. The ovaries of Gasterosteus aculeatus before, during and after the reproductive period. Proc. K. Ned. Acad. Wet. 62 : 225-237.

A NEW PROCEDURE FOR THE ISOLATION OF INTACT VITELLOGENIN FROM TELEOSTS

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Vitellogenin (VTG), the hepatically synthesized yolk protein precursor, is particularly difficult to obtain in a pure and unaltered form from many teleosts. Teleost VTGs are generally less phosphorylated than those of other vertebrates (de Vlaming et al., 1980), so that existing methods for VTG isolation which rely on its high surface charge, such as EDTA-Mg^{2+} precipitation or chromatography on DEAE-cellulose in Tris-Cl buffer with a NaCl gradient (Wiley et al., 1979), either fail to precipitate VTG or do not resolve it adequately from other blood proteins. Furthermore, teleost VTGs are highly susceptible to proteolysis during isolation, generally yielding phosvitin (PV)-like and lipovitellin (LV)-like cleavage products. The former peptide, which contains almost all the protein-phosphorous orginally associated with VTG, absorbs light at 280 nm either poorly or not at all (Jared & Wallace, 1968) and cannot be stained with protein-binding dyes such as Coomassie blue (unpublished); hence it is not detectable by the usual monitoring procedures. Analysis of the LV-like peptide without an appreciation of its proteolytic origin would lead one to conclude that plasma VTG is similar to yolk LV and hence is not proteolytically processed within the growing oocyte as has been found for other vertebrates (Wallace, 1978).

The problem is illustrated in figs. 1a and 1b, which represent chromatographic profiles of plasma from male and female Fundulus heteroclitus, an animal for which previously published methods have proven to be particularly inadequate. VTG, identified as the UV-absorbing [^{32}P]protein present in females but absent in males (fig. 1b, elution position 0.72), elutes in association with the penultimate protein fraction and is mostly degraded to yield a [^{32}P]PV-like peptide (fig. 1b, position 0.82), even though phenylmethylsulfonyl fluoride was added to the blood during collection. The problem was solved by injecting fish with the nontoxic proteolytic inhibitor, aprotinin (0.2 ml/10 gm fish; 17 TIU/ml; Sigma), 15 min prior to bleeding and by using a phosphate rather than NaCl gradient to develop the chromatograph (fig. 1c). VTG is well resolved from other plasma proteins and generally less than 10% is cleaved to yield the PV-like peptide.

References

de Vlaming, V. L., H. S. Wiley, G. Delahunty, & R. A. Wallace 1980. Goldfish (Carassius auratus) vitellogenin: Induction, isolation, properties, and relationship to yolk proteins. Comp. Biochem. Physiol. 67B: 613-623.

Jared, D. W., & R. A. Wallace 1968. Comparative chromatography of the yolk proteins of teleosts. Comp. Biochem. Physiol. 24: 437-443.

Wallace, R. A. 1978. Oocyte growth in nonmammalian vertebrates. In: The Vertebrate Ovary, Ed. R. E. Jones. Chr. 13: 469-502. Plenum, New York.

Wiley, H. S., L. Opresko, & R. A. Wallace 1979. New methods for the purification of vertebrate vitellogenin. Anal. Biochem. 97: 145-152.

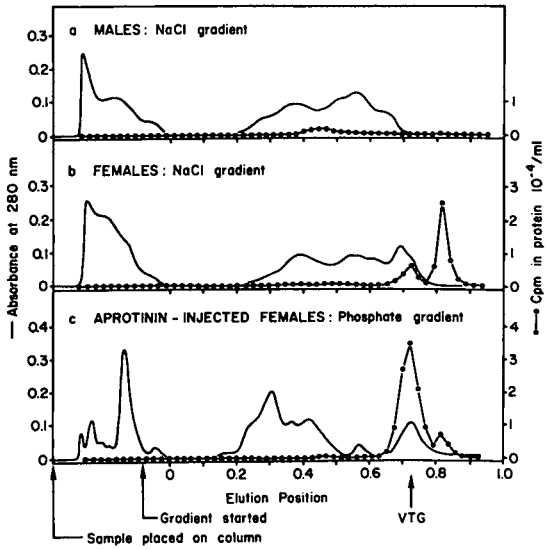

fig. 1. Chromatography on DEAE-cellulose of approx. 0.5 ml plasma from (a) males, (b) females, and (c) aprotinin-injected females. All animals were injected with 75 µCi[^{32}P]Pi 20 hr previously. After the plasma sample was placed on the column (30 ml bed volume) of microgranular DEAE-cellulose (Sigma), it was rinsed with starting buffer until most of the nonadherent protein percolated through. A 400-ml, 3-bottle gradient, which consisted of 134 ml starting buffer in each of the first 2 bottles and 132 ml limit buffer in the third bottle, was then pumped onto the column (flow rate = 2.4 ml/min). The effluent was monitored for absorbance at 280 nm and 100 µl samples of effluent fractions were placed on filter paper disks, processed to remove small molecular weight substances and lipid, and counted for protein-associated radioactivity. Starting buffer: a and b = 50 mM Tris-Cl, pH 7.5; c = 2.5 mM KH_2PO_4 - 10 mM K_2HPO_4. Limit buffer: a and b = 50 mM Tris-Cl, pH 7.5-0.35 M NaCl; c = 100 mM KH_2PO_4 - 400 mM K_2HPO_4. The column could be regenerated for another run by washing sequentially with 0.2 M Na_3 citrate-0.3% Brij-35, water, limit buffer, and starting buffer (100 ml each).

PHYSIOLOGICAL BASIS OF PRACTICAL MEANS TO INDUCE OVULATION IN FISH

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Summary

In order to better appreciate the best way in which techniques to induce spawning could be improved, a concise and critical review of their physiological basis has been made. The endocrine regulation of maturation and ovulation, the process of acquisition of ovarian receptivity and competence and the mechanism of actions of present treatments have been successively considered.
Keywords : rainbow trout, hypophysation, maturation, ovulation, review.

I. Introduction

In order to obtain eggs from some fish species, it would be useful to control different phases of oogenesis, mainly vitellogenesis and/or oocyte maturation-ovulation. One or the other or both of these phases may be disturbed by the artificial environment of fish farming which may impose stressful conditions on the fish (Billard et al., 1981a), or in which indispensable specific stimuli are sometimes lacking. Even when the whole gametogenesis can occur spontaneously it might be most useful to be able to control the ovulation time in order to synchronize groups of spawners and produce equal-aged fry, which should help fish-farm management.

This control is possible in two ways : either through the manipulation of environmental factors, or through the administration of exogenous substances directly acting upon physiological processes to favour, or even induce gonadal development. Today, inducing complete vitellogenesis appears to be realistic in farm practice only through environmental factors, when they can be manipulated. On the contrary, treatments with exogenous substances (especially hormones or hormonal substitutes), eventually in association with environmental control, have been widely used to induce the final steps of oogenesis(i.e. oocyte maturation and ovulation). These techniques, generally named "hypophysation", have been extensively reviewed (Pickford & Atz, 1959; Harvey & Hoar, 1980 among others), thus this paper will be limited to a concise analysis of the present knowledge about their physiological basis in order to better appreciate the best way in which they could be improved.

It may be useful to recall particularieties which could limit some comparisons between data obtained with different kinds of materials and methods, and to specify some definitions. Sexual endocrinology and intraovarian regulations may be somewhat different between fish in which a massive synchronous ovulation occurs after a long-lasting vitellogenesis (trout, carp, catfish),and fish which present a wide-spread breading season characterized by successive waves of oocytes at different stages of development, thus ovulating more or less regularly by "clutches" (medaka, zebrafish, killifish, gilt-head sea-bream). Concerning oocyte maturation and ovulation, these must be considered as two distinct processes which are normally linked but can be artificially dissociated (Jalabert, 1976; Iwamatsu 1978a; Goetz & Bergman, 1978). Oocyte maturation is more often morphologically characterized by a clarification of the yolk (due to the separate fusion of proteic and lipidic yolk), and finally by the germinal vesicle breakdown (GVBD). Ovulation sensus stricto is the mechanical expulsion of the oocyte out of the follicular envelops which can occur only after a phase of detachment between the oocyte and the follicular cells (Jalabert & Szöllosi, 1975). Finally, the interpretation of in vitro experiments must carefully take into account how and what kind of ovarian components were incubated : pieces of ovary, isolated follicles, oocytes surrounded only by granulosa cells, and/or naked oocytes. The distinction is never so easy : isolated follicles may retain some interstitial tissue which can be of importance (Iwamatsu & Katoh, 1978); and naked oocytes are usually obtained by mechanical dissociation, which may leave some granulosa cells, or by enzymatic dissociation, which may dommage the oocytes.

II. Endocrine regulation of maturation and ovulation

Though hypophysectomy, performed in comparison with ovulating intact fish, has clearly demonstrated that maturation and ovulation are under the control of the pituitary gland (Vivien, 1941; Yamazaki, 1962; Iwamatsu, 1978b), the roles of all the different hypophysial hormones are far from being well understood. However, it is known from different kinds of experiments that the glycoprotein gonadotropin (adsorbed on concanavalin A-sepharose) is a predominant and essential regulating factor. Thus, an antiserum to this hormone administered in vivo blocks the maturation process in salmon or in flounder, while an antiserum to a "carbohydrate-poor" protein claimed as vitellogenic gonadotropin, is without effect (Ng et al., 1980). Furthermore the homologous

glycoprotein induces, in vitro, intrafollicular oocyte-maturation in rainbow trout, common carp, goldfish (Jalabert, 1976) or sturgeon (Burzawa-gerard et al., 1976) and even in vivo ovulation in the hypophysectomized flounder (Idler & Ng, 1979). Morphological observations of the pituitary pars distalis (Eckengren et al., 1978) and measurements in plasma by means of radioimmunoassays confirm the physiological implication of this gonadotropin (GTH) in the regulation of the end of the sexual cycle. The plasma GTH levels reach their highest values during the periovulatory period in salmonids (Billard et al., 1978; Breton et al., 1982; Crim et al., 1975) and cyprinids (Billard et al., 1978; Breton et al., 1980). However, when serial samplings have been performed around the time of ovulation, the salmonids and cyprinids patterns appear to be different. In trout, the basal level rises slowly over several days to reach 10 to 20 ng/ml at ovulation (Breton et al., 1982; Fostier et al. 1978, 1981a), (fig.) then a high level is maintained (between 50 and 100 ng/ml, sometimes higher) during a period of several days, depending on the ovules retention in the peritoneal cavity (Jalabert & Breton, 1980). More accurately, a recent study (Zohar et al., 1982a) of the short-term profiles of plasma GTH in rainbow trout has shown a circardian rhythm in those levels characterized by large and slow fluctuations. The highest GTH concentration, which may reach about twice the basal level, occurs during the night and early morning In a cyprinid, the goldfish, the preovulatory basal level of plasma GTH also fluctuates (Hontela & Peter, 1978) but a true ovulating surge was detected, reaching at least ten times this basal level and lasting less than 12 hours (Breton et al., 1972; Stacey et al., 1979). Thus, if in salmonids the hypophyseal signal seems to be a relatively slow process, which could be accompanied by a progressive change in hormonal balance, in cyprinids this signal seems to be more like a switch.

The regulation of GTH secretion by the neuroendocrinological system has recently been reviewed (Peter & Crim, 1979). The gonadotropin releasing hormone (Gn-RH) activity in fish hypothalamus (Breton et al., 1975) has been confirmed (see the review by Ball, 1981). Though LH-RH and its analogues have been used with some success to induce ovulation in several species (see references in Donaldson et al., 1981a), high doses are usually necessary. This may be due to structural differences between LH-RH and the fish Gn-RH (King & Millar, 1980; Idler & Crim, 1982; Barnett et al., 1982). Furthermore, the hypophyseal sensitivity to LH-RH appears to change considerably throughout the sexual cycle. Thus in carp (Weil et al., 1975) and trout (Weil et al., 1978) females the highest sensitivity in vivo is found during the spawning period, and even, in the last species, the response to LH-RH increases during the very last preovulatory stages, while the pituitary GTH content remains unchanged. Such refractoriness might be due to the control of GTH release by a gonadotropin-release-inhibiting factor (GRIF), probably originating in the anterior preoptic region, as proposed in goldfish (Peter & Paulencu, 1980). It may be a dopamine type factor (Peter et al., 1982) though, in other respects, prostaglandins PGE_2 and $PGF_{2\alpha}$ inhibit GTH secretion in goldfish when injected into the hypothalamus (Peter & Billard, 1976).

The role of hypophyseal hormones other than GTH is more questionable (see the review by Fontaine, 1976). Probably ACTH can act though its control of interrenal gland, which produces corticosteroids hypothesized either as maturation inducing steroids (MIS) in the Indian catfish (Sundararaj & Goswami, 1977), or at least as potent modulators of GTH action upon the follicle (see part III). Marginal effects, in vitro, of mammalian thyrotropin (TSH) or prolactin have been sometimes reported (Iwamatsu, 1978b; Hirose, 1980), but the use of non piscine hormones, the possible contamination of TSH by LH or the homology between GTH and TSH (Fontaine, 1976) could explain such actions. However TSH might be efficient via the thyroid hormones (see part III), though data available on the level of plasma thyroid hormones during the spawning season are contradictory (Osborn et al., 1978; Sower & Schreck, 1982; Pickering & Christie, 1981).

Pineal has been suggested as playing a stimulatory role upon gonadal activity during spring in goldfish (De Vlaming & Vodicknick, 1978) by promoting a daily cycle in plasma GTH (Hontela & Peter, 1980). However, a study involved strictly with the oocytes maturation process in the medaka has shown neither the in vivo effects of pinealectomy, nor the in vitro direct action of melatonin (Iwamatsu, 1978c).

Analytical studies of the ovarian steroidogenesis and descriptions of hormonal profiles in plasma during the spawning period have confirmed the previous in vitro observations about the eminent role of some steroids (MIS) in the mediation of GTH action upon final maturation and ovulation. In salmonids, the preovulatory follicle is able to produce progestins (Fostier et al., 1981b; Suzuki et al., 1981a,b; Theofan, 1981), but also androgens, estrogens and corticosteroids (Suzuki et al., 1981a; Theofan, 1981; Zohar et al., 1982b). In the amago salmon, it has been proposed that both granulosa and thecal layers were involved in the synthesis of the MIS (i.e. 17α-hydroxy-20β-dihydroprogesterone = 17α,20β-OH-P) (Nagahama et al., 1982). Also identified in plasma (Idler et al., 1960; Campbell et al., 1980), this steroid shows a sharp rise at the precise time of oocyte maturation in rainbow trout (Fostier et al., 1981a; Scott et al., 1982; Wright & Hunt, 1982) (Fig.). This increase is characterized by large fluctuations (Zohar et al., 1982b). Steroids which act synergically with GTH upon oocyte maturation in vitro have also been measured (see part III)-

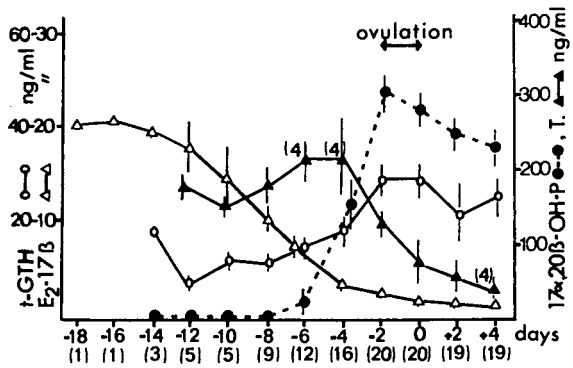

Figure. Periovulatory changes of plasma hormones levels in rainbow trout (means ± SE) Plasma gonadotropin (o o o), estradiol-17β (Δ Δ Δ), 17α-hydroxy-20β-dihydroprogesterone (● ● ●) and testosterone (▲ ▲ ▲) have been measured every two days in individual fish. Ovulation was detected at day 0. The numbers of sampled fish (in brackets) were the same for all the hormones except testosterone (4).

In rainbow trout, estradiol-17β (E2-17β)levels , which are high during exogenous vitellogenesis, decrease drastically before maturation (Fostier et al.,1978; Scott et al,1980) while testosterone levels, though decreasing still appear high (Scott et al.,1980) or even show a little peak before ovulation (Fig.). Using a cautious protocol of sampling, Pickering & Christie (1981) have confirmed an increase in cortisol concentration during this period. In goldfish,Cook, Stacey & Peter (1980, in Gen. Comp. Endocrinol. 40: 507-510) have described a preovulatory surge of cortisol analogous to the GtH surge.

To conclude if we consider all the different hormonal events recorded in vivo around the time of maturation and ovulation we must point out the difficulty in discriminating essential dominating factors and modulating ones. What is the primary signal which switches on irreversibly the whole hormonal process ? In trout the decrease of plasma E_2-17β, related to a fall in aromatase activity, may be controlled by GTH (Sire & Depeche, 1981; Zohar et al., 1982b), and could release a negative feedback upon the GTH secretion (Bommelaer et al., 1981) necessary to stimulate the MIS synthesis (Fostier et al., 1981b). Furthermore, the balance established between the steroids other than MIS (i.e. testosterone, E_2-17β, cortisol) could exert a positive synergic action (see part III). Thus, if such hormones act synergically by successive steps, the hormonal signal which has been received in vivo by the ovary before the beginning of an in vitro experiment, may be decisive on the further responses to different exogenous factors. That is to say that information on the accurate hormonal status before and at the time of sampling can help to interpret in vitro data. In other words, it may be a means of appreciating follicular receptivity and competence.

III. Acquisition of ovarian receptivity and competence

Before receiving the final signal(s) which trigger(s) maturation and ovulation, the somatic cells of the ovary and the oocytes themselves are involved in a preparation process which may be more or less lengthy depending on the species. Looking at the maturational response of oocytes in vitro, two main levels of sensitivity can be defined. 1. The "oocyte sensitivity" of naked oocytes to exogenous MIS. 2. The "follicular sensitivity" evaluated from the response of oocytes incubated within intact follicles (possibly with some ovarian interstitial tissue) to exogenous substances. When these are steroids, the "follicular sensitivity" may reflect the ability of the follicle (oocyte included) to metabolize them. Where gonadotropins are concerned, the "follicular sensitivity" integrates the sensitivity of somatic cell layers to the gonadotropin (through the stimulation of MIS secretion) and the primary sensitivity of the oocyte to MIS.

Table. Median efficient dose of hormones necessary to induce oocyte maturation in vitro in the follicles of different females sacrified at two different stages (see text). Relation with the estradiol level in plasma. Incubations carried out in vitro at 12°5, pH 7.9-8 for 72 h. as previously described (Jalabert, 1978).

Oocyte stage	Fish	Median Efficient dose (ng/ml) for		Estradiol in plasma of donnor fish
		s-GTH	17α20βP	
End of vitellogenesis (E.V)	1	393	101	30.0
	2	183	419	29.9
	3	91	87	17.2
	4	128	62	12.8
	5	60	190	19.3
G.V. peripheral (GVP)	6	18	21	4.3
	7	39	5.4	22.9
	8	40	20	2.8
	9	29	4.7	-
	10	15	-	6.6

The "follicular sensitivity" to steroids has been shown to depend on the follicular size (Iwamatsu, 1978a; Wallace & Selman, 1978; Hirose, 1980), on the season (Iwamatsu & Katoh, 1978), or on the hour of sampling in the daily spawning cycle of the Medaka (Hirose, 1972). It may also be related to the stage of germinal vesicle (GV) migration from the cen-

tre to the periphery of the oocyte in some species. For example, within the same strain of rainbow trout, we compared the median efficient dose (MED) of the MIS 17α-20β-OH-P for intrafollicular oocyte maturation in vitro, using follicles from different individuals in the same conditions of incubation, either when the oocytes showed the germinal vesicle (GV) well at the periphery (GVP, a stage estimated roughly at less that one week from the time of expected natural ovulation at 10°C), or when the GV could not yet be seen without fixation (stage EV = "end of vitellogenesis") estimated here at around 4 to 6 weeks prior to expected ovulation time, and corresponding to a high level of estradiol in the plasma of the same animals (Table), which might be interpreted as an indication that vitellogenesis is still going on. The oocytes at the EV stage already appeared to respond to 17α-20β-OH-P but the MED was then much higher than at the GVP stage, thus indicating lower sensitivity (Table). Moreover, injecting rainbow trout with 17α-20β-OH-P at about the same precocious stage induced oocyte maturation in vivo without ovulation, and although ovulation could then be induced in vitro, either manually or by action of prostaglanding $F_{2\alpha}$, developmental capacity of the eggs was low (Jalabert et al., 1978a). In the medaka, Oryzias latipes, which exbibits a precise daily rythm of spawning, the percentage of oocytes incubated within intact granulosa cells that reach GVBD after in vitro stimulated by progesterone, increases up to nearly 100 when the time at which they are taken draws near to 15 hours before expected natural GVBD; however, in these conditions maturation is rarely followed by ovulation, and oocytes cleave abortively when activated by insemination (Iwamatsu, 1974). Altogether this means that even though the oocytes are already able to exhibit very early a maturational response (GVBD) to an exogenous MIS, the follicle itself will not necessarily ovulate, because uncompletely differentiated, and even if ovules are obtained they will not necessarily be able to be fertilized or to develop normally.

Concerning the "follicular sensitivity" to gonadotropins,it depends, for a part, on the ability of a steroidogenic tissue to synthesize the MIS in response to GTH. This steroidogenic tissue appears generally to be located within the ovary in most of the species, as demonstrated by the positive action of gonadotropins upon intrafollicular oocyte maturation in vitro in adequate conditions (Jalabert, 1976; Wallace & Selman, 1980). However in the Indian catfish, Heteropneustas fossilis where the follicular sensitivity to gonadotropins alone in vitro appears extremely low, and where corticosteroids are potent inducers of GVBD it was hypothesized that the MIS might be produced by the interrenal (reviewed by Sundararaj & Goswami, 1977). In trout the ability of the ovary to synthesize the MIS in response to GTH was shown in vitro at the final stages of GV migration (Fostier et al., 1981b) and appeared all the more important as GV was more peripheral. This ability is certainly more precocious since intrafollicular oocytes at the EV stage are already able to reach GVBD in vitro although the follicles then require a higher dose of GTH (Table). In Atlantic salmon, Sakun (1966) was able to induce a maturational response, although characterized by the formation of abnormal metaphases, by injection of pituitary preparations, as early as 3 1/2 months prior to expected natural ovulation, but more precocious pituitary treatments only promoted an acceleration of vitellogenesis. In most species a transitory period probably occurs during which the ovary could possess a bipotentiality regarding steroidogenesis, oriented either to the control of vitellogenin synthesis (estrogens) or to the control of oocyte maturation and ovulation (MIS). Recent data in our laboratory tend to demonstrate that the modification in ovarian steroidogenesis could be amplified by the shape and intensity of the maturational gonadotropin (GTH) signal (Zohar et al., 1982 a, b). However, the ovarian competence and sensitivity is certainly the result of complex interaction between numerous factors. In rainbow trout E_2-17β lowers while testosterone and cortisol enhance the efficiency of GTH upon intrafollicular oocyte maturation in vitro (Jalabert, 1975) which may be partly related respectively to an inhibition or a stimulation of the production of MIS by the follicle (Jalabert & Fostier, unpublished data). In the catfish, the action of gonadotropins or cortisol upon intrafollicular oocyte maturation is reduced by E_2-17β and testosterone (Sundararaj et al., 1979). However, other ovarian functions than steroidogenesis, particularly related to the control of ovulation, are probably regulated by GTH. This could explain why hypophysectomized females of medaka injected with a MIS such as progesterone, undergo oocyte maturation but do not subsequently ovulate (Iwamatsu, 1978a). Besides, taking into account that a massive injection of gonadotropin in salmonids at precocious ovarian stages can induce oocyte maturation without subsequent ovulation (Jalabert et al., 1978b), as well as an injection of 17α,20β-OH-P, it may be hypothesized that the gonadotropin action necessary to bring about the convenient differentiation of ovarian functions related to ovulation must be progressive. This could explain the relative success of priming treatments to obtain ovulation in vivo (Jalabert et al., 1978b; Hunter et al., 1981; Donaldson et al., 1981a). Finally endocrine factors other than steroids or GTH by itself also participate in the regulation of the "follicular sensitivity" to gonadotropins. Thus, in the stellate sturgeon exposed to cold temperature, or kept in prolonged captivity prior to the onset of maturation, the follicular sensitivity to pituitary extracts in vivo or in vitro was impaired, but that it

was restored by injection of triiodothyronine (Dettlaff & Davydova, 1979). In Oryzias latipes, a low molecular weight factor from rabbit serum inhibits steroid-induced oocyte maturation in vitro, however such a factor was not so well characterized in medaka serum (Iwamatsu & Takama, 1980).

Environmental factors can modify the "follicular sensitivity" but their mechanism of action are far to be always well known. We have already mentioned the loss of ovarian sensitivity in sturgeons exposed to cold temperature (Dettlaff & Davydova, 1979). In Fundulus heteroclitus cessation of feeding eliminates the competence of follicles to respond to deoxycorticosterone in vitro, but human chorionic gonadotropin (HCG) injection or refeeding restores the response (Wallace & Selman, 1980). In grey mullets, which normally reproduce in seawater, modifications of ovarian steroidogenesis pathways were observed in fish confined in freshwater, in comparison with seawater fish. These modification were hypothesized to impair the synthesis and release of pituitary GTH, thus explaining the maturation/ovulation block actually observed in freshwater (Eckstein, 1975). Inversely, in coho salmon retained in sea-water before maturation, the pattern of plasma hormones was impaired and ovarian response to exogenous treatments appeared reduced compared to that of fresh water fish (Sower et al., 1982). Probably, osmoregulatory disturbance may also interfer with the process of ovarian hydratation which develops during maturation (Hirose et al., 1974; Oshiro & Hibiya, 1981).

In other respects, ovarian sensitivity, regarding oocyte maturation and ovulation, must not be considered exclusively from an endocrinological point of view, and cell-to-cell interractions within the ovary must be very important to harmonize the morphological and functionnal differentiation of the various cell types which cooperate in view of the final ovulation of mature oocytes able to be fertilized and develop normally. The granulosa cells probably assume numerous functions through their direct contact with the oocyte : not only do they cooperate to provide the oocyte with the MIS, but they might also the source of direct inhibiting factors of maturation if any, as seen by the increased efficiency of MIS in vitro after elimination of granulosa in the medaka (Iwamatsu, 1980). Moreover, oocytes of this species, taken in vivo after the endogenous stimulation necessary for further spontaneous maturation in vitro, acquire normal developmental activity only when surounded by the granulosa cell layer (Iwamatsu & Ohta, 1981) which seems to provide the maturing oocyte with "special factors" dependant upon gonadotropin stimulation. Ovulation implies the rupture of close relationships between the oocyte and the granulosa layer which were prevailing during vitellogenesis. In trout, this appears to occur during oocyte maturation, and to require stimulation by high levels of 17α-20βP in vitro (Jalabert, 1978). Thus, if a close relationship between oocytes and granulosa is necessary during a part of maturation, the follicular detachment should occur only after this period. This could explain the difference, observed in vitro, between the very low amount of 17α-20βP necessary to induce only maturation on one hand, and the very high doses necessary to bring about oocyte detachment on the other hand (Jalabert, 1978), and would be compatible with the progressive rise of 17α-20βP observed in vivo over at last 2 days (Fostier et al., 1981a) and the kinetics of secretion observed in vitro (Fostier et al., 1981b). Also inducing too abruptly a rise of the MIS by exogenous means could lead to dramatic repercussions upon the quality of the eggs. In extreme cases the ovulation of immature oocytes has even been observed, either in vitro after action of unappropriate MIS on the follicles(as seen by Goetz & Bergman, 1978) in the yellow perch, or in marginal temperature conditions (as cited by Dettlaff & Skoblina, 1969) in the sturgeon, confirming that oocyte maturation and ovulation are independant pocesses which can be artificially dissociated, as also shown in trout (Jalabert et al., 1972). Concerning the mechanical process of ovulation, this requires, in some species the contraction of follicular cells. In Oryzias latipes such cells containing an organized contractile system differentiate in the theca within only a few hours prior to ovulation (Pendergrass & Shroeder, 1976). In trout the morphology of thecal cells looks identical to authentic smooth muscle cells (Szöllösi et al., 1978). However, the presence of contractile cells in the follicle wall might not be required in species where maturation is accompanied by an important swelling of the oocyte and (or) the production of a jelly layer by the granulosa, and where the hydration process can provide the force to expel the oocyte as proposed in plaice (Oshiro & Hibiya, 1981). Whatever the mechanics of the ovulation process, this appears to require the local synthesis and action of prostaglandins, as has now been directly proved in the pond loach (Ogata et al., 1979), and possibly a mediation by catecholamines (Jalabert, 1976) which would suggest a role for an intraovarian innervation.

In comparison to the complexity of mechanisms which regulate the ovarian competence for maturation and ovulation, the criteria used in the current practice to choose brooders that will be submitted to hypophysation are very simplist, from the simple external observation of the abdomen aspect, to ovarian biopsy in order to check the follicular morphology (see Harvey & Hoar, 1980). In that case the criteria used are either the oocyte diameter, suitable in species such as the grey mullet (Shehadeh et al., 1973), or the position of the germinal vesicle, as in carp (Brzuska & Bieniarz, 1977) or the position of

the nucleoli in the GV as in cyprinids (Horvath, 1978). However, these criteria give only a partial indication and are very far, usually, from giving a 100 per 100 success. Even in trout, where we have a lot of experience, we know that the GV position is only an indicator which although it works well in more than 50 per 100 of cases cannot give an absolute certitude concerning the actual sensitivity, in vitro or in vivo.

IV. Mechanism of action of present treatments

Three main relays appear to follow one another between the stimulus of specific environmental factors and the start of oocyte maturation/ovulation process. Any treatment applied to the fish in order to induce successful maturation and ovulation must reach the oocyte and the follicle (ovulation), either directly or through the activation of one or several successive relays. They are : the Central Nervous System (CNS) and the Hypothalamus, the pituitary and the somatic tissues of the ovary. Each of these relays sends out a triggering information which may be composed of the release of a long lasting inhibiting signal and of the production of a stimulatory signal. Concerning methodologies for spawning control, mainly stimulating factors have been taken into account, i.e. : gonadotropins-releasing hormone (Gn-RH), gonadotropic hormone (GTH) and Maturation Inducing Steroids (MIS). However the competence of each relay to receive and send the specific stimulating information is regulated by other endocrine organs through secondary signals. The main one these is probably emitted by the ovary as a function of its state of development (feed-back).

Theoritically the most simple treatment would consist in a short-circuit of all relays by direct action of the MIS upon the oocyte. Taking into account the elements discussed in paragraph III, such an approach may encounter some risks : 1- inducing the maturation of non competent oocytes too precociously could be inefficient or produce eggs unable to develop normally. 2- eventhough the oocyte was competent, the treatment could be inefficient if the hypothesis was valid that a meiosis-inhibiting factor could be produced by the follicle and under gonadotropin control. 3- maturation might occur without subsequent ovulation, because of an insufficient functionnal differentiation of the follicle, under gonadotropin control, as observed, for example, in salmonids. In that case the negative feedback exerted by 17α-20βP upon the gonadotropin secretion (Jalabert & Breton, 1980) probably accentuated the gonadotropin deficiency. However, a convenient "priming" with a gonadotropin preparation was shown to bring a solution to the problem of ovulation, while the problem of oocyte competence for further development could be solved by the research of appropriate criteria. Practically, the successful ovulations obtained sometimes on non-hypophysectomized fish, using steroids alone, might be related to the fact that the fish were actually at a final stage before natural ovulation, but also that the steroids used, sometimes at high doses and which might not be specific MIS, could act synergically with the endogenous gonadotropin and/or be metabolized into specific MIS.

Hypophysation sensus stricto, i.e. the administration of various gonadotropic preparations, from crude pituitary extracts to gonadotropins from various zoological origins, have been the most widely utilized so far. Their action is primarely expected to occur directly upon the relay of ovarian somatic tissues, to induce simultaneously the synthesis of MIS, and the functionnal differentiation of the follicle for ovulation. In fact, several problems arise both from current practice and laboratory observations : 1. the problem of the zoological specificity of exogenous gonadotropic preparation has often been underlined (Burzawa-Gerard & Fontaine, 1972; Breton et al., 1973) but its consequences are difficult to evaluate for in vivo applications. 2. The problem of evaluating the actual activity of the different gonadotropins preparations used in practice (Yaron et al., 1982) is of general occurence. Moreover when crude pituitary extracts are used, they represent a complex mixture of various hormones which can interfere with the expected stimulation (see paragraph II). 3. Exogenous injections may promote a brutal surge of GTH (amoung other hormones when crude pituitary extracts are used). This artificial surge was observed in rainbow trout and coho salmon (Jalabert et al., 1978a, b), appearing completely different in shape to that which was found in naturally occuring ovulation (Fostier et al., 1982; Zohar et al., 1982a). In carp, although a pattern of GTH following hypophysation was also described (Jalabert et al., 1977; Weil et al., 1980), complete natural references are lacking to appreciate whether these variations are similar to the natural process. 4. It was reported that repeated injections of HCG could induce a "drug resistance effect" related to the production of antibodies against foreign proteins (The Freshwater Commercial Fish Artificial Propagation Work Group, 1977). To a lesser extent we might expect such a phenomenon to occur against crude pituitary extract.

Concerning the possible use of hypothalamic hormones acting upon the pituitary relay, the present limitations are related to several points : 1. The sensitivity of the pituitary even well loaded with GTH, may be GRIF-dependant. 2. The synthetic molecules of mammalian Gn-RH or analogues may be inappropriate in fish. 3. The catabolism of these molecules in vivo may be quick depending on the place of injection.

The mechanism of action of clomiphene citrate upon final maturation (recently revieved by Donaldson et al., 1982) is not completely

understood. Considering that estradiol appears to maintain a negative feedback on GTH release at this time (Bommelaer et al., 1981) clomiphene may probably act as an antiestrogen involving Gn-RH synthesis (Dixit, 1967; Viswanatan & Sundararaj, 1974).

V. Conclusion

Most of the present knowledge, although very incomplete, concerns the nature of trophic factors involved in the regulation of maturation/ovulation. The present use of these factors is purely empiric concerning the method of administration and generally based upon inaccurate criteria of ovarian development. Further progress involves :
- a more precise analysis of the shape of physiological trophic signals.
- a better knowledge of the mechanisms of negative regulation.
- a research of accurate criteria for ovarian competence.

References

Ball, J.N., 1981. Hypothalamic control of the Pars distalis in Fishes, Amphibian and Reptiles. Gen. Comp. Endocrinol. 44:135-170.

Barnett, F.H., J. Somn, S. Reichlin, I.M.D. Jackson 1982. Three luteinizing hormone-releasing hormone like substances in a teleost fish brain : none identical with the mammalian LH-RH decapeptide. Biochem. Biophys. Res. Commun. 105:209-216.

Billard, R., B. Breton, A. Fostier, B. Jalabert & C. Weil, 1978. Endocrine control of the teleost reproductive cycle and its relation to external factors : salmonid and cyprinid models. in Compar. Endocrinol. Gaillard P.T. and Boer LLH eds. Elsevier/North Holland Biomedical,Press-Amsterdam, 37-48.

Billard, R., C. Bry & C. Gillet, 1981. Stress environment and reproduction in teleost fish in "Stress and Fish" Edited by A.D. Pickering. Academic Press - London/N.Y.

Bommelaer, M.C., R. Billard & B. Breton, 1981. Changes in plasma gonadotropin after ovariectomy and estradiol supplementation at different stages at the end of the reproductive cycle in the rainbow trout (Salmo gairdneri R.). Reprod. Nutr. Develop. 21: 989-997.

Breton, B., R. Billard, B. Jalabert & G. Kann, 1972. Dosage radioimmunologique des gonadotropines plasmatiques chez Carassius auratus au cours du nycthémère et pendant l'ovulation. Gen. Comp. Endocr. 18:462-468.

Breton, B., R. Billard & B. Jalabert, 1973. Spécificité d'action et relations immunologiques des hormones gonadotropes de quelques téleostéens. Ann. Biol. Anim. Biochim. Biophys. 13:347-362.

Breton, B., B. Jalabert & C. Weil, 1975. Caractérisation partielle d'un facteur hypothalamique de libération des hormones gonadotropes chez la carpe. Gen. Comp. Endocrinol. 25:405-415.

Breton, B., L. Horoszewicz, K. Bieniarz & P. Epler, 1980. Temperature and reproduction in tench : effect of a rise in the annual temperature regime on gonadotropin level, gametogenesis and spawning. II. The female. Reprod. Nutr. Develop. 20:1011-1024.

Breton, B., A. Fostier, Y. Zohar, P.Y. Le Bail & R. Billard, 1982. Gonadotropine glycoproteique maturante et oestradiol-17β pendant le cycle reproducteur chez la truite fario (Salmo trutta) femelle. Gen. Comp. Endocrinol. in press.

Brzuska, E. & K. Bieniarz, 1977. Determination of the stage of ovarian maturity in female carps, in relation to injections of crude pituitary extracts (in Polish). Instytut Rybactwa Sroladowego, 105.

Burzawa-Gerard, E. & Y.A. Fontaine, 1972. The gonadotropins of lower vertebrates. Gen. Comp. Endocrinol., Supp. 3:715-728.

Burzawa-Gerard, E., B. Goncharov, A. Dumas & Y.A. Fontaine, 1976. Further biochemical studies on carp gonadotropin (c-GTH); biochemical and biological comparison of c-GTH and a gonadotropin from Acipenser stellatus Pall. (chondrostei). Gen. Comp. Endocrinol. 29:498-505.

Campbell, C.M., A. Fostier, B. Jalabert & B. Truscott, 1980. Identification and quantification of steroids in the serum of spermiating or ovulating rainbow trout. J. Endocrinol. 85:371-378.

Crim, L.W., E.G. Watts & D.M. Evans, 1975. The plasma gonadotropin profile during sexual maturation in a variety of salmonid fishes. Gen. Comp. Endocrinol. 27:62-70.

Dettlaff, T.A. & M.N. Skoblina, 1969. The role of germinal vesicle in the process of oocyte maturation in Anura and Acipenséridae Ann. Embryol. Morphol., Supp. 1:133-151.

Dettlaff, T.A. & S.I. Davydova, 1979. Differential sensitivity of cells of follicular epithelium and oocytes in the stellate sturgeon to unfavorable conditions, and correlating influence of triiodothyronine. Gen. Comp. Endocrinol. 39:236-243.

De Vlaming, V. & M.J. Vodicnik, 1978. Seasonal effects of pinealectomy on gonadal activity in the goldfish, Carassius auratus. Biol. Reprod. 19:57-63.

Dixit, V.P., 1967. Neurosecretion and feed back mechanism in clarias batrachus Linn. Ovariectomy and administration of exogenous sex hormones. Cellule 68:213-221.

Donaldson, E.M., G.A. Hunter & H.M. Dye, 1981a Induced ovulation in coho salmon (Oncorhynchus kisutch). II. Preliminary study of the use of LH-RH and two high potency LH-RH analogues. Aquaculture, 26:129-141.

Donaldson, E.M., G.A. Hunter & H.M. Dye, 1981b Induced ovulation in coho salmon (Oncorhynchus kisutch). III. Preliminary study on the use of the antiestrogen tamoxifen. Aquaculture 26:143-154.

Eckstein, B., 1975. Possible reasons for the infertility of grey mullets confined to

freshwater. Aquaculture 5:9-17.
Ekengren, B., J. Peute & G. Fridberg, 1978. Gonadotropic cells in the Atlantic Salmon, Salmo salar. Cell Tiss.Res. 191:187-203.
Fontaine, M., 1976. Hormones and the control of reproduction in aquaculture. J. Fish. Res. Bd. Canada 33:922-939.
Fostier, A., C. Weil, M. Terqui, B. Breton, & B. Jalabert, 1978. Plasma estradiol-17β and gonadotropin during ovulation in rainbow trout (Salmo gairdnerii). Ann. Biol. anim. Biochim. Biophys. 18:929-936.
Fostier, A., B. Breton, B. Jalabert & O. Marcuzzi, 1981a. Evolution des niveaux plasmatiques de la gonadotropine glycoproteique et de la 17α-hydroxy-20β-dihydroprogesterone au cours de la maturation et de l'ovulation chez la truite arc-en-ciel, Salmo gairdneri. C.R. hebd. Seances Acad. Sci. 293 D: 817-820.
Fostier, A., B. Jalabert, C. Campbell, M. Terqui & B. Breton, 1981b. Cinetique de libération in vitro de 17α-hydroxy-20β-dihydroprogesterone par des follicules de truite arc-en-ciel, Salmo gairdnerii. C.R. Acad. Sc. 292:777-780.
Goetz, F.W. & H.L. Bergman, 1978. The effects of steroids on final maturation and ovulation of oocytes from brook trout (Salvelinus fontinalis) and yellow perch (Perca flavescens). Biol. Reprod. 18:293-298.
Harvey, B.J. & W.S. Hoar, 1980. La reproduction provoquée chez les poissons : théorie et pratique. IDRC-Ottawa-48 p.
Hirose, K. 1972. Biological study on ovulation in vitro of fish. IV. Induction of in vitro ovulation in Oryzias latipes oocyte using steroids. Bull. Jap. Soc. Sci. Fish. 38:457-460.
Hirose, K., 1980. A bioassay for teleost gonadotropin using the germinal vesicle breakdown (GVBD) of oryzias latipes oocytes in vitro. Gen. Comp. Endocrinol. 41:108-114.
Hirose, K., T. Hirano & R. Ishida, 1974. Effect of salmon gonadotropin on ovulation in the ayu, Plecoglossus altivelis, with special reference to water balance. Comp. Biochem. Physiol. 47A:283-289.
Hontela, A. & R.E. Peter, 1978. Daily cycles in serum gonadotropin levels in the golfish effects of photoperiod, temperature and sexual condition. Can. J. Zool. 56:2430-2442.
Hontela, A. & R.E. Peter, 1980. Effects of pinealectomy, blinding and sexual condition on serum gonadotropin levels in the goldfish. Gen. Comp. Endocrinol. 40:168-179.
Horvath, L., 1978. Experiences in propagation of the common carp (Cyprinus carpio L.) out of the spawning season. Aqu. Hung. 1:66-72.
Hunter, G.A., E.M. Donaldson & H.M. Dye, 1981/1982. Induced ovulation in coho salmon (Oncorhynchus kisutch). I. Further studies on the use of salmon pituitary preparations. Aquaculture 26:117-127.
Idler, D.R., U.H.M. Fagerlund & A.P. Ronald, 1960. Isolation of pregn-4-ene-17α,20β-diol-3-one from the plasma of pacific salmon (Oncorhynchus nerka). Biochem. Biophys. Res. Commun , 2:133-137.
Idler, D.R. & B.T. Ng, 1979. Studies on two types of gonadotropins from both salmon and carp pituitaries. Gen. Comp. Endocrinol. 38:421-440.
Idler, D.R. & L.W. Crim, 1982. Gonadotropin releasing factor(s) (GTH-RF) from hypothalamus of winter flounder. Proccedings of the IXth International Symposium on Comparative Endocrinology - Hong-Kong. Abst. 20.
Iwamatsu, T., 1974. Studies on oocyte maturation of the medaka, Oryzias latipes. II. Effects of several steroids and calcium ions and the role of follicle cells on in vitro maturation. Annot. Zool. Japon, 47: 30-42.
Iwamatsu, T., 1978a. Studies on oocyte maturation in the medaka Oryzias latipes. V. On the structure of steroids that induce maturation in vitro. J. Exp. Zool., 204: 401-408.
Iwamatsu, T., 1978b. Studies on oocyte maturation of the medaka, Oryzias latipes. VI. Relationship between the circardian cycle of oocyte maturation and activity of the pituitary gland. J. Exp. Zool. 206:355-363.
Iwamatsu, T., 1978c. Studies on oocyte maturation of the medaka Oryzias latipes. VII. Effects of pinealectomy and mélatonin on oocyte maturation. Annot. Zool. Japon., 51: 198-203.
Iwamatsu, T., 1980. Studies on oocyte maturation of the medaka Oryzias latipes. VIII. Role of follicular constituents in gonadotropin and steroid induced maturation of oocytes in vitro. J. Exp. Zool. 211:231-239.
Iwamatsu, T. & T. Katoh, 1978. Maturation in vitro of oocytes of the loach, Misgurnus anguillicaudatus by steroid hormones and gonadotropins. Annot. Zool. Japon., 51:79-89.
Iwamatsu, T. & K. Takama, 1980. Inhibitory effect of rabbit serum factor on in vitro oocytes maturation of the teleost fish, Oryzias latipes. Develop. Growth. Differ. 22:229-235.
Iwamatsu, T. & T. Ohta, 1981. On a relationship between oocyte and follicle cells around the time of ovulation in the medaka, Oryzias latipes. Annot. Zool. Japon., 54:17-29.
King, J.A. & R.P. Millar, 1980. Comparative aspects of luteinizing hormone-releasing hormone structure and function in vertebrate phylogeny Endocrinol. 106:707-717.
Jalabert, B., 1975. Modulation par differents steroïdes non maturants de l'efficacité de la 17α-hydroxy-20β-dihydroprogesterone ou d'un extrait gonadotrope sur la maturation intrafolliculaire in vitro des ovocytes de la truite arc-en-ciel, Salmo gairdnerii. C.R. hebd. Seances Acad. Sci. 281D:811-814.
Jalabert, B., 1976. In vitro oocyte maturation and ovulation in rainbow trout, (Salmo gairdneri) northern pike (Esox lucius) and goldfish (Carassius auratus). J. Fish. Res. Bd. Canada 33:974-988.

Jalabert, B., 1978. Production of fertilizable oocytes from follicles of rainbow trout (Salmo gairdnerii) following in vitro maturation and ovulation. Ann. Biol. anim. Bioch. Biophys. 18:461-470.

Jalabert, B., B. Breton & C. Bry, 1972. Maturation et ovulation in vitro des ovocytes de la truite arc-en-ciel Salmo gairdneri. C.R. Acad. Sc. Paris 275, série D:1139-1142.

Jalabert, B. & D. Szöllösi, 1975. In vitro maturation of trout oocytes : effect of prostaglandins on smooth muscle-like cells of the theca. Prostaglandins 5:765-778.

Jalabert, B., B. Breton, E. Brzuska, A. Fostier & J. Wieniawski, 1977. A new tool for induced spawning : the use of 17α-hydroxy-20β-dihydroprogesterone to spawn carp at low temperature. Aquaculture 10:353-364.

Jalabert, B., B. Breton & A. Fostier, 1978a. Precocious induction of oocyte maturation and ovulation in rainbow trout (Salmo gairdneri): problems when using 17α-hydroxy-20β-dihydroprogesterone. Ann. Biol. anim. Bioch. Biophys. 18:977-984.

Jalabert, B., F.W. Goetz, B. Breton, A. Fostier & E. Donaldson, 1978b. Precocious induction of oocyte maturation and ovulation in coho salmon, Oncorhynchus kisutch. J. Fish. Res. Bd. Canada 35:1423-1429.

Jalabert, B. & B. Breton, 1980. Evaluation de la gonadotropine plasmatique t-GTH après ovulation chez la truite arc-en-ciel (Salmo gairdneri R.) et influence de la rétention des ovules. C.R. Acad. Sci., Série D 290: 799-801.

Nagahama, Y., G. Young & A. Kagawa, 1982. Steroidogenesis in the amago salmon (Oncorhynchus rhodurus) ovarian follicle : a two cell type model. Proceedings of the IXth International Symposium on Comparative Endocrinology, Hong-Kong.

Ng, T.B., C.M. Campbell & D.R. Idler, 1980. Antibody inhibition of vitellogenesis and oocyte maturation in salmon and flounder. Gen. Comp. Endocrinol. 41:233-239.

Ogata, H., T. Nomura & M. Hata, 1979. Prostaglandin $F_{2\alpha}$ changes induced by ovulatory stimuli in the pond loach, Misgurnus anguillicaudatus. Bull. Jap. Soc. Scient. Fish. 45:929-931.

Osborn, R.H., T.H. Simpson & A.F. Youngson, 1978. Seasonal and diurnal rhythms of thyroidal status in the rainbow trout, Salmo gairdneri R. J. Fish. Biol. 12:531-540.

Oshiro, T. & T. Hibiya, 1981. Water absorption of oocytes in the plaice, Limanda yokohamae during meiotic maturation and its role in rupturing follicles. Bull. Jap. Soc. Scient. Fish. 47:835-841.

Pendergrass, P. & P. Schroeder, 1976. The ultrastructure of the thecal cell of the teleost Oryzias latipes, during ovulation in vitro. J. Reprod. Fertil., 47:229-233.

Peter, R.E. & R. Billard, 1976. Effects of third ventricle injection of prostaglandins on gonadotropin secretion in goldfish, Carassius auratus. Gen. Comp. Endocrinol. 30:451-456.

Peter, R.E. & L.W. Crim, 1979. Reproducive endocrinology of fishes : gonadal cycles and gonadotropin in teleosts. Ann. Rev. Physiol. 41:323-335.

Peter, R.E. & C.R. Paulencu, 1980. Involvement of the preoptic region in gonadotropin release-inhibition in goldfish, Carassius auratus. Neuroendocrinology 31:133-141.

Peter, R.E., J.P. Chang & A.F. Cook, 1982. Neuroendocrine regulation of gonadotropin and growth hormone secretion in goldfish. Proceedings of the IXth International Symposium on Comparative Endocrinology- Hong-Kong. Abst. 64.

Pickering, A.D. & P. Christie, 1981. Changes in the concentrations of plasma cortisol and thyroxine during sexual maturation of the hatchery-reared brown trout, Salmo trutta L. Gen. Comp. Endocrinol. 44:487-496.

Pickford, G.E. & J.W. Atz, 1959. The physiology of the pituitary gland of fishes. New York Zoological Society. NY, 613 p.

Sakun, O.F., 1966. Transition to meiotic division of oocytes with uncomplete vitellogeny under the influence of hormonal stimulation in Salmo salar (Russian). Dokl. Akad. Nauk. 169:241-244.

Scott, A.P., V.J. Bye & S.M. Baynes, 1980. Seasonal variations in sex steroids of female rainbow trout (Salmo gairdneri Richardson). J. Fish. Biol. 17:587-592.

Scott, A.P., E.L. Sheldrick & A.P.F. Flint, 1982. Measurement of 17α,20β-dihydroxy-4-pregnen-3-one in plasma of trout (Salmo gairdneri R.). Seasonal changes and response to salmon pituitary extract. Gen. Comp. Endocrinol. 46:444-451.

Shehadeh, Z.H., C.M. Kuo & K.K. Milisen, 1973. Induced spawning of grey mullet Mugil cephalus L. with fractionated salmon pituitary extract. J. Fish. Biol. 5:471-478.

Sire, O. & J. Depeche, 1981. In vitro, effect of a fish gonadotropin on aromatase and 17β-hydroxysteroid dehydrogenase activities in the ovary of the rainbow trout (Salmo gairdneri Richardson). Reprod. Nutr. Dev. 21:715-726.

Sower, S. & C.B. Schreck, 1982. Steroid and thyroid hormones during sexual maturation of Coho salmon (Oncorhynchus kisutch) in seawater or freshwater. Gen. Comp. Endocrinol. 47:42-53.

Sower, S.A., C.B. Schreck & E.M. Donaldson, 1982. Hormone-induced ovulation of Coho salmon (Oncorhynchus kisutch) held in seawater and fresh water. Can. J. Fish. Aquat. Sci. 39:627-632.

Stacey, N.E., A.F. Cook & R.E. Peter, 1979. Ovulatory surge of gonadotropin in the goldfish Carassius auratus. Gen. Comp. Endocrinol. 37:246-249.

Sundararaj, B.I. & S.V. Goswami, 1977. Hormonal regulation of in vivo and in vitro oocyte maturation in the catfish, Heteropneustes fossilis (Bloch). Gen. Comp. Endocr. 32:17-28.

Sundararaj, B.I., S.V. Gosvami & V. Lamba, 1979. Some aspects of oocyte maturation in catfish. J. Steroid Biochem. 11:701-707.

Suzuki, K., Y. Nagahama & B. Tamaoki, 1981a. In vitro synthesis of an inducer for germinal vesicle breakdown of fish oocytes, 17α, 20β-dihydroxy-4-pregnen-3-one by ovarian tissue preparation of Amago salmon (Oncorhynchus rhodurus). Gen. Comp. Endocrinol. 45:473-481.

Suzuki, K., B. Tamaoki & K. Hirose, 1981b. In vitro metabolism of 4-pregnenes in ovaries of a freshwater teleost, the Ayu (Precoglossus altivelis): Production of 17α-20β dihydroxy-4-pregnen-3-one and its 5β-reduced metabolites, and activation of 3β-and 20β-hydroxysteroid dehydrogenases by treatment with a fish gonadotropin. Gen. Comp. Endocrinol. 45:533-535.

Szöllösi, D., B. Jalabert & B. Breton, 1978. Postovulatory changes in the theca folliculi of the trout. Ann. Biol. anim. Bioch. Biophys. 18:883-892.

The Freshwater commercial fish artificial propagation work groups in Fujian, 1977. Acta Biochim. Biophys. Sinica, 9:15-23.

Theofan, G., 1981. The in vitro synthesis of final maturational steroids by ovaries of brook trout Salvelinus fontinalis and yellow perch Perca flavescens. Ph. D. Dissertation - University of Notre Dame - USA

Viswanathan, N. & B.I. Sundararaj, 1974. Response of hypothalamohypophyseal ovarian system of the catfish Heteropneustes fossilis to administration of oestrogen and androgen. Anat. Pec. 178:525 abstr.

Vivien, 1941. Contribution à l'étude de la physiologie hypophysaire dans ses relations avec l'appareil génital, la thyroïde et les corps suprarenaux chez les poissons selaciens et teleostéens Bull. Biol. Fr. Belg. 75:257-309.

Wallace, R.A. & K. Selman, 1978. Oogenesis in Fundulus heteroclitus. I. Preliminary observations on oocyte maturation in vivo and in vitro. Dev. Biol. 62:354-369.

Wallace, R.A. & K. Selman, 1980. Oogenesis in Fundulus heteroclitus. II. The transition from vitellogenesis into maturation. Gen. Comp. Endocrinol. 42:345-354.

Weil, C., B. Breton & P. Reinaud, 1975. Etude de la reponse hypophysaire à l'administration de Gn-RH exogene au cours du cycle reproducteur annuel chez la carpe, Cyprinus carpio L. C.R. Acad. Sci. Paris, Série D 280:2469-2472.

Weil, C., R. Billard, B. Breton & B. Jalabert, 1978. Pituitary response to LH-RH at different stages of gametogenesis in the rainbow trout (Salmo gairdneri). Ann. Biol. anim. Bioch. Biophys. 18:863-869.

Weil, C. A. Fostier, L. Horvath, S. Marlot & M. Berscenyi, 1980. Profiles of plasma gonadotropin and 17β-estradiol in the common carp, Cyprinus carpio L., as related to spawning induced by hypophysation or LH-RH treatment. Reprod. Nutr. Dev. 20:1041-1050.

Wright, R.S. & S.M.V. Hunt, 1982. A radioimmunoassay for 4-pregnene-17α,20β-diol-3-one its use in measuring changes in serum level at ovulation in atlantic salmon, Coho salmon and rainbow trout. Gen. Comp. Endocrinol. 46:p.396, Abst. 145.

Yamazaki, F., 1962. Effects of hypophysectomy on the ovulation, oviposition and sexual behavior in the goldfish, Carassius auratus Bull. Fac. Fish. Hokkaido Univ. 13:39-46.

Yaron, Z., A. Bogomolnaya & V. Hilge, 1982. Application of an in vitro bioassay for fish GTH in improving harvest of carps pituitaries. Proceedings of the International Symposium on Reproductive Physiology of Fish. Wageningen - Netherlands.

Zohar, Y., B. Breton & R. Billard, 1982a. Short-term profiles of plasma gonadotropin levels in the female rainbow trout throughout the reproductive cycle. Gen. Comp. Endocrinol. 46:p. 396, Abst. 146.

Zohar, Y., B. Breton & A. Fostier, 1982b. Gonadotropic function during the reproductive cycle of the female rainbow trout, Salmo gairdneri, in relation to ovarian steroid secretion : in vivo and in vitro studies. Proceedings of the International Symposium on Reproductive Physiology of Fish, Wageningen, Netherlands.

EXPERIMENTAL MATURATION OF EUROPEAN SILVER EELS

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Abstract

Work on experimental maturation of eels has been made by us in our institute since 1958. In our work on HCG-matured male eels we have studied the effects of dose and environmental parameters (temperature, salinity etc.). In our experiments on female eels treated with carp pituitary extracts we arrived at a method which resulted in mature eggs and partly spawning. Also sexual behaviour was studied. Attempts at fertilization resulted in early embrynic development only. Finally a brief account is given of recent experiments on repeated maturation of males and of an attempt to compare the effects of carp-pituitary and salmon-pituitary extracts in maturation experiments with female eels.
Keywords: European Eel, maturation, gonadotrophins.

The Danish eel catches are bigger than those of other European countries. Economically evaluated the eel is our fish number three among fishes for consumption, only surpassed by cod and plaice. More than 2ooo papers on the biology of the eel have been written - but still we are almost ignorant about fundamental parts of its complex life-cycle. This is true for age and growth in nature, sex differentiation etc., but especially as far as its oceanic life concerns: the migration to the Sargasso Sea and the breeding in this distant area.

Eels leave European continental waters in a condition well adapted for migration (silver eels), but with quite immature gonads. We join their cycle in the Sargasso Sea where tiny larvae with rudiments of yolk sacs are found in the upper water layers of an apparently well-defined area. What lies between is unknown. An adult European eel was never caught in the Sargasso Sea or in the open ocean at all.

From European coastal waters we have a few quite unique reports (2 or 3) on sexually mature eels with fully developed spermatozoa and also a few female eels with large, but not normally developed ovaria. Such specimens are as rare as calves born with two heads and they only confirm the rule that silver eels delay their sexual maturation until their long journey is well over.

In the era before hormone treatment of eels was established eel-workers have made many laborious experiments in order to induce sexual maturity. The matter was not simple. Environmental parameters such as temperature, salinity, light, pressure and others have been changed more or less systematically and also ingenious exercisers have been invented in order to make the eel feel as if it were on migration or in the breeding area. None of these efforts have resulted in the slightest change in the eel's gonadal structure.

As early as 193o the late Anton Bruun and his team started hormone experiments with eels. You may say that these experiments are still running. Since about 1958 they were taken over by us.

Our intensions were (and still are) the following: by inducing sexual maturity we try in our laboratory to throw light on the almost completely unknown phases in the eel's life-story: the migration and the breeding biology. Our work is not an endocrinological work as such. We make use of the hormones rather as tools for provoking results which could contribute to our general understanding of the eel's natural history.

What is related by us in the following is mainly based on our two latest papers: that on male eels from 1967 and that on females from 198o. Finally we give some hitherto unpublished results on repeated maturation of male eels and a comparison between the effects of carp- and salmon-pituitary extracts upon female silver eels.

Successfull experimental maturation of male silver eels was achieved by French and Italian workers during the thirties of this century. The eels were treated with urin from pregnant women.

In our experiments we used a standardized preparation of HCG. Much effort was made in order to establish a proper dosage. We arrived at a dosage of 25o IU HCG/eel/week given until the eel

gave off sperm when stripped.

The full maturation cycle was described by us for male silver eels at body weights of about 8o g treated with the above mentioned "standard procedure" and under experimental "standard conditions" which were: a temperature of 14 ± 2°C, 27 per mille salinity and diffuse daylight. We charactherized the sexual cycle by seven developmental stages, stage 1 being the immature Syrski organ, stages 4 and 5 the fully ripe stages and stage 7 the post-spawned, depleted testes resembling the original Syrski organ very much. When the matured eel starts to give off sperm when stripped the developmental stage of testes will be close to stage 3.

This standard maturation formed a starting point for experiments in which we changed experimental environmental parameters such as light, salinity, temperature etc. Only temperature was seen to affect the rate of induced maturation. The relation between maturation rate and temperature was described as:

$$T = 13.2\ (1+\exp(-0.22(t-19.9)))$$

where T is the number of days elapsed for reaching stage 3 and t the temperature. Complete maturation was achieved in the interval 13.2 - 25.5°C only. A possible thermal optimum for the maturation process was indicated to about 2o°C and a biological zero was calculated to 11.o°C.

The results described make it likely that the eel matures at temperatures close to 2o°C. In the Sargasso Sea such temperatures are met with in the breeding season at depths of about 15o metres.

We have several reasons to believe that the European silver eels do not feed at all during their migration and spawning. Also we have strong reasons to suppose that they spawn only once during their life-time.

Recently we have been able to induce repetition of full sexual cycles (stt. 1-7) in male silver eels without intermediate feeding. These experiments (not earlier published) are mentioned shortly next.

Successfully repeated maturation was established in two different experimental runs started 6.2.1976 and 27.11.1978. Only the 1978-experiment is described here.

6o male silver eels (38 ± 2 cm long) were caught in the Baltic (Oct. 1978) and kept in seawater (15°C, 3o p.m.S) until 1978, Nov. 27th. This day (D1) they were injected with looo IU HCG and transferred to warm seawater (22°C). 283 days later (1979, Sept. 6th, D2) 15 out of the surviving 43 eels were re-injected with looo IU HCG.

In the period between D1 and D2 14 of the eels had died (and 3 escaped). Inspection of their gonads at death demonstrated that their sexual cycle from stages 1 to 7 was passed in about loo days. 14 eels we sacrified for control at the D2-day. They were all in stage 7.

The 15 re-injected eels were stripped for sperm 25 days after D2. Result: lo of the eels were re-matured. Stripping 4o days after D2 gave still 2 more ripe eels. The remaining 3 eels had not responded upon the second hormone treatment. They we sacrified 69 days after D2 showing gonads in stage 7.

Out of the 12 re-matured eels 3 died 29, 66 and 68 days after D2. Their development of testes ranged from stage 3 to stage 6-7. The 9 survivors were sacrified at D2 = 69. Developmental range: stage 5 to stage 7.

The induced repeated maturation described does not necessarily reflect the eel's natural habits. Fontaine, 1982, describes cases in which both male and female eels started feeding after induced spawning. He proposes a hypothesis of "land-locked" eel populations and concludes that further research is required. In our laboratory such work has been initiated this year. The intention of the workers (Dollerup and Graver) is to provoke feeding activity between two periods of induced maturity in male silver eels. Their experiments are followed up by studies of intestinal histology.

The paper by Fontaine et al., 1964, and the later appearing Japanese works inspired us to resume our work on female eels. Our results from the period 1973-79 are given in our paper 198o.

As in our work on the males we aimed to establish a "standard procedure" of maturation. We arrived at: 15 mg carp pituitary + 5oo IU HCG per eel (at body weights about 1 kg) given twice a week until fully mature. Experiments were carried out in seawater (3o p.m.S) at 23°C. We arrived at GSI-values up to 61. Attempts at fertilization, however, were successful only in a few cases, and the embryonic development ceased at very early stages (gastrula).

An important biological parameter was stated from the experiments: the fecundity was shown to range between o.7 and 2.6 million eggs for eels at body weights between o.5 and 2 kg.

The experimentally produced eggs (about 1.1 mm in diameter) gave us a background for seaking up possible eel eggs in the classic Danish material from the Sargasso Sea. We found none.

The sexual behaviour was studied by bringing matured eels together in separate tanks. The courtship was described between the sexes both of which had considerably enlarged eyes.

Ovaries and somatic bodies of matured female eels were analysed for contents of water, lipid and protein. Based on the calculated energy reserves a rough estimate of an energy budget was established. It was provided that the eel does not feed during migration.

The energy available for migration and spawning was calculated to cover the costs of a 4ooo km active migration. In his "new solution to the Atlantic eel problem" Tucker, 1959, suggests that the European eel will never reach the breeding area in the western Atlantic. If so, the reason is not the lack of sufficient energy reserves.

Since 1979 we have continued our maturation work on female eels. We have focused at a comparison between the effects of acetone dried carp and salmon pituitaries. Our background was the fact that the successful maturation and hatching results of Japanese workers were obtained by means of salmon pituitaries.

Unfortunately our experimental eels were affected strongly by diseases (Vibrio, etc.) during the last two seasons. The infection, however, did not seem to influence the developmental rate of the ovaries, but far the greater part of our eels died well before maturity.

Four experimental runs (9o eels) were carried out in which standard treatments with carp and salmon pituitary were run simultaneously. Very guardedly we give the conclusion that the two treatments have had very much the same effect.

References

Boëtius, I. & J. Boëtius, 1967. Studies in the European Eel, Anguilla anguilla (L.). Experimental induction of the male sexual cycle, its relation to temperature and other factors. Meddr. Danm. Fiskeri- og Havunders. N.S. 4: 339-4o5.

Boëtius, I. & J. Boëtius, 198o. Experimental maturation of female silver eels, Anguilla anguilla. Estimates of fecundity and energy reserves for migration and spawning. Dana 1: 1-28.

Fontaine, M., E. Bertrand, E. Lopez & O. Callamand, 1964. Sur la maturation des organes génitaux de l'Anguille femelle (Anguilla anguilla L.) et l'émission spontanée des oeufs en aquarium. Comp. Rend. Acad. Sci. 259: 29o7-291o.

Fontaine, M., N. Delerue-LeBelle, F. Lallier & E. Lopez, 1982. Toute les Anguilles succombent elles après la reproduction et frayent elles nécessairement en mer? Comp. Rend. Acad. Sci. 294: 8o9-811.

Tucker, D.W., 1959. A new solution to the Atlantic eel problem. Nature 183: 495-5o1.

APPLICATION OF LH-RH AND LH-RH ANALOGUES TO THE INDUCED FINAL MATURATION AND OVULATION OF COHO SALMON (ONCORHYNCHUS KISUTCH).

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Summary

Studies on the use of LH-RH analogues to induce ovulation in the coho salmon Oncorhynchus kisutch are reported. Two potent LH-RH analogues are shown to induce ovulation in coho salmon either alone or with a priming injection of partially purified salmon gonadotropin.
Keywords: salmon, Oncorhynchus, ovulation, LH-RH, gonadotropin.

Introduction

Since the first studies on controlled reproduction in teleosts were conducted a half century ago in South America (Houssay, 1930; Ihering, 1937) significant strides have been made towards more efficient and reproducible procedures. These "first generation" techniques involved the use of homologous or heterologous pituitary extracts from mature donor fish. Later refinements involved the use of preserved pituitary glands or partially purified gonadotropin preparations (Donaldson, 1973). Also during this period parallel procedures using mammalian gonadotropins especially HCG either alone or in conjunction with fish pituitary extracts were developed. Problems with the availability of sufficient fish pituitary glands and with standardization have led to the search for synthetic alternatives to gonadotropin and the development of "second generation" techniques for the induction of final maturation and ovulation. These techniques involve the regulation of the production and/or release of endogenous gonadotropin e.g. gonadotropin releasing hormones and antiestrogens or the provision of ovarian hormones normally produced directly or indirectly by gonadotropin stimulation e.g. progestins and prostaglandins.

The subject of the present paper is the development of one of these second generation techniques, i.e. the use of gonadotropin releasing hormones, for induced ovulation in the Pacific salmon. In our initial studies on this subject we investigated the use of the mammalian gonadotropin releasing hormone (LH-RH) alone and with a priming injection of partially purified salmon gonadotropin (SG-G100). We also investigated the use of two high potency LH-RH analogues preceded by priming injections of SG-G100 (Donaldson et al., 1981). In the sutdies presented here we report further studies on the use of high potency LH-RH analogues with the SG-G100 primer and also present studies on the use of high potency LH-RH analogues alone.

Materials and Methods

The experiments reported in this study were conducted at the West Vancouver Laboratory of the Fisheries Research Branch in the fall of 1979 and 1980.

The experimental animals used were adult 3 yr old coho salmon Oncorhynchus kisutch from the Capilano Salmon Hatchery, North Vancouver. Fish were transported by truck to the laboratory and held in 3 m fiberglas tanks under natural photoperiod. These tanks were supplied with aerated well water at 10.0-10.5°C.

The experiments conducted in 1979 and 1980 were initiated on Oct. 29 and Oct. 28, respectively. Fish were allowed to acclimate for a minimum of 3 days prior to treatment. Before treatment,fish were screened to select only those individuals which had not undergone germinal vesicle breakdown (GVBD). The procedures followed for the anesthetization, weighing, tagging and interperitoneal (i.p.) injection of fish have been described by Hunter et al. (1978). Similarily spawning procedures and methods used for the determination of egg survival to the eyed stage and to hatching have been reported by Hunter et al. (1978).

Physiological saline was used as the vehicle for all preparations at 0.4 ml/kg body wt. The partially purified salmon gonadotropin SG-G100 used was prepared according to procedures described by Donaldson et al. (1972) and Donaldson (1973) from chinook salmon pituitaries.

In the 1979 study, the synthetic LH-RH analogue D-Ala^6, des Gly^{10}-LH-RH(1-9) ethylamide, (LH-RHA DAla^6) was supplied by Ayerst Research Laboratories while the[D-Ser(Bu^t)^6]-LH-RH(1-9) ethylamide, analogue (LH-RHA DSer(Bu^t)^6) was supplied by Hoechst Canada Inc. The LH-RHA DAla^6 used in 1980 was supplied by Penninsula

Table 1. Induced ovulation in coho salmon using the gonadotropin releasing hormone analogues LH-RHA DAla^6 and LH-RHA DSer(Bu^t)^6 preceded by a priming injection of salmon gonadotropin (SG-G100) or LH-RHA.

Experiment	Adult females Number	Mean wt ± SD/kg	Injection protocol µg/kg			Cummulative % ovulation
			Day 0		Day 3	
1979 LH-RH primer	7	2.25 ± .83	6.6	DAla^6	33 DAla^6	100
	7	2.1 ± .35	20	DAla^6	100 DAla^6	100
	6+	2.3 ± .46	60	DAla^6	300 DAla^6	83.3
	7	2.18 ± .61	6.6	DSer(Bu^t)^6	33 DSer(Bu^t)^6	85.7
	7	1.82 ± .47	20	DSer(Bu^t)^6	100 DSer(Bu^t)^6	100
	7	2.49 ± .32	60	DSer(Bu^t)^6	300 DSer(Bu^t)^6	100
			Day 0		Day 3	
1979 SG-G100 primer	7	2.0 ± .53	100	SG-G100	30 DSer(Bu^t)^6	100
	7	2.25 ± .62	100	SG-G100	300 DSer(Bu^t)^6	100
	7	2.37 ± .73	100	SG-G100	30 DAla^6	100
	7	2.42 ± .61	100	SG-G100	300 DAla^6	100
	7	2.22 ± .46	100	SG-G100	Saline	100
	7	2.69 ± .82		Saline	Saline	85.7
			Day 0		Day 2,3,4	
1980 SG-G100 primer	8	2.5 ± .64	100	SG-G100	11 DAla^6	100
	8	2.2 ± .57	100	SG-G100	33 DAla^6	100
	8	2.15 ± .37	100	SG-G100	100 DAla^6	100
	8	2.06 ± .23	100	SG-G100	11 DAla^6	100
	8	2.14 ± .59	100	SG-G100	33 DAla^6	100
	8	1.95 ± .62	100	SG-G100	100 DAla^6	100
	8	1.95 ± .37	100	SG-G100	Saline	100
	8	1.91 ± .42		Saline	Saline	60

+ One fish accidentally killed.

Laboratories Inc., and Syndel Laboratories Ltd.

1979 LH-RH analogue primer

In 1979, 6 groups of 7 fish were administered priming injections of the LH-RHA DAla^6 or LH-RHA DSer(Bu^t)^6 at 6.6, 20 or 60 µg/kg body wt. These were followed on Day 3 by injections of the analogues at 33, 100 or 300 µg/kg body wt, respectively (Table 1).

1979 SG-G100 primer

On Day 0, 5 groups of 7 females were administered an i.p. injection of SG-G100 at 100 µg/kg body wt. On Day 3, one of these groups received a saline injection while the other 4 received 30 or 300 µg/kg injection of either the DSer(Bu^t)^6 or DAla^6 analogues. A sixth group was administered saline on both Day 9 and Day 3. This latter group served as a control for both studies conducted in 1979.

1980

In 1980, 7 groups of 8 fish were administered a priming injection of SG-G100 at 100 µg/kg body wt. On Days 2, 3 and 4, 6 of these groups received injections of the DAla^6 analogue at 11, 33 or 100 µg/kg body wt. The seventh group received saline on Days 2, 3 and 4. An eighth group received saline on Days 0, 2, 3 and 4 (Table 1).

Results

SG-G100 primer

The results of the experiments involving the use of the SG-G100 primer are presented in Table 1. Cumulative percent ovulations of test groups and SG-G100 and saline control groups for 1979 are presented in Figs. 1 and 2, respectively, while those conducted in 1980 are presented in Figs. 3 and 4, respectively. In 1979, groups administered the SG-G100 primer followed by either the DAla^6 or DSer(Bu^t)^6 analogues; reached 100% cumulative ovulations by either Day 9 or 12. However, the group which received the SG-G100 primer alone also reached the 100% level by Day 9. The saline control group was 57 and 71% ovulated by Days 9 and 12, respectively.

In 1980 the three groups administered 11, 33 or 100 µg of the DAla^6 analogue/kg body wt reached 100% cumulative ovulations on Days 15, 12 and 10, respectively. The groups administered the SG-G100 primer alone did not reach the 100% level until Day 15. On Day 15 60% of the fish in the saline

control group were ovulated.

LH-RH primer

Cumulative % ovulations of the groups administered a DSer(Bu^t)^6 or DAla^6 analogue primer followed by a second injection of the analogue at 5 x the priming dosage are presented in Fig. 5. Cumulative % ovulations of the saline control group are presented in Fig. 2. With the exception of the lowest dosage of the DSer(Bu^t)^6 analogue all test groups reached the 80% level of ovulations by Day 7 or 9 (Fig. 5). The group which received the SG-G100 primer alone reached 71 and 100% cumulative ovulations on Days 7 and 9, respectively (Fig. 2). The percentages of ovulated salmon in the saline control group on these 2 days were 43 and 57% (Fig. 2).

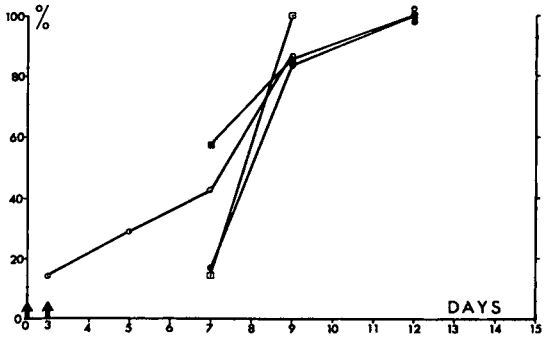

Fig. 1. Cumulative percent ovulation in adult female coho salmon administered i.p. injections of SG-G100 at 100 µg/kg body wt on Day 0 followed by a second injection of LH-RHA DAla^6 at 30 (⊙) or 300 (⊡) µg/kg or LH-RHA DSer(Bu^t)^6 at 30 (●) or 300 (■) µg/kg on Day 3. Arrows indicate injection days.

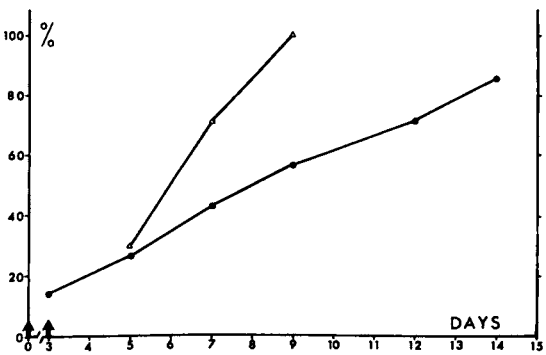

Fig. 2. Cumulative percent ovulation in adult female coho salmon administered i.p. injections of either SG-G100 (△) at 100 µg/kg body wt or saline (●) on Day 0 followed by saline injections on Day 3.

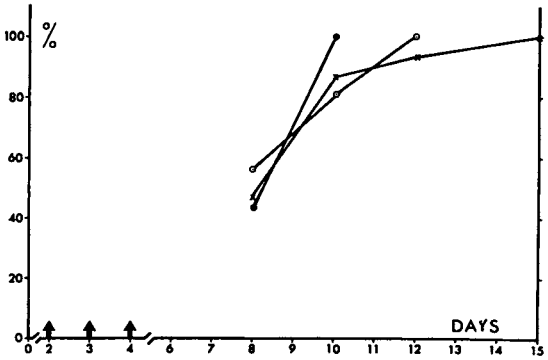

Fig. 3. Cumulative percent ovulation in adult female coho salmon administered i.p. injections of SG-G100 at 100 µg/kg body wt on Day 0 followed by injections of LH-RHA DAla^6 at 11 (×), 33 (⊙) or 100 (●) µg/kg body wt on Days 2, 3 and 4.

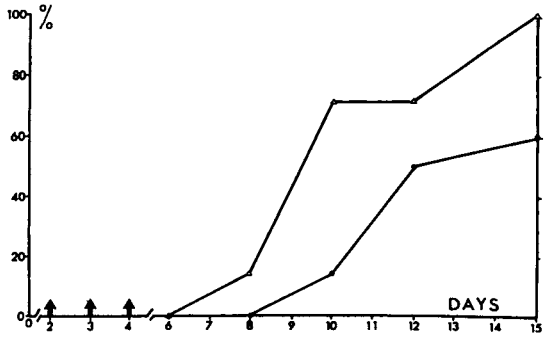

Fig. 4. Cumulative percent ovulation in adult female coho salmon administered i.p. injections of either SG-G100 (△) at 100 µg/kg body wt or saline on Day 0 followed by saline injections on Days 2, 3 and 4.

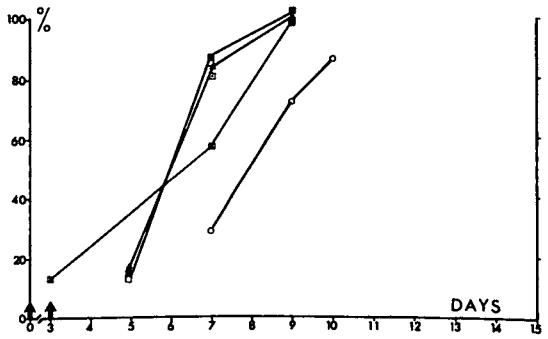

Fig. 5. Cumulative percent ovulation in adult female coho salmon administered i.p. injections on Days 0 and 3 of LH-RHA DAla^6 at 6.6-33 µg/kg (□), 20-100 µg/kg (⊠) or 60-300 µg/kg (⊡), or LH-RHA DSer (Bu^t)^6 at 6.6-33 µg/kg (○), 20-100 µg/kg (▲) or 60-300 µg/kg (■).

Survival of the eggs from all test groups to the eyed stage and to hatching ranged from 89-100% and 81-97%, respectively.

Discussion

In our initial studies on the induction of ovulation in coho salmon using LH-RH and its high potency analogues it was evident that LH-RH was not effective alone for the induction of ovulation. On the other hand, it was possible to induce ovulation with LH-RH analogues used in conjunction with an SG-G100 primer (Donaldson et al., 1981). In the above study the 100 µg/kg SG-G100 primer was not tested alone as it had been shown earlier to be below the critical dosage required to induce ovulation (Jalabert et al. 1978). It is evident from the current study that a single injection of 100 µg/kg SG-G100 is capable of inducing ovulation in coho salmon which are close to maturity with or without LH-RH analogue. However, in the 1980 experiment (Fig. 3) there appeared to be a dose related synergism between the SG-G100 and the LH-RHA DAla^6 in which the high dose (100 µg/kg LH-RHA DAla^6) group ovulated faster than the other two groups.Further evidence for the effectiveness of the SG-G100 primer followed by LH-RHA was provided by a study on the induced ovulation of coho salmon held in seawater or freshwater conducted in Oregon (Sower et al., 1982).

Furthermore, in the experiment in which the two analogues were tested alone (Fig. 5) all treatments except the low dosage of LH-RHA DSer(Bu^t)^6 were as effective as 100 µg/kg SG-G100 for inducing ovulation in the coho salmon. The study provides the first experimental evidence for induction of ovulation in a salmonid with LH-RH analogue alone and suggests that LH-RHA DAla^6 may be slightly more effective than LH-RHA DSer(Bu^t)^6. We have recently obtained experimental evidence for the difference in potency in the salmonid between LH-RH and its potent analogues. A single intraperitoneal injection of 0.2 mg LH-RH resulted in a plasma gonadotropin (GtH) concentration of 14 ng/ml at 1.5 hr which declined to a base line value of 5 ng/ml at 24-48 hr. On the other hand, injection of 0.2 mg LH-RHA DAla^6 resulted in a GtH concentration of 20 ng/ml at 1.5 hr and of 27 ng/ml at 24-48 hr. Fish in the former group had not undergone final maturation after 96 hr while most of those in the latter group had completed GVBD (Van Der Kraak et al., 1982). This data indicates that the effectiveness of the LH-RH analogue is correlated with the magnitude and duration of the increase in plasma GtH concentration which follows its administration. In recent studies conducted in 1981 we have obtained ovulation with a single injection of LH-RHA. A primer of LH-RHA followed by a single secondary injection of LH-RHA was even more effective. On the other hand, a combination of SG-G100 and LH-RHA in a single injection was more effective than a single injection of LH-RHA alone but less effective than two separate injections. These findings are currently being correlated with concommitant changes in the plasma gonadotropin profile (Van Der Kraak et al., 1981, unpublished). Collectively, the results indicate that the synthetic LH-RH agonists show considerable potential as second generation ovulation inducers in Pacific salmonids.

References

Donaldson, E.M. 1973. Reproductive endocrinology of fishes. Am. Zool. 13: 909-927.

Donaldson, E.M., F. Yamazaki, H.M. Dye & W.W. Philleo. 1972. Preparation of gonadotropin from salmon (Oncorhynchus tshawytscha) pituitary glands. Gen. Comp. Endocrinol. 18: 469-481.

Donaldson, E.M., G.A., Hunter & H.M. Dye, 1981. Induced ovulation in coho salmon (Oncorhynchus kisutch). II. Preliminary study on the use of LH-RH and two high potency LH-RH analogues. Aquaculture. 26: 129-141.

Houssay, B.A., 1930. Accion sexual de la hipofisis en los perces y reptiles. Rev. Soc. Argent. Biol. 6: 686-688.

Hunter, G.A., E.M. Donaldson, E.T. Stone & H.M. Dye, 1978. Induced ovulation of female chinook salmon (Oncorhynchus tshawytscha) at a production hatchery. Aquaculture. 15: 99-112.

Ihering, R. Von, 1937. A method for inducing fish to spawn. Prog. Fish-Cult. 34: 15-16.

Jalabert, B., F.W. Goetz & B. Breton, A. Fostier & E.M. Donaldson, 1978. Precocious induction of oocyte maturation and ovulation in coho salmon(Oncorhynchus kisutch). Fish. Res. Board Canada. 35: 1423-1429.

Sower, S.A., C.B. Schreck & E.M. Donaldson, 1982. Hormone induced ovulation of coho salmon (Oncorhynchus kisutch) held in sea water and fresh water. Can. J. Fish. Aquat. Sci. 39: 627-632.

Van Der Kraak, G., H.R. Lin, E.M. Donaldson, H.M. Dye & G.A. Hunter, 1982. Effects of LH-RH and des-Gly^{10}[D-Ala^6]LH-RH-ethylamide on plasma gonadotropin levels and oocyte maturation in adult female coho salmon (Oncorhynchus kisutch). Gen. Comp. Endocrinol. in press.

INDUCED BREEDING OF GREY MULLET, MUGIL CEPHALUS L.

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ICLARM Contribution No. 71

Abstract

This paper describes the present status of artificial propagation of the grey mullet (Mugil cephalus) including manipulation of breeding time and induced breeding. Predictable spawning can be induced by administration of piscine pituitary gonadotropin and human chorionic gonadotropin. The synthetic steroid, deoxycorticosterone (DOC) can also be used when oocytes have reached the subperipheral germinal vesicle stage (dosage 120 mg/kg fish) following a priming injection of gonadotropin, which is essential for bringing oocytes at yolk globule stage to final maturation. The results of using gonadotropin followed by DOC are compared with those using gonadotropins alone.
Keywords: induced-breeding, hypophysation, gonadotropin, deoxycorticosterone, Mugil cephalus.

Introduction

The initiation and course of gametogenesis is controlled by environmental and endocrinological factors. In females vitellogenesis is the main developmental phase and in many captive teleosts oocytes remain at the tertiary yolk globule stage. Their final maturation often requires exogenous hormone treatments. Hypophysation and exogenous hormone treatments have been widely used since Houssay's work (1931)—piscine pituitaries, mammalian gonadotropins, steroids and synthetic luteinizing releasing hormone (LH-RH)—but have always suffered from a lack of standardization between workers, particularly where spawning is induced by injections of fresh or preserved pituitary material. Lack of standardization can be mainly attributed to inadequate descriptions of the potency of pituitary preparations, the sexual maturity of recipients, and the dosage and injection procedures used.

Shehadeh et al. (1973a) and Kuo et al. (1974) described work with captive broodstock of grey mullet (Mugil cephalus). Attempts at induced breeding of grey mullet by administration of exogenous hormones were first made by Tang (1964). This paper summarizes recent progress using quantified gonadotropin preparations and the synthetic steroid deoxycorticosterone.

Oogenesis and environmental factors

Vitellogenesis in captive grey mullet begins towards the end of October in Hawaii and oocytes develop to the tertiary yolk globule stage as in the breeding season, though the rate of development varies among individuals. Vitellogenesis can be induced by manipulation of photoperiod and temperature regimes out-of-season: 6L/18D at 17-26°C initiates vitellogenesis after 7-9 weeks irrespective of preconditioning photoperiod; 6L/18D at a constant temperature of 21°C gives the best result for environmental advancement of vitellogenesis and prevents atresia. Vitellogenesis is accelerated at lower temperatures (17-19°C) but the quality of oocytes is questionable as their yolk deposition is less. Such eggs fail to develop beyond blastula stage. Oocyte development at 24°C can be accelerated by salmon gonadotropin injections (s-GTH) (Fig. 1).

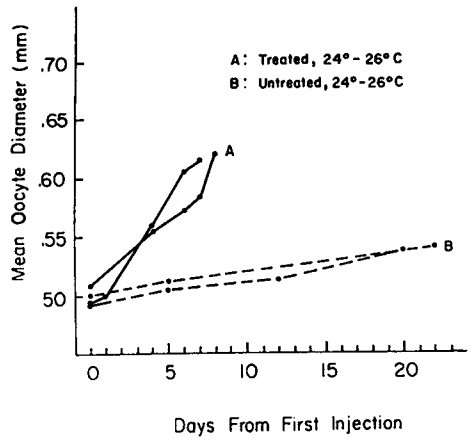

Fig. 1. Effect of s-GTH injections on oocyte development.

Induced breeding

1. The state of recipients

Recipient fish must be at the proper stage of ovarian development. Identification of this stage is critical. Various methods have been used: external anatomical characteristics, the microscopic appearance of oocytes (Sundararaj and Goswami, 1969), and physiological parameters associated with sexual maturation such as elevated plasma proteins and calcium concentration (Booke, 1964; Woodhead, 1968).

For grey mullet, however, the degree of sexual maturity is best expressed in terms of developmental stage of oocytes and their mean diameter.

Oocytes are removed by biopsy from unanaesthetised females using a polyethylene cannula of 0.8 mm 1 D inserted 6 to 7 cm (depending on length) into the ovaries through the oviduct (Shehadeh et al., 1973b). Oocytes from the mid-portion of the ovary are the most representative. Samples from the extremities should be avoided.

Hypophysation gives predictable spawning only if begun when the oocytes are at the tertiary yolk globule stage and the mean oocyte diameter is at least 0.6 mm and preferably larger than 0.65 mm.

2. Hormone treatments

The total dose of s-GTH required to induce spawning has been described elsewhere (Shehadeh et al., 1973a; Kuo et al., 1974; Kuo and Nash, 1975). It is inversely proportional to oocyte diameter and can be estimated from a regression line (Fig. 2) to be given in two injections: one-third of the total dose intramuscularly as a priming dose (which reinitiates final maturation) and the remainder at attainment of subperipheral stage, usually 24 hr later. Ovulation occurs after about 8 hr and oviposition after about 12 hr from the 2nd injection. Spawning behavior is initiated by the first release of a small number of ripe eggs which attract a male to respond and to release spermatozoa. Spawning in a 200-l

aquarium is very rapid, and is completed within 30 seconds from the first release of eggs. The first cleavage of the fertilized eggs can be observed in 55-65 minutes at 24°C (32‰ salinity).

The same procedure is followed using human chorionic gonadotropin (HCG). A total of 50 IU/g body wt is required in two injections. A priming injection (16.7 IU/g body wt) is used. The effectiveness of HCG depends upon its purity. Clinical grades (e.g., Antuitrin-Parke Davis and APL-Ayerst) are equally effective and spawning can be expected after about 10 hr. HCG-SIGMA, however, gave spawning after 12 hr and multiple oil-globule eggs were frequently observed (though these were fertilizable).

Administration of the priming dose, whether s-GTH or HCG, does not alter the mean diameter or diameter-frequency distribution of oocytes but at ovulation and oviposition, mean diameter increases dramatically to 0.825 and 0.923 mm, respectively, regardless of oocyte size at injection. Rapid increases in the water content, electrolytes and total osmolarity of oocytes occur.

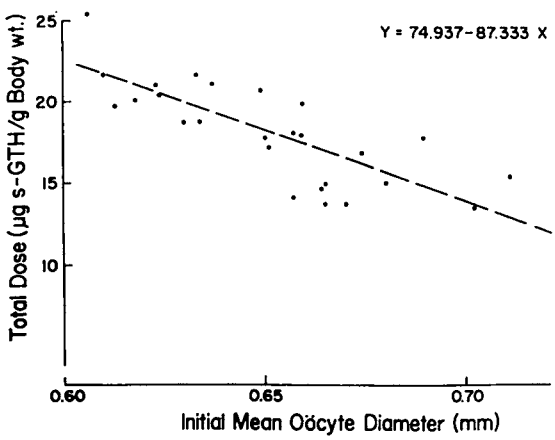

Fig. 2. Total dose of s-GTH required to complete final maturation.

A mixture of s-GTH and HCG for priming and spawning injections is also effective (Table 1): 1 mg s-GTH = 2250 IU HCG. Spawning can also be induced by a priming injection of acetone-dried carp pituitary homogenate (CPH: Stoller Fisheries, Iowa), 50 mg/kg followed by either s-GTH or HCG as above.

3. Confirmation of the necessity for exogenous hormone treatments

A female containing 0.668 mm mean diameter eggs was left with two running males for three days. No change in the oocyte size or frequency distribution was observed and no spawning occurred. The female was then given s-GTH doses as above and spawning occurred 10 hr after the spawning injection (9.19 g s-GTH/g body wt: total dosage, 14.85 µg/g body wt). This confirms that captive mature mullet are unable to complete final maturation and spawning without triggering by hormone administration. Induction of final oocyte maturation by manipulation of environmental conditions has yet to be determined.

4. Induced-breeding with Deoxycorticosterone (DOC)

Spawning was induced by a priming injection of either CPH or s-GTH, followed by a spawning injection of DOC at 50-157.1 mg/kg (Table 1). The response of recipients to DOC varied with the injection dose and spawning season, being more effective during the peak of the breeding season and with doses of about 100-130 mg DOC/kg. Spawnings were completed in 36 to 38 hr from the priming injection. Mean oocyte diameters were 0.823-0.831 mm and 0.923-0.944 mm at ovulation and oviposition, respectively. The responses of functional mature mullet females to repeated daily DOC injections at 50 and 100 mg/kg are shown in Fig. 3. No change in the oocyte diameter and appearance was observed unlike obvious increases when using s-GTH.

Further work with DOC

1. DOC binding in vivo

A female containing oocytes at the tertiary yolk globule stage was first injected with 4.7 mg/kg s-GTH, and the oocytes at subperipheral stage were obtained in 24 hr, when 100 µCi of ^{3}H-DOC was administered intramuscularly and the ^{3}H-DOC binding was followed for different subcellular fractions: a membrane fraction (precipitated at 800 x g), a mitochondrial fraction (precipitated at 10,500 x g) and the cytosol (supernatant at 10,500 x g). The highest binding level was noticed 15 min after the injection, followed by a steep decline. A binding peak was also observed at 6 hr (Fig. 4). In general, the DOC was mostly bound by the membrane fraction (31.8-80.5% of total binding) followed by mitochrondria fraction (13.3-52.8%).

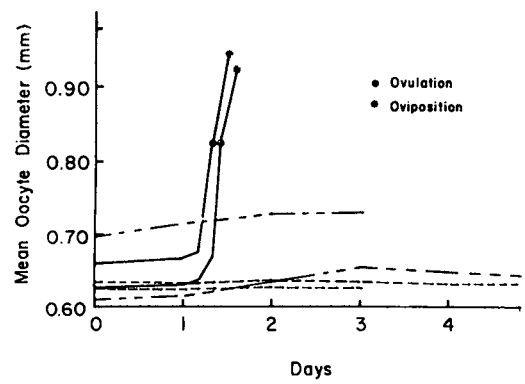

Fig. 3. Response of mature mullet females to injections of s-GTH and DOC. ——— : s-GTH, three times a week; — · · — : DOC, 50 µg/g daily; — — — : DOC, 100 µg/g daily.

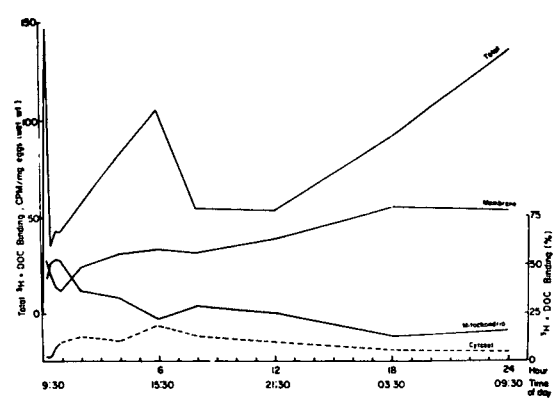

Fig. 4. DOC binding in vivo.

2. DOC binding with oocyte homogenate in vitro

Homogenates containing 20 mg oocytes and 40 ng ^{3}H-DOC were incubated at 24°C for various periods. Binding increased rapidly during the first hour and reached equilibrium after 4-5 hr, followed by a decline. The binding of ^{3}H-DOC (5 ng) with the membrane fraction of the oocytes is further shown in Fig. 5. The binding increases proportionally with the amount of oocyte tissue up to 100 mg homogenate. The binding capacity of oocyte homogenate (50 mg wet wt) increases linearly with the concentration of ^{3}H-DOC up to 5 ng, and does not differ between 4-hr and 5-hr incubation period.

To determine the specificity of the binding of the oocyte homogenate with DOC, homogenates containing 50 mg of oocytes and 2.5 ng of ^{3}H-DOC were incubated for 4 hr with various concentrations of cold hormones, hydrocortisone and corticosterone. The inhibition curves of the steroids examined were very similar (Fig. 6), though corticosterone seemed to compete slightly more in the binding with oocyte homogenate to ^{3}H-DOC. The specific binding of oocyte homogenate to ^{3}H-DOC reduced to 50% of total binding when the corticosterone and hydrocortisone concentration was increased beyond 10 μg and 100 μg, respectively.

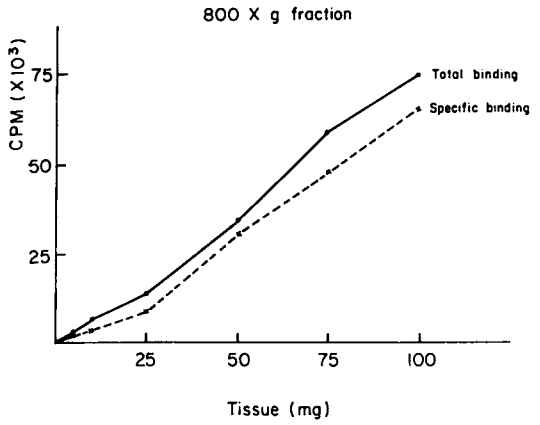

Fig. 5. Binding of DOC (5 ng) with membrane fraction of oocytes in vitro.

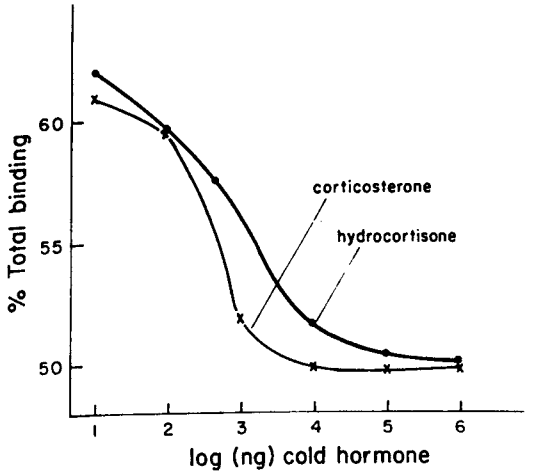

Fig. 6. Competitive binding of hydrocortisone and corticosterone with membrane fraction of oocytes.

Table 1. Injection dose, schedule and response of grey mullet females to gonadotropins and deoxycorticosterone (DOC).

Body wt (g)	Initial oocyte diameter (mm)	1st injection	2nd injection	Spawning hr after 2nd injection	Fertilization (%)
		A			
		s-GTH[1] (μg/g)	s-GTH (μg/g)		
872	0.651	5.7	11.4	12.0	98
980	0.711	5.1	10.2	11.8	83
714	0.623	7.0	14.0	12.3	92
1010	0.665	5.0	9.9	10.0	93
717	0.664	7.0	14.0	11.5	98
862	0.613	5.8	13.9	10.5	87
		B			
		HCG[2] (IU/g)	HCG (IU/g)		
759	0.645	19.6	30.4	15.0	98
908	0.621	19.3	38.6	13.2	90
1032	0.625	17.2	34.4	12.0	94
		C			
		s-GTH (μg/g)	HCG (IU/g)		
600	0.679	6.7	33.3	12.2	96
557	0.652	9.0	44.9	13.0	94
		D			
		CPH[3] (μg/g)	HCG (IU/g)		
900	0.632	55.6	33.3	10.5	95
750	0.646	66.7	33.3	10.0	91
900	0.640	55.6	33.3	10.2	90
1000	0.674	50.0	30.0	11.7	83
800	0.667	62.5	31.3	9.5	94
750	0.687	66.7	33.3	9.5	77
		E			
		CPH (μg/g)	DOC (μg/g)		
1000	0.704	50.0	100.0	12.0	68
850	0.662	58.8	117.7	12.5	86
1000	0.690	50.0	100.0	10.8	74
900	0.629	50.0	105.6	12.2	90
1100	0.635	50.0	95.5	12.5	92
900	0.634	55.6	111.1	11.4	91
1000	0.657	50.0	130.0	13.5	92
900	0.677	55.6	122.2	10.2	72
850	0.674	58.8	141.2	16.0*	73
700	0.707	71.4	157.1	16.2*	94
1100	0.611	50.0	136.4	20.3*	82
		F			
		s-GTH (μg/g)	DOC (μg/g)		
600	0.662	7.8	110.0	11.5	91
745	0.618	6.7	50.0	23.0	82
693	0.633	7.2	50.0	21.5	84
835	0.634	4.8	50.0	21.0	76

1. s-GTH: Salmon pituitary gonadotropin (SG-G100).
2. HCG: Human chorionic gonadotropin
3. CPH: Carp pituitary homogenate (acetone dried)
*Experiment was conducted toward the end of natural breeding season.

Discussion

Ovarian development in grey mullet can be accelerated by manipulation of temperature (17-21°C) or by injection of gonadotropin preparations, but without further hormone stimulation captive females will not complete the final oocyte maturation in captivity, and oocytes will undergo atresia and degenerate. Oocytes become responsive to exogenous gonadotropins and DOC when they have reached the subperipheral germinal vesicle stage.

The action of piruitary gonadotropins on final oocyte maturation is known to be mediated through steroid hormones (Jalabert, 1976). In vitro ovulation has been induced effectively by cortisone in Misgurnus fossilis (Kirshenblatt, 1959) and by deoxycorticosterone acetate (DOCA) in Heteropneustes fossilis (Goswami and Sundararaj, 1971). The ability of gonadotropins to promote corticosteroidogenesis has been demonstrated by Sundararaj and Goswami (1969, 1971). They proposed that gonadotropins may not act directly on the ovary to stimulate ovulation, but via the interrenal tissue by stimulating corticosteroid production. By contrast, final oocyte maturation can be induced in vitro by carp pituitary gonadotropin or progesterone in Salmo gairdneri (Jalabert et al., 1972). Progesterone or hydrocortisone in Oryzias latipes (Hirose, 1972), and in hypophysectomized goldfish in vivo (Yamazaki, 1965). More recently, Jalabert et al. (1977) concluded that the action of gonadotropins on final maturation is mediated through steroid hormones; 17α hydroxy-20β dihydroprogesterone (17α-20β P) appears to be the most likely natural mediator of oocyte maturation in rainbow trout, northern pike and goldfish (Fostier et al., 1973; Jalabert, 1976).

Spawning of grey mullet has been repeatedly induced by a priming injection of carp pituitary homogenate, s-GTH, or HCG, followed by a spawning injection of DOC. The priming injection merely stimulates the oocyte development from the tertiary yolk globule to the subperipheral germinal vesicle stage in 24 hr and this can be achieved only by treatments with a gonadotropin preparation.

The use of steroid hormones targeted at the gonads to induce final maturation and spawning after a priming dose of gonadotropin is a technique that merits further investigation. Steroids, such as DOC and 17α-20 βP, are relatively cheap and available as pure preparations. They could give culturists a useful method for timing the final stage leading up to spawning of captive broodstock.

Ootcytes are capable of not only synthesizing DOC but also of binding exogenous DOC which is detectable in a very short time after receiving in viyo administration. Furthermore, the exogenous DOC is bound in various subcellular fractions, though mostly in the membrane fraction.

References

Booke, H.E. 1964. Blood serum protein and calcium levels in yearling brook trout. Progr. Fish Cult 26:107-110.

Fostier, A., B. Jalabert, and M. Terqui. 1973. Action prédominate d'un dérivé hydroxyle de la progesterone sur la maturation in vitro des ovocytes de la truite arc-en-ciel, Salmo gairdneri. C.R. Acad. Sci. 277 Sci. Ser. D. 421-424.

Goswami, S.V., and B.I. Sundararaj. 1971. In vitro maturation and ovulation of oocytes of the catfish Heteropneustes fossilis Bloch. Effects of mammalian hypophysial hormones, catfish pituitary homogenate, steroid precursors and metabolites, and gonadal and adrenocortical steroids. J. Exp. Zool. 178: 467-478.

Hirose, K. 1972. Biological study on ovulation in vitro of fish. IV. Induction in vitro ovulation in Oryzias latipes oocytes using steroids. Bull. Jap. Soc. Sci. Fish. 38:457-461.

Houssay, B.A. 1931. Action sexuelle de l'hypophyse sur les poissons et les reptiles. C.R. Soc. Biol. 106:377-378.

Jalabert, B. 1976. In vitro oocyte maturation and ovulation in rainbow trout (Salmo gairdneri), northern pike (Esox lucius), and goldfish (Carassius auratus). J. Fish. Res. Board Can. 33: 974-988.

Jalabert, B., B. Breton, and C. Bry. 1972. Maturation et ovulation in vitro des ovocytes de la truite arc-en-ciel Salmo gairdneri. C.R. Acad. Sci. 275:1139-1142.

Jalabert, B., B. Breton, E. Brzuska, A. Fostier, and J. Wieniawski. 1977. A new tool for induced spawning. the use of 17α - hydroxy - 20β - dihydroprogesterone to spawn carp at low temperature. Aquaculture 10:353-364.

Kirshenblatt, I.D. 1959. Effect of cortisone on ovaries of the loach. Bull. Eksper. Biol. Med. USSR. 47:108-112.

Kuo, C.-M., C.E. Nash, and Z.H. Shehadeh. 1974. A procedural guide to induced spawning in grey mullet (Mugil cephalus L.). Aquaculture 3:1-14.

Kuo, C.-M., and C.E. Nash. 1975. Recent progress on the control of ovarian development and induced spawning of the grey mullet (Mugil cephalus L.) Aquaculture 5:19-29.

Shehadeh, Z.H., C.-M. Kuo, and K.K. Milisen. 1973a. Induced spawning of grey mullet Mugil cephalus L. with fractionated salmon pituitary extract. J. Fish. Biol. 5:471-478.

Shehadeh, Z.H., C.-M. Kuo, and K.K. Milisen. 1973b. Validation of an in vitro method for monitoring ovarian development in the grey mullet (Mugil cephalus L.). J. Fish. Biol. 5:489-496.

Sundararaj, B.I., and S.V. Goswami. 1969. Role of interrenal in luteinizing hormone-induced ovulation and spawning in the catfish, Heteropneustes fossilis (Bloch). Gen. Comp. Endocrinol. Suppl. 2: 374-384.

Sundararaj, B.I., and S.V. Goswami. 1977. Hormonal regulation of in vivo and in vitro maturation in the catfish, Heteropneustes fossilis (Bloch). Gen. Comp. Endocrinol. 32: 17-28.

Tang, Y.A. 1964. Induced spawning of striped mullet by hormone injection. Jap. J. Ichthyol. 12:23-38.

Woodhead, P.M.J. 1968. Seasonal changes in the calcium content of the blood of arctic cod. J. Mar. Biol. Assoc. U.K. 48:81-91.

EXPERIMENTS ON ARTIFICIAL MATURATION AND FERTILIZATION OF THE JAPANESE EEL (*ANGUILLA JAPONICA*)

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Summary

Gonadal maturation of Japanese eels was induced in females by injection of chum salmon pituitary homogenate and in males by injection of Synahorin or human chorionic gonadotropin. Milt was obtained after 6 injections and full-grown oocytes were obtained after 8-15 injections. However, only a small proportion of the oocytes of females underwent final maturation in response to this treatment. Final maturation and ovulation could be induced *in vitro* by steroid treatment but not by salmon gonadotropin (SG-G100) treatment.

Mature oocytes were spherical, white and transparent with a diameter of 1.0 mm. A high percentage of larvae hatched out from artificially-inseminated oocytes kept in seawater at 23°C. Hatching occurred 38-45 hr after fertilization and the newly-hatched larvae measured about 2.9 mm in body length; pectoral fins were not visible but the membranous fin was well developed. The larvae were kept at 23°C on the day of hatching. Subsequently, the larvae were maintained at 19°C. At 14 days after hatching, they were about 7.0 mm long; teeth, jaw and pectoral fins were developed. The larvae did not survive for longer periods. This may be due to the lack of suitable food and/or failure of the yolk sac to provide sufficient nutrition.
Keywords: Japanese eel, artificial maturation, gonadotropin, larvae.

Introduction

The life history of the eel is complex and the ecology of migration and spawning are still open to question. Although the breeding place of the European eel has been detected, the morphology and ecology of eel larvae, especially in preleptocephalus stages, remain to be investigated. Apart from these biological interests, in Japan it is important that a reliable supply of the larvae is established, since eel culture is extensive.

We and other investigators have succeeded in obtaining eggs and larvae of the Japanese eel, following artificial induction of maturation in aquaria. However, there remains numerous problems to be solved, as will become apparent in the present description of the experimental maturation of Japanese eels in Japan.

Observations and discussion

Experimental maturation of males

Because of the difficulty in obtaining male silver eels, cultivated males have been used in experimental maturation in Japan. Synahorin (mixture of hypophysial and chorionic gonadotropin; Teikoku Zoki) has been used as the gonadotropic hormone, but owing to suspension of its production, human chorionic gonadotropin (HCG: Teikoku Zoki or Sankyo Zoki) has been substituted (Motonobu *et al.*, 1976; Matsumaru and Ishii, 1979; Satoh, 1979).

Cultivated male eels (200-300g in body weight) were purchased from a commercial eel supplier in Hakodate. They were kept in circulating seawater tanks with a capacity of 5.5 gallons at 19°C. Intramuscular injections of Synahorin (50 RI/fish) or HCG (250 IU/fish) were given once a week. Fish were not fed throughout the experimental period.

The testis of cultivated eels prior to treatment had resting or dividing germ cells, in contrast to the testis of silver males which had spermatocytes. However, in cultivated males treated with 2-3 Synahorin injections, spermatocytes could be found. These males showed marked secondary sex characters and after 6 injections spermatozoa could be extruded by stripping the abdomen (Yamamoto *et al.*, 1972). Male eels treated with HCG showed similar development of the testis as to those treated with Synahorin.

However, these methods of experimental maturation of cultivated males are not totally satisfactory, particularly since these eels had sperm of high viscocity and of small quantity. In eels from which sperm could be extruded, the sperm ratio represented by the relative amount of spermatozoa to the total germinal element showed low values (50-75%) relative to other fish (e.g., more than 95% in the chum salmon, *Oncorhynchus keta*; Hiroi and Yamamoto, 1968). This low value seems to be related to incomplete spermatogenesis and spermiation. Since these processes are believed to be regulated by gonadal steroid hormones, particularly androgens and progestogens, the measurement of plasma steroid profiles during gonadotropin-induced testicular development may identify and help to solve some of these problems.

Experimental maturation of females

Experimental maturation of female silver Japanese eels has been carried out by using different methods. These methods can principally be classified as follows: methods

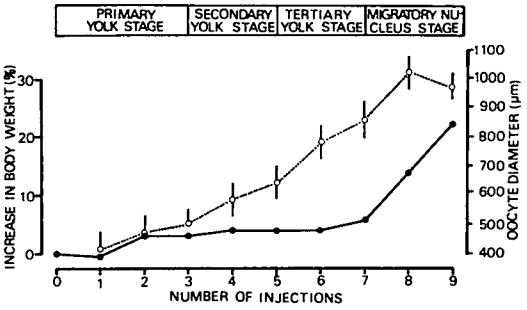

Fig. 1. Correlation between changes in body weight (solid line) and oocyte diameter (broken line) in female Japanese eels injected with chum salmon pituitary homogenate.

using Synahorin or HCG (Ochiai *et al.*, 1974; Satoh, 1979), and those using fish pituitary homogenate (Yamamoto *et al.*, 1974; Yamauchi *et al.*, 1976; Motonobu *et al.*, 1976; Matsumaru and Ishii, 1979), the latter being more effective than the former in obtaining mature eggs.

Silver eels were caught during their downstream migration in the Hiranuma river (Aomori prefecture) during October and November. Fish were transferred to Hakodate and raised in seawater tanks in a manner similar to that described for male eels. Intramuscular injections of chum salmon pituitary homogenate (2 pituitaries/500g BW) were given weekly.

Both body weight and oocyte diameter increased progressively during the treatment (Fig. 1). Vitellogenesis was completed after 6-8 injections and the oocyte diameter attained the maximum size of about 1.0 mm after 7-9 injections, when the germinal vesicle was situated at the periphery of the ooplasm near the animal pole (migratory nucleus stage). The average number of oocytes at this stage was about 1,300,000 per ovary. Serum vitellogenin levels assessed by the single radial immuno-diffusion technique (in collaboration with Dr. Hara) showed a marked increase from about 100 μg/ml vitellogenin to 5000-10,000 μg/ml after the first injection and apart from a tendency to decrease in the middle of the treatment these levels were essentially maintained throughout the experimental period. One exception to this pattern was in fish in which initial control values were very high; the treatment appeared to induce a slight increase. Body weight increased slowly until 9-10 injections had been administered and then sharply increased. In a small proportion of animals ovulation occurred 1-3 injections after the beginning of the sharp increase in body weight, but the majority did not undergo maturation and ovulation and body weight subsequently repidly declined. The increase in body weight seems to be directly related to hydration of

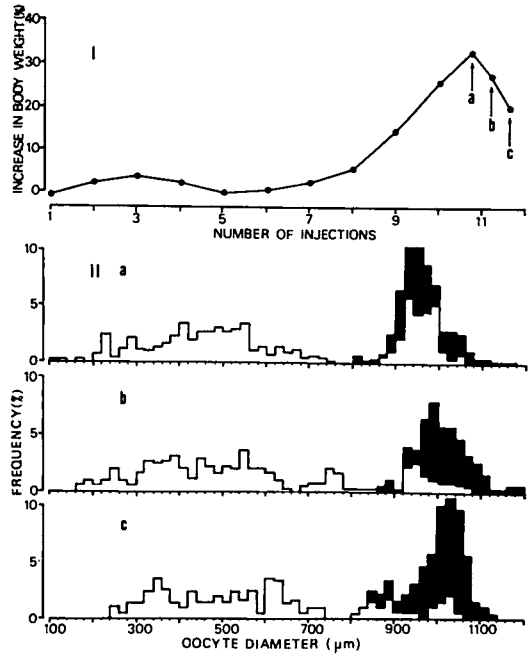

Fig. 2. Changes in body weight (I) and frequency distribution (II) of oocytes in female Japanese eels injected with chum salmon pituitary homogenate. Biopsy was carried out at the time of (a), 2 days after (b), and 5 days after (c) the peak in body weight. The shaded area in II signifies degenerated oocytes.

the oocytes, since the water content of the ovary increased in parallel with the rise in body weight. In contrast, the water content of the liver and muscle remained unchanged.

To help understand the failure of the majority of fish to undergo maturation and ovulation, the diameter of the oocytes at the time of the peak in body weight and during the decline in body weight was assessed by biopsy. As shown in Fig. 2, the ovary contained oocytes at various developmental stages, from the primary yolk stage to the migratory nucleus stage. A large percentage of the biggest oocytes were degenerated at the peak of body weight and this percentage increased as the body weight declined. It is not clear at present whether this size distribution of oocytes would naturally occur or if it is abnormal, induced by the hormone treatment. If it is a natural phenomenon, it may suggest that eels spawn more than once in a reproductive season.

Radioimmunoassay analysis of serum for various steroids during the treatment period showed that estradiol-17β (method of Kagawa *et al.*, 1981) levels remained low (1-2 ng/ml) in the period in which body weight remained essentially unchanged, and levels increased with the sharp increase in body weight; values were however stikingly inconsistent

between individuals, ranging between 2-10 ng/ml. These observations on Estradiol-17β levels are difficult to reconcile with the numerous report of increased Estradiol-17β levels associated with vitellogenesis and decreased levels in the post-vitellogenic period.

Full-grown immature oocytes could be induced to mature *in vitro* with various steroids, the most effective being progesterone. However, a subsequent experiment showed that 17α,20β-dihydroxy-4-pregnen-one was the most effective maturation-inducing steroid. 17α-Hydroxyprogesterone levels were undetectable (less than 30 pg/ml) in the of both ovulated and non-ovulated females. Since progestogens are known to be directly involved in the induction of final oocyte maturation in a number of teleosts, the lack of evidence for progestogen synthesis in eels which underwent final oocyte maturation is difficult to explain, particularly as 17α-hydroxyprogesterone is effective for the induction of final maturation in eel oocytes *in vitro*. In contrast, partially purified chinook salmon gonadotropin (SG-G100) at a concentration of 1 and 10 μg/ml had no effect on maturation *in vitro*. This *in vitro* data suggests that the oocytes have the capacity to respond to steroidal maturational agents, and that the follicle however is in some way deficient, and cannot sythesize maturational steroids in responese to gonadotropin. Nonetheless ultrastructural observations on the follicle revealed special thecal cells of active appearance which possessed characteristics of steroid secreting cells. The failure of SG-G100 to induce final oocyte maturation may be due to several possibilities including a specificity of the follicle for eel gonadotropin and/or the incomplete development of the follicle with regard to the synthesis of maturation inducing steroid(s). As estradiol-17β levels are high during hydration of the oocyte there is also the possibility of inhibition of maturation by this steroid by an action on the follicle, as reported in rainbow trout (Jalabert, 1976), if estradiol-17β is produced in response to SG-G100 *in vitro*.

In several species, it appears that a preovulatory surge of gonadotropin is necessary for final oocyte maturation and ovulation to occur. Cytological evidence suggests that this is also the case in the eel. After a series of injections of pituitary homogenate, pituitary GTH cells accummulated hormone granules which disappeared immediately after ovulation (Fig. 3). In contrast eels which did not ovulate possessed GTH cells displaying heavy granulation. These results suggest that the processes controlling vitellogenesis and oocyte maturation may not be sequential in the eel and that suitable hormone treatment for accelerating vitellogenesis is not necessarily effective for maturation and ovulation. The potential use of LH-RH as an agent for induced maturation and ovulation, by causing an GTH surge was explored but efforts were unsuccessful. However, there are reports of a positive effect of LH-RH on maturation and ovulation in Japanese eels, although the success rate was low (Motonobu *et al.*, 1976; Satoh, 1979). Further investigation may however allow the present method of experimental maturation to be refined to include this agent.

As the foregoing indicates, a number of problems remain to be overcome in the experimental maturation of Japanese eels. Further work on the physiological changes that occur after treatment over long periods with gonadotropic preparations should help identify the shortcomings of the present method.

Development of eggs and larvae

The spherical ripe eggs obtained were transparent with a diameter of about 1.0 mm. They were inseminated in large glass petri dishes by the dry method with sperm collected from 2-3 males induced to mature by hormone treatment. A suitable temperature for development of eggs seems to be in the range of 20°-25°C, judging from rate of development of eggs from 3 hr after fertilization to hatching. Hence, the development of the fertilized eggs in normal seawater was observed at 23°C (Fig. 4-a,b). These eggs kept at 23°C hatched out 38-45 hours after fertilization.

The optimum rearing water temperature showed a tendency to decrease with time after hatching and larvae were reared at 23°C on the day of hatching and subsequently main-

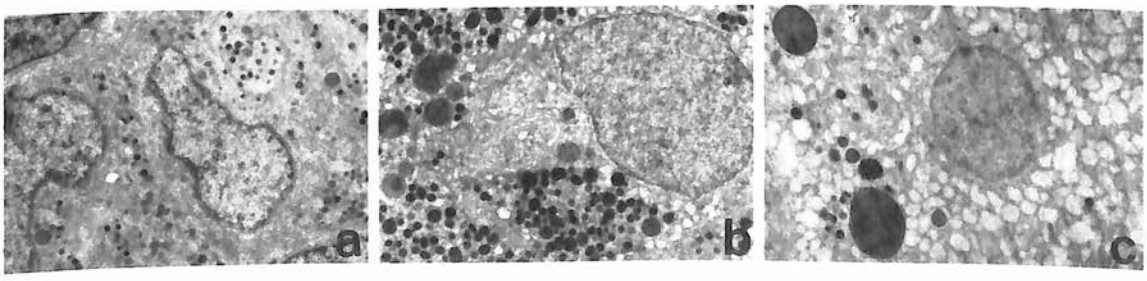

Fig. 3. Electron micrographs of gonadotrophs in the pituitary gland of female Japanese eels injected with chum salmon pituitary homogenate. a, initial control; b, eel with ovary containing oocytes at the migratory nucleus stage; c, eel soon after ovulation. a-c, × 4800.

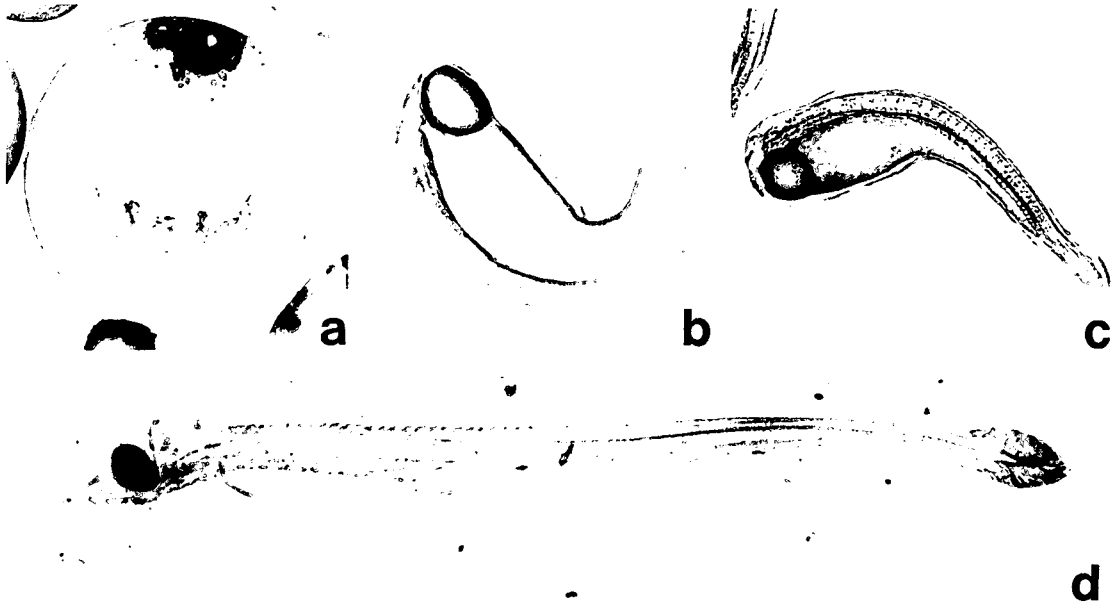

Fig. 4. Living eggs at: a, eight-cell stage, × 37; b, heart formation stage, × 37. Living larvae: c, just after hatching, × 23; d, 14th day after hatching, × 19.

tained at 19°C.

The larvae survived for 14 days. Newly-hatched larvae were about 2.9 mm in body length, and they measured 7.0 mm long by 14 days after hatching; teeth, jaw and pectoral fins were developed (Fig. 4-c,d). The larvae did not survive for longer periods. Satoh (1979) reported that larvae given a certain zooplankton could survive for longer periods, up to 17 days. The mortality of larvae may thus be related to the lack of suitable food. Related to this is the possibility that the short survival of larvae may be linked to an insufficient development of the yolk supply during vitellogenesis. This suggestion arises from the biopsy data during oocyte growth which revealed oocytes in all stages of development and from the observation that the survival period of larvae varied between individual females, ranging from 5-14 days. This has also been reported by other workers. The causes of larval mortality may thus be rooted in the vitellogenic period.

Conclusion

Spermatogenesis and vitellogenesis can be stimulated by gonadotropic preparations but subsequent "maturation process" of the germ cells (spermiation and final oocyte maturation) only occur in a very low proportion of animals. Preliminary physiological data suggests that a lack of development of gonadal somatic elements involved in steroidogenesis may account for these observations. Larvae appear to develop normally for up to 14-17 days and then die. Two possible and related factors have been tentatively identified, lack of suitable external food supply, and insufficient supply of nutrients from the yolk sacs, possibly due to incomplete vitellogenesis.

References

Hiroi, O. & K. Yamamoto, 1968. Studies on the maturation of salmonid fishes. I. Changes in the testis of the chum salmon, *Oncorhynchus keta*, during anadromous migration. Bull. Fac. Fish. Hokkaido Univ., 19:173-184.

Jalabert, B., 1976. *In vitro* oocyte maturation and ovulation in rainbow trout (*Salmo gairdneri*), northern pike (*Esox lucius*), and goldfish (*Carassius auratus*). J. Fish. Res. Bd. Can. 33:974-988.

Kagawa, H., K. Takano & Y. Nagahama, 1981. Correlation of plasma estradiol-17β and progesterone levels with ultrastructure and histochemistry of ovarian follicles in the white spotted-char, *Salvelinus leucomaenis*. Cell Tissue Res., 218:315-329.

Marumatsu, Y. & T. Ishii, 1979. Studies an artificial maturation of female Japanese eels. Bull. Chiba Pref. Fish. Exp. Stn. 3:13-17. (in Japanese).

Motonobu, T., K. Yamashita & H. Oka, 1976. A memoir on the hatched larvae of the Japanese eel, *Anguilla maponoca*, from the adults matured artificially. Bull.

Shizuoka Pref. Fish. Exp. Stn. 10:87-90. (in Japanese).
Ochiai, A., S. Umeda & M. Ogawa, 1974. On the acceleration of maturity of the catadromous female eel by hormone injection, and changes in the liver and blood character. Bull. Jap. Soc. Sci. Fish., 40(1):43-50. (in Japanese with English summary).
Satoh, H., 1979. Towards the complete cultivation of Japanese eels. Iden, 33(11):23-30. (in Japanese).
Yamamoto, K., O. Hiroi, T. Hirano & T. Morioka, 1972. Artificial maturation of cultivated male Japanese eels by synahorin injection. Bull. Jap. Soc. Sci. Fish., 38(10):1083-1090. (in Japanese with English summary).
Yamamoto, K., T. Morioka, O. Hiroi & M. Omori, 1974. Artificial maturation of female Japanese eels by the injection of salmonid pituitary. Bull. Jap. Soc. Sci. Fish., 40(1):1-7. (in Japanese with English summary).
Yamauchi, K., M. Nakamura, H. Takahashi & K. Takano, 1976. Cultivation of larvae of Japanese eel. Nature, 263(5576):414.

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GONAD DEVELOPMENT IN FOOD FISHES OF KUWAIT

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Summary

A knowledge of the biology of food fishes including reproduction, is important for the successful management and mariculture of these organisms. The silvery croaker, *Otolithes argenteus (Fam.Sciaenidae)*, the silvery grunt, *Pomadasys argenteus (Fam. Pomadasyidae)*, and the yellow-finned black porgy, *Acanthopagrus latus (Fam.Sparidae)*, are among the most important food fishes in Kuwait. In our studies, aspects of the reproductive biology of the three species are examined and some of the results presented here.

Maturation of the gonads, in the three species, which can be divided into eight arbitrary stages, following a distinct sequential pattern over one annual reproductive cycle (Fig. 1). Immature and resting gonads are found predominantly in the late summer and early winter months. During the breeding season, from January to April, gonads in the maturing and spawning stages of development occur.

The seasonal changes in the gonadosomatic index (GSI) correspond closely to the maturation stages in the three species. The GSI is low in the non-breeding season and maximal in February and March during the spawning period (Fig. 2).

Five-stages of oogenesis can be distinguished in the ovaries of the three species. Small oogenia, closely associated to the lamellar membranes, and oocytes in the primary growth phase predominate in the resting gonads. Oocytes in early vitellogenesis and active vitellogenesis are found mostly in maturing gonads. Hydrated, mature eggs occur just prior to spawning. In the post-ovulatory gonads the lamellae are collapsed and oocytes undergoing breakdown and resorption occur.

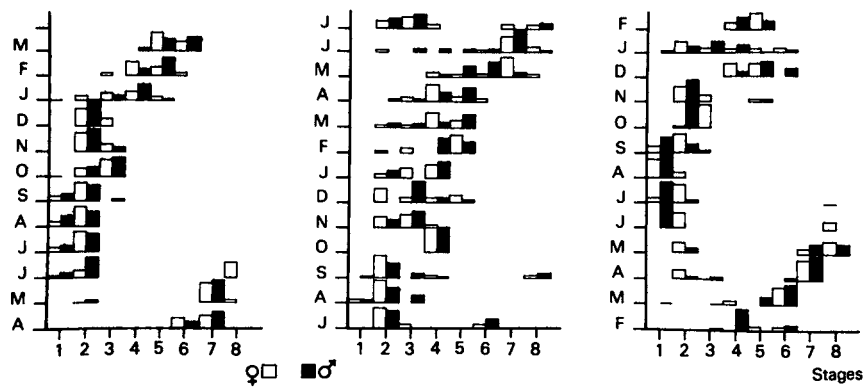

Fig. 1: Seasonal changes in the maturation stages (1-8) of the gonads.

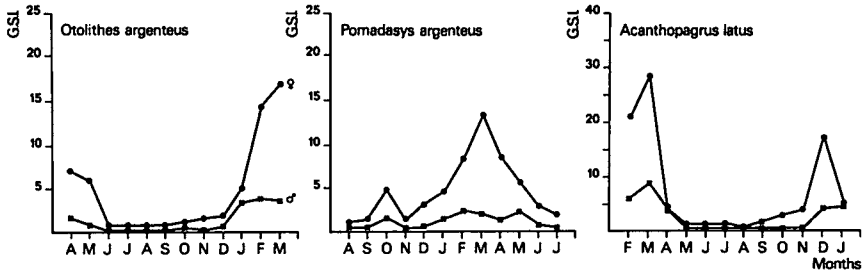

Fig. 2. Changes in the GSI in male and female fish.

The distribution of oocytes of different sizes within the ovary is a good indicator of the spawning habits of fish. Oocyte development in all three species examined here is of the "group-synchronous" type. A hetero-genous population of small oocytes give rise to a synchronous population of larger oocytes released in the spawning season.

ON SOME PATTERNS OF REPRODUCTIVE PHYSIOLOGY IN MALE TELEOST FISH.

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Two main types of testicular structure, associated with different patterns of spermatogenesis, spermatology and reproductive physiology, can be identified in teleost fish. A tubular type characterizes the testis of the Poecilidae (e.g. guppy) in which spermatogenesis is continuous. Type A spermatogonia (G_A) are restricted to the proximal blind end of the tube in which they divide and organize into cysts (G_B) that move to the distal extremity. The spermatids adhere tightly to the Sertoly cells and, after spermiation, the spermatozoa remain packed together in spermatozeugma. Spermiogenesis is complicated, and the most complex spermatozoa are found in teleost fish. They have highly condensed chromatin, an elongated head, a large mid-piece with glycogen stores and a highly modified centriolar complex. Sperm motility is very long (a few hours or days) and sperm survival can reach a few months in the ovarian cavity of the female (internal fertilization). Seminal fluid secretion is very limited, being restricted to the efferent duct; this seems to be related to the existence of spermatozeugma which probably constitute a very efficient way of transferring the sperm to the female.

In the lobular-type testis, the seminiferous tube is often anastomosed into a network of connective tissue originating from the testicular capsule. In histological sections, this network appears in the form of a lobe, hence the term "lobule". In addition, this type of structure seems to be homologous to the lobule of the mammalian testis and may correspond to a primitive form of testicular structure.

These lobules have a permanent layer of Sertoli cells with G_A at the inner periphery and a central lumen in which the spermatozoa are released from the cysts at the end of spermatogenesis. The cysts are rather stationary, showing only a slight centripetal displacement during spermatogenesis. The spermatozoa are free, moving from the lobule to a short, non-secretory efferens duct and then to a sperm duct. The seminal fluid originating from the Sertoli cells and the secretory epithelium of the sperm duct is abundant. Sperm morphology is very simple. The sperm head is spherical or slightly elongated and the mid-piece is quite reduced. The process of spermatogenesis is a subcontinuous process in some species as carp, in which spermiation may occur nearly all the year round, provided that such environmental conditions as temperature are satisfactory. In many other species such as trout, spermatogenesis is seasonal and discontinuous, even if the environmental conditions are satisfactory, and a new spermatogenetic cycle starts only when the spermatozoa from the previous cycle have been released from the testis.

The efficiency of spermatogenesis is quite variable in fish. The maximal value of the GSI varies from 0.1% in Leporinus (Godinho, personal communication) to 10% or sometimes more in salmonids (see Table). Annual spermatogenetic production, expressed in number of spermatozoa per g of body weight for interspecies comparison, varies from 100 to 7000 millions in the species listed on the table. The GSI in females is more stable (20-30%) but fecundity is very variable because of the great differences in egg size. When spermatogenetic production is expressed in 10^6 spermatozoa per egg laid by a female of similar size, the ratio varies from 3500 for trout to only 1.65 for Leporinus. The minimal number of spermatozoa that need to be inseminated to fertilize one egg is much lower, being about 2000 when fertilization is internal and about 10000-30000 when it is external. In the latter case, the value is optimistic because insemination would be carried out in a diluent. Approximately 10 times more spermatozoa would be needed in fresh water.

Table. Efficiency of reproduction in some teleost fish species

Species	GSI (maxi) ♂	♀	Spermatogenetic production 10^6 spz/g b.w	Fecundity egg/g b.w	Egg diameter mm	Sperm motility min	No of spermatozoa per egg laid (10^9)	per egg insemimated
Trout	10	25	7000	2	4	1	3 500	30 000
Carp	8	30	4000	100	1.2	10	40	10 000
Pike	2	20	600	30	2	2	20	30 000
Leporinus	2	20	100	90	1.1	--	1.65	--
Guppy	5	20	2700	30	1,5	120	90	2 000

ULTRASTRUCTURE OF TESTIS ORGANIZATION AND RESTING CELLS IN Liza aurata Risso 1810, (Teleostei, Mugilidae)

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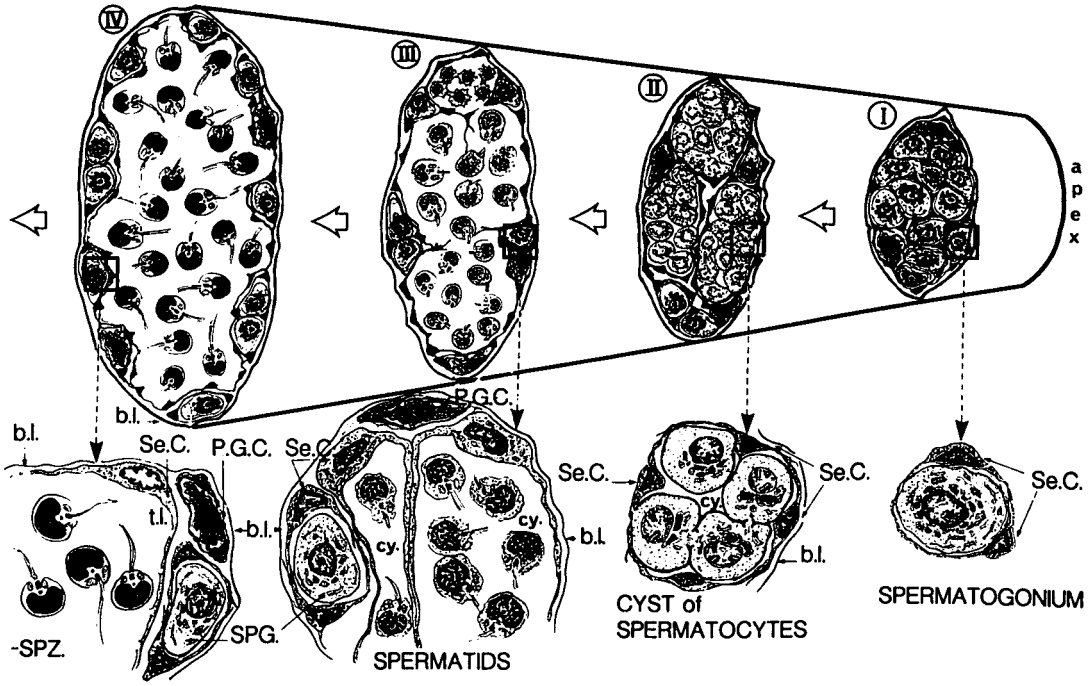
Summary

In Teleosts, the tubular structure of the testes is now well established (Grier et al, 1980; Grier, 1981).

The testis of Liza aurata is composed of seminiferous tubules bordered by basal lamina, Inside the tubules are germ cells and Sertoli cells (outside the tubules are interstitial cells or Leydig cells). Germ cells are gathered in seminiferous cysts in which they differentiate synchronously.

At the onset of spermatogenetic activity, spermatogonia divide several times and enter meiotic prophase. The nest of these cells is surrounded by Sertoli cells, which constitutes a cyst.

Grier et al (1980) and Grier (1981) describe two different tubular testis-types. In the "unrestricted spermatogonial testis type", typical of most Teleosts, spermatogonia are located along the entire length



ORGANIZATION OF A SEMINIFEROUS TUBULE

b.l. = basal lamina; cy. = cyst; PGC. = Primordial Germ Cell; Se.C. = Sertoli Cell; SPG. = Spermatogonium; SPZ. = spermatozoa; t.l. = tubule lumen.

These cysts, bordered by Sertoli cells, contain primary spermatocytes, secondary spermatocytes, spermatides at different stages and spermatozoa. Spermatogonia individually surrounded by Sertoli cells are not gathered in cysts; they occur along the entire length of the tubule and in the apical part of the tubule immediately beneath the epithelium which borders the gonad.

of the tubule. In the "restricted spermatogonial testis-type", typical of Atheriniformes, spermatogonia are totally restricted to the distal terminus of the tubule beneath the tunica albuginea. In Liza aurata, it seems difficult to think that the testes are an intermediate type between those described by Grier (1981). It is possible that, apart

from Atheriniformes, Teleosts present seminiferous tubules with parietal and apical spermatogonia as in Liza aurata.

Moreover, in golden grey mullet, there is no germinal epithelium as is generally admitted for Teleosts. At any moment of the gonadal cycle, in addition to spermatogonia we observe primordial germ cells (PGC), which are undifferentiated cells, characterized by a high nucleus cytoplasm ratio, an abundance of ribosomes and a scarcity of cytoplasmic organelles. These resting cells differentiate into spermatogonia as shown through transitional stages. Spermatogonia divide very actively and colonize the seminiferous tubule after the spawning period. The germ stock of the gonad is constituted by spermatogonia as dynamic elements and by PGC as latent ones.

References

Grier H.J., 1981. Cellular organization of the testis and spermatogenesis in Fishes. Amer.Zool., 21, 345-357.

Grier H.J., Linton J.R., Leatherland J.F., de Vlaming V.L., 1980. Structural evidence for two different testicular types in Teleost fishes. Amer. J. Anat., 159, 331-345.

EFFECTS OF HUMAN CHORIONIC GONADOTROPIN (HCG) ON MATURATION AND OVULATION OF OOCYTES IN THE CATFISH *CLARIAS LAZERA (C & V)*.

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Administration of HCG is necessary to provoke maturation and ovulation of oocytes. Measurement of dosage of the inducing agent (HCG), correct timing of the latency and selection of relevant criteria for ovulation and maturation of oocytes is a major problem in standardizing procedures for breeding experiments. Selection of adult females for artificially induced breeding was based on extrusion of postvitellogenic oocytes (Ø > 1 mm) after pressure on the abdomen. Upon intramuscular hormone injection, the females were stripped.

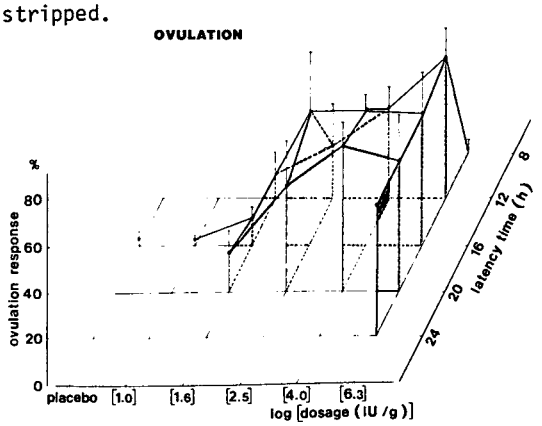

Fig. 1. Ovulation responses with S.D. and latency time to HCG in *C.lazera*.

The effect was studied of HCG and latency time of hormone injection on ovulation of oocytes of adult brood-fish 10-12 months old and of mean weight 150 g. The ovulation response of fish, 5 fish per treatment, kept at 25 °C was defined as the weight of the stripped eggs divided by total weight of ovary estimated after stripping and autopsy of the brood-fish. An optimum effective dosage of about 2.5 IU per gram bodyweight with a latency time of about 16 h at 25 °C was found.(Fig.1.) Samples of about 200 eggs per female were subsequently fertilized with fresh milt and incubated in Petri dishes with stagnant water. Between 30 an 40 h after fertilization at 30 °C, the eggs hatched. Maturation response of female brood-fish was defined as the number of normally developed larvae divided by total number of eggs incubated. An optimum effective dosage of about 2.5 IU/g at a latency time of about 16 h at 25 °C was found.(Fig.2.)

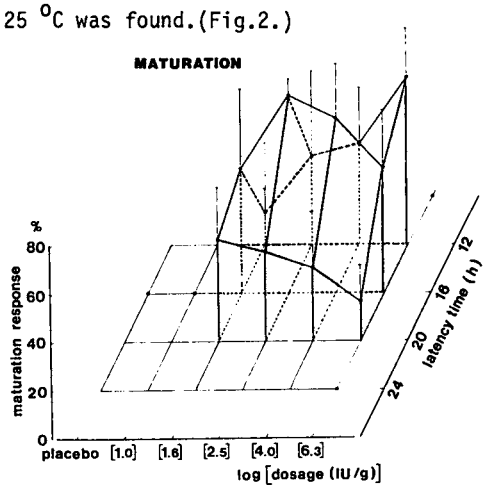

Fig. 2. Maturation responses (see Fig. 1.)

In a second trial, a dosage-response curve for ovulation and maturation was estimated for hypophysectomized and sham-operated fish at a constant latency time of 16 h and temperature 25 °C. These two parameters were investigated for a future development of a biological assay of gonadotropin in pituitary tissues. The brood-fish were stripped four weeks before the trial, during that period, a uniform stock of postvitellogenic oocytes was built up. They were injected 8 h after the operation and stripped according to the first trial. At a dosage of 4 IU/g, the ovulation and maturation response curves reached a maximum of about 70 and 80 %, respectively. The curves for hypophysectomized and sham-operated fish were similar in shape. This means that the tedious procedure of hypophysectomy is not necessary to develop a biological assay for gonadotropin.

EFFECT OF TRIIODOTHYRONINE(T_3) AND SOME GONADOTROPIN AND OF STEROID HORMONES ON MATURATION OF CARP(CYPRINUS CARPIO L.) OOCYTES IN VITRO

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Summary

The effects of triiodothyronine (T_3) as well as of gonadotropin and steroid hormones on carp oocyte maturation in vitro were investigated with the use of ovarian fragments (from 5 females) containing the oocytes after completed vitellogenesis. Twenty-four hours before the start of ovarian fragment culture, females were injected carp hypophysial homogenate (chh) at a dose of 0.5 mg/kg body weight.

The ovarian fragments from all females were divided into 2 groups:

Group I- Oocytes incubated in medium supplemented with gonadotropin or with one of the steroid hormones without T_3
- nine subgroups, one of them without any hormone control,

Group II- Oocytes incubated in medium supplemented with gonadotropin or with one of the steroid hormone + T_3
- nine subgroups, one of them with T_3 alone.

The percentage of mature oocytes in subgroups with T_3 + steroid hormone or with T_3 + gonadotropin hormone was nearly twice as high as that in the subgroup incubated with only steroid or only with gonadotropin hormones (without T_3)(Fig. 1).

Differences between first and second groups proved to be statistically significant (p 0.01).

It may indicate that inside the oocytes or inside granulosa cells there are T_3 receptors.

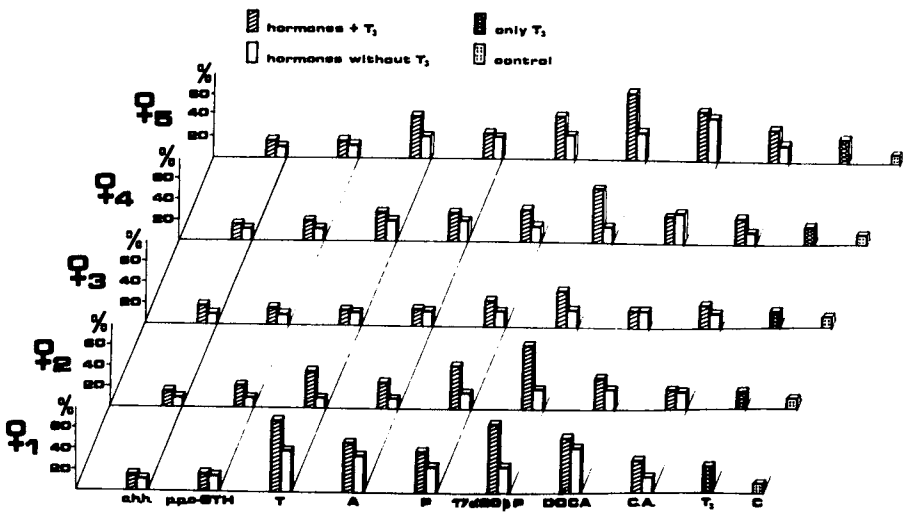

Fig. 1. Percentage of mature carp oocytes obtained in the media with T_3 and in the media without T_3 (chh-carp hypophysial homogenate; pp.c-GTH-partly purified carp gonadotropin hormone; T-testosterone; A-androsterone; P-progesterone; 17α20βP-17α20β dihydroprogesterone; DOCA-desoxycorticosterone acetate; CA-cortisone acetate; T_3-triiodothyronine alone; C-control).

GONAD MATURATION OF FEMALE CAPELIN (MALLOTUS VILLOSUS VILLOSUS)

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Summary

Developing capelin oocytes has been studied using both histological and total preparations. A new maturity scale has been constructed and maturity of female capelin is assessed according to morphological structures in fresh or conserved oocytes, using low power microscopy. Maturity by age and length has been studied using data collected using the new maturity scale. A comparision is made between estimates of spawning stock of female capelin using traditional and new criteria of maturity. The growth rate of oocytes has been estimated, and predictions of maturity by time can be made.

Introduction

The study of the maturity cycle of female capelin was initiated due to uncertainities in methods for the estimation of the size of the spawning stock. These estimations are carried out in September, about 6 months prior to the time the fish begin to spawn. At this time the gonads are small and stage of sexual development is difficult to assess. The purpose of the study, carried out at the Institute of Fisheries Research in Bergen, and the Institute of Fisheries in Tromsø, was to present a maturity scale which could be used in the field, and would, more precisely predict "spawners" during early stages of development.

Results and discussion

The maturity scale

Oocyte morphology and histology has been studied and a maturity classification was developed from the appearance of whole oocyte preparations using low power microscopy (Forberg, 1983, in press). Immature capelin, or class I, have only first growth phase oocytes. I define them as maturing when yolk vesicles appear in the cytoplasm, in class IIa-IIb. During class IIIa-IIIc, fat vesicles appear in the cytoplasm, and during class IV rapid accumulation of solid yolk takes place. Class V describes fish with ovulated oocytes, class VI are spawning, class VII are spent, and class VIII are spent/recovering capelin.

Growth rate of oocytes

Increase in mean diameter ($\bar{D}$) of second growth phase (SGP) oocytes was studied in material from 1978 and 1979. SGP oocyte growth is initially slow (class II-III) and the data were well fitted to a linear growth function during June - October:

$\bar{D} = 44.74 + 1.03t$, $r^2 = 0.72$, $n = 17$
(t = time in days from 1.1.1978).
Oocyte growth rate then increases (class IV-V) and the data were well fitted to an exponential growth function during November - March: $\bar{D} = 201.4\ e^{0.001t}$, $r^2 = 0.96$, $n = 36$.

Maturity rate by length and age of the fish

Maturity data, using the new scale, were collected during September-October 1977-80. Fish less than 12.0 cm were immatures. The proportion of maturing fish increases with increasing size, and with increasing age within intermediate size groups (12.0-13.0cm). Fish larger than 14.0 cm were all maturing. The rate of maturity increases with increasing fish size, and with age within size groups smaller than 16.0 cm.

Estimations of the spawning stock

Traditional method of maturity classification appears to give a large underestimate of the total number of the potential spawners (Table 1).

Table 1. Total stock, and spawning stock of female capelin, in numbers x 10^{-10}, using five different definitions of the potential spawning stock: a) "maturing" by traditional classification; b) class IIa or more mature; c) class IIb or more mature; d) class IIIa or more mature; e)class IIIb or more mature.

year	total stock ≥ 12 cm	spawning stock a	b	c	d	e
1977	13	4	9	7	5	4
1978	13	4	10	8	6	4
1979	16	1	10	7	3	2
1980	18	6	14	12	10	7

References

Forberg, K. G. 1983. Maturity classification and growth of capelin oocytes. J. Fish. Biol. (in press).

ULTRASTRUCTURE OF CELLULAR AND NON-CELLULAR LAYERS SURROUNDING THE OOCYTE OF A TELEOSTEAN GOBIUS NIGER L. : PRELIMINARY OBSERVATIONS

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SUMMARY

From the early previtellogenic stages oocytes of Gobius niger are surrounded by a single layer of flattened follicular cells and separated by a basal lamina from several layers of thecal cells which progressively decrease to form a single very thin thecal layer.

At Stage II, the follicular epithelium separates from the oocyte surface forming a narrow space filled with a slightly floculated material. Microvillosities protrude from the oocyte surface into this space. An electron-dense material is then deposited between the oocyte microvillosities and constitutes the beginning of the zona radiata. From this stage on, numerous mitochondria and Golgi complexes are present in the peripheral ooplasm. They produce smooth dense-cored vesicles which deposit the electron-dense material by exocytosis on the external surface of the oocyte. Microvillosities protrude from the follicular cells towards the oocyte. The zona radiata is gradually built up.

At Stage II^+, the zona radiata consists of two electron-dense layers, the higher homogenous and the lower finely reticulated, perforated by pores through which the oocyte and follicular microvillosities pass. With the formation of the zona radiata, bristle-coated vesicles appear at the base of the oocyte microvillosities. They are pinocytotic and collect the exogenous vitellogenin which reaches the oolemma by passing between follicular cells, then along microvillosities towards the base of the zona radiata. Exogenous vitellogenesis occurs very early at the same time as endogenous vitellogenesis. Between the follicular cells lacunae appear in which a material subsequently transformed into adhesive filaments is deposited.

At Stage III, follicular cells appear as a continuous epithelium alternating with transversal sections of adhesive filaments. These filaments constitute a longitudinal network which covers the oocyte and is attached to the zona radiata only at the animal pole. As the adhesive filaments are formed the ultrastructure of the follicular cells takes on a secretory aspect.

During Stage III and IV_a, the adhesive filaments develop. Their outer part is deeply indented, with pin-like formations.

At maturation (Stage IV_b), the follicular cells separate from the surface of the zona radiata which quickly fills in and frees the adhesive filaments. During spawning the adhesive filaments break at the vegetative pole and later attach the animal pole of the oocyte to a support.

The zona radiata can be considered as a primary envelope and the adhesive filaments as secondary formations.

STUDIES ON THE BREEDING BIOLOGY OF THE EEL (ANGUILLA JAPONICA TEMMINCK & SCHLEGEL)

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Summary

Methods of identifying the sex of eels at the seaward migrating period, ultrastructure of pituitary of female eels, development of gonads and artificial inducement of sexual maturity in male and female eels were studied. The results indicated that the eels collected at the seaward migrating period can be induced sexual maturation of both sexes by means of hormonal injections under pond conditions.

External characteristics of male and female eels at seaward migrating periods, by comparing the diameter of eyes, interorbital space, the shape of snout, etc, are sufficiently reliable for the identification of both sexes. These morphological dimensions when calculated with the aid of distinguish function, confirm the differences between the two sexes. Consideration of morphological differences of the 400-600 mm size group, permits separation of the sexes with 90 % accuracy. Plotting of size distribution separates naturally one sex from the other, those above 600 mm are nearly all female, and those below 400 mm are almost all male. Because the body weight (W) of eel varies as some power of body length(L), that is $W=aL^b$, the relationship between body length and body weight of both sexes may be expressed as $W=0.0000001285L^{3.015}$. The regression coefficient of these two variates of eel between 400-600 mm in body length is different for male and female. The former is 0.887, that is, W=0.887L-280.20, and the later, 1.150, that is, W=1.150L-376.73. In the case of the condition factor, the male is found to be 1.15±0.062, the female 1.42±0.053, differences of both figures are significant. So, the external characteristics and sex distinction of male and female eels can be used for the selection of breeders in the induced breeding of eel.

The neurohypophysis of eel is composed of neurosecretory fibres, pituicytes and capillaries. The general characteristics of the neurosecretory fibres are typical of those of fishes, a few were myelinated. Some multilamellate bodies were present in neurohypophysis. Two types of fundamentally different neurosecretory fibres can be clearly distinguished. Type A neurosecretory fibre contain spherical membrane-bound vesicles measuring 1250-1750 Å in diameter, type B contain smaller vesicles measuring 450-1000 Å in diameter. Most of the neurosecretory fibres do not make direct contact with the endocrine cells but are separated from them by a basement membrane. There are synaptic junctions between neurosecretory fibres and pituicytes. Six different cell types(prolactin cell, ACTH cell, TSH cell, GtH cell, GH cell and the secretory cell of meta-adenohypophysis) can be recognized in the adenohypophysis by the characteristics of ultrastructure and the size of the granules they contain. At the level of ultrastructure, prolactin cells, ACTH cells and TSH cells can be demonstrated in high activity during the seaward migrating period, while GtH cells had not yet in a functional state.

Fully mature male and female eels were induced by injection with common carp pituitaries plus HCG and LHRH-A, des-Gly^{10}(D-Ala^6)LH-RH-ethylamide. The eels were kept in ponds with artificial sea water at a temperature of 15-20°C. From November to next March, after 6-10 injections with 10-20 days interval between each injection(4-5 mg carp pituitary + 1500-2000 IU HCG + 40-50 ug LHRH-A/kg body weight), most of females were matured. Mature males were obtained by 4 or 5 injections with lower dosage(0.5-1.0 mg carp pituitary + 500-800 IU HCG/kg body weight).

The oogenesis and spermatogenesis as well as the development of ovary and testis under artificial inducement were also investigated histologically and cytologically. The development of testis is composed of six stages: 1) early multiplication of spermatogonia, 2) late multiplication of spermatogonia, 3) growth and maturation of spermatocytes, 4) initial appearance of spermatozoa, 5) complete maturation of spermatozoa, 6) degeneration of spermatozoa. The oogenesis is composed of six phases: 1) oogonia, 2) oocyte with follicular epithelium consist of a single layer of follicle cells, 3) oocyte with adipose vesicles, 4) oocyte filled with yolk, 5) oocyte with migratory nucleus, 6) degeneration of oocyte. Based on the six phases of oogenesis, the development of ovary is also composed of six stages correspondingly.

This investigation showed it is possible to induce sexual maturation in the male and female eel by keeping them in artificial sea water and giving hormonal injections.

EFFECT OF ENDOSULFAN ON BIOCHEMICAL CHANGES IN OVARY AND GSI OF TWO SPECIES OF FRESHWATER TELEOST

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Summary

Endosulfan a chlorinated insecticide was daily exposed to two species of freshwater teleost, Clarias batrachus and Heteropneustes fossilis during the preparatory phase (February-April) for a period of 30 days. Pesticide at a concentration of 0.0001 ppm significantly increased the glycogen content of ovary in C. batrachus and H. fossilis. The activity of acid and alkaline phosphatase, total and free cholesterol and ascorbic acid and the value of gonadosomatic index was unaffected during this phase in the two species of fresh water teleost. It seems that endosulfan at this dose level failed to induce bio-chemical changes in ovary during preparatory phase.

Endosulfan (6, 7, 8, 9, 10, 10a hexachloro, 5, 5a, 6, 9, 9a hexahydro-6, 9 methano 2, 4, 3-benzedioxathiepin-3-oxide) is a non systemic contact insecticide and acaricide is used extensively for the control of variety of plant pests. Endosulfan was found to cause the death of fish in the Rhine river during 1969 (Walker, 1971). Several workers have reported varied toxicological effects of endosulfan in species of animals like mice, rat and rabbit (Nath et al., 1978; Dikshith et al., 1978; Dikshith and Datta, 1978; Dikshith et al., 1982a, b (unpublished)). Very little work is available on endosulfan toxicity of fish (Krishna Gopal et al., 1981; Mohanaranga Rao and Murty, 1980).

Adult female C. batrachus and H. fossilis were kept under laboratory conditions for two weeks before experimentation and fed with commercial fish food and liver on alternate days. Single concentration of endosulfan 0.0001 ppm) was added daily after changing the aquaria water. On completion of day 30, fish were killed, ovary was taken out immediately and weighed individually. Gonadosomatic index (GSI) was calculated by multiplying the ovary weight with 100 and quotient being divided by body weight. There was no change in the GSI of the two species of freshwater teleost after exposure with endosulfan when compared to control animals, 10% homogenate of ovary was made and estimation of the biochemical parameters were conducted as per the standard procedures. It has been observed that endosulfan at the dose used, could not produce changes in ovarian biochemistry of C. batrachus and H. fossilis during preparatory phase when compared to respective control values. Singh and Singh, (1980) have also reported no change during preparatory phase in total lipids and cholesterol values in ovary of H. fossilis due to cythion and Hexadrin. Kapur et al., (1978) have reported a decrease in 3B-HSD level after fenitrothion treatment in the ovary and testis of Cyprinus carpio. In the present study, endosulfan treatment could significantly enhance glycogen contents of ovary both in C. batrachus and H. fossilis. The ATPase activity in ovary was slightly decreased in H. fossilis. It is apparent from these findings that endosulfan during this phase of reproductive cycle could not bring any drastic changes in the ovarian biochemistry even after a prolonged treatment of 30 days.

Higher concentration of endosulfan if given for the same period may produce some changes in ovary of two species of fish.

References

C. Walker (1971). Environmental pollution by chemicals. Hutchinson.

G. Nath, K.K. Datta, T.S.S. Dikshith, S.K. Tandon and K.P. Pandya (1978). Toxicology 11, 385.

H. Singh and T.P. Singh (1980). Environmental poll. 23, 9.

K. Kapur, K. Kamaldeep and H.S. Toor (1978). Bull. Environ. Cont. Toxicology. 20, 438.

R.B. Raizada, K.K. Datta and T.S.S. Dikshith (1982). Drugs and chemical Toxicology (communicated).

T.S.S. Dikshith, G. Nathand, K.K. Datta (1978). Ind. J. Exp. Biol. 16, 1000.

T.S.S. Dikshith and K.K. Datta (1978). Bull. Environ. Cont. Toxicology, 20, 826.

T.S.S. Dikshith, R.B. Raizada, K.K. Datta and B.S. Kapalia (1982). Drugs and chemical Toxicology (communicated).

SPERMIATION RESPONSE OF CARP TO HOMOLOGOUS PITUITARY GLAND

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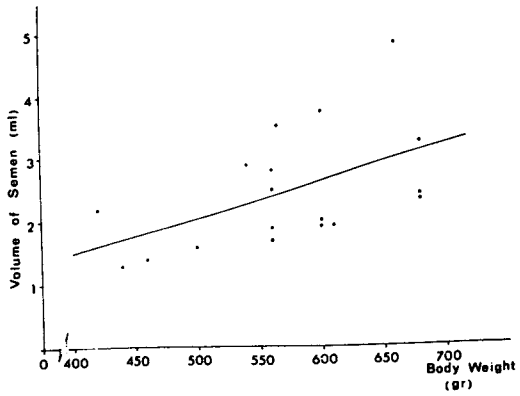
Israeli carp reach sexual maturity at the age of 6 months and differ in this respect from other carp populations. Maturation-inducing potency in Israeli carp pituitaries were previously tested by the frog spermiation assay (Yashouv et al., 1970). The purpose of this study was to determine gonadotropic potency in carp pituitaries, by the spermiation response in the homologous species.

Pond temperature was recorded daily during the period of pituitaries collection in order to assess possible relationship between ambient temperature and gonadotropic potency in the glands.

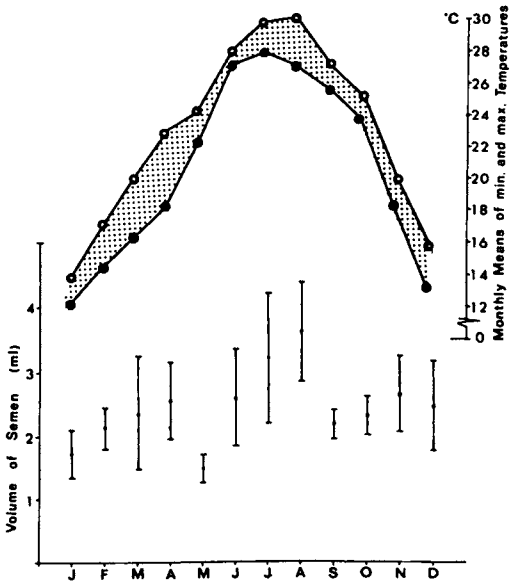
Recipient fish (500 ± 100 gr v.w.) were kept 48 hrs at 16°C prior to treatment to reduce variability in response among individual fish. The test was performed on 13 groups of equal size male carp. Fish of 12 groups were injected with pituitary homogenate (1 gland/kg) collected in another month, while the fish in the control group were injected with saline. Twenty-four hours later the fish were weighed and stripped 3 times. The semen from each fish was collected and its volume recorded. The SNK a posteriori test (Sokal & Rohlf, 1969) was applied for multiple comparisons among means of semen volumes of various months.

Another group of 18 fish was injected with homologous pituitary homogenate and stripped the next day in order to estimate the association between individual volume of semen and corresponding body weight (Fig. 1).



Correlation between volume of semen stripped and body weight of carp treated with homologous pituitary homogenate (1 gland/1 kg b.w.) y=0.0572x-0.82307 ; r = 0.5003.

The seasonal fluctuations of pond water temperature did not show any significant correlation with the pituitaries' gonadotropic potency (Fig. 2).



Spermiation response of carp to homogenate of homologous pituitaries collected at monthly intervals (mean ± SEM ; n-14). Maximal and minimal temperature of pond water during the same year.

No significant difference in spermiation response was noted among the groups treated with pituitaries collected along 12 consecutive months as tested by the SNK test. Semen released by males treated with pituitaries was more fluid than by the control fish.

As a consequence of the present study we suggest that carp pituitaries collected in Israel at any time during the year may be suitable for induction of spawning.

References:

Sokal, R.R. & Rohlf, F.J., 1969. Biometry. W.H. Freeman and Co., San Francisco. 776 p.

Yashouv, A., Nussbaum, M., Berner-Samsonov, E. & Abraham, M., 1970. Relative potencies of carp pituitaries collected for spawning induction. Bamidgeh, 20(4): 125-127.

INDUCED SPAWNING OF THE SHARPTOOTH CATFISH *CLARIAS GARIEPINUS* WITH NOTES ON TECHNIQUES EMPLOYED IN THE REMOVAL OF EGG ADHESIVENESS

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SUMMARY

The use of mammalian gonadotrophic hormones and pituitary glands to spawn the catfish *Clarias* has been demonstrated by a number of research workers. Because of the adhesive properties of the eggs, hormone-injected females are usually allowed to spawn naturally in specially prepared ponds. Where species of *Clarias* are artificially spawned, the fertilized eggs are usually deposited onto nylon screens, to which the eggs stick after they come into contact with water. These screens are then submerged in well-oxygenated water until the eggs hatch. Alternatively, fertilized eggs are deposited in a monolayer in plastic trays in the presence of fresh water. These screens and trays take up considerable space and are impractical if *Clarias* is to be spawned on a large scale. The continuous removal of dead eggs to avoid mould formation is also a nuisance.

Adaptations of techniques followed for the removal of egg adhesiveness in the common carp by using urea (Woynarovich, 1962; Rothbard, 1978), as well as full cream powdered milk (Soin, 1977), was adapted and used successfully for *Clarias gariepinus* (Schoonbee *et al.*, 1980). Eggs so treated were then hatched in breeding jars (Fig. 1). The work described on the induced spawning and hatchery procedures by these authors can be seen as the first step towards the large scale culture of *C. gariepinus* as a pond fish. These results also indicate that the sharptooth catfish is not difficult to spawn artificially when a mixture of carp pituitary and HCG is employed. Indications are that if sufficient pituitary material from *Clarias* can be obtained, only this may be required to spawn this fish.

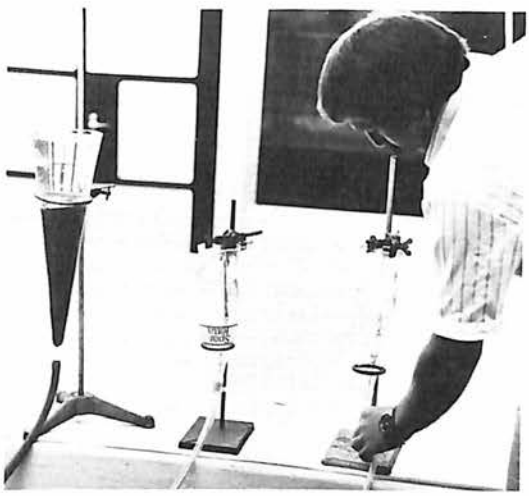

Fig. 1. Small effective breeding jars can be made from 1ℓ cordial bottles. Note how densely the eggs of *Clarias gariepinus* pack.

Table 1. Results of induced spawning experiments with the sharptooth catfish *Clarias gariepinus*

Date and season	No. of females used	Hormone injection times and dosages†			Remarks
		Day 1		Day 2	
18 October 1979 (late spring)	8	15h30 350 I.U. Puberogen + 1 gland/kg	22h30 150 I.U. Puberogen + 1 gland/kg	18h30 4 females, complete spawn 2 females, partial spawn	Gonads of 2 males used per female spawner. Male gonads removed and milt stripped over eggs.
	1*	15h30 1.3 gland/kg	19h30 1.3 gland/kg		*Clarias* pituitaries used. Fish spawned in tank within 10 h of first injection.
1 November 1979 (early summer)	2	10h30 250 I.U. Puberogen + 1 gland/kg	13h30 250 I.U. Puberogen + 1 gland/kg	08h30 Complete spawn of both females, 22 h after first injection	Only one male of approximately 4 kg used to fertilize eggs. Approx. 80% fertilization success.
4 December 1979 (mid-summer)	4	08h30 750 I.U. Pregnyl/kg	13h30 1 gland/kg	08h00 3 females, complete spawn 1 female, no spawn. Gonads underdeveloped.	

*Pituitary glands from female *C. gariepinus* donors, preserved in alcohol. In all other cases pituitary glands were obtained from common carp. †Dosages are given per mass of spawning fish.

INDUCTION OF OVULATION IN H. FOSSILIS-ANALYSIS OF SEX RELATED DIFFERENCES IN THE POTENCY OF HOMOLOGOUS AND HETEROLOGOUS PITUITARY EXTRACTS

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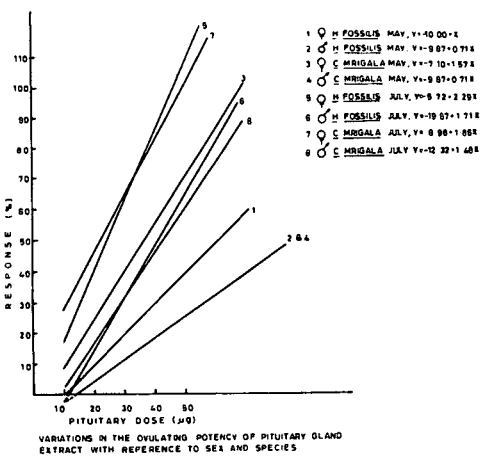
In order to make the induced breeding technique more successful and economic, attempts are continuously being made to standardize the doses of pituitary gland extract for inducting spawning in important food fishes (Jhingran, 1975). Reports on comparative ovulating potency of homologous and heterologous pituitary gland extracts from male and female fishes are meagre and debatable. Effect of homologous and heterologous pituitary extract collected from male and female specimens on induction o spawning has been described here.

Pituitary glands from mature specimens of either sex of Cirrhinus mrigala and Heteropneustes fossilis were collected separately during prespawning (May) and spawning (July) phases of reproductive cycle. Glands were dried in chilled aectone immediately after removal. Mature female specimens of H. fossilis, collected from ponds (Varanasi, India) in June and were hypophysectomized as per technique of Singh (1969) and used as recipients for evaluation the ovulating potency of different pituitary extracts. Various doses of bovine LH (5, 10, 15, 20 and 25µg/fish) and extracts of acetone dried pituitary glands (10, 20, 30, 40 and 50 µg/gish) were injected to hypophysectomized H. fossilis and ovulation was checked by stripping 24 hrs after the injection.



VARIATIONS IN THE OVULATING POTENCY OF PITUITARY GLAND EXTRACT WITH REFERENCE TO SEX AND SPECIES

Ovulating potency of pituitary glands collected in July from either sex of H. fossilis and C. mrigala was significantly greater than those of May. Homologous pituitary gland extracts from female fish were more effective than homologous extracts of males in inducing ovulation and heterologous extracts of both sexes. Bovine LH at the tested doses failed to induce ovulation in hypophysectomized H. fossilis.

The above findings suggest that (1) homologous pituitary extract is more effective than heterologous one, in inducing ovulation in H. fossilis, (ii) ovulating response of pituitary glands of both species collected during July indicates presence of higher gonadotrophic potency in the glands than those of May, (iii) there is a sex related difference in ovulating potency in both the species studied which is higher in female than male, (iv) least effectivesness of bovine LH may be due to the distant phylogenetic relationship.

References

Jhingran, V.G., 1975. "Fish and Fisheries of India, Hindustan Publishing Corporation (India).

Singh, T.P., 1969. Observation on the effects of gonadal and adrenal cortical steroids upon thyroid gland in hypophysectomized catfish, Mystus vittatus (Bloch). Gen. Comp. Endocrinol., 12: 556-560.

Acknowledgement

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REPRODUCTION REGULATED BY EXTRAOCULAR PHOTORECEPTOR IN A SEASONALLY BREEDING FISH, ORYZIAS LATIPES

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Summary

Enucleation of bilateral eyes(OPX) in teleost, Oryzias latipes, enhances gonadal development, e.g. increase of gonosomatic index(GSI), break down of germinal vesicle and completion of oocyte maturation, during a period from prespawning(March) through spawning(April to August)(Fig.1). Since blinded female, furthermore, responds to daily long or short photoperiod in regard to an increase of GSI and progress of oocyte maturation, and shows diurnal locomotor activity being synchronous to the daylight cycle, it emphasizes the presence of extraocular photoreceptor.

Pinealectomized(PINX) female revealed suppressed gonad(Fig.1,2), even in a long daylight condition, and without synchronization of locomotor activity to the daylight cycle. Therefore, it is highly probable contribution of the pineal in the photoreceptive mechanism with respect to progonadal function.

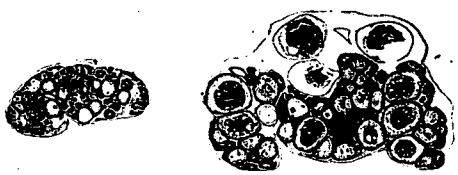

Fig. 1. Ovaries of PINX(left) and OPX.

Photoreceptive outer-segment organization of the pineal, and pineal innervation of Habenula commissure(Urasaki,'74) and the third ventricular ependyma(Sathyanesan and Sastry,'82) likely indicate a presence of pineal role in mediating photic stimulation for hypophyseal gonadotropic-gonadal axis.

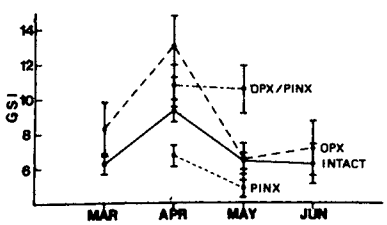

Fig. 2. Seasonal difference of GSI.

The present study was carried out for aim at possible role of pineal in regulating gonadal development and locomotor activity with respect to spawning behaviour. Suppressive effect of pinealectomy to increase of GSI induced by long photoperiod, was without effect on ovulation and oviposition. On the view point of GSI value correlating to plasma estradiol level(Yaron et al.,'77), ovulation and spawning after pinealectomy may not depend oocyte maturation in regarding hormonal level.

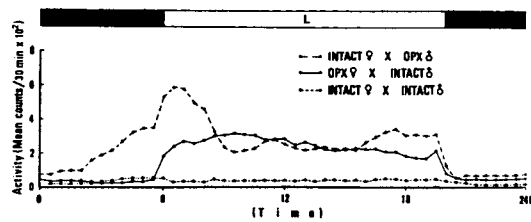

Fig. 3. Actograph of a couple of fish.

Blinded fish revealed GSI at high, but ovulation and spawning, suppressed and without punctuality(Urasaki et al. '81). Moreover, the blind female showed a actogram completely synchronous to LD cycle, and, it is also given in Fig.3. Therefore, it likely indicate the pineal play a role in oocyte maturation, but not in spawning mechanism.

References

Sathyanesan, A.G. & V.K.S. Sastry, 1982. Pineal innervation of the third ventricular ependyma in the teleost, Puntius sophore(Hams). J. Neural Trans. 53: 187-192.

Urasaki, H. et al., 1981. Photoperiodicity of reproduction modulated by extraocular photoreceptive mechanism in Oryzias latipes. Bull. Lib. Arts & Sci. Sch. Med. Nihon Univ. 9: 1-21.

Yaron, Z. et al., 1977. Occurrence and biological activity of estradiol-17β in the intact and ovariectomized Tilapia aurea(Cichlidae, Teleostei). Gen.Comp.End. 33: 45-52.

SATURABLE GONADOTROPIN BINDING SITES IN THE TESTIS AND OVARY OF IMMATURE CHUM SALMON.

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Summary

There is abundant evidence that peptide hormones exert their effects on target cells by binding to specific high-affinity receptor sites in the plasma membrane. Peptide hormone receptors have commonly been identified by competitive binding studies involving the uptake of radiolabelled and unlabelled hormone by the target tissue when incubated *in vitro*. By use of this approach gonadotropin receptors have been described in gonadal tissues from all vertebrate groups with the exception of fish. A similar approach was adopted to characterize the properties of gonadotropin binding sites in gonadal tissues obtained from immature chum salmon (*Oncorhynchus keta*).

A necessary prerequisite to competitive binding studies involves evaluation of the biological properties of salmon gonadotropin (s-GtH) subjected to radiolabelling. Iodinated s-GtH was prepared by the lactoperoxidase method using KI supplemented with 100,000 cpm ^{125}I instead of carrier free ^{125}I routinely used for iodinations. The iodination mixture was then serially diluted and aliquots were assayed for their ability to stimulate testosterone production in minced testis pieces incubated *in vitro*. The potency of the iodinated preparation was compared with intact s-GtH and separate preparations of iodinated s-GtH in which either KI or H_2O_2 had been omitted. Based on the incorporation of 44.5% of the ^{125}I it was calculated that s-GtH was iodinated to an average molar ratio of 0.88 mol iodine/mol s-GtH. The dose response curves for intact s-GtH and the various iodinated preparations were indistinguishable suggesting the lack of an effect of iodination on the biological activity of s-GtH.

Initial competition studies were conducted by incubation of ^{125}I-s-GtH and increasing amounts of unlabelled SG-G100 with the 3000 x g particulate fraction obtained from chum salmon testis or ovary homogenates. The presence of saturable ^{125}I-s-GtH binding sites was demonstrated by greater than a 50% reduction in the uptake of radiolabelled hormone in the presence of 0.1 and 10 ug SG-G100. Saturable binding sites could not be demonstrated in non-gonadal tissues including the liver, kidney and muscle. The demonstration of saturable binding sites was dependent on both time and temperature. Binding was higher at 20° than 8°C and increased with the time of incubation for 20 hr. Competition for ^{125}I-s-GtH binding sites was evident at low levels of s-GtH with significant displacement of bound hormone occurring with less than 2 ng s-GtH. Gonadotropins of mammalian origin had only a weak effectiveness in competing for ovarian gonadotropin binding sites; s-GtH having at least 300 x the potency of ovine LH and FSH.

The affinity and capacity of ovarian gonadotropin binding were determined by Scatchard analysis following correction for the maximal binding activity of the labelled hormone preparation (Dufau and Catt, 1978). Data suitable for Scatchard analysis was obtained following incubation of ovarian tissue with either a constant amount of ^{125}I-s-GtH and increasing amounts of unlabelled hormone or with increasing amounts of radiolabelled hormone. Saturable gonadotropin binding was due to a single class of high-affinity binding sites with an equilibrium association constant of 2.5 and 3.1 x 10^9M for the two methods, respectively. By both methods the binding capacities were about 40 pg/mg wet weight of tissue.

The properties of saturable gonadotropin binding sites in chum salmon show a close parallelism to the properties described for gonadotropin receptors in other vertebrate groups. However, the conventional definition of a hormone receptor embodies the dual functional properties of recognition plus translation of the hormone-receptor interaction into a specific biological response. Further studies are necessary to establish the physiological significance of these sites in the salmon.

References

Dufau, M.L. and K.J. Catt, 1978. Gonadotropin receptors and the regulation of steroidogenesis in the testis and ovary. Vitam. Horm. 36: 461-592.

GROWTH HISTORY AND REPRODUCTION IN ATLANTIC COD (*Gadus morhua*)

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Summary

Growth and reproductive condition of mature and potentially first spawning cod were followed under laboratory conditions for a period of 10 months (June to March). These fish exhibited natural reproduction with respect to the timing of spawning, percent maturation-at-length and age, and relative fecundity. Preliminary analysis indicates that immature cod which had the potential to spawn for the first time had different growth histories with respect to their ultimate reproductive state. Cod in this category which did mature exhibited better early summer growth than did those which did not mature. In mature females, fecundity was related to initial body size (June weight) and growth during June to September. These results are discussed in relation to the egg maturation cycle.

Gadoids, including Atlantic cod (*Gadus morhua*), exhibit considerable variability in relative fecundity, age and size at maturity, egg size and spawning time. This species is widely distributed in the North Atlantic and much of this variability can be attributed to inter-stock differences (May, 1967; Oosthuizen and Daan, 1974). However, intra-stock shifts in length-at-maturity over time have been noted for the North Sea (Oosthuizen and Daan, 1974) and Gulf of St. Lawrence cod (Lett, 1978); in the latter case over a relatively short period (15 y). Similarly, differences in relative fecundity have been observed among cod stocks and individuals of the same stock. Year-to-year variation within stocks probably occurs also. It has often been suggested that this variation may be an adaptive response to fishery-induced changes in population density. A compensatory increase in egg production during periods of low population density would be a potentially important aspect of the stock-recruitment process.

Woodhead and Woodhead (1965) investigated the reproductive cycle in Barents Sea cod and concluded from consideration of the egg maturation cycle that feeding condition (and hence growth) in the summer prior to spawning could influence fecundity in the next spawning season.

The purpose of the following study was to determine, under laboratory conditions, if the growth pattern in the year prior to spawning was related to maturation rate in potentially maturing cod or to fecundity in mature cod.

Methods

Cod, 39 to 70 cm in length were caught by otter trawl in Passamaquoddy Bay, transported to the laboratory, and held in 2-m diameter tanks, where they were kept under natural photoperiod at 4 or 8°C and subjected to a water current of about 0.2 to 0.5 body length. The fish were fed 3 times a day on a prepared diet consisting of ground flounder, herring meal, shrimp meal, herring oil and binder. Apart from the spawning period when appetite declined markedly, the cod were fed prescribed rations varying from 0.5% body weight d^{-1} to satiation. The fish were tagged and their weights and lengths were recorded every 2 months (June to March). At the end of the experiment, the gonads were removed and fecundity was determined in all females having ripe ovaries.

Results

Although there was considerable variability within tanks, growth increased with increasing ration, plateauing at a ration level of about 1% d^{-1}.

The cod showed normal reproduction with respect to the timing of gonad growth, size and age at maturity and relative fecundity. No direct effect of temperature on reproduction was observed. The length at which 50% of the males and females matured was 42 and 52 cm, respectively. The percent of males maturing at ages 3, 4 and 5 was 70, 95 and 100, while the corresponding values for females were 50, 70 and 100. Two groups of fish were distinguished those with initial weights suggesting they had the potential to mature during the experimental period, and mature (previously spawning) fish. Preliminary results indicate that in potential first spawners, different growth patterns were observed between fish that did mature and fish that did not mature (the initial weights of these two categories were not significantly different). Males which did mature weighed significantly more in all growth periods than nonmaturing males (Fig. 1).

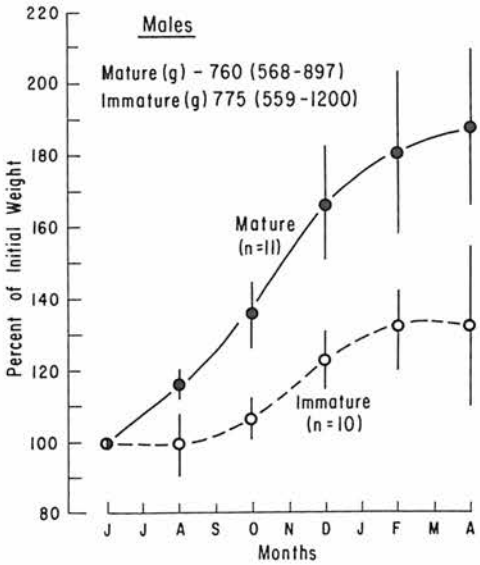

Fig. 1. Growth pattern of potentially first spawning males during the year prior to spawning. Starting weights are indicated. Bars denote 95% C.I.

Only during the first growth period (June to July) was growth in females which ultimately matured, significantly better than in those which did not mature. Thereafter, there was no significant difference in size indicating that non-spawning females partially recovered from the poorer early growth (Fig. 2).

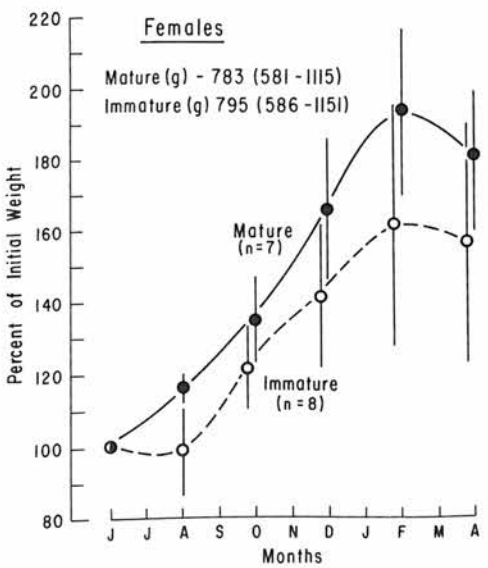

Fig. 2. Growth pattern of potentially first spawning females during the year prior to spawning.

Since eggs are committed to mature as early as 11 months prior to spawning, I tested the hypothesis that fecundity was a function of initial size (June weight) plus growth during various periods prior to spawning. Regression analysis was used with fecundity as the dependent variable. The equation with June weight as the only independent variable gave an r^2 of 0.647. When a second independent variable was added (June-July growth), the r^2 increased to 0.756. With June to September, June to November, or June to January growth as the second independent variable, the corresponding r^2 values were 0.821, 0.822, 0.823, respectively. Since there was no improvement in fit when growth after September was included in the equation it was concluded that the equation with June to September growth adequately represented the model. This relationship is presented in Fig. 3.

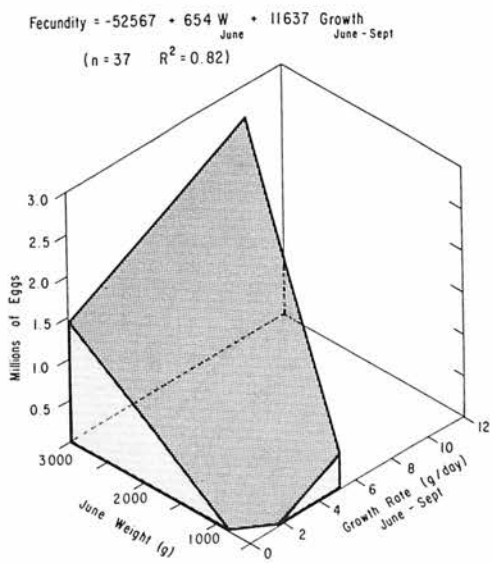

Fig. 3. The relationship between fecundity, June weight and June to September growth.

Discussion

The above data suggest that feeding conditions and growth, several months prior to spawning, may greatly influence the probability of maturation and the number of eggs produced in the following year. In the case of potential first spawners, maturation commitment is made relatively early in the summer. This may explain why Hislop et al. (1978) were unable to demonstrate a ration effect on maturation in haddock. Their study was initiated in November, about 4 months prior to spawning. It is conceivable that feeding rate or

growth did not directly influence maturation rate but that some other factor (such as stress from capture) may have had a coincidental but separate effect on growth and maturation. However, all fish were held for at least one month prior to experimental use and they showed no obvious signs of ill health.

Relationships between fecundity and ration have been demonstrated for several species although the mechanisms involved may differ. In Salmo gairdneri, restricted food supply resulted in a high proportion of atretic eggs (Scott, 1962), but in Poecilia reticulata (Hester, 1964), Pseudopleuronectes americanus (Tyler and Dunn, 1976), and Gadus morhua, differential recruitment of oocytes may be involved. My results indicate that the relationship between growth and fecundity was not a confounded function of gonad growth since ovaries did not appreciably increase in weight until after October. The growth period having the greatest influence on fecundity corresponded to that period in the egg maturation cycle in which oocytes reached the circumnuclear ring phase (maturation commitment). In this respect, the data are consistent with the hypothesis of Woodhead and Woodhead (1965).

If the relationship between fecundity, June weight and June to September growth applies to the natural situation it can be concluded that, over the size and growth range observed in this experiment, initial size had the greater influence on fecundity. However, much of the individual variability in relative fecundity, encountered in nature, might be explained by differences in summer growth.

Acknowledgements

I am indebted to Drs J. S. Scott and D. F. Markle and to Messrs P. Hurley and P. Koeller who made many helpful suggestions to improve this manuscript. Mrs. T. Parker and Mr. W. McMullon assisted in the preparation of text and figures.

References

Hester, F. J. 1964. Effects of food supply on fecundity in the female guppy, Lebistes reticulatus (Peters). J. Fish. Res. Board Can. 21(4): 757-766.

Hislop, J. R. G., A. P. Robb, and J. A. Gauld. 1978. Observations on effects of feeding level on growth and reproduction in haddock, Melanogrammus aeglefinus (L.) in captivity. J. Fish. Biol. 13(1): 85.

Lett, P. F. 1978. A comparative study of the recruitment mechanisms of cod and mackerel, their interaction, and its implication for dual stock management. Ph.D. Thesis, Dalhousie University, 125 p.

May, A. W. 1967. Fecundity of Atlantic cod. J. Fish. Res. Board Can. 24(7): 1531-1551.

Oosthuizen, E., and N. Daan. 1974. Egg fecundity and maturity of North Sea cod, Gadus morhua. Neth. J. Sea Res. 8(4): 378-397.

Scott, D. P. 1962. Effect of food quantity on fecundity of rainbow trout, Salmo gairdneri. J. Fish. Res. Board Can. 19(4): 715-731.

Tyler, A. V., and R. S. Dunn. 1976. Ration, growth, and measures of somatic and organic condition in relation to meal frequency in winter flounder, Pseudopleuronectes americanus, with hypotheses regarding population homeostasis. J. Fish. Res. Board Can. 33: 63-75.

Woodhead, A. D., and P. M. J. Woodhead. 1965. Seasonal changes in the physiology of the Barents Sea cod, Gadus morhua L. in relation to its environment. I. Endocrine changes particularly affecting migration and maturation. Int. Comm. Northwest Atl. Fish. Spec. Publ. No. 6: 691-715.

ENVIRONMENTAL FACTORS IN FISH REPRODUCTION

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Summary

Environmental factors act both as selective forces in the evolution of reproductive strategies and as proximate factors during the life-time of individuals. They affect both quantitative and qualitative components of reproduction by modifying the partitioning of time and resources between reproductive and non-reproductive activities. The confident prediction of the effects of such factors requires an understanding of the physiological mechanisms by which the effects are mediated. Because of the complexity of the environmental and physiological components, such an understanding will require careful experimentation with attention paid to the appropriate controls and statistical analysis.
Keywords: feeding, fecundity, gametes, timing.

Introduction

An ecologist studying fish reproduction is interested in the quantitative relationships between environmental factors and the reproductive output of the fish. The aim is to predict the reproductive success from a knowledge of the prevailing environmental conditions. If the predictions are to be made with confidence, the physiological mechanisms that mediate between the factors and the reproductive output must be understood. Most physiological studies on the effects of environmental factors have investigated their role in determining the timing of the breeding season. Because the majority of such studies have been on temperate species, the roles of temperature and photoperiod in synchronizing the timing of breeding in relation to the seasonal cycle have received most attention (de Vlaming, 1974).

Although the timing of reproduction relative to seasonal variations is an important element of the reproductive strategy of a species, it is only one of the components. The life-time reproductive output of a fish will depend on several life-history characteristics including: age at first maturity, interval between successive breeding seasons, number of breeding attempts during a breeding season, fecundity per breeding attempt, size and quality of the gametes and the effectiveness of any parental care. Environmental factors may influence each of these, and the goal of this review is to consider some of these influences, as far as possible within the framework of a bio-energetics model of fish reproduction.

Bioenergetics model of reproduction

In the bioenergetics model, the individual fish is viewed as a device that converts food as an input into reproductive products as an output. The neuro-endocrine system is conceptualised as a mechanism that achieves an adaptive partitioning of the resources made available by feeding between reproduction and the competing demands of somatic maintenance and growth, in the face of fluctuating environmental conditions, the most crucial of which is the food supply itself. In parallel with the input/output model of energy being partitioned between the gonads and the soma, there is a time budget model in which the neuro-endocrine system is conceptualised as a mechanism that achieves an adaptive partitioning of time between reproductive and non-reproductive behavioural activities. Measurements of the energy costs of the activities allows translation between the two models.

There are relatively few studies on the energy costs of reproduction in fishes (Wootton, 1979). Reproductive effort (R.E.) can be defined as the ratio of the energy content of the eggs (or sperm) produced in a given time period to the energy content of the food consumed in that period. Female medaka (*Oryzias latipes*) had an R.E. of about 5-25% depending on ration and temperature (Hirshfield, 1980). The viviparous *Poeciliopsis occidentalis* in natural populations had R.E.s estimated to range between 3.1 and 9.8% (Constantz, 1979). For female sticklebacks, *Gasterosteus aculeatus*, the R.E. in females that were not changing in weight was 14-27% (Wootton, 1977). Glebe & Leggett (1981) estimated that in semelparous *Alosa sapidissima* and salmonids more than 70% of the total energy reserves was used in the spawning migration whereas in iteroparous *A. sapidissima* and salmonids less than 60% was used. These figures suggest the significant energy costs associated with reproduction and emphasise the importance of the physiological mechanisms that ensure these costs are expended at ecologically appropriate times and in appropriate environmental conditions.

Environmental effects on feeding

Environmental factors may limit the reproductive output not because of any direct effect on the reproductive system but because of their direct effects on the rate of food consumption and thus on the materials available for reproduction,

Temperature has a strong effect on the rate of food consumption in fishes (Elliott, 1981). In G. aculeatus there is a monotonic, positive relationship between the maximum rate of food consumption and temperature over the rate 5.0-19°C (Wootton et al, 1980). At low temperatures, the difference between the maximum ration the fish consumes and its maintenance ration is much narrower than at higher temperatures, the scope for growth increases with temperature (Allen & Wootton, 1982), hence the scope for the transfer of material to the gonads. In a natural population of G. aculeatus during the winter, the rate of food consumption was close to the maintenance requirements and only a small proportion of the energy income was invested in the gonads, whereas in spring the maintenance costs formed a much smaller proportion of the energy income and there was a rapid growth in gonad size in the females (Wootton et al, 1980; Allen & Wootton, 1982).

The effect of photoperiod on the rate of food consumption has received relatively little systematic study. Many species tend to restrict their normal feeding activity to certain periods during the diel cycle, and with seasonal changes in the relative length of the light and dark components of the cycle, the time available for feeding can vary. In G. aculeatus, feeding in February and December was restricted to the relatively few hours of daylight, whereas in May, feeding took place over virtually a 24h period (Allen, 1980). Thus even if photoperiod had no direct effect on the reproductive system of G. aculeatus, the effects of variations in food consumption imposed by variations in the time available for active feeding could influence both the timing and quantitative aspects of reproduction.

Water quality may also influence the rate of food consumption and so the resources available to be partitioned into reproduction, e.g. feeding rate in G. aculeatus seems to be depressed at low pH (Faris, unpub).

In assessing the importance of environmental factors in influencing the reproductive biology of fishes, it is necessary to distinguish between direct effects and indirect effects which are mediated through the influence of the factors on feeding.

Neuro-endocrine effects on feeding

The neuro-endocrine system that controls reproduction may also affect the feeding motivation of the fish. Experiments with exogenous androgens such as 11-ketotestosterone in salmonids and carp have shown that hormonal treatments can increase growth rates, appetite and the food conversion efficiency (Matty & Lone, 1979; Fagerlund et al, 1979). Some protogynous sex-reversing fish show a spurt in growth during the change from female to male (Shapiro, 1979). As female G. aculeatus come to the end of the breeding season, their ad lib food intake declines (Fletcher, unpub), which also suggests that the neuro-endocrine system that controls reproduction exerts some control over feeding motivation.

Thus environmental factors which act directly on the reproductive system may also cause changes in feeding behaviour which are mediated by the reproductive system.

Age of first reproduction

Short generation times can generate high rates of numerical increase, so that a fish can increase its genetic representation in the next generation by shortening the time it takes to become sexually mature. In a strongly seasonal environment, there may be a restricted period of time in which breeding can take place successfully so that the minimum age of first reproduction is constrained.

Minimum age of first reproduction is genetically determined, although in a few cases it is possible to infer the environmental factors that have led to the evolution of an observed age. A field study of Poecilia reticulata in Trinidad has implicated predatory fish as an important factor, for in populations in which adult guppies were heavily predated, the size and by implication the age at first reproduction was reduced (Reznick & Endler, 1982).

In most fish species age and size are confounded because of the indeterminate nature of fish growth and it is likely that within a given population, it is size rather than age which determines when reproduction occurs. Alm (1959) concluded that within a given population the faster growing fish tended to mature at an earlier age than the slower growing fish. Growth rate is particularly susceptible to effect of environmental factors such as food and temperature. In G. aculeatus a smaller proportion of females fed on a low ration became sexually mature than females fed on medium or high rations. Most populations of G. aculeatus reach sexual maturity at the age

of a year, but in some populations maturity may be delayed by a year (Wootton, 1976), although the factors which cause this delay are not known. Food shortage may cause an increase in the age of maturity because the critical size is reached after a longer period of time, but the long-term selective effect of chronically low food levels may be to select for a small size at maturity and hence a decrease in the age at maturity. Populations of G. aculeatus in infertile waters can reach sexual maturity at one year but do so at a small size (Wootton et al, 1978).

In the cyprinodonts Xiphophorus maculatus and X. variatus the social environment influences the age of first reproduction. Larger males tend to delay the maturation of small and behaviourally subordinate males (Sohn, 1977; Borowsky, 1978). In female Sarotherodon mossambicus, a cichlid, social isolation delayed the first spawning by about 10 days (Silverman, 1975).

The physiological mechanism which controls the onset of sexual maturation for the first time in relation to age and size is not understood, but the onset of maturation does represent a crucial change in the pattern of partitioning of both time and materials between reproductive and non-reproductive activities.

Sex ratios

Populations in which the sex ratio is biased towards females will have higher total reproductive outputs than populations with more equal sex ratios if all the females have access to the fertilizing capacity of the males. Two mechanisms through which environmental factors can influence sex ratios that do not seem to depend on differential mortality rates have been reported. In the Atlantic silverside, Menidia menidia, a species with a life span of a year, the environmental temperature influences the sex ratio of the progeny of some females. Warm temperatures tend to bias the sex ratio towards males, the critical period for sex determination apparently occurring late in larval development (Conover & Kynard, 1981). Sequential hermaphroditism has been reported for many species (Shapiro, 1979) and in many cases this is associated with a biased sex ratio. In protogynous species, the sex ratio is biased towards females. Two major hypotheses have been proposed to account for the initiation of sex reversal: the developmental hypothesis and the social control hypothesis. The former postulates that sex reversal is initiated when the fish reach a critical size or age. If size is crucial then environmental factors such as food supply may have an important influence on the rate at which the critical size is reached. The second hypothesis suggests that it is the social environment that determines when sex reversal takes place. In protogynous species, the removal of the male in the group results in the sex reversal of one of the females, often the largest. The mechanism by which the social control of the reversal process is maintained is controversial. Unfortunately there seem to have been no systematic experimental studies on the effects of resource levels such as food on the process of sex reversal. Too little is known of the physiological control of sex reversal for a predictive model of the process to be developed.

Fecundity

The lifetime fecundity of a female is the product of the fecundity per spawning, the number of spawnings per breeding season and the number of breeding seasons. Each of these components is likely to be dependent on environmental factors. In those species which deposit all the eggs produced per breeding season over a short time interval, and in semelparous species that are total spawners, any environmental effects on fecundity will be exerted prior to that spawning. In partial or serial spawners, environmental factors may modify fecundity within a breeding season.

The detection of environmental effects on fecundity is complicated in fish because there is a positive relationship between fecundity and body size. Fish usually show indeterminate growth so that as a female grows her fecundity will tend to increase. Appropriate statistical and experimental techniques must be used to partition out the effects of a change in body size on fecundity from any direct effects of environmental factors on fecundity. Any environmental effects on growth in size will probably be reflected in changes in fecundity.

Once the effects of size on fecundity have been removed, the effects of the rate of food consumption on fecundity can be defined. Finally, the effects of other environmental factors on fecundity which cannot be accounted for solely in terms of the effects of those factors on the rate of food consumption can be analysed. Only Hirshfield's (1980) study on O. latipes in which the effects of ration and temperature were analysed using covariance techniques have approached this complete analysis. O. latipes females spawn daily for as long as four months. At 25^{o} and 29^{o}C a positive correlation between the daily rate of egg production and ration was found, whereas at 27^{o}C, there was no significant correlation. The rations consumed at 27^{o} and 29^{o}C were com-

parable, but at 25°C the highest ration consumed was similar to the lowest ration used at the higher temperatures, a striking example of the effect of temperature on food consumption and hence fecundity (Hirshfield. 1980). The apparent interaction between temperature and ration at the higher temperatures raises the possibility that temperature has a direct effect on the reproductive system of this species, but the major effect of temperature is probably mediated through its effect on food consumption.

In G. aculeatus the breeding season lasts two or three months in late spring and summer, with the females spawning at intervals of a few days. The effect of ration and body size on fecundity per spawning was analysed using mutiple regression techniques (Fletcher & Wootton, unpub). The two predictor variables accounted for 55% of the variance in fecundity and there was no significant interaction between them. The standardised regression coefficients indicated that body size was about twice as important as a predictor variable than ration. This result confirms and extends that of Wootton (1977) who noted the importance of body size, but using a smaller number of replicates had not detected a significant effect of ration.

In G. aculeatus and in O. latipes at 27° and 29°C, there was an inverse relationship between R.E. and ration. Thus the lower fecundity at the lower rations nevertheless involved partitioning a higher proportion of the food consumed into egg production. Fish on low rations were sacrificing growth to maintain their reproductive output.

Cichlasoma nigrofasciatum females showed a decline in the fecundity per spawning when kept on low rations. This decline was relatively much greater than was noted in G. aculeatus in which the effect of ration on fecundity per spawning was weak (Townshend & Wootton, unpub).

The marine gadoid, Melanogrammus aeglefinus, is a partial spawner with an extended breeding season. Females on a ration close to maintenance retained the trait of partial spawning, but at each spawning produced far fewer eggs than females on high rations. The fecundity of the experimental fish was compared with the fecundity of wild fish of the same length. Females on the low ration were producing an average of 16.9% of the expected number of eggs, while females on the high ration produced an average of 89.1% of the expected number (Hislop et al, 1978).

In contrast, female G. aculeatus on low rations almost maintained the fecundity per spawning but had fewer spawnings than females on higher rations. Thus the fecundity over the breeding season was highly correlated with ration (Wootton, 1977). This response to food level means that the female G. aculeatus can track food level in terms of their egg output. Young G. aculeatus start eating similar food items to the adults relatively soon after hatching so that the food availability experienced by the adults is likely to be a good predictor of that experienced by their offspring.

The physiological mechanism by which food supply affects fecundity is not known. Nor is it known to what extent different stages of oogenesis are sensitive to food shortage. Atresia and the rate of recruitment of vitellogenic oocytes have been suggested as mechanisms for controlling the number of eggs. In the cyprinid Phoxinus phoxinus, there is some evidence that both mechanisms may operate but at different stages in the annual reproductive cycle. Females collected in autumn and held at 10°C and a 12L12D photoperiod for six weeks had a higher proportion of their oocytes in the stage of vesicular yolk formation if they were fed than if they were starved. The proportion of atretic oocytes was very low in both fed and starved fish. When females were collected in spring and held at 7°C and a photoperiod of 14L8D, the fed fish had a higher proportion of oocytes with granular yolk than starved females, but the latter had a higher proportion of atretic oocytes. In summer conditions of 15°C and 16L8D, starved females lost all their oocytes containing granular yolk and the bulk of their oocytes were in the pre-vitellogenic stages. Since eggs were not spawned during the summer experiment, it was assumed that the ovaries of the starved females had undergone massive atresia during the six weeks of starvation (Mehsin, 1982). Histological studies on the ovaries of wild and experimental M. aeglefinus suggested that the low fecundity of fish on low rations was not due to a change in the rate of atresia but a reduction in the number of oocytes developing that season. In wild fish the number of oocytes being resorbed varied at different stages of oogenesis which suggested that adjustments were continually made to the number of oocytes undergoing maturation. The upper limit of potential fecundity may have been determined by the November prior to the spring in which spawning took place and variations in the food supply then caused reductions from this upper limit (Robb, 1982).

The social environment may influence fecundity (Dahlgren, 1979; Kapur, 1981), but the experimental design for detecting such an effect must show that it is not solely a result of changes in the rate of food consumption as a result of the social environment. In female G. aculeatus housed in pairs in 131 tanks, the aggressiveness of one

of the females frequently prevented access by the other female to the available food, and the subordinate female did not produce eggs. But this result is probably an experimental artefact, in natural populations females are frequently seen feeding in schools and may not form strong dominance relationships in such a situation (Wootton, 1976). It has been suggested that population density can affect the fecundity and fertility of female P. reticulata. Dahlgren (1979) found some evidence that density did affect fecundity and fertility when a constant weight of food was fed but the results were ambiguous. When food was supplied in excess, no effects of density on either fecundity or fertility were detected (Dahlgren, 1980c). Social inhibition of the onset of sexual maturation seems to be a more significant factor than the social modulation of fecundity.

Pathogens particularly parasites interfere with egg production by females. G. aculeatus females infested with the pleurocercoid stage of the cestode Schistocephalus solidus often fail to spawn, and heavily infested females have significantly smaller ovaries than uninfested or lightly infested fish (Wootton, 1976). M. aeglefinus infested with the copepod Lernaeocera branchialis had significantly lower fecundities than uninfested fish of the same length. This significant difference was maintained when the comparison was for females of a given gutted weight (Hislop & Shanks, 1981). Both G. aculeatus and M. aeglefinus show a clear relationship between fecundity and food supply and it is probable that the effects of parasitism in these species are a result of the parasites intercepting resources that should be invested in egg production. But in some species the parasite may interfere directly with the reproductive system of the host to inhibit gonadal development so that the parasites are not competing with the gonads for resources. Disentangling the direct and indirect effects of parasitism on the reproductive system will require careful physiological analysis.

Aspects of water quality such as temperature, pH and level of contamination by pollutants are known to be related to fecundity (Wootton, 1979; Billard, 1981; Gerking, 1980). Gerking's comprehensive study of the cyprinodont Cyprinodon n. nevadensis suggested that reproduction was more sensitive to water quality than other components of the life history. There was a relatively narrow range of optimum temperatures for egg production, and the latter was also sensitive to acid water, whereas there was a relatively wide range of salinities over which egg production was maintained. Since the rate of food consumption is related to temperature and possibly also to pH, it is not clear to what extent water quality exerted its effect on reproduction through its effect on the resources available to be partitioned into gonadal production and to what extent it had a direct effect on the reproductive system. Oogenesis seemed to be sensitive to water quality, but it is also sensitive to food supply.

The effects of environmental factors on the rate of gamete production by male fish have received little attention because of the difficulty in quantifying sperm. In male G. aculeatus the testes are small and probably less sensitive to food levels than the ovaries (Stanley, unpub).

There is a need for a systematic experimental study of the factors which determine the potential fecundity of fishes and the mechanisms by which the actual fecundity is determined including the relative roles of atresia and the rate of recruitment of populations of maturing oocytes.

Gamete quality

Environmental effects on gamete quality have received less attention than their effects on fecundity. Two aspects of egg quality are egg size and chemical composition. In G. aculeatus there was a significant but weak correlation between egg diameter and ration and a positive relationship between egg diameter and the length of the fry at hatching. No significant relationship was found between fry length and the number of days that fry could survive after hatching when starved (Fletcher, unpub). No significant effect of ration on the chemical composition of the eggs was detected. Apart from a slight effect of ration on egg diameter, female G. aculeatus maintained both the size and quality of their eggs in the face of low food supply. Dahlgren (1980a,b) detected no significant effect of different levels of protein or non-protein in the diet on the diameters of ova in P. reticulata.

Some field studies have suggested that environmental factors, including food supply, can have detectable effects on egg quality. In Rutilus rutilus, Kuznetsov & Khalitov (1978) claimed that the amount of fat in the ovaries was higher under favourable feeding conditions, although they found little effect on the weight or diameter of the eggs. In Coregonus albula, the chemical composition of the eggs and their dry weight were related to the lake from which the females were taken. The lake in which fish growth was slowest had the poorest quality eggs (Kamler & Zuromska, 1979). But in these studies the absence of experimental studies makes it difficult to identify the causal basis for the effects reported.

At high temperature, C.n. nevadensis produced a number of small soft egg shells containing no yolk, while at low pH the percentage of abnormal eggs was increased (Gerking, 1980). In Gambusia affinis exposure of females to freshwater in their first week of life caused them to produce heavier than average new born compared with females that were kept in brackish water (Stearns, 1980). In freshwater the G. affinis had to meet the energy costs of osmo-regulation but this did not explain why they produced larger offspring.

The physiological mechanisms by which the size and quality of eggs are controlled are obscure, and so it is difficult to predict the possible effects of environmental factors. The evidence suggests that in the face of environmental fluctuations, fish tend to conserve egg quality rather than fecundity.

Reproductive behaviour

Behaviour associated with the successful reproduction is likely to be sensitive to environmental factors. The reproductive behaviour of male G. aculeatus is complex, it includes territorial defence, nest-building, courtship and parental behaviour. An experimental analysis of the effect of density of males and food ration on some aspects of male behaviour indicated that density was a crucial factor in territorial behaviour and nesting. In a given fixed area, the proportion of males holding a territory and building a nest was lower at higher male densities, and there was a greater chance of a nest being destroyed. Low food rations also caused a reduction in the proportion of males holding a territory and building nests, but had no effect on the proportion of nests destroyed. When males were transferred from the groups to isolation, a high proportion of the males that had received high rations then built nests, even if they had not been successful in the group. In contrast, only a small proportion of the fish that had received a low food ration built nests in isolation (Stanley & Wootton, unpub). Isolated males on low rations spent less time fanning eggs, although their success in hatching a single clutch of eggs was not different from males on higher rations. Food shortage alters the time and probably the energy partitioning of male sticklebacks, but the physiological control mechanisms are not known.

In C.n.nevadensis the range of temperatures over which males showed active chasing of the female was wider than the optimum range for egg production (Gerking, 1980). But, in general, the relative sensitivity of the behavioural components of reproduction to environmental factors such as temperature and pH is not known.

The environmental requirements for successful spawning may, in some species, be rigorous and subtle. Aquatic vegetation is a stimulus for ovulation in Carassius auratus but is an essential cue for spawning (Stacey et al, 1979). P. phoxinus has proved difficult to breed reliably in aquaria, although manipulation of the photoperiod and temperature can stimulate gametogenesis (Scott, 1979). Kapur (1979) noted the difficulty of obtaining spawning in some of the Indian carps in captivity.

Termination of the breeding season

In fish, fecundity is a function of body size, but breeding involves the commitment of resources to spawning that could have been committed to increasing body size. Termination of breeding permits the allocation of resources to somatic growth. G. aculeatus held under permissive conditions of photoperiod and temperature alternates between reproductively active and inactive phases, although the period between the start of successive phases of reproductive activity is less than the year that would separate successive breeding seasons in natural conditions (Baggerman, 1980). Thus there is evidence for an endogenous control of reproductive cycling in G. aculeatus and probably other species too (Sundararaj, 1978). In G. aculeatus environmental factors can modify the length of the reproductively active phase. Females kept on low rations have shortened reproductive phases with premature regression of the ovaries (Wootton, 1977). In the closely related Culaea inconstans, the end of the breeding season coincided with the time when the water temperature went above 20^{o}C for prolonged periods in summer (Reisman & Cade, 1967). Both food shortage and high temperatures caused gonadal regression in Gillicthys mirabilis (de Vlaming, 1974), while food shortage caused rapid regression in the ovaries of P. phoxinus held at 15^{o}C (Mehsin, 1982).

The changes in energy partitioning during gonadal regression would be of considerable interest especially if the physiological mechanisms controlling the changes could also be elucidated. The relative importance of endogenous and exogenous factors in the process needs clarification.

Timing of breeding

Although the control of timing of breeding seasons has received much attention from physiologists, there have been few ecological studies which show why relatively precise control of timing is essential.

Nevertheless, in temperate species, the period of time in which the majority of young fish become free-swimming is relatively short compared with the time potentially available.

Many species have wide north-south geographical ranges, so that a species may experience a wide range of seasonal patterns of photoperiod and temperature. Some species may also establish breeding populations in environmental regimes which they have not experienced in their evolutionary history, e.g. the breeding populations of S. trutta in upland equatorial streams of Kenya. Thus species may be flexible in their use of cues to ensure the correct time of breeding.

Several cues may be available for timing and some may be more reliable than others, but in many cases the cues tend to be confounded. In high latitudes, low temperatures, short photoperiods and low food availability may occur together in the same season. In its evolutionary history, a species may never have encountered these factors varying independently, and so may respond to a complex of cues rather than a single factor. P. phoxinus hides under stones during the day in winter. Only after the temperature has risen above 8°C do the fish become active in daytime and start to experience the longer photoperiods characteristic of spring which stimulate vitellogenesis in the ovaries (Scott, 1979). If fish are presented with cues or combinations of cues that do not occur naturally and have not been encountered during their evolutionary history, then the physiological response may, in functional terms, be nonsensical. Such responses may not throw light on the normal control of timing. In experiments in which fish are exposed to various combinations of factors such as temperature and photoperiod, careful thought has to be given to what stimulus combinations represent control conditions.

The best indication that the role of environmental factors in the timing of a breeding season is reasonably well understood is that the timing can be precisely controlled by the experimental manipulation of factors. Some progress in achieving this has been made for G. aculeatus (Baggerman, 1980) and Salmo gairdneri (Whitehead et al, 1978; Billard, 1980). In both species, one a late spring the other a late winter spawner, there is evidence that different stages in recrudescence of the gonads are sensitive to different environmental conditions. The phases of gametogenesis that require the least investment of materials appear to be the most independent of environmental conditions, whereas the stages in the reproductive cycle which are more demanding of resources whether for gametogenesis or the behavioural aspects of reproduction are more sensitive to the timing cues of photoperiod and temperature.

The relative importance of temperature, photoperiod and other factors as timing cues varies between taxa (de Vlaming, 1974). It is not clear what the relationship is between such variation and the ecological requirements of the adults, eggs and fry, but the ecology of the early ontogenetic stages may be important in determining which cues are most appropriate. The rate of development of eggs is particularly sensitive to temperature, high temperatures permit rapid escape from the egg to the free-living stages. In species in which there is no parental care and the eggs are not laid in a refuge from predators, a period of increasing temperatures may be the most suitable cue for the timing of the final stages of oogenesis. In species with parental care such as G. aculeatus, photoperiod may be a more reliable timing cue.

Disentangling the effects of temperature, photoperiod and other factors on the timing of reproduction requires consideration of the environmental conditions and the ecology of the species over the normal reproductive cycle. G. aculeatus fry hatched in June and then kept on a 16L8D photoperiod at 20°C do not become sexually mature (Baggerman, 1980). The first month after hatching is a period of rapid somatic growth (Allen, 1980). In autumn, spermatogenesis and oogenesis to the stage of vesicular yolk formation take place in this period of declining temperatures and photoperiod. At low rations, somatic growth still has priority over gonadal growth while at high rations the growth of the liver is particularly high. At this stage, exposure to a photoperiod of 16L8D at 20°C will induce sexual maturation within 65 days, but natural populations will not experience such conditions. Shorter photoperiods do not induce sexual maturation. Over the winter, the gonads, especially the ovaries continue to mature slowly even at low rates of food consumption. As the winter progresses, sexual maturation after the fish are exposed to 20°C can be stimulated by shorter and shorter photoperiods, so that by the end of February, a photoperiod of 8L16D is sufficient, but under such conditions the length of the period of active sexual activity is short. In natural conditions, the lengthening photoperiod and the increasing temperature of spring permit sexual maturation, then the long photoperiod and warm temperature of late spring and summer maintain an extended breeding season. Throughout the annual cycle, until the onset of spawning, the gonads seem to be relatively insensitive

to fluctuations in the food supply, the liver and the carcase buffer the effect of variations in food supply. Thus photoperiod and temperature cues are used to control the timing of reproduction, but the number of spawnings after the onset of breeding is a function of food availability (Baggerman, 1980; Wootton, 1977; Allen & Wootton, in press).

Breeding in the female G. aculeatus makes heavy demands for energy and materials because of the high cytoplasmic production in the form of eggs, while breeding in the male makes heavy demands on energy because of the complex reproductive behaviour. Baggerman (1980) has suggested that changes in sensitivity to photoperiod during the annual cycle of G. aculeatus depend on changes in a photoreactivity threshold, the timing of which depends on a circadian rhythm in sensitivity to light. It would be interesting to know if there are also changes in feeding motivation correlated with changes in the threshold, so that the energy demands of sexual maturation are met. Such a correlation is suggested by the observation that at the end of a breeding season, the mature G. aculeatus become refractory to long photoperiods (Baggerman, 1980), and also show a decline in the ad lib food consumption (Fletcher, unpub).

In G. aculeatus a relatively endogenous cycle of recrudescence and regression of the gonads has its timing modulated by temperature-dependent photoperiodic responses, but the quantity of reproduction is primarily determined by food supply.

Conclusion

Because of the potential complexities in the relationships between environmental factors and reproduction, both careful experimental design and statistical analysis are required in the elucidation of these relationships. Many of the relationships are size-dependent such as the relationship between fecundity and size, so that adequate techniques for controlling for size-dependence must be used. The experimental design should also permit the detection both of the main effects of the experimental factors and their interactions. Careful attention must also be paid to the appropriate level of replication. Some experiments are difficult to interpret because while the experiment is essentially a nested-design with a group of fish in a tank all given the same treatment, the subsequent analysis treats each fish as an independent replicate, so that any "tank" effects are confounded with "treatment" effects. Such "tank" effects may be important in species in which social interactions such as dominance hierarchies develop.

Physiological experiments should also take into account the ecology of the species so that meaningful combinations of the relevant environmental factors can be presented (Scott, 1979). The geographical origin of the experimental population should also be considered, for there may be significant inter-population differences in the response to combinations of environmental factors. Any differences would be relevant to the prediction of the effects of environmental factors on reproduction, but may also yield some insight into the physiological mechanisms through which the factors have their effects.

The bioenergetics model of fish reproduction could form a valuable framework within which the physiological analyses are synthesised. The model centres attention on an environmental factor that is frequently ignored or inadequately controlled in experiments, that is the rate of food consumption. Given the energy and material costs of reproduction, food consumption and the subsequent partitioning of energy and materials between reproductive and non-reproductive activities represent major processes which will be influenced by environmental factors and the physiological mechanisms that mediate the effects of the factors.

References

Allen, J.R.M., 1980. The estimation of the natural feeding rate of the three-spined stickleback (Gasterosteus aculeatus L.) (Pisces). Ph.D. Thesis, University of Wales.

Allen, J.R.M. & R.J. Wootton, 1982. The effect of ration and temperature on the growth of the three-spined stickleback, Gasterosteus aculeatus L. J. Fish Biol. 20:409-422.

Alm, G., 1959. Connection between maturity, size and age in fishes. Rep. Inst. Freshw. Res. Drottningholm 40:5-145.

Baggerman, B. 1980. Photoperiodic and endogenous control of the annual reproductive cycle in the teleost fishes. In: M.A. Ali (Ed.): Environmental physiology of fishes. Plenum, New York. p. 533-567.

Billard, R., 1980. Les possibilities de controle de la reproduction chez les poissons par modulation des facteurs de l'environnement et manipulations hormonales. Cah. Lab. Hydrobiol. Montereau 10: 11-32.

Billard, R., C. Bry & C. Gillet, 1981. Stress, environment and reproduction in teleost fish. In: A.D. Pickering (Ed.): Stress and fish. Academic Press, London. p. 185-208.

Borowsky, R.L., 1978. Social inhibition of maturation in natural populations of Xiphophorus variatus (Pisces: Poeciliidae).

Science 201:933-935.
Conover, D.O. & B.E. Kynard, 1981. Environmental sex determination: interaction of temperature and genotype in a fish. Science 213:577-579.
Constantz, G.D., 1979. Life history patterns of a livebearing fish in contrasting environments. Oecologia 40:189-201.
Dahlgren, B.T., 1979. The effects of population density on fecundity and fertility in the guppy, Poecilia reticulata (Peters). J. Fish Biol. 15:71-92.
Dahlgren, B.T., 1980a. The effects of three different dietary protein levels on the fecundity in the guppy, Poecilia reticulata (Peters). J. Fish Biol. 16:83-98.
Dahlgren, B.T., 1980b. The influence of three dietary non-protein levels on fecundity and fertility in the guppy Poecilia reticulata. Oikos 34:337-346.
Dahlgren, B.T., 1980c. Influences of population density on reproductive output at food excess in the guppy, Poecilia reticulata (Peters). Biol. Reprod. 22: 1047-1061.
de Vlaming, V.L., 1974. Environmental and endocrine control of teleost reproduction. In: C.B. Schrek (Ed.): Control of sex in fishes. Virginia Polytechnic and State University, Blacksburg, VA. p. 18-83.
Elliott, J.M., 1981. Some aspects of thermal stress on freshwater teleosts. In: A.D. Pickering (Ed.): Stress and fish. Academic Press, London. p. 208-245.
Fagerlund, U.H.M., D.A. Higgs & J.R. McBride, 1979. Influence of feeding a diet containing 17a-methyl testosterone or testosterone at two ration levels on growth, appetite and food conversion efficiency of underyearling coho salmon (Oncorhynchus kisutch). Proc. World. Symp. Finfish Nutr. Fish Feed Tech. 1:221-230.
Gerking, S.D., 1980. Fish reproduction and stress. In: M.A. Ali (Ed.): Environmental physiology of fishes. Plenum, New York. p. 569-587.
Glebe, B.D. & W.C. Leggett, 1981. Latitudinal differences in energy allocation and use during the freshwater migrations of American shad (Alosa sapidissima) and their life history consequences. Can. J. Fish. Aquat. Sci. 38:806-820.
Hirshfield, M.F., 1980. An experimental analysis of reproductive effort and cost in the Japanese medaka, Oryzias latipes. Ecology 61:282-292.
Hislop, J.R.G. & A.M. Shanks, 1981. Recent investigations on the reproductive biology of the haddock Melanogrammus aeglefinus, of the northern North Sea and the effects on fecundity of infection with the copepod parasite Lernaeocerca branchialis. J. Cons. int. Explor. Mer. 39:244-251.
Hislop, J.R.G., A.P. Robb & J.A. Gauld, 1978. Observations on the effects of feeding level on growth and reproduction in haddock, Melanogrammus aeglefinus (L.) in captivity. J. Fish. Biol. 13:85-98.
Kamler, E. & H. Zuromska, 1979. Quality of eggs and production of Coregonus albula (L.) in three Masurian Lakes. Pol. Arch. Hydobiol. 26:595-623.
Kapur, K., 1981. Spawning of major Indian carps - a review. Acta hydochim hydobiol. 9:163-182.
Kuznetsov, V.A. & N. Kh. Khalitov, 1978. Alterations in the fecundity and egg quality of the roach, Rutilus rutilus in connection with different feeding conditions. J. Ichthyol. 18:63-70.
Matty, A.J. & K.P. Lone, 1979. The effect of androgenic steroids as dietary additives on the growth of carp (Cyprinus caprio). Proc. Worl. Maricul. Soc. 10: 735-745.
Mehsin, K.A.A., 1982. Some effects of food supply on the annual reproductive cycle of female Phoxinus phoxinus (L.). Ph.D. Thesis, University of Wales.
Reisman, H.M. & T.J. Cade, 1967. Physiological and behavioural aspects of reproduction in brook stickleback, Culaea inconstans. Amer. Midl. Nat. 77:257-295.
Reznick, D. & J.A. Endler, 1982. The impact of predation on life history evolution in Trinidadian guppies (Poecilia reticulata). Evolution 36:160-177.
Robb, A.P., 1982. Histological observations on the reproductive biology of the haddock, Melanogrammus aeglefinus (L.). J. Fish Biol. 20:397-408.
Scott, D.B.C., 1979. Environmental timing and the control of reproduction in teleost fish. Symp. zool. Soc. Lond. 44:105-132.
Shapiro, D.Y., 1979. Social behaviour group structure and the control of sex reversal in hermaphroditic fish. Adv. Study Behav. 10:43-102.
Silverman, H.I., 1978. The effects of visual social stimulation upon age at first spawning in the mouth brooding fish Sarotherodon (Tilapia) mossambicus (Peters). Anim. Behav. 26:1120-1125.
Sohn, J.J., 1977. Socially induced inhibition of genetically determined maturation in the platyfish, Xiphophorus maculatus. Science 196:199-201.
Stacey, N.E., A.F. Cook & R.E. Peter, 1979. Spontaneous and gonadotropin induced ovulation in the gold fish Carassius auratus L.: the effects of external factors. J. Fish Biol. 15:349-362.
Stearns, S.C., 1980. A new view of life-history evolution. Oikos 35:266-281.
Sundararaj, B.I., 1978. Environmental regulation of annual reproductive cycles in the catfish Heteropneustes fossilis. In: I. Assemacher & D.S. Farner (Eds.): Environmental endocrinology. Springer-Verlag, Berlin. p. 26-27.
Whitehead, C., N.R. Bromage, J.R.M. Forster & A.J. Matty, 1978. The effects of alter-

ations in photoperiod on ovarian development and spawning time in the rainbow trout (Salmo gairdneri). Ann. Biol. anim. Bioch. Biophys. 18:1035-1043.

Wootton, R.J., 1976. The biology of the sticklebacks. Academic Press, London.

Wootton, R.J., 1977. Effect of food limitation during the breeding season on the size, body components and egg production of female sticklebacks (Gasterosteus aculeatus). J. Anim. Ecol. 46:823-834.

Wootton, R.J., 1979. Energy costs of egg production and environmental determinants of fecundity in teleost fishes. Symp. zool. Soc. Lond. 44:133-159.

Wootton, R.J., J.R.M. Allen & S.J. Cole, 1980a. Energetics of the annual reproductive cycle in female sticklebacks, Gasterosteus aculeatus L. J. Fish Biol. 17:387-394.

Wootton, R.J., J.R.M. Allen & S.J. Cole, 1980b. Effect of body weight and temperature on the maximum daily food consumption of Gasterosteus aculeatus L. and Phoxinus phoxinus (L.): selecting an appropriate model. J. Fish Biol. 17:695-705.

CONTROLLED REPRODUCTION IN THE ROACH (RUTILUS RUTILUS L.)

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Summary

Timing of reproduction in teleosts, determined principally by environmental factors, generally ensures that favourable conditions are available for larvae. However in fish culture artificial advancement of spawning is a valuable technique, particularly in extending the growing season for fry. Manipulation of photoperiod and temperature has been used to accelerate gametogenesis and spawning in the roach, as part of a study on culture of this species. 48 fish of mixed sex (60-120g) were introduced to aquaria in late autumn. Experimental groups were subjected to a photo-period accelerating at twice the natural rate (which was used for controls) from November, with both groups kept in spring-like water temperature (9-16°C). Experimental groups spawned in early February, some 13-16 weeks before the normal spawning period (May/June) of local wild populations. Only one control fish spawned, and then poorly in response to hypophysation. Experimental females showed a surge in plasma calcium (indicative of vitellogenesis) prior to spawning, but not the controls. Evidently photoperiod is the principal factor synchronizing reproduction in the roach though temperature has a supportive role.

Keywords: roach, reproduction, photoperiod, advanced spawning, gametogenesis.

Introduction

The mechanisms controlling reproduction in teleosts ensure that favourable conditions (e.g., temperature and food supply) are present when larvae emerge. Thus most species use regular, predictable changes in the environment, daylength and temperature for example, to cue reproduction; these factors probably interact with endogenous rhythms. This principle may be exploited by man in order to control the reproduction of captive species. Environmental regulation of reproduction has been frequently reviewed (e.g. de Vlaming, 1972; Billard & Breton, 1978; Crim, 1982) and has been shown to differ according to species. Endogenous rhythms are important in some fish, e.g., the catfish Heteropneustes fossilis (Sundararaj et al., 1978), which occupy environments with little seasonal change in light and temperature. In temperate climes, seasonal changes in daylength particularly, and temperature, become more pronounced, and fish of these regions use them to regulate reproductive cycles. For example, daylength plays the major role in synchronizing reproduction in salmonids, whereas both daylength and temperature may be important in many cyprinids (de Vlaming, 1972). Hence, strategies for controlling reproduction artificially, must be based on natural regulating mechanisms. Thus, time of spawning can be considerably modified by manipulating photoperiod for rainbow trout Salmo gairdneri (Whitehead et al., 1978) and water temperature for carp Cyprinus carpio (Kossman, 1975).

This study concerns the roach, a freshwater cyprinid of temperate regions which has been rarely studied previously. Commercial demand by anglers in Britain has prompted the investigation of culture techniques including induced spawning, which has been shown to be straightforward in ripe fish (Worthington et al., in press). However, spawning may be advanced only a few weeks by this method alone, and in order to extend the growing season of fry significantly, the acceleration of gametogenesis itself is required. Since photoperiod and temperature both influence reproduction strongly in roach (Jafri, 1980) both were manipulated in this study in an attempt to accelerate gametogenesis and thereby advance spawning.

Materials and Methods

48 roach of mixed sex (60-120g) from Severn-Trent Water Authority's coarse fish farm were introduced to two 140Ol recirculating systems, each equipped with an ultraviolet steriliser, heater, settling tank and biological filter. Each aquarium was provided with artificial spawning substrate. All fish were individually identified by subcutaneous injection of alcian blue dye, then maintained under the following regimes:

		Advanced photoperiod	Natural photoperiod
n		24	24
Temperature	°C	9-16	9-16
Photoperiod		8L:16D to 16L:8D	Natural

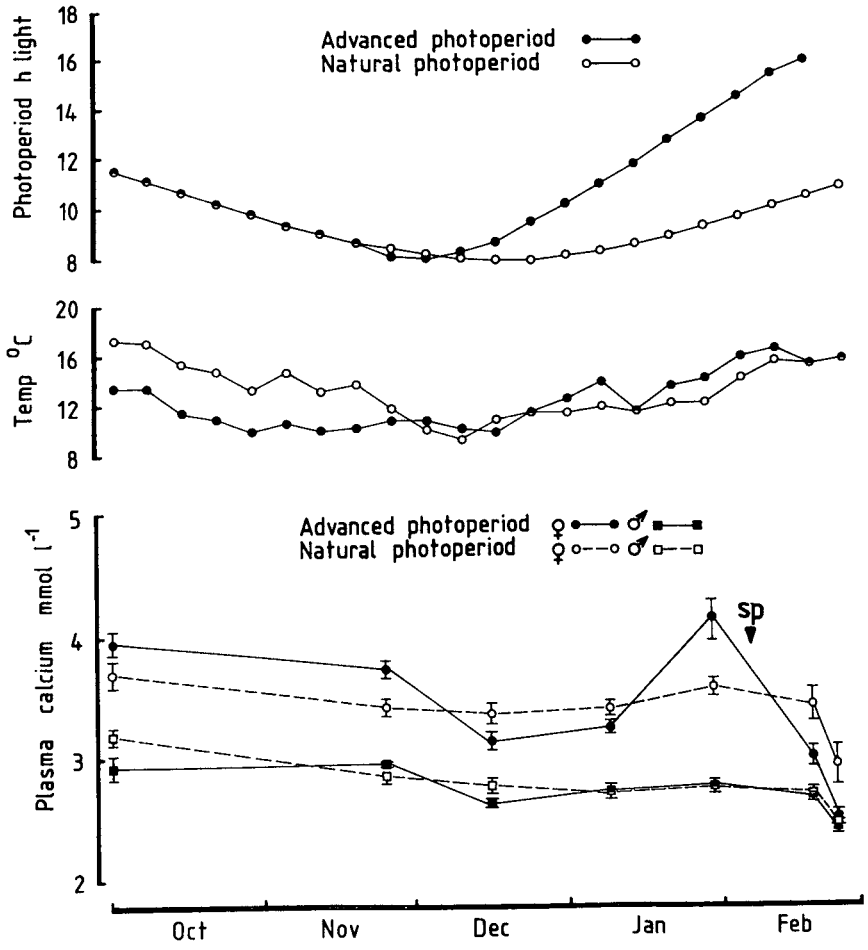
The advanced photoperiod began with 8L:16D in late November, accelerating thereafter at twice the natural rate to 16L:8D scheduled for late February. Natural photoperiod

Table. Reproductive events recorded from roach maintained under different photoperiod regimes (advanced: ♂ n=8, ♀ =16; natural: ♂=9, ♀=15).

	Advanced Photoperiod				Natural Photoperiod			
Date	♀ Releasing Ovulated Eggs (N)	♂ With Tubercles (N)	♂ Releasing Semen (N)	Spawning	♀ Releasing Ovulated Eggs (N)	♂ With Tubercles (N)	♂ Releasing Semen (N)	Spawning
5.2.82	0	7	3	-	0	7	0	-
7.2	2	8	4	√	0	7	0	-
8.2	0	8	4	-	0	7	0	-
10.2	1	8	7	√	0	7	2	-
11.2	1	8	7	√	0	7	2	-
14.2	1	6	6	√	0	7	0	-
17.2	2R	4	2	-	0	3	1	-
19.2			Administered CPE 7.5 - 12.5 mg kg^{-1}					
20.2	0	4	6	-	0	3	3	-
21.2	1	4	6	√	1	3	3	√•

R = Resorbing female
• = Poor spawning

Figure. Plasma calcium values ($\bar{x}$ ± s.e.) for roach maintained under the indicated photoperiod and water temperatures.



corresponded to sun-rise and sun-set (GMT). Temperatures were maintained above 9°C. Photoperiod was adjusted weekly, and temperatures recorded daily.

Blood samples were taken from the caudal vasculature at 4 then 3 weekly intervals. Plasma was analysed by fluorometric titration for total calcium (indicative of vitellogenin, Elliot et al., 1979). During blood sampling, fish were examined for the development of tubercles, spermiation, or ovulation. To check the state of gonad maturity all fish were hypophysised with carp pituitary extract (CPE) given as 3 I/P injections (12.5mg kg^{-1} over 24h).

Results

Data on photoperiod, temperature and plasma calcium are shown. Precise temperature matching of the two groups was not possible with the available facilities and the very severe winter. On 29 January females in the "advanced" group were observed to be very rotund and near spawning. Subsequent reproductive events are summarized in the table. The advanced fish spawned spontaneously on four occasions beginning 7 February. One female from each regime ovulated and spawned after hypophysation. Both of these spawnings gave viable eggs although hatching was better in experimental (70-80%) than control fish (30-40%). Overall, viability of larvae was excellent. Marked changes in plasma calcium were confined to the females under advanced photoperiod, which increased greatly between 8 and 29 January ($P < 0.001$). Then during spawning, calcium fell sharply ($P < 0.001$). No such changes were observed in controls. Prespawning calcium levels in males were consistently lower than in females. Levels in all groups declined markedly ($P < 0.05$) between 18 and 23 February.

Discussion

The group maintained under the compressed natural photoperiod spawned 13 to 16 weeks before a local, wild population, which spawned between 19 May and 9 June (Worthington et al., unpubl.). Similar studies on annually spawning teleosts show that spawning can be advanced 11 weeks in coho salmon Oncorhynchus kisutch (Macquarrie et al., 1978) and 12 weeks in rainbow trout, Salmo gairdneri (Whitehead et al., 1978). However, the fish in the present study were only manipulated for 10 weeks, as opposed to the 33 months for coho salmon and 6 months for rainbow trout. It is likely that the prespawning period offers the greatest potential for advancing spawning in cyprinids, because gonad activity is low or retarded over winter (Billard & Breton, 1978).

It is clear that all of the environmental requirements for complete gametogenesis and spawning were fulfilled in the experimental group, by the spontaneous nature of spawning. The control group did not spawn spontaneously despite the fact that temperature surpassed the spawning threshold of 14°C (K. Easton, pers. comm.). However, one control female did ovulate and spawn sparingly after hypophysation, though the hatching rate was very poor. This may have been due to poor egg quality or fertilization. Overall, it seems that whilst daylength is the major environmental factor synchronizing the prespawning and spawning stages of the reproductive cycle of the roach, temperature plays a modulatory role. Unfortunately, incomplete temperature control prevents further interpretation of this aspect. It is considered that a period of short daylength and low temperature is probably required, because roach kept under long day and 16°C during the prespawning phase show gonad regression (Worthington et al., unpubl.). Thus, short daylength and low temperature may be stimulatory as in another cyprinid Mirogrex terrae-sanctae (Yaron et al., 1980) in which high temperatures block vitellogenesis, although this species is a winter-spawner.

It is noteworthy that all fish in the control group matured within one week of each other, when the photoperiod was 14.5L:9.5D (corresponding to mid-May, the start of the natural spawning season). This group synchrony is essential in a species which spawns annually in shoals, and indicates the precision of the mechanism controlling reproduction. However, because the aquaria were arranged in parallel in a recirculating system, it is possible that pheromones released from early maturing fish induced spawning behaviour and maturation in fish in other tanks, as suggested by evidence from the goldfish (Partridge et al., 1976).

The increases in plasma calcium in females due to vitellogenesis are lower than those characteristic of salmonids, presumably because these species have much larger, more yolky eggs, requiring a higher level of vitellogenesis. Despite the differences in absolute values, the gonad patterns are similar to those seen in the rainbow trout subjected to compressed photoperiod cycles (Whitehead et al., 1978). In contrast, the peak seen in the compressed cycle roach group is more acute compared to the control group, and also a wild population, where plasma calcium for three months prior to spawning was elevated (Worthington et al., unpubl.). This indicates that vitellogenesis during the prespawning period was accelerated under a regime of increasing daylength and temperature.

Further experiments are needed in order to define more precisely the environmental requirements for reproduction in roach. Never-theless, application of the environmental regime described would facilitate a 30-40% extension of the growing season of 0+ roach.

Acknowledgement

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References

Billard, R. & B. Breton, 1978. Rhythms of reproduction in teleost fish. In: J.E. Thorpe (Ed.): Rhythmic activity of fishes. Acad. Pres, London. pp. 31-53.

Crim, L.W., 1982. Environmental modulation of annual and daily rhythms associated with reproduction in teleost fishes. Can. J. Fish. Aquat. Sci. 39:17-21.

Elliott, J.A.K., N.R. Bromage & C. Whitehead, 1979. Effects of oestradiol-17β on serum calcium and vitellogenin levels in rainbow trout. J. Endocrinol. 83:54-55.

Jafri, S.I.H., 1980. The control of reproduction in the roach. Ph.D. thesis. University of Liverpool.

Kossman, H., 1975. Reproduction experiments on carp (Cyprinus carpio). EIFAC 25:122-126.

Macquarrie, D.W., J.R. Markert & W.E. Vanstone, 1978. Photoperiod induced off-season spawning of coho salmon (Oncorhyncus kisutch). Ann. Biol. anim. Bioch. Biophys. 18:1051-1058.

Partridge, B.L., N.R. Liley & N.E. Stacy, 1976. The role of pheromones in the sexual behaviour of the goldfish. Anim. Behav. 24:291-299.

Sundararaj, B.I., P. Nath & V. Jeet, 1978. Role of circadian and circannual rhythms in the regulation of ovarian cycles in fishes: a catfish model. In: P.J. Gaillard and H.H. Boer (Ed.): Comparative Endocrinology. Elsevier/North Holland, Amsterdam. pp. 137-140.

de Vlaming, V.L., 1972. Environmental control of teleost reproductive cycles: a brief review. J. Fish Biol. 4:131-140.

Whitehead, C., N.R. Bromage, J.R.M. Forster & A.J. Matty, 1978. The effects of alterations in photoperiod on ovarian development and spawning time in the rainbow trout (Salmo gairdneri). Ann. Biol. anim. Bioch. Biophys. 18:1035-1043.

Worthington, A.D., N.A.A. Macfarlane & K.W. Easton. The induced spawning of roach, Rutilus rutilus L., with the antioestrogens clomiphene and tamoxifen. J. Fish Biol. (in press).

Yaron, Z., M. Cocos & H. Salzer, 1980. Effects of temperature and photoperiod on ovarian recrudescence in the cyprinid fish Mirogrex terrae-sanctae. J. Fish Biol. 16:371-382.

THE SENSITIVITY OF REPRODUCTION IN FISHES TO STRESSFUL ENVIRONMENTAL CONDITIONS

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Abstract

Temperature, salinity and acidity have a pronounced influence on reproduction of the desert pupfish, Cyprinodon n. nevadensis. Reproductive performance was evaluated using dual criteria of egg production and egg viability. Some observations were also made on eggs during their maturation in the ovary. Tests were performed under conditions that were optimal for reproduction as well as sublethal environments above and below the optimum. Oogenesis proved to be sensitive to stress and underlay the reduced production and viability of eggs that was observed under stressful conditions. The reproductive tolerance limits (50% of peak performance) for the three stressful factors employed were considerably narrower than the lethal limits for larvae and adults.

Tests designed to reveal physiological compensation of the reproductive process to temperature and pH stress were negative. Evidently the reproductive tolerance limits are fixed, i.e. they cannot be shifted by prolonged exposure to stressful environments.

Keywords: fish reproduction, stress on reproduction, oogenesis, egg production, reproductive performance, temperature, ph, salinity.

Introduction

This paper will review the work in my laboratory on the effects of temperature, salinity and acidity on reproduction in the desert pupfish, Cyprinodon n. nevadensis. Departures from optimum conditions for these three factors cause a reduced reproductive performance as measured by egg production and egg viability. Under these conditions the fish is considered to be under stress. We have called it reproductive stress because the other vital functions are affected little, if at all, and the circumstances under which it occurs do not lead to premature death. The stresses that we have applied are systemic in nature, affecting the physiology of the whole animal, in contrast to specific poisons that act directly on the developing egg or sperm.

The term reproduction is used in a restricted sense to include oogenesis, egg production and egg viability. Some material on larval survival tests is included to show that the earlier stages in the reproductive process are more sensitive to stress than is the organism which hatches from the egg.

The pupfish, a member of the family Cyrinodontidae, was chosen as the experimental animal because it lays eggs nearly every day for a sustained period of time (14 weeks in one experiment), it is small and resistant to disease, and its eggs can be collected and cultured easily. This species is adapted to water of high temperature, high salinity and high alkalinity which must be taken into account when comparing its responses with other species. Two other cyprinodonts, the flagfish, Jordanella floridae, and the sheepshead minnow, Cyprinodon variegatus, and a member of the family Cyprinidae, the bluntnose minnow, Pimephales promelas, have been used for most of the reproductive studies similar to those reported here (Craig & Baksi, 1977; Hansen et al., 1977; Mount, 1973).

Oogenesis effect

Temperature stress

The oogenesis effect is the negative effect of a stressful environment on some stage of development while the egg is in the ovary. We first recognized this effect when egg viability was compared at two temperature regimes: (1) success of hatching of eggs laid by females at 28 C (optimum temperature) and incubated above and below that temperature, and (2) success of hatching of eggs laid and incubated at the same temperature, i.e. not transferred to another temperature. The latter regime subjects the egg to a constant temperature in the ovary as well as during incubation. This experimental design more closely resembles the situation in nature than the first which is designed to test the developmental tolerance of eggs all laid at a uniform temperature. The two types of experiments yielded strikingly different results both above and below the optimum temperature. For example, at 22C the former experiment gave 75% of a normal hatch while the latter gave 1%; about the same disparity was found at 34C. Temperature stress was evidently responsible for the disparity, causing some change in the egg while it developed in the ovary. This view was strengthened when we observed that acclimated females laid numerous eggs (5 eggs $g^{-1}day^{-1}$) at 32C but hatching success was only 15%. Many eggs reached maturity, therefore, but only a few possessed the qualities required for hatching. Small, fragile eggs with soft chorions and little or no yolk were observed. Mean yolk diameter was reduced to 1.02 at 32C from 1.08 mm at 20C. The relatively high egg production coupled with poor quality at 32C has been

observed several times among the variety of experiments on temperature effects that we have performed (Shrode & Gerking, 1977; Gerking et al., 1979).

Investigators working in Ontario, Canada, on smallmouth bass, Micropterus dolomieui, have been among the foremost advocates of the influence of temperature on reproductive success in nature. Examining the population dynamics of the bass, Christie & Regier (1973) explained year to year variations in recruitment by the unpredictable occurrences of low summer air temperatures. Two effects were noted: desertion of nests by males when the temperature fell below 14C during spawning and poor growth of young-of-the-year at low temperatures resulting in abnormal overwinter survival. These ideas were later expanded upon by Shuter (1980). Forney (1972) felt that recruitment was dependent upon the average temperature during the June spawning season. These authors do not entertain the possibility of a direct influence of temperature on the gonads. We suggested (Shrode & Gerking, 1977) that the temperature experience of females at critical times during egg maturation might explain some of the order of magnitude differences in year class strength in natural populations. This point of view is backed up by Kaya (1973a) who demonstrated that temperature has a direct action on the gonads independent of pituitary and gonadal hormones. Kaya (1973b) also concluded that gonad regression after the spawning period was more rapid at high temperatures (24-25 compared with 10.5C) regardless of the photoperiod (4 and 15 hours of light). The relative importance of photoperiod and temperature, when applied together, on teleost reproduction is not entirely clear (see review by de Vlaming, 1975), but the fact that temperature has some influence on gametogenesis is not disputed.

pH stress

The oogenesis effect is also evident in acid-stressed fish. Ruby et al. (1977) classified seven stages of oogenesis in the flagfish, subjected the fish to a graded series of acidities ranging from pH 6.7 to 4.5, and examined the ovaries histologically after 20 days of exposure. The number of early stages (Stages 0-1) increased, or "piled up", and the number of the late stages (Stage 6) were reduced as compared with the same stages of development in the controls at pH 6.7. The oocytes apparently have difficulty progressing through the maturation process. Primary vitellogenesis occurs at Stage 4, and the authors felt that vitellogenesis and RNA protein synthesis leading to yolk formation were both inhibited by acid stress. Stage 4 corresponds to the beginning of true vitellogenesis in teleost fish (Wallace, 1981), which involves the following events: (1) sequestering of hepatically derived vitellogenin, a yolk precursor, (2) transfer of vitellogenin from blood to yolk spheres, (3) enhancement of vitellogenin transfer by pituitary gonadotropin and (4) fusion of yolk spheres. Thus, the blockage of oogenesis due to acid stress may be caused by an inhibition of the synthesis of vitellogenin in the liver or to a depressed secretion of gonadotropin which enhances the vitellogenin transfer.

Atresia, or egg resorption, was observed by Ruby et al. (1977) in varying amounts at all pH levels including the control. We also observed a similar response in the pupfish ovary. At pH 8.3 (control) there were 343 oocytes of which 18 were mature; at pH 5.5 oocytes numbered 269 with 12 mature; and at pH 5.0, 255 oocytes and 8 mature. The percentage of mature oocytes judged to be abnormal were: 12% at pH 8.3, 24% at pH 5.5 and 60% at pH 5.0. Presumably these abnormal eggs are resorbed. Ball (1960) felt that environmental stress was the principal cause of atresia, although he observed the phagocytic resorption of eggs in ovaries of normal fish as well. This corresponds with observations on the pupfish and the flagfish. An outstanding example of atresia in nature is provided by Beamish (1974) who observed mature eggs in the ovaries of acid-stressed white suckers, Catostomus commersoni, but these eggs were not deposited.

Egg production

The number of eggs spawned is a reflection of the number that successfully progress through the final stages of oogenesis. Since egg production can be studied without killing the animals and preparing the ovaries for microscopic analysis, this measure of reproductive output was used most often. An aquarium divider between males and female pupfish is removed once a day for 21 days; courting behavior ensures; the female, followed by the male, dives into a black yarn mop on the bottom of the tank; and one or more eggs are laid individually and fertilized on the threads. The adhesive eggs can be removed from the threads with tweezers and transferred to incubating dishes.

Temperature stress

Egg production exhibited a graded response to temperature stress above and below the optimum. The number of eggs were counted daily at 10 temperatures ranging from 18 to 36C at two degree intervals. They were expressed as eggs per spawning, eggs $g^{-1}day^{-1}$ and percentage of the total number of days (21) on which spawning occurred. All three criteria gave the same result, and only eggs $g^{-1}day^{-1}$ are reported here. The optimum temperature was 30C with an egg production of 8 eggs g^{-1} day^{-1}, and 50% of this value was achieved at 24 and 32C. Since the eggs produced at 32C were of poor quality (see above), the toler-

ance limits for reproduction were arbitrarily established at 50% of peak egg production and egg viability. These limits were observed at 24 and 30C, far narrower than the lethal limits of 2 and 44C. Upon comparing this narrow band of favorable temperatures with conditions at the locality from which the fish were collected, Saratoga Springs in Death Valley, California (Deacon, 1968), successful spawning, judged on the basis of temperature alone, extends from April to November. Rarely are temperatures exceeding the upper limit for reproduction experienced during this period. The upper limit for reproduction in the pupfish was the highest known for teleosts until Smith & Chernoff (1981) reported that Cyprinodon sp and Gambusia cf. senilis "...appear to constitute breeding populations" at 39.2-43.8C in a thermal stream near San Diego, Chihuahua, Mexico.

Salinity stress

The euryhaline cyprinodonts have the highest tolerance to salinity of any teleost group. For example, the sheepshead minnow survives in water up to 142 p.p.t. (Simpson & Gunter, 1956). The reproductive salinity tolerance limits of the pupfish proved to be broad, ranging from 0.5 to 20 p.p.t. Reproductive performance dropped rapidly above and below these levels. The lethal tolerance range, however, is even broader, extending from 0.1 to 53 p.p.t. (Lee & Gerking, 1980). The pupfish obviously is able to reproduce successfully over only a fraction of this range. Kinne (1964) states that this is the case for several organisms, but little work on fishes has been done for comparison with these results. Kinne & Kinne (1962) found that successful hatching occurred in C. macularius up to 70 p.p.t. in contrast to the 20 p.p.t. observed in our experiments. Of the little additional related information in the literature, there is some debate about the effect of low salinity on reproduction in the euryhaline mullets, Mugil cephalus and M. capito. Abraham et al. (1966, 1967) claim that vitellogenesis is inhibited in fresh water, but Eckstem & Eylath (1970) report that reproduction is not disturbed if these species are transferred to fresh water as fry and are reared to maturity in this environment.

pH stress

Pupfish reproduction is severely inhibited when the pH is lowered only a modest amount from the level of its habitat, pH 8.3. Egg reproduction was halved at pH 7.0 (8.75 eggs $g^{-1}day^{-1}$ at pH 8.3; 3.86 eggs $g^{-1}day^{-1}$ at pH 7.0), and reproductive output dropped steadily to pH 5.0 at which level virtually no eggs were laid. Egg viability was reduced to half the control value at about pH 7.0, and no eggs hatched below pH 6.0. In view of the fact that the LC_{50} of larval pupfish was pH 4.7, it is clear that reproduction responds more quickly and more severely to acid stress than do other vital functions. This result is supported by Craig & Baksi (1977) using the flagfish and by Mount (1973) with the fathead minnow. Flagfish reproduction, for example, was halved at about pH 6.0 from the control level at pH 6.7, while the lethal point was pH 4.5. The pupfish is adapted to live in more alkaline water than these other species, but the difference between the level of pH causing reproductive stress and that resulting in death is the same in every species tested in this fashion.

Acclimation

Reproduction is one of the most complex vital functions, involving many elements which must mesh together in order to produce viable eggs in sufficient quantity, such as the brain-pituitary-adrenal-gonad complex, neurological mechanisms and meiotic chromosomal re-arrangements. If fish reproduction adapts to a stressful environment, or compensates for the physiological disturbance it suffers, by producing a normal number of viable eggs, then the spectrum of environments would be broadened over which successful reproduction could be achieved. This response would have both theoretical and practical significance; survival value would be enhanced and physical-chemical habitat alterations would not be as detrimental as would otherwise be indicated.

Temperature

We have tested the ability of pupfish reproduction to adapt to temperatures that result in subnormal egg production and viability. Fish were exposed for a period of time to an environment known to reduce the reproductive output; egg production and viability were measured; and this output was compared with that of fish which did not have the benefit of such exposure, i.e. fish which were transferred immediately from normal to sublethal conditions. The reverse was done also. Fish acclimated to the sublethal environment were transferred and tested immediately under normal conditions.

Since we had no guidance about how long reproductive acclimation to temperature might take, an entire generation from mature female to mature female was exposed to the same temperature (Gerking et al., 1979). Thus, oogenesis, the egg, the larva and finally the sexually mature adult had the same temperature experience. Broods were reared at two sublethal temperatures, 24 and 32C, and at the 28C optimum. Egg production was measured at the rearing temperature and also at the temperature to which they had not been exposed for a whole generation. Thus the 24C fish were spawned at that temperature and at 28 and 32C; the 32C fish at 24, 28 and 32C;

the 28 fish at 24, 28 and 32C. No adaptation was observed. For example, the 32C acclimated fish laid the same number of eggs at its accustomed temperature (ca 1.4 eggs $g^{-1}day^{-1}$) as did those reared at 24 and 28C, and this held for the fish acclimated at 24 and 28 also. All groups laid nearly 9 eggs $g^{-1}day^{-1}$ at 28C whether they had been reared at that temperature or not. Egg viability tests were not helpful in interpreting acclimation experiments, since rearing eggs at 32C was disadvantageous no matter what the previous thermal history of the females had been.

Acidity

Reproductive adaptation to pH was attempted by exposing several pairs of fish for two successive three-week periods to pH 8.3 (control) and to pH 6.3, a sublethal condition which leads to low egg production and viability. Reproductive output was measured during the second three-week period. Then a reciprocal transfer was made and a five-week measurement of reproductive output was repeated on each group (Gerking & Lee, 1982). If compensation had occurred in the pH 6.3 group, its egg production should have been greater than that of the group transferred from the control pH which did not have the benefit of prior exposure to the low pH. This did not occur, however. The reproductive outputs of the two groups were not statistically distinguishable (pH 6.3 = 5.27 ± 4.12; transferred from pH 8.3 to 6.3 = 4.77 ± 4.06 eggs $g^{-1}day^{-1}$). When the pH 6.3 group was transferred to the control pH, an unexpected result occurred. Egg production did not recover to the control level as it had when the fish were released from temperature stress, but remained at the level characteristic of the sublethal exposure. Control egg production was about 8.0 eggs g^{-1} day^{-1}, but the group transferred from pH 6.3 to 8.3 was 5.5 eggs $g^{-1}day^{-1}$. This suggests that possibly some damage to the reproductive process had been produced by the exposure to the sublethal pH. This outcome repeated the result of a similar, earlier experiment, so we are reasonably certain that the observation was not an anomaly. There is always the possibility, of course, that the five-week period was not sufficient for recovery to take place. Thus, under the limitations of our experiments pupfish reproduction did not adapt to an exposure to sublethal pH, and was disturbed to such a degree that recovery to normal was not evident when the fish were released from acid stress.

References

Abraham, M., N. Blanc & A. Youshouw, 1966. Oogenesis in five species of grey mullets (Teleostei, Mugilidae) from natural and landlocked habitats. Israel J. Zool. 18: 155-172.

Abraham, M., A. Youshouw & N. Blanc, 1967. Induction experimentalle de la ponte chez Mugil capito confin en eau douce. C.r. hebd. Séanc. Acad. Sci. Paris. 265:818-821.

Beamish, R.J., 1974. Growth and survival of white suckers (Catostomus commersoni) in an acidified lake. J. Fish. Res. Board Can. 31:49-54.

Christie, W.J. & H.A. Regier, 1973. Temperature as a major factor influencing reproductive success of fish - two examples. Rapp. et Proces-verbeaux de Réunions. Cons. Intern. pour l'explor. de la Mer. 164:208-218.

Craig, G.R. & W.F. Baksi, 1977. The effects of depressed pH on flagfish reproduction, growth and survival. Water Res. 11:621-626.

Deacon, J.E., 1968. Ecological studies of aquatic habitats in Death Valley National Monument with special reference to Saratoga Springs. Final Report under contract 14-10-0434-1989, U.S. Forest Service.

de Vlaming, V.L., 1972. Environmental control of teleost reproductive cycles. J. Fish Biol. 4:131-140.

Eckstein, B. & U. Eylath, 1970. The occurrence and biosynthesis in vitro of 11-ketotestosterone in ovarian tissue of the mullet, Mugil capito, derived from two biotypes. Gen. Comp. Endocrin. 14:396-403.

Forney, J.L., 1972. Biology and management of smallmouth bass in Oneida Lake, New York. N.Y. Fish and Game J. 19:132-154.

Gerking, S.D. & R.M. Lee, 1982. Survival and reproduction of the desert pupfish (Cyprinodon n. nevadensis) after acclimation to acid water. J. Exp. Zool. (accepted for publication).

Gerking, S.D., R. Lee & J.B. Shrode, 1979. Effects of generation-long temperature acclimation on reproductive performance of the desert pupfish, Cyprinodon n. nevadensis. Physiol. Zool. 52:113-121.

Hansen, D.J., S.C. Schimmel & J. Forester, 1973. Arochlor 1254 in eggs of sheepshead minnows: effect on fertilization success and survival of embryos and fry. 27th Ann. Conf. S. E. Assn. Game and Fish Commissioners. p. 420-426.

Kaya, C.M., 1973a. Effects of temperature on responses of the gonads of green sunfish (Lepomis cyanellus) to treatment with carp pituitaries and testosterone propionate. J. Fish. Res. Board Can. 30: 905-912.

Kaya, C.M., 1973b. Effects of temperature and photoperiod on seasonal regression of gonads of green sunfish, Lepomis cyanellus. Copeia 1973:369-373.

Kinne, O., 1964. The effects of temperature and salinity on marine and brackish water animals II. Salinity and temperature combinations. Oceanogr. mar. Biol. Ann. Rev. 2:281-339.

Kinne, O. & E.M. Kinne, 1962. Rates of

development of embryos of a cyprinodont fish exposed to different temperature-salinity-oxygen combinations. Can. J. Zool. 40:231-253.
Lee, R.M. & S.D. Gerking, 1980. Reproductive performance of the desert pupfish (Cyprinodon n. nevadensis) in relation to salinity. Env. Biol. Fish. 5:375-378.
Mount, D.I., 1973. Chronic effect of low pH on fathead minnow survival, growth and reproduction. Water Res. 7:987-993.
Ruby, S.M., J. Aczel & G.R. Craig, 1977. The effects of depressed pH on oogenesis in flagfish, Jordanella floridae. Water Res. 11:757-762.
Shrode, J.B. & S.D. Gerking, 1977. Effects of constant and fluctuating temperatures on reproductive performance of a desert pupfish, Cyprinodon n. nevadensis. Physiol Zool. 50:378-389.
Simpson, D.G. & G. Gunter, 1956. Notes on habitats, systematic characters, and life histories of Texas saltwater Cyprinodontes. Tulane Stud. Zool. 4:115-134.
Smith, M.L. & B. Chernoff, 1981. Breeding populations of cyprinodontoid fishes in a thermal stream. Copeia 1981:701-702.
Wallace, R.A., 1981. Cellular and Dynamic aspects of oocyte growth in teleosts. Am. Zool. 21:325-343.

Added references

Shuter, B.J., J.A. MacLean, F.E.J. Fry & H.A. Regier, 1980. Stochastic simulation of temperature effects on first year survival of smallmouth bass. Trans. Am. Fish. Soc. 60:53-61.
Ball, J.N., 1960. Reproduction in female bony fishes. Symp. Zool. Soc. London. 1:105-137.

THE ROLES OF LIGHT AND TEMPERATURE IN THE REPRODUCTIVE CYCLES OF THREE BITTERLING SPECIES; RHODEUS OCELLATUS OCELLATUS, ACHEILOGNATUS TABIRA AND PSEUDOPERILAMPUS TYPUS

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Summary

In Rhodeus ocellatus ocellatus which continues cyclic spawning from spring to summer, the gonadal maturation was induced by raising temperature above 10 °C in early spring. Contrastedly, the gonadal regression at the termination of spawning season was effected solely by shortening the daylength below 13L in early autum, when the natural temperature was well above 20 °C. If the regressed fish were reared under a photoperiod over 14L, they could be brought soon into recrudescence and oviposition could be started in 4 weeks.

In Acheilognathus tabira, a spring-spawner, the lower critical temperature for maturation was about 13 °C, while the gonadal involution, unlike in R. ocellatus, was caused by temperature rising beyond 25 °C in early summer. However, if the fish kept spawning under moderate temperatures, they were found to develop photoperiodism, which resembled that of R. ocellatus, in a few months. During autumn, the gonadal recrudescence proceeded only slowly owing to the then shortening daylength.

On the other hand, in Pseudoperilampus typus which is an autumn-spawner, the gonadal maturation was brought about by the photoperiod shortening below 13L. Moreover, the gonadal regression was induced by the temperature falling below about 15°C.

These results are compared with well studied cases of other cyprinids and possible rules in the environmental regulation of teleostean reproductive cycle are discussed.

Keywords: reproductive cycle, bitterling, photoperiodism, critical temperature, recrudescence.

Introduction

Among cyprinids, the bitterling group is characterized by its various types of spawning periods, i.e., spring-, autumn-, and spring to summer-spawning types, respectively. Therefore, they are ideal for comparative studies on the environmental factors involved in the annual reproductive cycle of teleost. Until now, however, very few experimental works have been conducted on the bitterlings (Verhoeven & van Oordt, 1955; Nishi & Takano, 1979).

We have selected 3 bitterling species which exhibit different spawning types: Rhodeus ocellatus ocellatus, spring to summer; Acheilognathus tabira, spring; Pseudoperilampus typus, autumn. This paper reports on the environmental regulation of the reproductive cycle in these species clarified by a series of experiments. In these experiments, adult fish in different phases of their annual reproductive cycle were subjected to various photoperiod and temperature regimes, which were similar to their natural environmental conditions.

Rhodeus ocellatus ocellatus

It has been reported earlier that the spawning period of this fish extends from late March to mid-September (Asahina et al., 1980). Male and female GSIs (gonad weight x 100/ body weight) depict a sudden rise, a peak in May and/or a plateau, and then an abrupt decline. Along with yolk accumulation in the ovary in March, the female ovipositor grows to about the anal fin height and continues cyclic elongation throughout the breeding season. Toward the season's end, the ovipositor dwindles, concurrent with ovarian involution. In the prespawning season, the testicular lobules contain cysts filled with spermatogonia and spermatocytes. The testis undergoes shrinkage and spermatogenesis stops abruptly at the season's end.

Initiating factor of spawning season

The bitterling collected in mid-January were exposed to several combinations of temperatures (10, 16 or 22 °C) and photoperiods (7, 9, 12 or 16L -- L/D cycle in 24 hr period). They were fed mainly with tubifex worms. Freshwater mussels as spawning beds were also placed in the aquaria.

Within 4 weeks of rearing, GSIs increased rapidly at both 16 and 22 °C, regardless of photoperiod, and some females started cyclic oviposition. GSIs showed no significant change at 10°C. After raising temperature to 13°C, however, a rapid increase of GSI and initiation of oviposition were induced in all the photoperiod groups.

Apparently, the gonadal maturation in the

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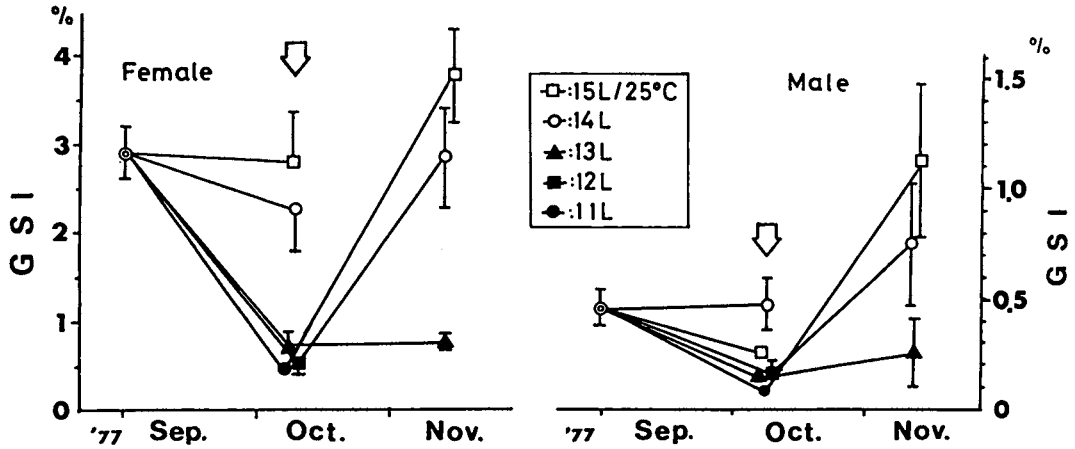

Fig. 1. Effects of various photoperiods on GSI of Rhodeus ocellatus ocellatus during late- and post-spawning periods. In two groups, photoperiod were changed on the 36th day (arrows). Temperature was kept at 25°C. Symbols and bars, mean ± S.E.M.

prespawning period depends on the temperature rising above 10 °C. Increasing daylengths during this period seem to have little effect.

Terminating factor of spawning season

Fish collected in late August were used. About half of them had regressing gonads. Experimental fish were first divided into 5 groups with different photoperiods; 11, 12, 13, 14 or 15L. The temperature was maintained at 25°C.

Within a span of 5 weeks, oviposition cycle resumed in both the 14 and 15L groups, whereas no oviposition occurred and female GSIs decreased significantly in the remaining groups. However, by following lengthening of photoperiods from 11 and 12L to 15 and 14L, respectively, ovarian recrudescence was soon induced and oviposition commenced eventually. GSIs increased markedly. In contrast, neither oviposition nor GSI increase was observed in the group kept at 13L throughout the experiment (Fig. 1).

These results indicate that the photoperiodism as affecting the gonadal activity develops during the course of breeding period, and manifests itself when the natural daylengh approaches the critical level between 13 and 14L. This phenomenon undoubtedly confirms that the natural spawning period of this species terminates so abruptly in September, when the water temperature is still warm.

Development of photoperiodism

From similar experiments conducted monthly, this photoperiodism was found to develop in July. To identify factors responsible for its development, bitterling having just started spawning were brought in April under 4 different conditions at either 15 or 25°C and with or without mussel. Photoperiod was fixed at 16L. In the absence of mussel, the fish soon stopped oviposition cycle, although high levels of GSI were maintained. In September or 5 months later, the photoperiod was shifted to natural daylengths.

In response to the shift of photoperiod, GSIs decreased rapidly in either of the 25 °C groups, with or without mussel. This demonstrated that the development of photoperiodism was not ascribable to continuation of spawning cycles. In addition, when the temperature was changed to 15 °C, some females kept spawning for 2 more months, indicating delayed manifestation of photoperiodism. On the other hand, spawning continued for a duration of one month, when the temperature was raised from 15 to 25 °C. This indicated that there was insufficient development of photoperiodism at 15°C.

Therefore, the temperature can affect considerably both the development and the manifestation of photoperiodism. The development of photoperiodism is presumed to be induced, however, by some innate mechanism, probably related to maintenance of full gonadal maturation, since it takes place under regular, favorable conditions.

Decline of photoperiodism

The responsiveness to photoperiod seems to be lost before the following spring. Possible involvement of temperature in this phenomenon was investigated. Regressed fish caught in November were kept on the regimes of 5, 10 or 15 °C at 10L for 9 weeks. Subsequently, each group was divided into 2 subgroups, 10 or 14L at 15°C, to be reared for another 5 weeks.

At the termination of rearing, gonadal recrudescence was most advanced, but

photoperiodic response was least clear in the 15 to 15°C subgroups. Even the subgroup kept at 10L throughout the experiment had a marked increase in GSI. In the 5 to 15°C and 10 to 15°C subgroups, the photoperiodism persisted rather distinctly.

Additional experiments showed that the recrudescence under short daylength was completed in 2 to 3 months at higher temperatures. It appears likely, in nature, the photoperiodism becomes weak enough or lost by mid-winter so that the rising temperature in early spring takes over the control of maturation. The importance of this finding has to be clarified by further investigation.

Acheilognathus tabira

Judging from the ovipositor elongation and presence of ovulated eggs in the ovary, the spawning period of this fish extends from late April to late June (Shimizu & Hanyu, 1981). Gonads remain involuted during high summer months, when most oocytes are at peri-nucleolus stage and male germ cells are all spermatogonia. In the first maturational phase starting in September, formation of yolk vesicles and spermatocytes occurs, accompanying slow increase of GSI and egg diameter. In the second maturational phase starting in March or early April, accumulation of yolk globules and active spermatogenesis proceed with concomitant rapid increase of GSI and egg diameter.

Initiating factor of spawning season

The bitterling maintained in an outdoor pond were used. In late February, experimental fish were brought under conditions with combinations of temperatures (8, 13 or 16°C) and photoperiods (11 or 15L). After 60 days, GSIs of both sexes were greatly increased in the 16°C groups, regardless of photoperiod. This increase was associated with active spermatogenesis in males and heavy accumulation of yolk in females. Some females had ovulated eggs. Under 13 °C, male GSIs increased considerably, but females GSIs showed no significant increase. GSI changed scarcely in the 8°C groups.

Thus the rising temperature is considered to be the only initiating factor of spawning season in this species, as also observed in R. ocellatus. The critical temperature is almost 13°C, although a slight difference exists between sexes.

Terminating factor of spawning season

Fish continuing cyclic oviposition in late May were exposed to several combinations of temperatures (22, 26 or 30 °C) and photoperiods (8, 12 or 15L). During 3 weeks' exposure, both male and female GSIs decreased greatly in 30 °C groups, while GSIs and gonadal histology changed little in 22 °C groups (8, 12 and 15L). The decrease in GSI was less at 26°C than at 30°C.

These results indicate that the termination of the spawning season in this species is caused by the temperature rising beyond the upper critical level of about 25 °C. On the other hand, it was also found that the photoperiodism could develop in this species. Thus, fish kept under 22°C-15L for 2 months commencing from May responded to shortening photoperiod (to 11L) and gonadal regression occurred rapidly. It may be possible for this species to terminate spawning in this way in some cool habitats.

Factors involved in recrudescence

Regressed fish with oocytes at peri-nucleolus stage in early September were maintained on regimes of 22°C-8L, -11L, -15L or 27°C-15L. After 6 weeks, a slight increase in GSI and concomitant formation of both yolk vesicles and spermatocytes were observed in all the 22 C groups, whereas hardly any gonadal change occurred in the 27 °C group. After another 4 weeks, however, only the 22 °C-15L group revealed high gonadal activity with sharply increased GSI. The remaining groups did not undergo any further changes.

It is therefore concluded that, the first phase of gonadal recrudescence is induced by the lowering temperature in autumn, but the second phase of recrudescence does not occur because of shortening daylenghs of this season. The latter situation is very similar to that of R. ocellatus in autumn.

Pseudoperilampus typus

This bitterling species spawns in autumn. A detailed study of its annual reproductive cycle is prevented by difficulties in sampling except for the larval schooling stage in spring.

Initiating factor of spawning season

Larvae collected in May were reared in an outdoor pond and brought up to the adult size by mid-summer. In late August, experimental fish were transferred to artificial conditions of temperatures (18, 25 or 28 °C) combined with photoperiods (12, 13, 14 or 15L).

After one month, male and female GSIs were markedly increased in the 12L groups, regardless of temperature, whereas they were hardly augmented in both the 14 and 15L groups. Under 13L-18 °C, only part of the females showed an increase in GSI, although most males showed an uniform increase.

This species is, therefore, a short day fish which matures in response to the shortening of daylength below 13L.

Terminating factor of spawning season

Fish were first made to start their spawning cycle under10 L-19°C in October, and then in early November, they were divided into 4 groups on regimes of 10L at 8, 12, 15 or 19°C. After about 6 weeks, male and female GSIs were reduced in the 8 and 12 °C groups. Part of fish showed decrease in GSI in the 15 °C group. At 19°C, females continued cyclic spawning and high levels of GSI were maintained.

Thus, the termination of spawning season can be attributed to the temperature lowering below about 15°C.

Discussion

It has often been pointed out that both photoperiod and temperature are important in regulating reproductive cycles of some teleosts inhabiting the temperate zone (Sundararaj & Vasal, 1976; Peter & Crim, 1979). The gonadal activity of the bitterling is also affected significantly by photoperiod and temperature, but in a different way. In this group, unlike in other cyprinid fishes so far reported, the two factors are not simultaneously effective, one dominates over the other in some phases and vice versa in the remaining phases of their annual reproductive cycles.

As described above, Rhodeus ocellatus, spawning from spring to summer, matures depending solely on the rising temperature in early spring and undergoes regression responding exclusively to the shortening daylengh in early autumn. Spring-spawning Acheilognathus tabira behaves in the corresponding manner in spring, and can do so again for the termination of its spawning period, provided this lasts longer under cool temperatures. Autumn-spawning Psudoperilampus typus matures in response to the shortening daylength in early autumn and regresses depending on the lowering temperature in late autumn.

Similar alternation of dominant factors during annual reproductive cycle may take place in species other than the bitterlings. Thus, the goldfish, Carassius auratus, has been reported to fully mature in winter depending only on the temperature (Yamamoto et al., 1966). We have succeeded in inducing the goldfish to remature and spawn during autumn by exposing them to long photoperiod combined with warm temperature which is only slightly higher for the season (Hanyu et al., unpublished).

The occurrence of postspawning refractoriness has been indicated in cyprinids such as Rhodeus amarus (Verhoeven & van Oordt, 1955) and Notropis bifrenatus (Harrington, 1957), although de Vlaming (1975) has recently reported that Notemigonus crysoleucas shows no such refractoriness. Interestingly, R. ocellatus neither exhibits refractoriness while A. tabira clearly does, as mentioned earlier. In the latter, after a strong suppression by high temperature during summer, the first phase of recrudescence proceeds under lowering temperature regardless of photoperiod. This instance suggests that the refractoriness has some causal relation to high temperatures, especially in fish whose upper critical temperature for maturation is relatively low.

Lastly mention should be made of the gonadal responsiveness to long photoperiod-warm temperature in late summer and autumn . The responsiveness seems to be rather common in the spring-spawning cyprinids, although it does not play a positive role under short daylength present in autumn. It can, however, be utilized for artificial control of "out of season" sexual maturation in these fishes, and should be searched for in fishes other than cyprinids.

References

Asahina, K., I. Iwashita, I. Hanyu & T. Hibiya, 1980. Annual reproductive cycle of a bitterling, Rhodeus ocellatus ocellatus. Bull. Japan. Soc. Sci. Fish. 46: 299-305

de Vlaming, V.L., 1975. Effects of photoperiod and temperature on gonadal activity in the cyprinid teleost, Notemigonus crysoleucas. Biol. Bull. 148: 402-415.

Harrington, R.W.Jr., 1957. Sexual photoperiodicity of the cyprinid fish, Notropis bifrenatus (Cope), in relation to the phases of its annual reproductive cycle. J. Exp. Zool. 135: 529-553.

Nishi, K. & K. Takano, 1979. Effects of photoperiod and temperature on the ovary of the bitterling, Rhodeus ocellatus ocellatus. Bull. Fac. Fish. Hokkaido Univ. 30: 63-73.

Peter, R.E. & L.W. Crim, 1979. Reproductive endocrinology of fishes: gonadal cycles and gonadotropin in teleosts. Ann. Rev. Physiol. 41: 323-335.

Shimizu, A. & I. Hanyu, 1981. Annual reproductive cycle of a spring-spawning bitterling, Acheilognathus tabira. Bull. Japan. Soc. Sci. Fish. 47: 333-339.

Sundararaj, B.I. & S. Vasal, 1976. Photoperiod and temperature control in the regulation of reproduction in the female catfish Heteropneustes fossilis. J. Fish. Res. Board Can. 33: 959-973.

Verhoeven, B. & G.J. van Oordt, 1955. The influence of light and temperature on the sexual cycle of the bitterling, Rhodeus amarus. Proc. Akad. Sci. Amst. C58:624-634.

Yamamoto, K., Y. Nagahama & F. Yamazaki, 1966. A method to induce artificial spawning of goldfish all through the year. Bull. Japan. Soc. Sci. Fish. 32: 977-983.

INVESTIGATIONS INTO THE IMPORTANCE OF DAYLENGTH ON THE PHOTOPERIODIC CONTROL OF REPRODUCTION IN THE FEMALE RAINBOW TROUT

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Summary

Exposure of female rainbow trout to photoperiods of constant length produced a similar sequence of changes of gonadotropin, oestradiol 17B and vitellogenin as seen under natural seasonal cycles. Constant long days or long days until June 21 followed by short days significantly advanced spawning, whereas 12L: 12D produced similar timings to the controls. These results confirm that a seasonally-changing daylength is unnecessary for reproductive development in the trout and support a circadian or circannual mechanism of photosensitivity with differing light cues at different times of the annual cycle (Photoperiod, Spawning, Trout, Endocrine Control).

Introduction

It is now well recognised that the reproductive cycle of salmonid fish is controlled by modifications in levels of hormones from the hypothalamic-pituitary-gonadal axis induced by cues from the external environment in particular seasonal changes in daylength (Billard et al, 1978). Thus, under conditions of constant temperature and rate of feeding, spawning of rainbow trout can be advanced or delayed by accelerating or slowing down respectively the seasonal changes in daylength into shorter or longer periods of time than the one year of the natural cycle (Bromage et al, 1982a). It has been suggested that the increasing and decreasing components of a natural seasonal cycle may be required for appropriate reproductive development in fish (Erikson & Lundquist, 1980). However, in other animals and plants on which studies of light measurement have been conducted, reproductive responses occur to absolute changes in daylength as a result of mechanisms involving either circadian or circannual phases of photosensitivity or 'hour glass' measurement and do not require the progressive seasonal changes in daylength which are seen in natural cycles (Follett & Davies, 1975). Whether such mechanisms operate in salmonid fish is not known. Thus, in the present work the nature of the photoperiodic cues involved in reproductive development in female salmonids, and also the neuro-endocrine mechanisms by which this control is achieved, are further investigated by studying the effects of light cycles of constant length on the timing of spawning and the levels of gonadotropin, oestradiol-17B and calcium as an index of vitellogenin in the rainbow trout.

Materials and methods

Five groups of 3 yr-old rainbow trout, of Danish origin with a natural spawning time of Jan-Feb were maintained in light-proof 800l tanks, each with a single 40-watt fluorescent tube (200 lux at the water surface) controlled by time clocks adjusted weekly and exposed to the following light cycles:
(1) Simulated normal seasonal light cycle (Control); (2) Normal seasonal (increasing) light cycle until 21 June followed by constant 16-hour light: 8-hour dark (16L:8D);
(3) Constant 12L: 12D throughout the year;
(4) Constant 16L: 8D (Long day);
(5) Constant 16L: 8D until the summer solstice (June 21) followed by 8L: 16D (Long-Short).

The water temperature was constant at 9°C with a dissolved oxygen of 100% saturation in the effluent and a pH of 6.6. The fish were fed at a level of 0.5% body weight/day with BP Nutrition Mainstream diet. All fish were blood sampled at the start of the experiment in Feb and each month thereafter. Methods for sampling and subsequent assay of gonadotropin, oestradiol and calcium are described in Bromage et al (1982a). Fish were also examined each month for signs of sexual maturation. Spawning was said to have occurred when ripe eggs were expelled after gentle hand pressure on the abdomen, i.e. stripping. Differences between means were tested statistically by either a Student's t-test or an F-test if the variances were dissimilar.

Results

In the control fish, spawning occurred in late Jan, at the same time as similar groups of fish maintained in outside tanks under ambient conditions. In contrast, fish exposed to long days until the 21 June followed by short days as well as those under constant long days spawned in Oct and Nov respectively, some 14 and 10 weeks earlier than the controls. The two groups of fish under 12L: 12D and a normal seasonal cycle until 21 June + long days spawned in early and late Jan respectively.

Under all regimes a similar profile of changes of gonadotropin, oestradiol 17B and calcium was shown although the timing and duration of these changes was necessarily modified by the differences in daylength to produce the clear differences in spawning time. These are considered in more detail below:-

Gonadotropin (see Fig 1): The lowest or basal levels of this hormone were shown at the beginning of the experiment in Feb and during the mid-cycle of each of the experimental and control groups. In April/May there were increases in gonadotropin which in all groups except those under the increasing + long regime were significantly different from basal values($p < 0,05$) These increases lasted for 2-4 months and in each case was followed by a reduction to basal values which occurred more quickly under the long to short regime. The return to low levels during mid-cycle in all photoperiod treatments was then followed by much more rapid and significant ($p < 0.01$) increases in gonadotropin and in all cases initiation of these spikes of activity occurred before the time of spawning, although levels continued to rise after completion of this process.

Oestradiol-17B (see Fig 2): Under all photoperiod regimes low serum oestradiol values were maintained from Feb to June, although during this period there were modest changes in some of the cycles. Between June and Aug more significant increases were shown ($p < 0.001$) and these occurred earlier in fish which had been exposed to longer light regimes earlier in the year, i.e., 12L: 12D, Long-Short and 16L: 8D. Peaks in serum levels of this hormone were subsequently shown in the Autumn (Sept-Dec) and they were at their highest in those fish which had received longer days or at least not a decreasing or short photoperiod after the summer solstice. The lowest peak level attained and the one of the shortest duration occurred in the fish on long days until June 21, followed by a direct switch to short days. In all treatments the levels of oestradiol were falling towards basal values before the beginning of the increases in gonadotropin which occurred at the time of spawning.

Calcium (see Fig 3): Basal levels of calcium were found until May in the two groups of fish under 16L: 8D, until July-Aug under 12L: 12D, and Sept under the increasing + long regime. After these dates significant increases in serum levels occurred ($p < 0.001$) up to peak heights which under each regime usually preceded the time of spawning by 2-3 weeks.

Discussion

It is clear from the present results that spawning occurs in the rainbow trout exposed to light regimes of constant length and that seasonally-changing daylength is not essential for the cueing and modulation of reproductive development. This confirms other studies (Bromage et al, 1982b) from this laboratory and similar results in other species of teleost (Baggerman, 1972; Sundararaj & Vasal, 1976).

The finding that the time of spawning is unrelated to the total number of hours of light or dark received by the different groups of fish strongly indicates the presence of a circadian or circannual-based mechanism for photoperiodic response rather than the 'hour-glass' seen in some animals (Lees, 1972). Circadian phases of photosensitivity have also been demonstrated in the stickleback (Baggerman, 1972) and catfish (Sundararaj & Vasal, 1976) although in these species environmental factors other than light are of similar or greater importance in the cueing of development.

The earlier spawning times of fish exposed to longer days earlier in the year suggest that the trout is a 'long-day animal', although the further acceleration of spawning by the switching of fish from long to short days at the time of the summer solstice provide evidence of an additional short-day requirement later in the cycle. It is probable that the extended period of egg development in salmonids, when com-

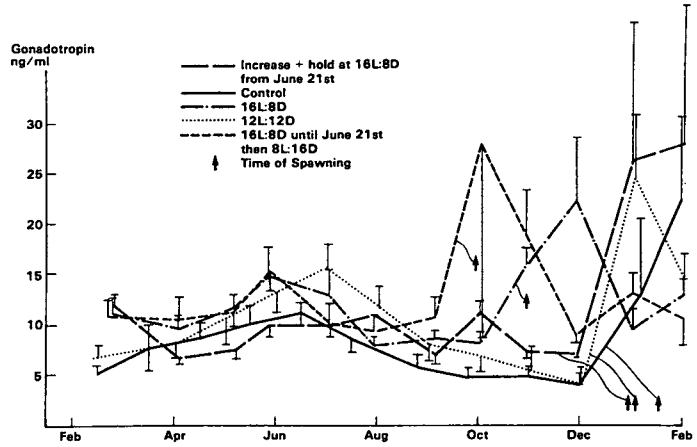

Fig. 1 Changes in serum gonadotropin (mean ± SEM, n=6) under the five photoperiod regimes.

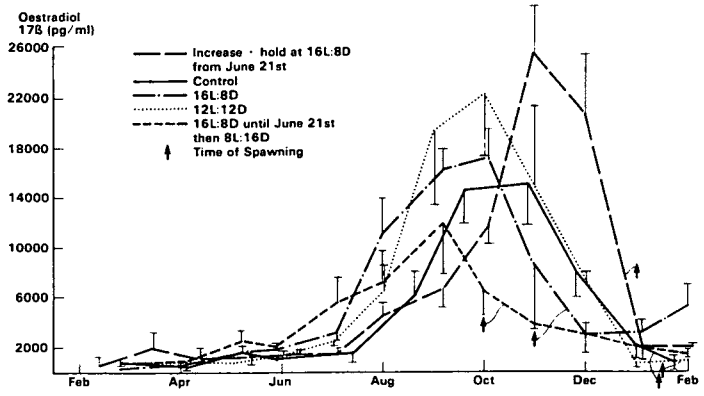

Fig. 2 Changes in serum oestradiol-17B (mean ± SEM, n=6) under the five photoperiod regimes.

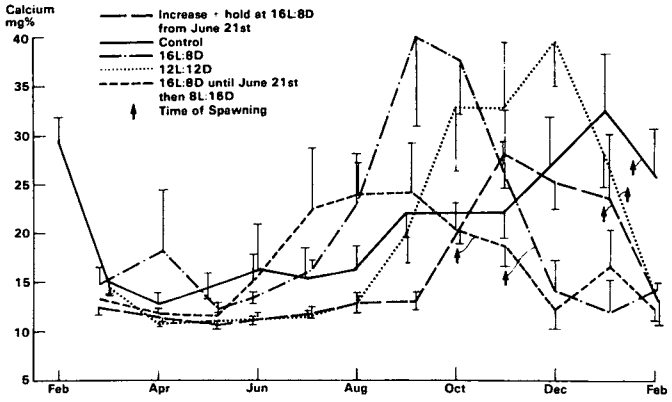

Fig. 3 Changes in serum calcium (mean ± SEM, n=6) under the five photoperiod regimes.

pared with other fish, requires more than one photoperiod cue during the annual cycle. The failure of either constant long or short days to arrest development in the trout (Bromage et al, 1982b) argues against this hypothesis, although there is evidence in other animals for movement of phases of photosensitivity in the absence of expected environmental triggers (Gwinner, 1973). It is also possible that the egg and developing follicle have a programmed life and that after this point has been reached, feedback mechanisms may provide the signal for final oocyte maturation and spawning.

Turning now to the neuroendocrine mediation of the photoperiodic response, one sees a similar sequence of changes of gonadotropin, oestradiol-17B and vitellogenin under all regimes. Such profiles have also been reported for fish on seasonally-changing light cycles (Bromage et al, 1982a). The first measurable change under all regimes was an increase (early in the cycle) of gonadotropin which probably initiates subsequent alterations in oestradiol (Bromage et al 1982a). The apparent failure of the different photoperiod regimes to affect the timing of onset of this early change in gonadotropin is not unexpected as the experiment was not begun until Feb. However, there were differences in duration of this increase which was shortened in fish which were moved from long to short days in June. Furthermore, the curtailment of gonadotropin secretion by exposure to short days was paralleled by reductions in the peak oestradiol level subsequently achieved in the same group of fish. In contrast levels in fish on longer days (16L: 8D or 12L: 12D) after June 21 were higher and maintained for longer periods of time. Subsequently, gonadotropin levels under all regimes were reduced in mid-cycle when oestradiol levels were rapidly increasing.

The timing of the second and much larger increase in gonadotropin was clearly modified by photoperiod manipulation. Such increases which are thought to be concerned with the control of final oocyte maturation and ovulation, have also been reported by other workers (Billard et al, 1978). In all regimes oestradiol had reached basal levels at this time. The maintenance of high levels of gonadotropin after spawning, although probably due to the marked reduction in steroid feedback which occurs after ovulation, may also be important in the control of early egg development for the next cycle.

References

Baggerman, B., 1972. Photoperiodic responses in the stickleback and their control by a daily rhythm of photosensitivity. Gen.Comp.Endo.Supp 3:466-476.

Billard, R., B. Breton, A.Fostier, B.Jalabert & C.Weil, 1978. Endocrine control of the teleost reproductive cycle and its relation to external factors: Salmonid and Cyprinid models. In P.Gaillard & H.Boer (Ed): Comparative Endocrinology. Elsevier Press, Amsterdam.

Bromage, N., C. Whitehead & B.Breton, 1982a. Relationships between serum levels of gonadotropin, oestradiol 17B and vitellogenin in the control of ovarian development in the rainbow trout. Gen. Com.Endo. 47: 366-376.

Bromage, N., C. Whitehead & J.Elliott, 1982b. Daylength requirements and the control of reproduction in the female rainbow trout. Gen.Comp.Endo.46: 391.

Eriksson, L. & H. Lundqvist, 1980. Photoperiod entrains ripening by its differential effect in salmon. Naturwiss 67: 202.

Follett, B. & D. Davies, 1975. Photoperiodicity and the neuroendocrine control of reproduction in birds. Symp.Zool. Soc.Lond., 35: 199-224.

Gwinner, E., 1973. Circannual rhythms in birds: their interaction with circadian rhythms and environmental photoperiod. J.Repro.Fert.Sup. 19: 51-65.

Lees, A., 1973. Photoperiodic time measurement in the aphid Megoura viciae. J.Ins.Phys. 19: 2279-2316.

Sundararaj, B. & S. Vasal, 1976. Photoperiod and temperature control in the regulation of reproduction in the female catfish. J.Fish.Res.Bd.Can. 33: 959-973.

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INFLUENCE OF TEMPERATURE ON GONAD DEVELOPMENT IN A STRONGLY PHOTOPERIODIC SPECIES, *GASTEROSTEUS ACULEATUS* L.

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Gonad maturation in the stickleback is mainly dependent on the photoperiod, but is modulated by temperature in at least two different ways, as shown below.

Gonad recrudescence begins in late summer and phase 1(sperm production;oöcyte growth up to vacuolisation)is completed by December. Development of phase 2(Leydigcell development and nest building;further oöcyte growth and ovulation)begins in Jan./Febr.and is completed around mid-April,when breeding begins.

It was found that development of phase 2 can be initiated only when the photoperiod is able to overcome the photoreactivity threshold.This threshold is high in September(when phase 2 can be initiated only by photoperiods longer than 14L-10D)and declines to very low in Jan./Febr.(when phase 2 can be initiated by photoperiods as short as 9L-15D).As shown below,the height of the threshold depends on the range of the photo-inducible phase, which is determined by a daily rhythm of photosensitivity.

Figure 1 shows the response of fish(caught in nature in Oct./Nov.)when exposed to a set of skeleton photoperiods(II-VI)in which the total amount of light was 8 hours,divided into a main light period of 6 hours,plus a two hour light pulse at various times in the dark period.It can be seen that the fish did not respond,or only very little to light between 6-12 hrs(I-III),but were quite responsive between 12-19 hrs(IV-V),with a peak between 14-16 hrs.The period between 12-19 hrs is the photo-inducible phase. Figure 1 thus explains why in Oct./Nov. development of phase 2 can be initiated only by photoperiods longer than 12L-12D,because only in those cases the photo-inducible phase is exposed to light.

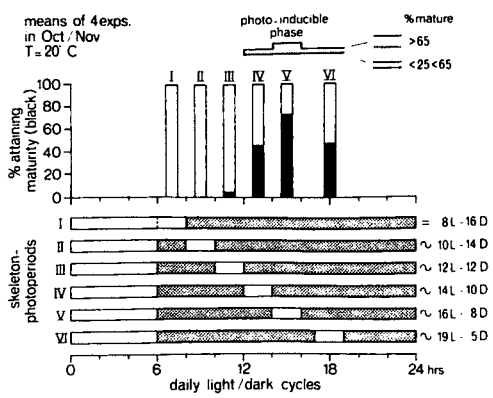

Fig. 1.Determination of the range of the photo-inducible phase by means of a series of skeleton photoperiods.

By repeating the experiment of fig.1 at different times between late summer and spring, it was found that the range of the photo-inducible phase gradually extends into the earlier hours of the day;fig.2. This figure thus explains the decrease of the photoreactivity threshold between late summer and spring.

By keeping fish(hatched in June)from August under a constant photoperiod(8L-16D) at two different temperatures,15° and 20°C, it was found that the range of the photo-inducible phase under 20° did not extend as far into the earlier hours of the day as happened under 15°,the latter equalling the extension occurring under natural conditions (fig.2).

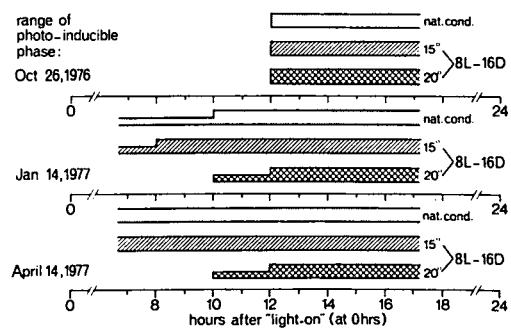

Fig.2.Extension of the range of the photo-inducible phase under different external conditions between autumn and spring.

The conclusions is that the lower temperatures of winter favour the extension of the photo-inducible phase into the earlier hours of the day,allowing phase 2 to start its development in Jan./Febr.,when the natural day lengths are still very short.From that time(as other experiments showed)the rate of its development is favoured by both longer photoperiods and higher temperatures.

References.

Baggerman,B.1972.Photoperiodic responses in the stickleback and their control by a daily rhythm of photosensitivity.Gen.Comp.Endocrinol.Suppl.3, 466-476.

Baggerman,B.1980.Photoperiodic and endogenous control of the annual reproductive cycle in teleost fishes.In:Environmental Physiology of Fishes.Ed.M.A.Ali. 533-567. Plenum Publ.Corp., New York.

EFFECTS OF PHOTOPERIOD AND TEMPERATURE ON REPRODUCTION OF MALE THREE-SPINED STICKLEBACK DURING WINTER

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Photoperiods have a marked, though season- and temperature-dependent influence on the reproduction of the three-spined stickleback Gasterosteus aculeatus L. (Baggerman, 1957, Borg, 1982). Spermatogenesis is active from the end of the breeding season in late summer to late autumn. The kidney of the male hypertrophies in the breeding season and secretes a "glue" that is used in the building of the nest. This hypertrophy is androgendependent.

In order to study environmental effects on androgen-production and spermatogenesis, and their relation to each other, sticklebacks were subjected to photoperiods of Light: Dark (LD) 16:8 or 8:16 h and 20° or 9° C for 30 days in December-January. The activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and glucose-6-phosphate dehydrogenase (G-6-PD), two enzymes necessary for production of active sex steroids were investigated histochemically. Also the histology of testes and kidney were studied.

3β-HSD reactions were carried out for 3 h at 37° C in phosphatebuffer with NAD and nitrobluetetrazolium (NBT) added. Epiandrosterone, first dissolved in dimethylformamide served as substrate. G-6-PD incubations were carried out for 15 min at 37° in Trisbuffer, with NADP, NBT and polyvinylalcohole added. Glucose-6-phosphate was used as a substrate. In both types of reactions a blue formazan stain was found in the cytoplasm of interstitial cells. The intensity of reactions was arbitrarily, and blindly, classified as 0, +, ++ or +++.

The most intense reactions of both enzymes were found in LD 16:8 20° C, the only group where also red breeding colours and reproductive behaviour were observed. The low temperature groups were intermediate, and the LD 8:16 20° group had significantly fainter reactions of both enzymes than all other experimental groups.

Initially the testes contained spermatozoa together with spermatids and spermatocytes, or spermatozoa almost alone. A quiescent spermatogenesis with few cells other than spermatozoa dominated in LD 16:8 20° and in the low temperature groups, but was not found in LD 8:16 20°. Most fish in LD 8:16 20° displayed a vigorous spermatogenesis with large numbers of spermatogonia and also other stages. This condition was not found in any other group, including the initial controls.

Both LD 16:8 groups had significantly higher second proximal renal tubule epithelia (KEH) than both LD 8:16 groups. The KEH in LD 8:16 20° was also significantly lower than in LD 8:16 9°, and lower than in the initial controls.

Androgen-stimulation was most marked in the LD 16:8 20° C group, and least in LD 8:16 20° C. An active spermatogenesis was only present in the latter group. Thus, there was a negative correlation between spermatogenesis and steroid-production. These effects are probably due to androgen-inhibition of spermatogenesis, which has previously been demonstrated at/after the breeding season in this species (Borg, 1981).

References

Baggerman, B. 1957. An experimental study on the timing of breeding and migration in the three-spined stickleback (Gasterosteus aculeatus L.). Arch. Néerl. Zool. 12: 105-317.

Borg, B. 1981. Effects of methyltestosterone on spermatogenesis and secondary sexual characters in the three-spined stickleback (Gasterosteus aculeatus L.). Gen. Comp. Endocrinol. 44: 177-180.

Borg, B. 1982. Extraretinal photoreception involved in photoperiodic effects on reproduction in male three-spined stickleback, Gasterosteus aculeatus. Gen. Comp. Endocrinol. 47: 84-87.

REPRODUCTIVE BEHAVIOR AND ENDOCRINE ACTIVITY OF THE PUFFER, *FUGU (= SPHEROIDES) NIPHOBLES*, OCCURRING ON THE COAST OF SADO ISLAND IN THE JAPAN SEA

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Summary

The puffer, *Fugu (= Spheroides) niphobles*, is a very common species of fish along the coast of Japan. Its peculiar spawning behavior linked with the moon or tidal phases is well known on the Pacific coast. Since there is a great difference in the water level and its amplitude between the Pacific coast and the Japan Sea coast, it is needed to elucidate the reproductive activity of this puffer occurring on the Japan Sea coast. This study was therefore designed to know still unclear problem of this species on the Japan Sea side with special regard to maturation and endocrine activity.

On the basis of gonosomatic index and histological examination of both sexes, it is estimated that the peak of maturation is early June in the male and late June in the female. The spawning occurred almost every evening during a period of fifty minutes before and after sunset in the breeding season lasting from early June to late July (fig. 1). The result obtained may intimate that there is not so clear-cut correlation between spawning and lunar periodicity on the coast of Sado Island. This phenomenon may be owing to minor spring tide and daily high tide.

The GTH cells in the adenohypophysis showed a hyperfunctional figure such as hypertrophy and degranulation with various grade of vacuolization in the peak of the gonadal maturation and spawning. The AF stainable material demonstrated in the preoptico-hypophyseal system was very abundant in April and May, but it became scanty in June, suggesting an acute release of neurohypophyseal hormone. Toward the postspawning season, a gradual increase in the material of this system is seen. The cell of the nucleus lateralis tuberis showed only a faint dilation prior to the peak of gonadal maturation. Seasonal change in the thyroid gland is distinct and its highest activity is recognized in April and May just prior to the sexual maturation. During a period from June to October, the activity is declined to a rather mild or quiescent condition. Histological changes in the thyroid gland coincided roughly with the morphometrical changes in the TSH cells. The ACTH cell-interrenal axis also showed a seasonal change, but not so direct correlation is seen between this axis and gonadal maturation.

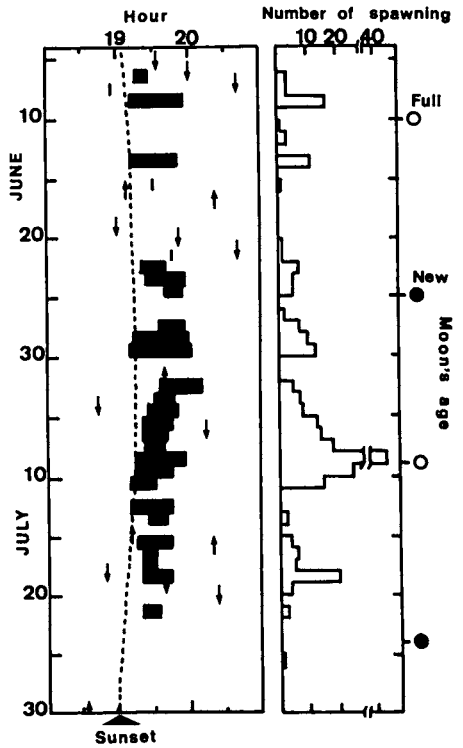

fig. 1. Observation of puffer's spawning on the coast of Sado Island, with time of full (↑) and low (↓) tide, duration and number (frequency) of spawnings.

SEXUAL STIMULI ASSOCIATED WITH INCREASED GONADOTROPIN AND MILT LEVELS IN GOLDFISH

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In many male mammals and birds, exposure to sexual stimuli results in acute elevations of circulating luteinizing hormone (LH) and testosterone (T). Previous work in our laboratory showed that a similar phenomenon exists in the male goldfish (*Carassius auratus*). Males placed with stimulus pairs of spawning goldfish had "Con A II" or "maturational" gonadotropin (GtH) concentrations and expressible milt volumes that were significantly elevated as compared to males kept in all-male groups; stimulatory effects on GtH and milt persisted for at least 2 and 24 h, respectively, at 14°C (Kyle et al., 1979). In the present experiment, we determined the sensory cues needed to evoke these rapid physiological changes.

Methods

Experimental males were initially hand-stripped of milt and either isolated in 65 litre glass aquaria at 20°C, placed in contact with a pair of spawning goldfish, or separated from the pair with a perforated, transparent partition. This spawning pair consisted of a mature male and either a prostaglandin F_2alpha (PG)-injected female or male; PG treatment induces qualitatively normal female sexual behaviour in both male and female goldfish (Stacey, 1981). After 2 h, the experimental males were anesthetized, stripped of milt, bled, and killed for determination of the gonadosomatic index (GSI). Expressed milt was aspirated into micropipettes, centrifuged, and the volume of milt and packed sperm measured; the ratio of these volumes gave the percentage of seminal plasma in each sample.

Results

Males placed in contact with either a hetero-sexual or homosexual (where a PG-treated male played the female role) spawning pair showed significantly increased GtH and milt levels over those values for isolated fish. In contrast, no increases occurred if contact with the spawning pair was prevented, even though visual and chemical cues could be perceived (Fig. 1). The percentage of seminal plasma and GSIs were not different among treatment groups.

Some of the males placed in contact with spawning fish failed to court or spawn during the 2 h test. When the data for the combined "contact" group were divided into sexually active and inactive categories and compared with the isolated controls, the sexually active males had significantly higher GtH and milt levels than either the inactive or isolated fish (Fig. 2).

Discussion

While the sight, sound, or smell of a female can cause rapid, transient elevations of LH and T in some male mammals and birds, visual and chemical stimuli alone from a spawning female goldfish, either PG-treated (present study) or ovulated (unpublished results), did not elevate GtH or milt levels in the male. These elevations only occurred in males that were placed in contact with a spawning pair (even when that pair contained a PG-treated male playing the female role) and were sexually active during the test.

In contrast, males that did not court or spawn had GtH and milt levels not different from isolated fish. This suggests that male sexual activity and elevations in milt and GtH are concurrent events which may share a common activational mechanism in the brain.

In summary, the performance of male sexual behaviour is correlated with the rapid elevation of GtH concentrations and expressible milt volumes, which may act as a positive feedback system to maintain sexual readiness.

Fig. 1. Milt volumes, GtH concentrations after 2 h of stimulation, and GSIs (mean ± S.E.) of male goldfish that were isolated from (I), in contact with (C), or separated (S) from either a heterosexual (♀) or homosexual (♂) pair of spawning fish. GtH values were combined to yield three groups: I, C, and S.

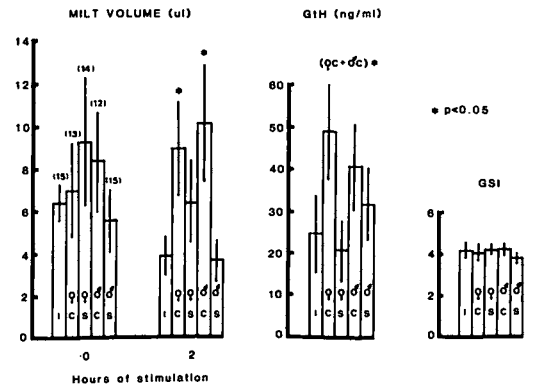
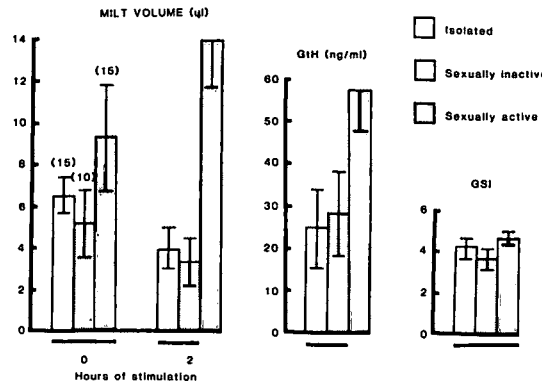


Fig. 2. Milt volumes, GtH concentrations after 2 h of stimulation, and GSIs (mean ± S.E.) of isolated, sexually inactive, and sexually active male goldfish. Those values underlined by the same bar are not significantly different at $p<0.05$.



References

Kyle, A.L., N.E. Stacey, R. Billard, and R.E. Peter. 1979. *Amer. Zool.* 19: Abstr. 554.

Stacey, N.E. 1981. *Amer. Zool.* 21: 305-316.

CHANGES IN PLASMA LEVELS OF GONADOTROPIN AND THYROID HORMONES DURING SPAWNING IN THE WHITE SUCKER (*CATOSTOMUS COMMERSONI*).

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Summary

Plasma gonadotropin and thyroid hormone levels were measured in white suckers during their spawning migration. Gonadotropin levels were low in both males and females prior to spawning, and increased to peak levels in spermiated males and ovulated females. Levels then declined in spent fish returning to the lake. Thyroxine levels were highest in prespawning animals, and tended to decline over spawning, with highest levels and diurnal variations found in males. Triiodothyronine levels were constant in both sexes.

Introduction

The white sucker (*Catostomus commersoni*) is abundant and widely distributed in North American lakes and rivers. In the spring of 1981 and 1982, adult suckers were captured during their spawning migration at a number of sites along a small stream draining into Lac Ste. Anne, Alberta. Blood samples were taken from the caudal vasculature of each fish within 15 minutes of netting. Sex and gonadal condition were determined by external morphology and (in 1981) oocytes were aspirated from unovulated females and checked for germinal vesicle migration (GVM) and breakdown (GVBD). Blood from each fish was assayed for gonadotropin (GtH), total triiodothyronine (T_3), and total thyroxine (T_4). Blood GtH was measured using a radioimmunoassay for carp gonadotropin. T_3 and T_4 were also measured by RIA.

Results and Discussion

Gonadotropin

GtH levels in spermiated male suckers were uniformly low prior to the appearance of ovulated females, at which time levels increased dramatically and remained high throughout spawning with no significant diurnal variation (fig. 1a). Spermiating males on the spawning grounds in 1982 also had significantly higher GtH levels than spent males caught returning to the lake (means ± s.e.= 11.3 ± 1.0 vs. 4.4 ± 0.3). High GtH levels in spawning males may in part be a response to ovulated females; on May 12, 1981, GtH levels of spermiating males caught in the presence of ovulated females (23.4 ± 3.1) were significantly higher than those of spermiating males caught in an area devoid of females. (4.4 ± 1.1) GtH levels in females were correlated with ovulation. Levels were lowest in prespawning fish, and remained low throughout the spawning season in preovulatory fish (fig. 1b). GtH increased significantly in fish with GVM and GVBD, reaching peak levels in ovulated fish and decreasing with completion of oviposition and return to the lake (levels in returning fish= 5.2 ± 0.5). No consistent diurnal variation in plasma GtH was seen.

Thyroid Hormones

T_3 levels in both sexes showed very little variation of any kind during spawning. Mean T_3 levels ranged from 1.8 to 5.0 ng/ml in both males and females. There were significant diurnal variations in T_4 levels in male suckers. In prespawning animals, highest levels were found at 0300 hrs (10.2 ± 1.6 ng/ml) and lowest at 1500 hrs (4.3 ± 0.6). In spawning animals, T_4 levels were elevated at 1500 hrs (11.0 ± 1.2), but means ranged from 5.9 to 6.7 ng/ml at all other times. These peaks in T_4 levels correspond to times of greatest migratory and

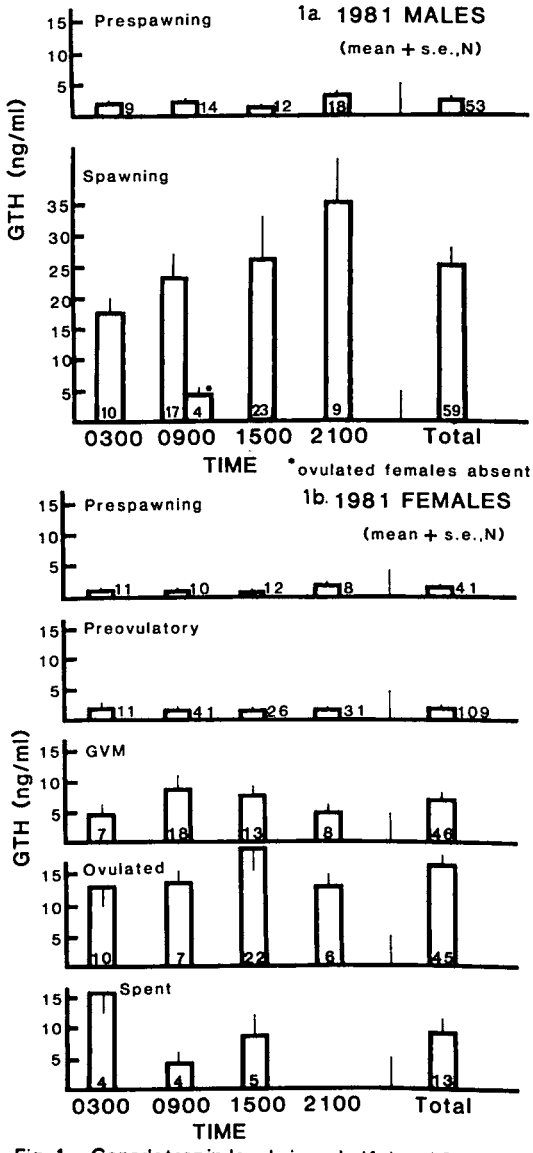

Fig. 1. Gonadotropin levels in male (1a) and female (1b) suckers at four different times of day during the 1981 spawning season.

spawning activity and may therefore reflect increased metabolic demands at these times. There was no apparent diurnal variation in T_4 levels in female suckers. In general, T_4 levels in females were lower than those in males, and tended to decline with reproductive stage (highest = 5.0 ± 0.3 in prespawning females declining gradually to a low of 2.1 ± 0.4 in spent females). No observable changes in T_4 levels were seen associated with the initiation or cessation of female spawning activity, although higher levels at the start of spawning support a role for thyroid hormones in ovarian maturation.

MANIPULATION OF SPAWNING ACTIVITY IN RAINBOW TROUT BY LIGHT PROGRAMS

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The spawning in rainbow trout coincides with short days of winter.

The purpose of this experiment was to enhance the frequency of spawning by way of artificial light programs.

Eighty male and 60 female trout were evenly and randomly allotted to 8 fish tanks located in a dark basement and supplied with well water of constant quality and temperature (10 ± 1°C). Two tanks each were subjected to an artificial light year of 12, 9, 6 or 3 months, respectively. All fish were anaesthetized and checked for mature germ cells at two-week intervals, during peak periods females were checked weekly. Quantity and quality of germ cells, sperm motility, egg size and hatching rate were determined.

It was possible to reduce the spawning interval of males and females from 12 months to 9 or 6 months (Fig. 1 and 2). In females the period between the first and the last fish spawning was reduced from 14 to 8.5 to 6 weeks, respectively. The output of sperm and eggs was normal, and there was not much difference between groups (Table 1). Eggs obtained from the 9 and 6 months group were slightly smaller but the quality of both male and female germ cells was normal.

The 3 months group started to spawn very early and the spawning season lasted for almost six months regardless of the continuing light program (Fig. 1 and 2). The individual response was erratic and unpredictable. The eggs were smaller, but both male and female germ cells were functionally normal (Table 1).

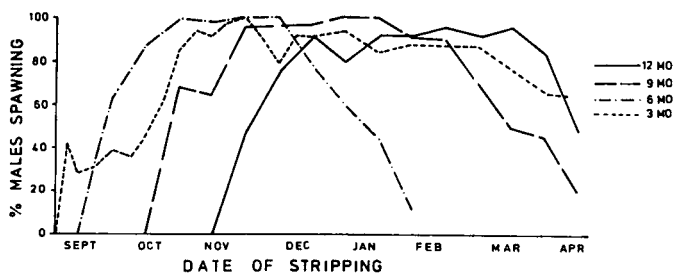

Fig. 1: Proportion of milters spawning in response to the respective light program

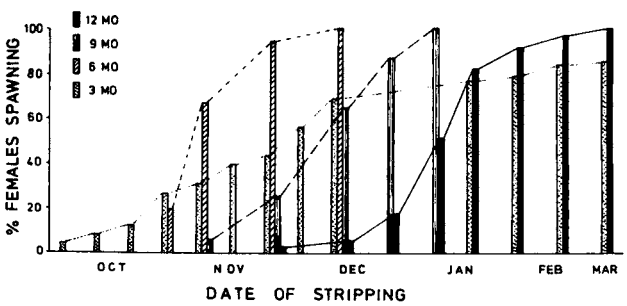

Fig. 2: Proportion of females spawning in response to the respective light program

Table 1: Sperm output, egg weight and fertilization capacity of germ cells collected under different light programs

Length of artif. year (mo)	Sperm/male ($X10^9$)	Weight of 100..... green eggs (g)	Weight of 100..... eyed eggs (g)	Eyed eggs (%)	Hatching rate (%)
12	336	7.3	10.6	87.9	84.0
9	399	6.4	11.9	88.1	84.0
6	308	6.0	10.5	93.5	91.8
3	335	5.4	8.3	94.2	91.6

LONG PHOTOPERIODS STIMULATE GONAD DEVELOPMENT IN RAINBOW TROUT

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Summary

Experiments with various photoperiods revealed that long constant daylengths induced precocious maturation in male under-yearling rainbow trout. Ovarian development in two year old female trout was also stimulated by similar daylength, with ovulation and plasma levels of testosterone showing cycles of approximately 6 months. Experiments in progress on a commercial fish farm have also demonstrated that long photoperiods can be used to advance spawning up to six months and thus ensure a supply of out-of-season eggs.

Materials and methods

1. The influence of daylength on precocious maturation was investigated in two experiments. In the first, under-yearling trout were kept at 13.5°C on seven different photoperiods: 9 h, 14 h, 19 h, natural (N), simulated natural (SN) and changing 4 to 20 h and 20 to 4 h (1 h/week). When the increasing and decreasing daylength came to an end, they were continued at constant 20 h and 4 h, respectively. In the second experiment, only 9 h, 14 h, 19 h and SN were used.

2. In a preliminary experiment, a small number of mature female trout were kept on a constant 18 h daylength.

3. In a current experiment on a commercial fish farm, started in June 1981, two year old female trout (n = 42) have been kept on a constant 18 h photoperiod (natural day light extended by artificial light) at a constant 10°C. Control fish are maintained under natural illumination.

Results

Precocious maturation in males was only induced by constant long day length (Table 1). When the increasing photoperiod reached 20 L:4 D after 16 weeks no fish had spermiated. However, after 6 weeks maintained on constant 20 h day length, 87% of the males were running. Fertilization rates in excess of 90% were obtained using the milt from these fish.

Early maturation in salmonids is frequently associated with rapid growth of the under-yearlings. However, on the 19 h and 20 h photoperiods, growth was consistently less than on SN ($p < 0.01$, student's t-test). The stimulatory influence of long daylength on precocious male maturity is thus not primarily dependent on fast growth rate.

Ovarian development in 2 year old female trout was also stimulated by constant 18 L: 6 D. Ovulation and plasma levels of testosterone showed a 6 month cycle and a few fish produced viable eggs three times within 12 months.

In the strain under test, which normally matures December-January, spawning was slightly delayed (January-February 1982) by the 18 L:6 D photoperiod. One third of these fish ovulated again in July 1982, and plasma testosterone levels indicate that over 75% of the fish will have ovulated by September. Ovulation occured shortly after testosterone levels exceeded 200 ng/ml. Testosterone levels in control fish in July were around 10 ng/ml.

Discussion

The stimulatory effect of long photoperiods runs contrary to the current hypothesis that short days are required to accelerate gonadal maturation in rainbow trout. A reproductive cycle of 6 months duration under constant long day conditions has also been reported in brook trout, stickleback and dab and it is possible that under these conditions two gametogenic cycles are running concurrently and out of phase.

We have demonstrated that long constant photoperiods can be applied to rainbow trout to obtain ovulation at 6 month intervals. The apparatus required is cheap and easy to operate, offering a simple, commercially applicable method for obtaining good quality, out-of-season eggs.

Table 1. % precocious males on different photoperiods

	9 h	14 h	19 h	Inc.	Dec.	SN	N
Expt. 1	5.8	15.7	95.0	86.7	20.1	15.0	10.0
Expt. 2	7.7	17.6	83.8	-	-	16.7	-

INFLUENCE OF SALTWATER SPAWNING AND STRESS ON QUALITY OF SEX PRODUCTS OF CHUM SALMON (ONCORHYNCHUS KETA)

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Summary

Chum salmon enter fresh water (FW) for spawning often in an advanced stage or after completion of sexual maturation. Therefore, holding of broodstock in sea water (SW) appears to be a feasible management option for the enhancement of chum salmon. However, captured fish maturing in SW have both poor gamete fertility and high prespawning mortality. To identify the underlying causes we measured characteristics of sex products of chum salmon held in FW and SW and also investigated the effect of stress on plasma cortisol concentrations.

Maturing salmon caught at sea were tranferred to tanks with FW or SW (28‰ salinity). Stress was applied to one SW group by lowering the water level in the tanks from 80 to 15 cm for one hour daily over 8 days, starting two days prior to the first recorded ovulation. Blood samples for cortisol-RIA taken from SW fish after 6 days of stress treatment were compared with unstressed controls and pre-stress values.

Females were spawned within two days of ovulation. Gamete fertility was measured by crossing with pooled gametes of the opposite sex collected from fish of the same stock returned to the river.

In the FW (n = 19), SW-unstressed (n = 55) and SW-stressed (n = 42) fish, prespawning mortality was 26%, 27% and 40%, respectively. Fish in all groups reached sexual maturity within 7 days. Among males which were not sacrificed for semen collection 100% mortality was reached 10 days earlier in SW than in FW fish. Gamete characteristics of stressed and unstressed fish in SW were similar. Therefore, values from these groups were pooled for comparison with FW fish.

Females: Significant differences between SW (n = 31-32) and FW (n = 7-8) groups were found (see Table).

In SW females fertility was negatively correlated with pOsm ($r = -0.68$, $P < 0.01$), Na^+ ($r = -0.47$, $P < 0.02$) and K^+ ($r = -0.42$, $P < 0.02$).

Males: (no. of observations SW: 24-34, FW: 4-6, $\bar{x} \pm SD$). No pure milt was obtained from SW-males since large amounts of liquid, accumulating in the posterior intestine, contaminated the samples. This was confirmed by high concentrations of Mg^{++} (SW: 63.5 ± 20.3 mM, FW: 1.52 ± 0.55 mM, $P < 0.001$) and Ca^{++} (SW: 1.53 ± 0.75 mM, FW: 0.84 ± 0.52 mM, $P < 0.01$) in seminal plasma, perhaps reflecting secretion of bivalent ions via the intestine. Sperm fertility rates in SW and FW fish were similar (SW: 38.2 ± 39.9%, FW:53.3 ± 29.8%), but motility (score 0-5) was low in SW (0.9 ± 1.1) in contrast to FW fish (3.2 ± 1.2). Motility was correlated with fertility in SW males ($r = 0.53$, $P < 0.01$).

Plasma cortisol levels in stressed and unstressed fish were similar (pooled data: females 28.7 ± 14.3, n = 26, males 14.8 ± 8.8, n = 33) but significantly different from values measured 6 days earlier (11.4 ± 8.3, n = 9 and 8.0 ± 3.5, n = 14, respectively).

The following conclusions may be drawn: High fertility (> 80%) occured in 42% of SW-maturing female chum salmon. Poor fertility was directly correlated with osmoregulatory malfunction. The breakdown in osmoregulation may be correlated with the degeneration of osmoregulatory tissues which occurs during sexual maturation in Pacific salmon (Williams et al., 1977). Our data suggest that the resulting high osmotic pressure of the ovarian fluid is the cause of decreased fertility of females held in SW.

Stress, as applied, did not affect plasma cortisol concentrations or gamete characteristics.

	Salt water $\bar{x} \pm SD$	Fresh water $\bar{x} \pm SD$	P
Fertility %	55.2 ± 40.0	93.6 ± 7.1	0.02
Egg diameter (cm/20 ova)	13.8 ± 0.5	14.4 ± 0.5	0.02
Ovarian fluid/ ovarian mass (%)	5.0 ± 2.2	5.8 ± 2.9	NS
Ovarian fluid pH	8.7 ± 0.2	8.8 ± 0.1	NS
Ovarian fluid pOsm	394 ± 44	311 ± 51	0.001
Ovarian fluid Na^+ (mM)	176 ± 23	141 ± 24	0.001
Ovarian fluid K^+ (mM)	5.3 ± 3.5	3.2 ± 0.5	NS
Ovarian fluid Ca^{++} (mM)	2.6 ± 0.7	1.5 ± 0.6	0.001
Ovarian fluid Mg^{++} (mM)	2.9 ± 1.0	1.1 ± 0.3	0.01

References

Williams, P.V., et al. 1977. Investigation of prespawning mortality of 1973 Horsefly River sockeye salmon. Int. Pac. Salmon Fish. Comm. Progr. Rep. 37: 37 pp.

BASIC AND APPLIED RESEARCH ON FISH REPRODUCTION: A BALANCE?
(Final address on behalf of the Organizing Committee)

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Mr. Honorary President, Ladies and Gentlemen, Dear Friends,

First of all I should like to confess that it is both a pleasure and an honour, as well as a stress to be the last speaker in a long row of such distinguished speakers. The more so, when I scrutinized the title of my contribution: Is there a balance between basic and applied research in fish reproduction?

We have had, ladies and gentlemen, a wonderful event here.

The fact that some 90 contributions were submitted to this conference, and that about 150 scientists participated, representing not less than 30 nations, clearly illustrates the enormous interest in this particular field: Fish Reproductive Physiology.

The idea as well as the concept for this symposium originated at the Paimpont Meeting in 1977 in France and our colleague Prof.Dr. P.G.J.W. van Oordt (University of Utrecht, The Netherlands) advocated to devote a future meeting to an integration of basic and applied aspects of fish reproductive physiology. I take it, that the interest of all of you in coming to and participating in this meeting is a sign that this combination has worked quite well throughout this particular week.

Coming to the topic of this short address - is there a balance? - I must admit that while attending this meeting I have wondered how many types of balances there could be.

Of one I am sure, because that has been revealed to us already during the reception at "Kasteel Groeneveld" last Tuesday; the organizing committee faces a deficit; a negative balance.

Speaking now in financial terms it may be added that the totalized financial input in this meeting amounts to - and I hope that in the meantime all of you are familiar with the Dutch currency - about 1 million Dutch Guilders (U.S.$ 350.000.-). Taking into account that in any agricultural project the rentability reaches hardly 10%, the trout production of, for instance, France has to increase next year with some 1-2 million kg in order to reach another type of balance.

We may also look for a balance in our own field of research on reproduction and look for benefits of this meeting. In doing so I shall openly admit that I have learned a lot in this meeting.

This address started with a confession and I do like to make one more. I, myself, am an aquaculturist from profession and in listening to the speakers who demonstrated the complex mode of actions and interactions of releasing factors, precursors, hormones and pheromones, I often wondered, how, for goodness sake, we succeeded in getting these creatures reproduced. And this was more especially the case after that most comprehensive and excellent review of Dr. R. Billard (Institut National de la Recherche Agronomique, Jouy-en-Josas, France), our honorary President, on the present status in the control of reproduction of fish in aquaculture, where he elaborated on the fact that, although hypophysation procedures are widely and successfully practised to induce reproduction in fish, this may represent the administration of a wrong hormone in the wrong dose at the wrong time.

I think we all agree, that the artificial induction of reproduction in fish by injection of hormonal substances can be regarded as shooting with a gun on a mosquito and we are desperately in need of more accurate and dependable methods in order to guarantee a safe and continuous supply of young fish of different species for aquacultural purposes.

Quite an important part of fish culture still relies on the supply of fry or fingerlings from the wild, which of course can fluctuate quite considerably.

- In South-East Asia the culture of milkfish (Chanos chanos) not only provides for an important contribution to the human diet, but also offers hundreds of thousands of jobs in rural areas. However, this aquacultural enterprise totally depends on catch of milkfish fry in the wild and from year to year this catch may fluctuate regionally by a factor 10.
- The output volume of eel in Japan dropped considerably in the years '70 and this was correlated with a strong decline in the catch of elvers. This independable supply is responsible for the fact that sometimes prices for elvers go skyhigh and reach a few hundreds of U.S. dollars per kg of elvers.
- Although the situation starts to improve, during the last decade only somewhat more than half of the available fishpond area in Central Africa was in full operation, which was mainly due to inadequate availability of young fish.

The important role of fish reproduction in aquaculture can be illustrated by mentioning just two "historical" facts.

- After Remy and Gehin rediscovered the artificial reproduction of fish and implemented this in fish farming activities in the Elsace in 1843, the culture of trout, pike and other species became a well established activity.
- After the introduction of the so-called "hypophysation" in fish culture during the years '50 the farming of for instance chinesian herbivorous fish species became common use throughout the world.

With these points in mind I should like to make a few remarks.

Is there a balance? Yes and No!

Yes, because, when looking at the papers both of the Paimpont Meeting and this event, we learn that 80%, respectively 60% of the papers are dealing with species of which either the farming is an established enterprise or which represent promising candidates for aquaculture.

No, because of a lot of labour input obviously goes into the research of species (mainly Salmonids), which from an aquacultural point of view do not form too much difficulties in reproductive management on fish farms.

There is certainly a strong need for further research in reproductive management for example on milkfish, on eel, on mullet, on Indian carps, on Latin-American Characids and on quite a few Asian and African Siganids and Groupers.

Basic research and applied research sometimes do stand apart in the sense that the former confines itself to the question "how it is controlled" and the latter's interest is in "how it can be governed to obtain a tool in the enhencement of aquaculture".

In respect to this I like to recall Dr. R. Billard, who said in his introductory lecture: "The type of knowledge required in reproductive physiology of fish depends on the type of aquaculture".
I completely agree with him and I am sure he will agree with a slight alteration of the sentence, based on the above mentioned points: "The type of aquaculture depends on the knowledge available in reproductive physiology of fish". Only then fish reproductive management finds a sound basis.

This lays a claim both on applied and basic researchers of which I hope we are all aware.

Personally I found a gap in this meeting, especially in the discussions. The question "how it can be governed and used in aquaculture praxis" has not been put forward too often.

I am sure that in future events like this we will increasingly learn each other's research aims and applications better and in doing so be able to close this gap.

Ladies an gentlemen, in looking back I like to propose another title for this address. I should like to omit the questionmark; I even should like to omit a dot at the end in order to ensure that this dialogue is not ended but can be continued in the next meeting at St. John's, Canada in 1987.

In this respect we can all be grateful to the Canadian delegation, which informed us that they are willing to organize a follow-up to this meeting in 5 years time.

At the end of this symposium I would like to express many thanks to our session chairmen and chairladies who so ably conducted their sessions.

Our sincere thanks go to all those, who contributed by giving lectures, by submitting posters, by participating in the discussions and by providing company.

We like to acknowledge gratefully the interest that the Minister of Agriculture and Fisheries took in this event and materialized in such a superb reception at "Kasteel Groeneveld".

Ladies and Gentlemen, this event could not have taken place without the enormous efforts of both Dr. H.J.Th. Goos (University of Utrecht, The Netherlands) and Dr. C.J.J. Richter (State Agriculture University of Wageningen, The Netherlands). Since we do have elected an honorary chairman in the person of Dr. R. Billard, I like to recommend Dr. Goos and Dr. Richter to be nominated honorary secretaries retro-actively from August 2nd, 1982.

On behalf of the participants I also want to thank the students both from The Netherlands and from abroad, not only for contributing to the presentation of illustrations, but also because their presence and interest is the best guarantee for the continuation of this important work.

I like to recall the outstanding cooperation of the direction and the staff of the International Agricultural Centre (IAC) by providing us with this nice ambience for our meeting.

It is a pleasure to be sure that I speak on behalf of all participants in addressing our sincere appreciation for all the work done and still to be done by Mrs. Ada van Ingen, Mrs. Ellen van de Wetering and Mrs. Liesbeth Hotke.

Dear participants, let us finally thank each other for coming here, let us thank each other for our most enjoyable company and, looking forward to our next meeting, let us declare

"This Symposium is closed".

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