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Organisateurs : R. BILLARD
Mme Jacqueline MARCEL
et les membres du Laboratoire
de Physiologie des Poissons
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Sexual differences in Salmon gonadotropin

par B. BRETON, P. PRUNET, Pierrette REINAUD

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Summary. Salmon (*Oncorhynchus tschawytscha*) GTH's were purified from a mixture of pituitaries of both sexes or from separate male and female glands. Some of their biochemical, immunological and biological properties have been compared. These hormones differed essentially in amino acid composition, especially lysine, histidine and half cystine residues, and by the presence of α-amino (or α-aminoiso) butyric acid in male and GTH II preparations. The same differences were found between GTH I-II and GTH male-female. Observed biochemical differences were sex-linked ; they did not influence immunological and qualitative biological effects, but could determine the affinity of the GTH of each sex or its specific receptors.

Introduction.

Recently, Idler *et al.* (1975*a*, *b*) isolated two gonadotropins (GTH) by chromatography on DEAE cellulose. One of these two GTH acted preferentially to stimulate ovarian c-AMP production in immature rainbow trout ; the other stimulated testicular production of c-AMP. This was the first demonstration of the existence of two gonadotropins in the teleost pituitary using apparently different biological assays. Burzawa-Gérard (1973) in the carp, Donaldson *et al.* (1972) in the salmon, and Burzawa-Gérard *et al.* (1975) in the sturgeon reported only one GTH, but it had never been shown that two different GTH were present when total gonadal tissues were used in studying *in vitro* c-AMP secretion by immature testis or ovary under pituitary stimulation.

In this work we have compared gonadotropins isolated either from a mixture of pituitaries of both sexes or from isolated male and female pituitaries. Our object was to determine if the differences reported by Idler were sex-linked.

Material and methods.

Salmon (*Oncorhynchus tschawytscha*) pituitary glands were collected at the Cowlitz and Spring Creek hatcheries in the state of Washington (USA) during the 1975 spawning season.

The purification procedure was carried out according to Idler's method with a slight modification. The method included saline extraction in a Tris 0.05 M NaCl

buffer, pH 7.6 ; affinity chromatography on concanavaline A sepharose in the same buffer ; gel filtration on ultrogel ACA 54 in Tris 0.05 NaCl 0.15 M, pH 7.6 ; ion-exchange chromatography on DEAE cellulose biogel A using a linear elution gradient of NH_4HCO_3 from 0.03 M to 0.25 M, pH 8.0. The last step was gel filtration on ultrogel ACA 54 in 0.01 NH_4CO_3 buffer, pH 8.0.

Biological activities were measured according to Jalabert *et al.* (1974). The following parameters were studied on the different preparations :

— Kd and apparent molecular weight after ultrogel ACA 54 chromatography ;

— electrophoretic pattern in polyacrylamide gel electrophoresis (PAGE) using 7.5 p. 100 acrylamide gel ; migration was done at pH 9.1 under 2.5 mA per tube during 3 hrs ;

— immunological properties were studied either by immunoelectrophoresis and Ouchterlony immunodiffusion with an antibody produced against total salmon pituitary extract, or by radioimmunoassay (RIA). The technique was the same as that used for carp GTH (Breton *et al.*, 1971). Each preparation was studied in competition reactions to the others with several antibodies to s-GTH. Antibodies were produced against pure t-GTH (Breton *et al.*, 1976), partially purified salmon GTH (SG-G 100) (Donaldson *et al.*, 1972) and the pure female salmon GTH prepared in this experiment. Displacement curve slopes were studied by covariance analysis according to Snedecor and Cochran (1957).

Pituitary gonadotropin contents as measured either by RIA or bioassay were determined both in male and female pituitaries during a complete reproductive cycle to see if the immunological and biological properties of male and female GTH allowed similar determination of GTH pituitary levels using the same RIA system and a female bioassay for both sexes. Ten male and female rainbow trout were killed for

TABLE 1

Some characteristics of different salmon GTH's

Characteristics	Hormones			
	I	II	female	male
(1) DEAE-c chromatography elution molarities	0.082	0.103	0.085	0.110
(2) Ultrogel ACA 54 chromatography : Kd	0.30	0.31	0.305	0.30
(3) Gel electrophoresis : band Rf	0.315-0.366 0.387	0.375-0.422 0.45	0.41-0.47	0.37-0.44
(4) Arbitrary units of specific activity.....	20	20	20	11

(1) : DEAE-c Diethylaminoethyl cellulose.
(2) : Kd : distribution coefficient.
(3) : Rf : electrophoretic mobility.
(4) These units are defined as the dose ratio inducing 50 p. 100 oocyte maturation for the hormone and a stantard crude pituitary glycoprotein extract.

determination each month throughout the reproductive cycle ; pituitary gonadotropin content was measured by RIA and bioassay. The results were analyzed by studying the correlations between the GTH values obtained by the two methods.

Results.

When gonadotropins were purified from a mixture of pituitaries of both sexes, two active fractions I and II were obtained after DEAE cellulose chromatography. Isolated male and female pituitaries gave only one peak of activity. The specific activities of the 4 hormones are given in table 1. I and II have similar activities, but GTH isolated from male pituitaries had half of the specific activity of the gonadotropin extracted from female pituitaries. Some biochemical characteristics of the 4 hormones are shown in table 1. The immunological properties are the same for all the preparations ; immunoelectrophoresis and immunodiffusion showed only one precipitation line for each hormone with the antibody produced against total salmon pituitary extract ; the lines in the immunodiffusion test did not cross. In the RIA studied there are no statistical differences between the slope of the competition curves for hormone pairs obtained with the same antibody (table 2).

TABLE 2

RIA studies with different s-GTH preparations using two antibodies

Antibody raised against	Labelled s-GTH	Standard	Slope	Zero intercept
t-GTH	♀	♀	— 1.021 3 NS	— 2.006 7
	♂	♂	— 0.999 8	— 1.8683
	♂	♂	— 1.083 1 NS	— 1.499 6
	♂	♀	— 1.128 6	— 1.761 2
SG-G100	♀	♀	— 1.228 9 NS	— 0.930 5
	♀	♂	— 1.185 9	— 0.695 4
	♂	♂	— 0.947 9 NS	— 1.172 9
	♂	♀	— 0.993 0	— 0.995 3
t-GTH	I	I	— 0.971 0 NS	— 0.994 02
	I	II	— 1.020 6 NS NS	— 1.183 5
	I	♀	— 0.993 8	— 0.938 2
	II	III	— 1.144 3 NS	— 0.913 0
	II	I	— 1.184 7 NS NS	— 0.928 8
	II	♂	— 1.099 4	— 1.041 8

The aminoacid composition of the 4 hormones was also analyzed after 24-hr hydrolysis at 110 °C in HCl 6N.

Table 3 shows the aminoacid compositions of the preparations compared with those of the hormone prepared by Pierce *et al.* (1976) from the pituitary of the same species. The differences between I and II and male and female hormones were located in the lysine, histidine, half cystine and isoleucine residues. They were the same in the two pairs of hormones. Except for the half cystine residues, there was no important difference between s-GTH II, s-GTH male and Pierce's preparation.

TABLE 3

Amino acid compositions of different salmon gonadotropin preparation residues/200 residues.

	s-GTH Pierce 1976	s-GTH I from a mixture	s-GTH II of pituitaries	s-GTH from isolated ♀ pituitaries	s-GTH from isolated ♂ pituitaries
Lysine	11.8	19.7	11.8	20.4	12.4
Histidine	5.4	10.2	7.6	9.6	6.4
Arginine	4.8	10.3	10.	11.6	9.6
Aspartic Acid	19.6	18.7	18.3	20.	18
Threonine	22.4	20.3	20.5	18	21.4
Serine	11.8	10.2	11.7	10.2	13.2
Glutamic Acid	20.6	17.3	18.5	16.8	18
Proline	16.6	15	16	16	16.8
Glycine	9.4	9.1	8.7	8.6	9.8
Alanine	7.6	7.5	6.3	7.4	6
Half Cystine	24.2	8.5	16.4	6.8	15.2
Valine	13.0	14.2	14.1	14	16.4
Methionine	4.2	5.0	4.8	5.2	4.2
Isoleucine	6.2	7.8	10.5	8.2	11.6
Leucine	8.2	10.8	11.5	11.6	12
Tyrosine	8.0	10.3		9.8	
Tyrosine + α-amino (iso) butyrique acid			11.4		10
Phenylalanine	5.4	5.4	6.0	4.9	6.6

The I-female pair shows differences with this last preparation similar to those of pairs I-II and male-female. In addition, a compound was found in the s-GTH II-male pair that could be either α-aminobutyric acid or α-aminoisobutyric acid, as demonstrated after calibration of the aminolyzer. This product was not present in the other two hormones or in Pierce's preparation.

Comparisons of pituitary GTH levels by radioimmunoassay and bioassay gave a good correlation between the two values for the female but not for the male pituitary (table 4). The only statistically different results in the male were obtained during active spermatogenesis and spermiation at 5 p. 100 confidence limit when GTH pituitary content reached its maximum level.

Table 4

Correlations between GTH level measured by radioimmunoassay and biological assay in the pituitary of rainbow trout during the reproductive cycle.
In the two methods GTH contents were expressed in μg t-GTH-mg fresh tissue

Stage of vitellogenesis (mean diameter of oocytes)	0.5 mm	0.5-1	1-1-5	1-5-2	2-3	3-4	VG+	Global
Correlation between RIA s-GTH and s-GTH	0.98	0.52	0.71	0.54	0.70	0.83	0.97	0.77
Statistical significance..	** 1 p. 100	* 5 p. 100	** 1 p. 100	NS	* 5 p. 100	* 2 p. 100	* 5 p. 100	** 1 p. 100

Stage of spermatogenesis.....	I	II	III	IV	V	VI	VII	VIII	Global
	resting spermatogenesis			active spermatogenesis-spermiation					
Correlation between RIA s-GTH and BD s-GTH	0.006 3	— 0.41	— 0.99	0.55	0.85	0.41	0.89	0.39	0.16
Statistical significance .	NS	NS	NS	NS	* 5 p. 100	NS	* 5 p. 100	NS	NS

The RIA system used pure t-GTH (Breton *et al.*, 1976) and the antibody was produced against this preparation. The technique was the same as that described for carp GTH (Breton *et al.*, 1971) ; here anti t-GTH was used at 1/2 10^{-5} concentration.

Discussion.

Most of the biochemical characteristics show similarities between GTH I-female and GTH II-male. The most important differences were noticed in their aminoacid compositions, and also in their electrophoretic mobilities, but they did not induce specific immunological properties.

Of the two factors isolated by Idler (1975*b*), one (corresponding to our first peak of activity) was preferentially active on ovarian c-AMP production in immature trout, and the other (corresponding to our fraction II) sitmulated c-AMP formation by the testis. Our results demonstrated biochemical identity between GTH I-female and GTH II-male. Thus, GTH from male and female pituitaries can be isolated from a mixture of pituitary on the basis of their different biochemical properties. Such sex-linked differences have already been reported for mammalian gonadotropins. They generally affect hexose and salic acid content. We have not measured these components in our preparations but there is another difference between male and female salmon GTH, never reported in mammals i.e. the presence of either α-aminobutyric or α-aminoisobutyric acid in the male hormone. Presently, we have no idea concerning the possible role of this compound. The biochemical differences between male and female GTH do not induce immunological differences, but they seem to have biological effects. GTH pituitary measurement during the whole reproductive cycle gave a good correlation between RIA and bioassay results for the female but not for the male des-

pite the fact that the corresponding purified GTH has the same immunological prc-perties. This situation must be compared to the fact that highly purified male GTH has half the specific activity of the female gonadotropin when assayed on a female receptor as bioassay. On the other hand, Upadhyay (1977) was not able to demonstrate qualitative differences in the *in vivo* action of the male and female hormones at the ultrastructural level. They both initiated complete spermatogenesis in immature rainbow trout, but failed to induce total vitellogenesis after 10 weeks of treatment. Quantitative analysis remains to be done.

In conclusion, there may be some biochemical sex-linked differences in salmon gonadotropin isolated using *in vitro* trout oocyte maturation assay. These divergencies would permit chromatographic separation and could determine the affinity of each GTH for its specific receptors (ovaries or testis) without affecting their qualitative properties. They are of the same type and do not represent two distinct GTH's. These results do not reject the further existence of another GTH in fish pituitary.

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Résumé. Les GTH de saumon (*Oncorhynchus tschawtscha*) ont été purifiées soit à partir d'un mélange d'hypophyses, soit à partir d'hypophyses mâles et femelles séparées. Certaines de leurs propriétés biochimiques immunologiques et biologiques ont été comparées. Ces hormones diffèrent essentiellement dans leurs compositions en acides aminés aux niveaux de leurs résidus en lysine, histidine, hémicystine et par la présence d'acide α-aminobutyrique ou α-aminoisobutyrique dans les préparations mâle et dans GTH II purifiée à partir d'un mélange d'hypophyses. Les mêmes différences sont retrouvées à la fois entre les préparations I, II et mâle, femelle. Les différences observées sont liées au sexe, et n'influent pas sur les propriétés immunologiques. Elles pourraient déterminer l'affinité de chaque GTH pour leurs récepteurs spécifiques.

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Interaction of salmon gonadotropin subunits : spectroscopic studies

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Summary. Pituitary gonadotropins of female and male pacific salmon *Oncorhynchus tshawytscha* were prepared separately. The two preparations exhibited different sedimentation coefficients (2.8 and 2.3, respectively) but similar circular dichroism spectra indicative of low α helix and high β sheet contents. Both hormones gave a difference spectrum (1 750 M^{-1} cm^{-1} at 287 nm) characteristic of perturbed tyrosine and phenylalanine residues when dissociated at acid pH. These results suggest that fish and mammal gonadotropins exhibit the same general folding of their polypeptide chains and undergo the same conformational transition when their subunits associate ; however, fish gonadotropin subunits reassociate at considerably faster rates.

Introduction.

The presence of two distinct gonadotropins in fish is still an open and interesting question. A single gonadotropin having both lutropin and follitropin activities was found in carp (Billard *et al.*, 1970 ; Burzawa-Gérard, 1971, 1974*a* ; Sundararaj *et al.*, 1976) and in salmon (Donaldson *et al.*, 1972), although physicochemical, biological or immunological evidence for two distinct gonadotropins has been presented (Idler *et al.*, 1975*a* ; Pierce *et al.*, 1976).

As those of mammalian origin, fish gonadotropins are composed of two dissimilar subunits (Donaldson *et al.*, 1972 ; Burzawa-Gérard, 1974*b* ; Burzawa-Gérard *et al.*, 1975) which can be dissociated in acid condition with loss of biological activity. This activity loss in mammalian hormones is accompanied by a specific conformational change of the subunits which is association-dependent (see review in Garnier, 1978). From experiments reported below, it is shown that two gonadotropin preparations, one from female salmon (s GTH_1), the other from male salmon (s GTH_2), also undergo the same reversible conformational transition during the association-dissociation process of their two subunits.

Material and methods.

The gonadotropins were prepared from separate female and male pituitary glands of pacific salmon *Oncorhynchus tshawytscha*, according to Idler *et al.* (1975*a*). Assayed *in vitro* for trout oocyte maturation (Jalabert *et al.*, 1974), both preparations exhibited the same potency and were two times more active than a sample obtained from Idler and prepared according to his method (Idler *et al.*, 1975*b*).

Protein concentrations were determined by a Lowry assay with bovine serum albumin as standard.

Ultracentrifuge experiments were carried out in a Spinco Beckman ultracentrifuge model E.

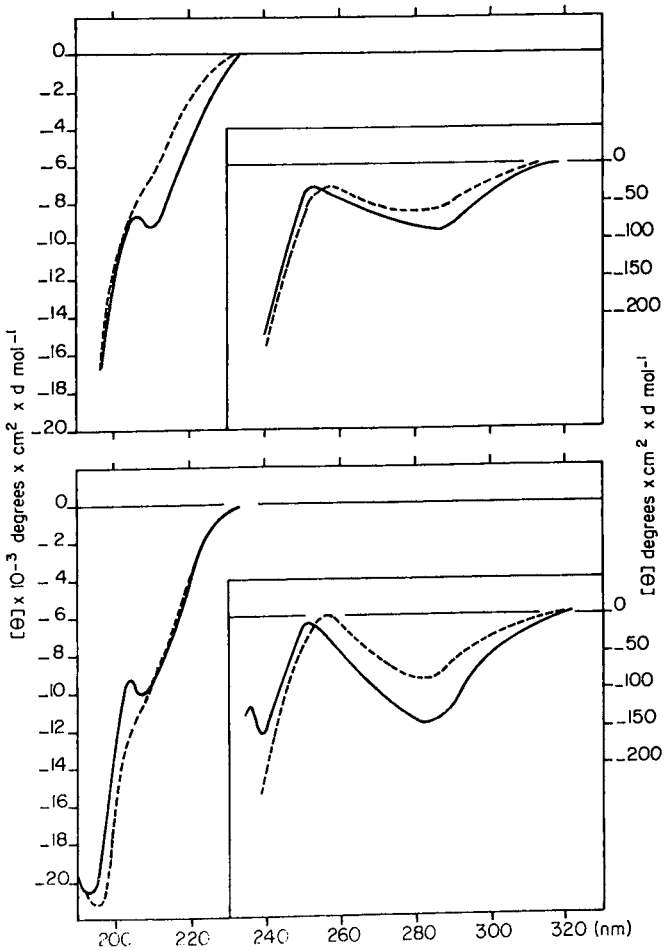

FIG. 1. — *CD spectra of s* GTH_1 (bottom) *and s* GTH_2 (above) *assuming a mean molecular weight per residue of* 141.

——— : native hormone ;

– – – – – : dissociated hormone (pH 2.2).

Circular dichroism spectra were recorded with a Jouan Dichrograph III ; light paths were 0.5 to 0.01 cm. Mean molecular weight per residue was assumed as 141.

Optical densities and U. V. difference spectra were recorded in a Cary 118 spectrophotometer with a 1 cm light path cuvette in a thermostated holder. Kinetics of dissociation and reassociation of hormones were followed at 287 nm.

Results and discussion.

The two preparations were found to differ essentially by their sedimentation coefficient ($S_{20,w}$) of 2.8 for s GTH_1 (0.5 mg/ml) and 2.3 for s GTH_2 (0.7 mg/ml) at neutral pH, 0.1 M NaCl. Both exhibited the same absorbance spectra with a maximum at 275-276 nm characteristic of tyrosine residue with no tryptophane contribution.

They had similar circular dichroism (CD) spectra (fig. 1) with minima at 280 nm, 210 nm (s GTH_2) or 208 nm (s GTH_1) and 193 nm; s GTH_1 had another small band at 234 nm. All these bands were already reported for mammalian gonadotropins and they are indicative of low α helix and high β sheet contents. These CD spectra suggest that the general gonadotropin folding has been conserved through evolution from fish to mammal.

Other interesting common features are the blue shift of the far U. V. CD, the decrease of the CD band at 280 nm (fig. 1) and the appearence of difference spectra (1 750 M^{-1} cm^{-1} at 287 nm) characteristic of perturbed tyrosine and phenylalanine residues (fig. 2) when the hormones are dissociated at acid pH. By raising the pH again to neutral, the two subunits reassociated with recovery of most of the native CD and loss of the difference spectrum. This suggests that salmon gonadotropins undergo the same conformational transition as mammalian gonadotropins when their two subunits associate.

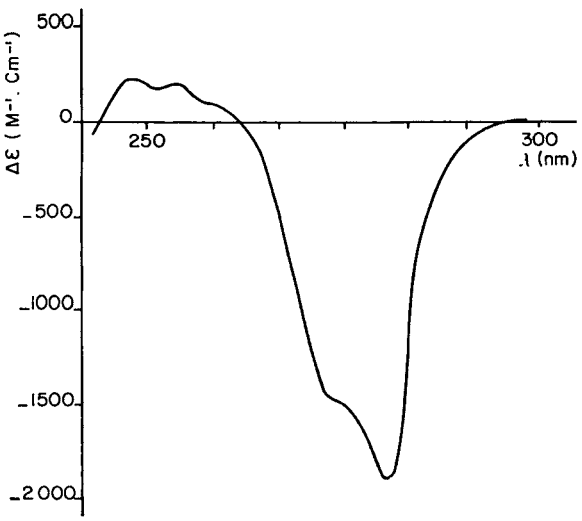

FIG. 2. — *UV difference spectrum of s-GTH_1 upon dissociation at pH 2.2*
Reference : s-GTH_1 at pH 5.6.

Association-dissociation processes were followed with time (fig. 3) ; contrary to mammalian hormones, they were found to be much more rapid processes. For example, reassociation of sGTH at neutral pH from acid pH at 37 °C was 5 to 10 times faster than association of o-lutropin subunits or 20 to 50 times faster than h-chorio-gonadotropin reassociation (Pernollet *at al.*, 1976).

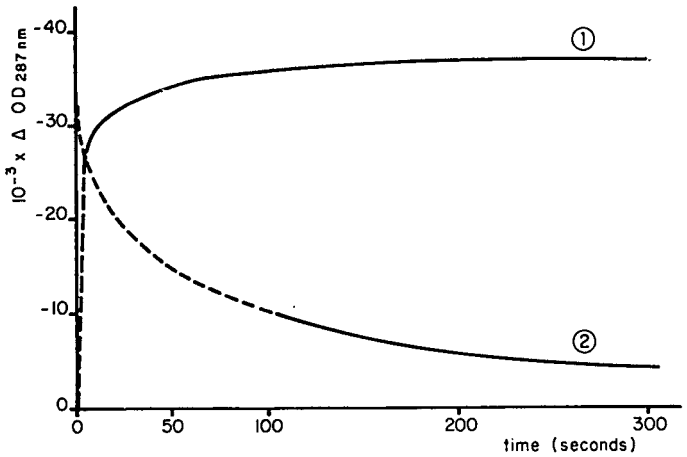

FIG. 3. — *Change with time of the absorbance at 287 nm of s-GTH$_1$ during dissociation of the subunits at pH 2 (curve 1) and re-association at pH 6.4 (curve 2).* Temperature 37 °C, 0.1 M NaCl. Dashed parts correspond to extrapolated absorbance change to zero time.

These observations strengthen the hypothesis that subunit association and subsequent conformational change are necessary steps *in vivo* for the formation of active gonadotropin.

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Résumé. A partir d'hypophyses du saumon *Oncorhynchus tshawytscha* femelle et mâle deux préparations de gonadotropine ont été obtenues. Elles ne diffèrent que par leur coefficient de sédimentation respectivement 2,8 et 2,3. Leurs spectres de dichroïsme circulaire sont semblables et indiquent une faible teneur en hélice α et une forte teneur en structure β. A pH acide, ces deux hormones se dissocient en leurs sous-unités, donnant naissance à un spectre de différence (1 750 M^{-1} cm^{-1} à 287 nm) caractéristique de résidus tyrosines et phénylalanines perturbées. Ces résultats suggèrent que les gonadotropines de poisson et de mammifère présentent la même conformation générale de leurs chaînes polypeptidiques et qu'elles subissent la même transition conformationnelle quand leurs sous-unités s'associent à la différence que les sous-unités de gonadotropine de poisson s'associent à des vitesses beaucoup plus grandes.

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Etude par radioimmunologie des propriétés des immunsérums de l'hormone gonadotrope de la carpe *(Cyprinus carpio)* et de ses sous-unités

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avec la collaboration technique de Michèle VALLENS, Yvette LANGLOIS, Aléna DUMAS

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Summary. *Radioimmunological properties of antisera from carp gonadotropin* (c-GTH) *and its subunits.*

Anti-sera against carp gonadotropin (c-GTH) and its subunits SU I and SU II were produced in rabbits (IS-c-GTH, IS-SU I and IS-SU II). The radioimmunological titers were determined when antiserum binding dilution was 50 p. 100 of the ^{125}I-labelled glycoproteins. The titers of IS-c-GTH were 1/750 000, 1/300 000 and 1/500 000, respectively with c-GTH, SU I and SU II. With SU I, the titer of IS-SU I was 1/100 000 ; this antiserum in excess bound only 25 p. 100 of the c-GTH and no SU II. With c-GTH and SU II, the titer of IS-SU II was 1/270 000 ; it was 1/13 000 with SU I. With these antisera no binding was observed with rat hormones (LH, FSH, TSH, PRL), but IS-c-GTH and IS-SU II weakly bound rLH. Displacement studies were also carried out ; the cross-reactions in each system were as follows :

IS-c-GTH $\leftrightharpoons$ ^{125}I-c-GTH 100 p. 100 with SU II (for 0.1 ng) 0.01 p. 100 with SU I
IS-SU I $\leftrightharpoons$ ^{125}I-SU I 20 p. 100 with c-GTH (for 0.1 ng) 0 p. 100 with SU II
IS-SU II $\leftrightharpoons$ ^{125}I-SU II 25 p. 100 with c-GTH 0 p. 100 with SU I.

No displacement was observed in any test with rat hormones, even at high doses (100 ng). c-GTH and its subunits were very antigenic in rabbit and thus we could separately test each subunit.

L'hormone gonadotrope de la carpe (c-GTH) est composée comme toutes les hormones hypophysaires glycoprotéiques de deux sous-unités. Cette hormone, ainsi que ses sous-unités (SU I et SU II), ont été obtenues à un degré élevé de pureté (Burzawa-Gérard, 1971, 1974). Toutefois, il existe un équilibre entre l'hormone et ses sous-unités et de ce fait toutes les préparations de c-GTH contiennent des sous-unités libres en faible proportion (Burzawa-Gérard, 1974).

Les méthodes de dosages radioimmunologiques décrites jusqu'à présent mettent en jeu la GTH de carpe (Breton *et al.*, 1971, 1972, 1973) ou celle d'un saumon (CRIM *et al.*, 1973). Des résultats importants ont pu être apportés en ce qui concerne la varia-

tion des taux plasmatiques en GTH au cours des cycles de reproduction du saumon ou du cyprin. Toutefois, il est probable, tout au moins en ce qui concerne la c-GTH, que des systèmes assez complexes aient été mis en jeu. En effet, l'hormone marquée peut également contenir des sous-unités marquées et les immunsérums des anticorps, non seulement contre l'hormone, mais aussi contre ses sous-unités.

Il apparaît donc nécessaire de préciser les propriétés radioimmunologiques des sous-unités comparées à celles de l'hormone. Certains résultats préliminaires ont été brièvement décrits (Burzawa-Gérard *et al.*, 1977).

Matériel et méthodes.

a) *Hormones utilisées.* La c-GTH et ses sous-unités sont obtenues selon les techniques précédemment décrites (Burzawa-Gérard, 1971, 1974). Les hormones gonadotropes de rat (r-LH, r-FSH) sont préparées par Jutisz et Ribot ; l'hormone thyréotrope de Rat (r-TSH) et la prolactine de Rat (r-PRL) sont fournies par le NIAMD.

b) *Techniques radioimmunologiques.* Les immunsérums anti-c-GTH (IS-c-GTH) anti SU I (IS-SU I) et anti SU II (IS-SU II) ont été obtenus chez le lapin après 4 injections de c-GTH ou 5 injections des sous-unités émulsionnées dans l'adjuvant de Freund complet. Les immunsérums obtenus sont gardés congelés ou lyophylisés jusqu'au moment de leur utilisation (Kerdelhué *et al.*, 1971).

La c-GTH, SU I et SU II sont marquées à l'iode (^{125}I) par la chloramine T selon la technique de Greenwood *et al.*, (1963) dans les conditions utilisées dans le cas des hormones gonadotropes de rat (Kerdelhué *et al.*, 1971). Une filtration sur Sephadex G 75 permet d'éliminer l'iode libre des glycoprotéines iodées (seule sera utilisée la fraction correspondant au maximum de la radioactivité). L'activité spécifique des solutions marquées est d'environ 100 à 150 μC/mg.

La réaction radioimmunologique est réalisée en tampon phosphate (0,1 M, pH 7,4) contenant de l'albumine sérique de bœuf (0,1 p. 100) dans un volume final de 520 μl. Les essais sont réalisés en triplicas et incubés 48 h à 4 °C. Le complexe hormone-anticorps est isolé par immunoprécipitation ; dans ce but on ajoute du sérum de mouton anti-γ-globuline de lapin et on incube 24 h à 4 °C. Le précipité formé est séparé par centrifugation (1 000 g) et le surnageant éliminé par aspiration. La radioactivité du précipité est déterminée par un compteur γ en nombre de coups par mn (cpm).

c) *Techniques biochimiques.* L'analyse des produits iodés est réalisée par filtration sur Sephadex G 100 et par électrophorèse analytique sur gel de polyacrylamide.

Résultats.

A. *Marquage de la c-GTH et de ses sous-unités.*

La filtration sur Sephadex G 100 d'une fraction de c-GTH marquée montre la présence de deux pics. Le premier correspond au coefficient d'exclusion de l'hormone ($K_D \simeq 0{,}30$), le second $K_D \simeq 0{,}45$ n'est pas différent de celui des sous-unités (fig. 1). Les sous-unités SU I et SU II marquées sont elles-mêmes exclues pour un coefficient d'exclusion ($K_D \simeq 045$) voisin de celui des sous-unités natives (fig. 1). Ces résultats

ont été confirmés par électrophorèse analytique sur gel de polyacrylamide. La proportion des sous-unités présentes dans les solutions de c-GTH marquées est augmentée.

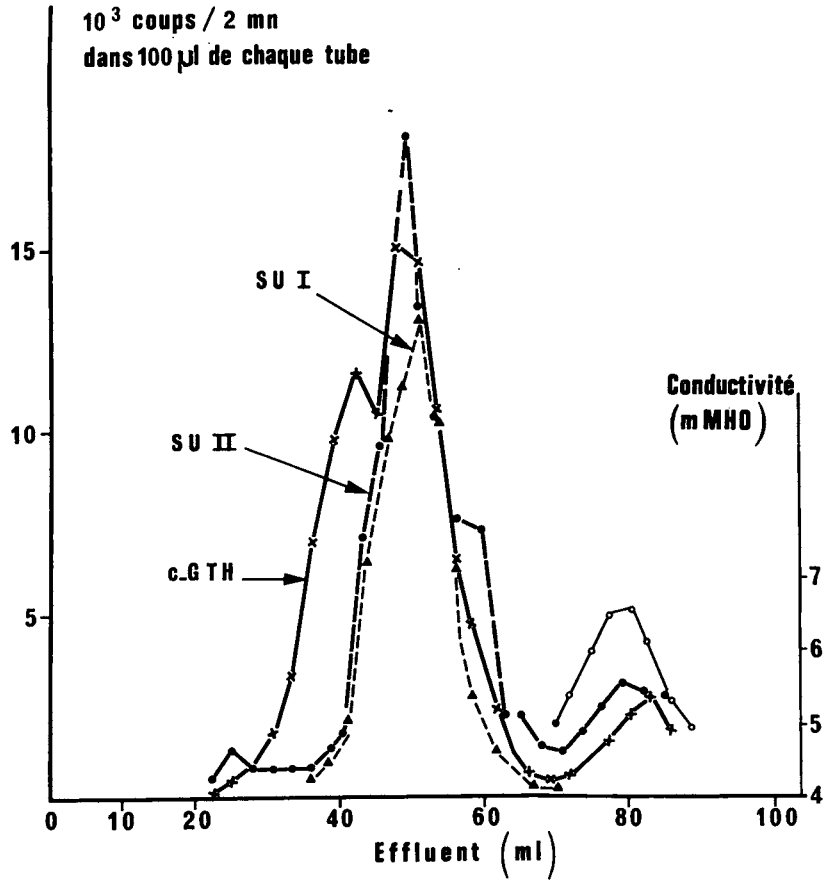

FIG. 1. — *Etude par filtration sur Sephadex G 100 des solutions de c-GTH SU I et SU II marquées obtenues après Sephadex G 75.*

Colonne 1,2 × 70 cm ; débit 17 ml/h 1,6 ml/fraction. Tampon tris H-Cl 0,05 M, pH 7,7.
10 µl de chaque solution radioactive sont dilués dans 1 ml de NaCl 0,5 M, auxquels sont ajoutés 1 mg de c-GTH, SU I ou SU II selon qu'il s'agit de ^{125}I-c-GTH, ^{125}I-SU I ou ^{125}I-SU II.
×——× ^{125}I-c-GTH ; ▲---▲ ^{125}I-SU I ; ●——● ^{125}I-SU II ; o——o Conductivité (m MHO).

B. *Etude des immunsérums anti c-GTH et anti sous-unités.*

a) Le titre radioimmunologique est représenté par la dilution finale de l'immunsérum considéré qui permet d'obtenir 50 p. 100 de liaison avec l'antigène marqué. Les titres de l'IS-c-GTH sont respectivement de 1/750 000, 1/300 000 et 1/500 000 avec c-GTH, SU I et SU II (fig. 2). Le titre de l'IS-SU I vis-à-vis de SU I est de 1/1 000 000 par contre il n'y a pas de titre définissable pour la c-GTH et SU II. L'IS-SU I en excès ne

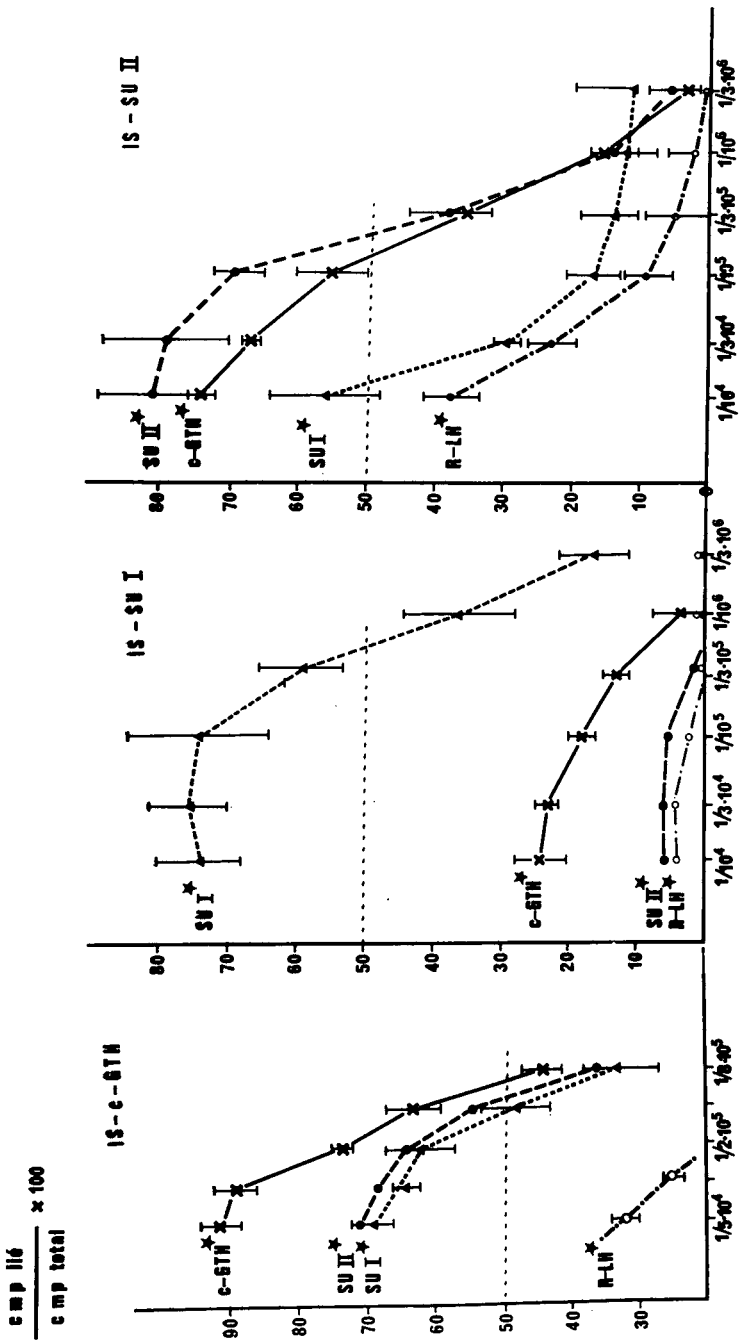

FIG. 2. — *Etude de l'affinité de liaison des immunsérums* (IS-c-GTH, IS-SU I et IS-SU II) *pour diverses glycoprotéines marquées* (^{125}I-c-GTH : 12 150 cpm, ^{125}I-SU I : 13 700 cpm, ^{125}I-SU II : 12 250 cpm). Chaque essai est réalisé en triplica.
×——× ^{125}I-c-GTH ; ▲---▲ ^{125}I-SU I ; ●——● ^{125}I-SU II ; ○—·—○ ^{125}I-R-LH.

$\frac{\text{cpm lié}}{\text{cpm total}} \times 100 \pm$ limites pour $p = 0{,}05$.

lie que 25 p. 100 de la c-GTH et pratiquement pas SU II (fig. 2). Les titres de l'IS-SU II vis-à-vis de la c-GTH et de SU II ne sont pas sensiblement différents 1/270 000 ; vis-à-vis de SU I le titre est de 1/13 000 (fig. 2). Aucun de ces trois immunsérums ne possède de sites de liaison pour la r-FSH, la r-TSH, mais les immunsérums IS-c-GTH et IS-SU II lient faiblement la r-LH (fig. 2).

b) Le déplacement de la liaison de l'antigène marqué à l'immunsérum correspondant a été étudié pour chacun des trois systèmes à l'aide de l'antigène lui-même ou d'autres glycoprotéines (hormone ou sous-unités et hormones hypophysaires de rat). Dans chaque cas la dilution de l'immunsérum entraînant 50 p. 100 de liaison de l'antigène marqué a été utilisée.

1. Système IS-c-GTH $\leftrightharpoons$ ^{125}I-c-GTH (fig. 3). Le pourcentage de liaison en l'absence d'hormone non marquée est de 49,3 (46,3-52,3), il est de 37,3 (36,2-38,4) pour 0,01 ng de c-GTH. La sensibilité de ce système est donc inférieure à 10 pg. SU II déplace l'équilibre de ce système, mais de manière non parallèle à c-GTH (covariance entre les droites obtenues après la transformation Logit-Log (dose) pour la c-GTH marquée et la SU II marquée : $F_{cov.} = 14{,}99$ (dl = 1-18) $p < 0{,}01$). Mais la réaction croisée est pratiquement nulle avec SU I (0,01 p. 100).

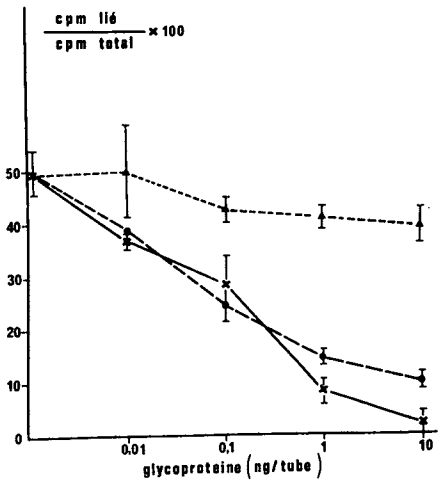

FIG. 3. — *Déplacement de la liaison de ^{125}I-c-GTH à l'IS-c-GTH par c-GTH, SU I et SU II.* Chaque essai est réalisé en triplica. IS-c-GTH dilution finale de 1/500 000. ^{125}I-c-GTH : 12 150 cpm. ×——× c-GTH ; ▲- - -▲ SU I ; ●——● SU II.

$\overset{\text{cpm lié}}{\underset{\text{cpm total}}{\ast}} \times 100 \pm$ limites pour $p = 0{,}05$.

2. Système IS-SU I $\leftrightharpoons$ ^{125}I-SU I (fig. 4). Le pourcentage de liaison en l'absence de la sous-unité (SU I) non marquée est de 49,3 (44,5-54), il est de 43,0 (39,6-46,4) pour 0,01 ng de la SU I. La c-GTH déplace aussi cette liaison, mais la transformation en Logit-Log (dose) montre que les droites ne sont pas parallèles ($F_{cov.} = 17{,}15$ (dl 1-14) $p < 0{,}001$). SU II même à des doses élevées (100 ng) n'est pas capable de la déplacer.

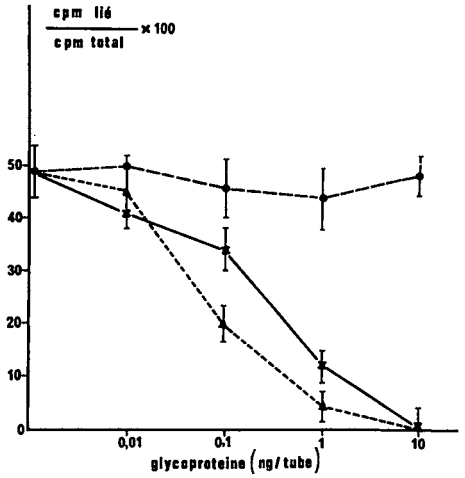

FIG. 4. — *Déplacement de la liaison de ^{125}I-SU I à l'IS-SU I par c-GTH, SU I et SU II.*
Chaque essai est réalisé en triplica. IS-SU I dilution finale 1/400 000. ^{125}I-SU I : 13 700 cpm.
×——× c-GTH ; ▲---▲ SU I ; ●——● SU II.

$$\frac{\text{cpm lié}}{\text{cpm total}} \times 100 \pm \text{limites pour } p = 0.05.$$

3. Système IS-SU II ⇋ ^{125}I-SU II (fig. 5). Le pourcentage de liaison en l'absence de la sous-unité (SU II) non marquée est de 28,6 (24,9-32,3), il est de 18,3 (16,1-20,5) pour 0,01 ng de la SU II. La sensibilité est donc bien inférieure à 10 pg. Dans ce système, la

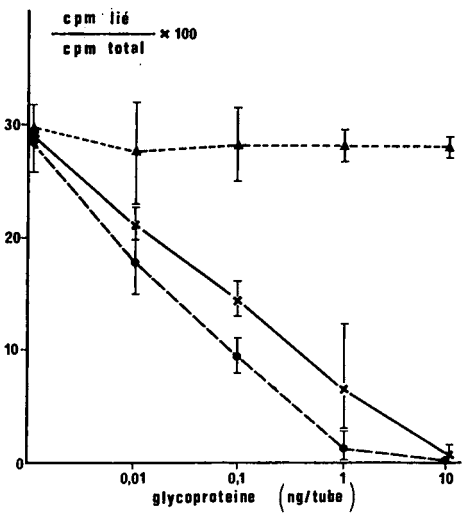

FIG. 5. — *Déplacement de la liaison de ^{125}I-SU II à l'IS-SU II par c-GTH, SU I et SU II.*
Chaque essai est réalisé en triplica. IS-SU II dilution finale 1/400 000. ^{125}I-SU II : 12 250 cpm.
×——× c-GTH ; ●——● SU II ; ▲---▲ SU I.

$$\frac{\text{cpm lié}}{\text{cpm total}} \times 100 \pm \text{limites pour } p = 0,05.$$

c-GTH est également capable de déplacer la liaison. La transformation en Logit-Log (dose) montre que dans ce cas les droites obtenues avec la SU II et la c-GTH marquées sont parallèles ($F_{cov.} = 3,08$ (dl 1-14) $p > 0,05$). La réaction croisée est voisine de 20 p. 100 SU I ne la déplace sensiblement pas, même à des doses élevées (100 ng).

Etudiées dans chacun de ces trois systèmes, r-LH, r-FSH, r-TSH et r-PRL ne manifestent aucune réaction croisée, même à des doses élevées (100 ng).

Discussion.

La c-GTH, SU I et SU II se révèlent être très antigéniques chez le lapin comme le soulignent les titres élevés des immunsérums anti c-GTH, anti SU I et anti SU II chez cette espèce. Un résultat analogue a été observé pour la c-GTH par Breton *et al.* (1971) chez le cobaye. La sensibilité des trois systèmes radioimmunologiques qui ont été développés ici est élevée.

La technique de marquage à la chloramine T ne modifie apparemment pas les caractéristiques biochimiques (coefficient d'exclusion sur Sephadex G 100, comportement électrophorétique) des sous-unités. Par contre une importante dissociation de la c-GTH en ses sous-unités est observée sous l'influence de l'iodation et les solutions de ^{125}I-c-GTH sont enrichies en sous-unités marquées. En conséquence, l'affinité de la c-GTH iodée pour l'IS-SU I et plus particulièrement pour l'IS-SU II peut être surestimée. Il en est de même pour la détermination des réactions croisées de SU I et SU II dans le système IS-c-GTH $\leftrightharpoons$ ^{125}I c-GTH.

Même si elle est surestimée, la réaction croisée entre c-GTH et SU II est bien démontrée par nos résultats. Dans ces conditions et dans la mesure où la SU II libre serait présente dans le plasma ou l'hypophyse, la détermination radioimmunologique du taux d'hormone proprement dite dans ces matériels, par l'emploi de l'IS-c-GTH peut conduire à un résultat inexact. Chez les mammifères et dans certaines conditions physiologiques, il a été montré que les taux plasmatiques en sous-unités libres pouvaient être élevés (Vaitukaitis, 1977).

En ce qui concerne les poissons téléostéens, la recherche de la présence des sous-unités libres dans le plasma n'a pas encore été abordée. D'un point de vue biochimique, nous avons observé que certaines caractéristiques de l'équilibre entre la c-GTH et ses sous-unités sont différentes de ce qui est observé dans le cas des hormones glycoprotéiques hypophysaires mammaliennes ; la dissociation et l'association sont en particulier beaucoup plus rapides et ceci pourrait avoir une signification physiologique (Fontaine et Burzawa-Gérard, 1978). L'application des résultats présentés dans ce travail ouvre l'accès à ces recherches. Les immunsérums obtenus à partir des sous-unités se révèlent être bien plus spécifiques que celui obtenu à partir de la c-GTH. En particulier il n'y a pas de réaction croisée entre les deux sous-unités de la c-GTH. Ce résultat permet donc le dosage sélectif de l'une ou l'autre sous-unité dans la mesure où la c-GTH elle-même est écartée.

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Immunological relatedness of gonadotrophins of various fishes as shown by radioimmunoassays

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Summary. Pituitary extracts and plasmas of 35 species of fish were tested in two radioimmunoassay (RIA) systems, a salmon-salmon homologous RIA and a salmon-carp heterologous RIA, in which the same antiserum, raised against salmon gonadotrophin, SG-G100, was employed. In the homologous RIA, most salmonid species tested, except for the powan and ayu, cross-reacted in a manner identical with that of the standard, SG-DEAE-3. Non-parallelism of inhibition curves were found in 13 non-salmonid species while 3 others showed non cross-reaction. In the heterologous RIA, all cyprinids, except the rudd, and all salmonids, except the ayu, as well as 9 other species, gave inhibition curves parallel to that of the standard purified carp gonadotrophin. These results may indicate that immunological properties of fish gonadotrophins do not correspond to knowr phylogenetic relationships of fishes.

Introduction.

The objective of this study is to evaluate, from an immunological viewpoint, the concept of species specificity of fish gonadotrophins on the basis of cross-reactivity of pituitary extracts and, when available, plasmas of 35 species of fishes in two RIA systems for teleost gonadotrophins namely, a salmon-salmon homologous RIA and a salmon-carp heterologous RIA, in each of which the same antiserum against salmon (*Oncorhynchus tshawytscha*) gonadotrophin (SG-G100 ; Donaldson *et al.*, 1972) was employed.

Materials and methods.

a) *The RIA systems.* — The development of these two RIA systems has been already presented (Tan, 1976*a*). Full technical details and a validation as complete as possible of these two systems have been described (Tan, 1976*b*).

b) *Preparation of samples.* — Pituitaries were homogenised in 0.05 M phosphate-buffered saline (pH 7.5, 0.9 p. 100 NaCl and 0.1 p. 100 sodium azide in 0.05 M

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phosphate buffer). Each homogenate was left at 4 °C overnight and then centrifuged at 1 000 g for 20 min. at 4 °C. Pituitary extracts, usually prepared at a concentration of 1 pituitary/ml, were stored at — 20 °C and analysed within 10 days of being prepared. Plasma samples were stored at — 20 °C prior to analysis.

c) *RIA analysis.* — A post-precipitation double antibody RIA technique was employed. Purified carp gonadotrophin, which was equipotent to NIH-LH in the frog

TABLE 1

Comparison of parallelism of inhibition curves of plasma and pituitary extracts of various fish species with that of the standard hormone in both radioimmunoassays

Species	Homologous RIA		Heterologous RIA	
	Plasma	PE	Plasma	PE
Oncorhynchus spp. (3 species)	p	p	p	p
Salmo gairdneri				
(*a*) wild	p	p		p
(*b*) hatchery reared	np	np		
Salmo trutta	p	p	p	p
Salmo salar				
(*a*) parr	p	p		
(*b*) adult	p	p		p
Salvelinus alpinus	p	p		p
Coregonus lavaretus	np	p(np) *		p
Plecoglossus altivelis		np		np
Esox lucius	-ve	np		p
Poecilia latipinna				p
Gadus morhua	-ve	np		
Pollachius pollachius		np		p?
Trisopterus luscus		-ve		p
Anguilla anguilla		np		np
Protopterus sp.		np		p
Pterophyllum scalare		np		p
Pleuronectes platessa		np		np
Dicentrarchus labrax	-ve	np (-ve) **		np
Perca fluviatilis				-ve
Morone saxatilis				p
Ambloplites rupestris				p?
Mugil labrosus		-ve		
Ictalurus punctatus		np		p
Scomber scomber	np	np		np
Cyprinus carpio	np	np		p
Carassius auratus		np	p	p
Carp-goldfish hybrid		np	p	p
Puntius gonionotus				p
Rutilus-rutilus				p
Scardinius erythrophthalmus				np
Catostomus commersoni				p
Petromyzon marinus		np		p
Lampetra fluviatilis				p
Scyliorhinus canicula		-ve		-ve

Abbreviations : PE pituitary extract ; RIA radioimmunoassay ; p, inhibition curve of sample being parallel to that of the standard ; np, inhibition curve not parallel to standard curve ; -ve, no cross reaction ; p? parallelism questionable due to incomplete inhibition curve.

* Infrequent parallelism between standard and sample observed.

** Infrequent cross reaction observed.

spermiation bioassay (Fontaine, personnal communication), and salmon gonadotrophin, SG-DEAE-3 which elicited an almost 100 p. 100 response in the goldfish spermiation bioassay at a dose of 0.3 μg/10 mg body weight (Donaldson *et al.*, 1972) were used for radioiodination and as standards.

Ten-20 serial dilutions of a known quantity of pituitary extract were analysed in duplicate so as to obtain inhibition of binding of the label to the antibody over a range of 10-90 p. 100. Plasma samples were assayed at more than 3 serial dilutions.

Results.

A comparison of parallelism of inhibition curves of plasma and pituitary extracts of various fish species with those of the standard hormone in both RIAs are shown in table 1.

In the homologous RIA, the inhibition curves of both pituitary extracts and plasma of all *Oncorhynchus* spp., adults and precocious male parr of the Atlantic salmon, *Salmo salar*, adults of *Salmo trutta*, *Salvelinus alpinus* and wild *Salmo gairdneri* were parallel with that of the standard, SG-DEAE-3. However, samples from hatchery-reared *Salmo gairdneri* gave non-parallel inhibition curves ; similarly non-parallelism of inhibition curves were found with pituitary extracts of the powan, *Coregonus lavaretus*, and the ayu, *Plecoglossus altivelis*, as well as 13 other non-salmonid species, while 3 species showed no cross-reaction. Thus samples of only some salmonids appear to cross-react in a manner identical to that of the standard in the homologous RIA.

In the heterologous RIA, pituitary extracts of all cyprinids and salmonids tested gave inhibition curves that were parallel to that of the standard, a purified carp gonadotrophin (BG2-116 or BG4-240), except for those of *Plecoglossus altivelis* and *Scardinius erythrophthalmus*. Nine other species, including 2 species of cyclostomes, *Petromyzon marinus* and *Lampetra fluviatilis* showed parallel inhibition curves while 4 others showed non-parallel inhibition curves. No cross-reaction was observed with the pituitary extracts of *Perca fluviatilis* and *Scyliorhinus canicula* at the dosage tested. The heterologous RIA therefore appears to be less species-specific than the homologous RIA.

Discussion.

The data indicate that even with the use of two RIA systems employing the same antiserum, the degree of immunological cross-reactivity of samples from various species depends on the nature of the purified hormone used as the label and standard. It is important to realise that these immunological observations are based on the ability of the test samples and standard to inhibit the binding of the labelled hormone on the particular sites on the antibodies. Thus in general, though not without reservation, if identical antigenic determinants are present in both the unknown samples and the standard hormone, the various inhibition curves of test samples and the standard hormone should be parallel whereas if the inhibition curves of the test samples from a particular species are not parallel with that of the standard hormone, then identical antigenic determinants are absent.

In the homologous RIA, the majority of the salmonids tested showed inhibition curves which were parallel to that of the standard hormone. Similar findings have

been reported by Crim *et al.* (1975), who employed another salmonsalmon homologous RIA which was separately developed. Non-parallelism of inhibition curves was, however, not reported by these workers. The non-parallel inhibition curves obtained from pituitary extracts of the hatchery-reared fish though not from the wild-caught, *Salmo gairdneri* emphasizes the need for caution in evaluating the immunological reactivities of similar species derived from different sources. Similar non-parallelism observed in 13 other non-salmonid species further reflects the zoological specificity of this homologous RIA system. Similar observations have been reported by Breton *et al.* (1973) using a carp-carp homologous RIA.

This is the first time that common antigenic determinants have been demonstrated between the pituitary extract of a cyclostome and the gonadotrophin of a teleost. This is particularly interesting since there is no evidence for the presence of thyroid stimulating hormone in the Agnatha (Sage, 1973), though a gonadotrophin has been shown to be present in the pituitary on the lamprey investigated in the present work (Dodd *et al.*, 1960).

The above findings, though based on a limited number of species, do not appear to be consistent with known phylogenetic relationships (Greenwood *et al.*, 1966). It is, however, not surprising that within a group as diverse as the fishes, certain antigenic determinants in the gonadotrophin molecule are common to a number of species.

While parallelism between the inhibition curves of a pituitary extract and the standard hormone is a prerequisite for the estimation of potency by RIA, more rigorous validation procedures must be performed before the RIA is employed for a particular species, these should include an investigation of the possibility that non-gonadotrophic glycoproteins may interfere in the system. This is particularly essential in the heterologous RIA in view of the wide cross-reactivity observed with pituitary extracts of various species. Furthermore, in many of the species tested, there remains a need to determine whether the RIA values correspond to the biological activity of the samples.

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Résumé. Les extraits hypophysaires et les plasmas de 35 espèces de poissons sont testés dans deux systèmes de dosage radioimmunologique (RIA), un système homologue saumon-saumon et un système hétérologue saumon-carpe, dans lequel le même antisérum, contre la gonadotropine de saumon SG-G100, est employé. Dans le RIA homologue, pour la plupart des espèces de salmonidés testées, excepté le Lavaret et le Plecoglossus, la réaction croisée est identique à celle obtenue avec le standard SG-DEA-3. Un non-parallélisme des courbes d'inhibition est observé pour 13 espèces non salmonidés, tandis que 3 autres ne montrent pas de réaction croisée. Dans le RIA hétérologue, tous les cyprinidés excepté le Rotengle et tous les salmonidés, excepté le Plecoglossus, de même que 9 autres espèces, donnent des courbes d'inhibition parallèles à celles du standard, la gonadotropine de carpe purifiée. Ces résultats montrent que les propriétés immunologiques des gonadotropines de poisson sont sans relation avec ce qui l'on sait de leurs relations phylogénétiques.

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Studies on the purification of gonadotrophin from dogfish (*Scyliorhinus canicula* L.) pituitary glands

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Summary. Gonadotrophin (GTH) was purified from the dogfish (*Scyliorhinus canicula*) by first preparing a glycoprotein fraction followed by chromatography on Con A-Sepharose and CM-cellulose. GTH and TSH activities were assayed using chicks and quail. Although CM-cellulose produced two peaks of GTH activity, their biological activities were similar, suggesting that the dogfish produces one GTH which is particularly LH-like.

Introduction.

While it is now widely accepted that two gonadotrophins, resembling mammalian LH and FSH, are produced by the pituitaries of birds, reptiles and amphibians, most work with bony fishes suggests the presence of only one gonadotrophin in this class of vertebrates (e. g. Burzawa-Gérard, 1971 ; Donaldson *et al.*, 1972 ; Burzawa-Gérard *et al.*, 1975 ; Breton *et al.*, 1976). This conclusion is, however, being questioned in recent publications (e. g. Idler *et al.*, 1975 ; Pierce *et al.*, 1976 ; Farmer and Papkoff, 1977). No information exists on the gonadotrophin(s) of the cartilaginous Elasmobranch fishes and the present study presents some results on the purification of this hormone from the lesser spotted dogfish (*Scyliorhinus canicula*).

Materials and methods.

Pituitary glands were collected from fresh, sexually mature dogfish caught in the middle of the breeding season when pituitary gonadotrophin content is at its highest (Sumpter, 1976), and stored at — 20 °C. Two batches of material were processed, each containing *ca.* 1 000 lobes. Both contained approximately equal numbers of each sex. Only the ventral lobes were processed, as this is the main, if not the only, pituitary lobe containing gonadotrophin.

An initial glycoprotein extract (GTN) was prepared essentially according to Stockell Hartree (1975). Affinity chromatography on Con A-Sepharose was then carried out according to Idler *et al.* (1975) except that 1 mM $MnCl_2$ and 1 mM $CaCl_2$ were added to the buffers. Ion-exchange chromatography of the adsorbed gonadotro-

phic fraction (Con A-2) was carried out on CM-cellulose (Whatman CM-32) according to Stockell Hartree (1975).

Gonadotrophic activity was assayed both by estimating the uptake of radioactive phosphorus into the testes of day-old chicks (Follett and Farner, 1966), and by measuring androgen release from isolated quail testicular cells (Maung and Follett 1977). Thyrotrophin was estimated by the uptake of phosphorus into the thyroids of day-old chicks (Scanes and Follett, 1972). Mean potencies and 95 p. 100 confidence limits were calculated by standard bioassay statistics.

Results.

All the gonadotrophic and thyrotrophic activity in the ventral lobe was precipitated in the glycoprotein fraction (GTN). Following chromatography of this GTN on Con A-Sepharose, most emerged unadsorbed (Con A-1), but a small peak of adsorbed material was eluted by a buffer containing glucoside. This second peak (Con A-2) contained the vast majority (*ca.* 90 p. 100) of the gonadotrophin.

The TSH activity was also largely bound to Con A-Sepharose, although there did appear to be some separation from the GTH activity as only 60 p. 100 of the TSH activity recovered was in Con A-2. With high recoveries of gonadotrophin and the elimination of much inactive protein, the increase in potency averaged 17-fold after Con A-Sepharose chromatography.

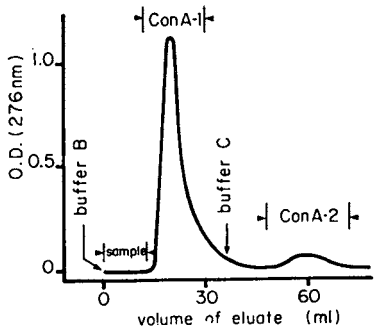

FIG. 1.

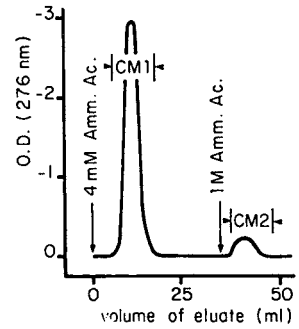

FIG. 2.

FIG. 1. — *Elution profile of* 500 *mg GTN on a Con A-Sepharose column* (1.25 × 15 cm). The flow rate was 10 ml/hr. Fractions were pooled as indicated. Buffer B was 0.05 M Tris-Cl, pH 7.7 ; 0.5 M NaCl ; 0.2 mM dithiothreitol ; 1 mM $MnCl_2$; 1 mM $CaCl_2$. Buffer C contained 0.15 Mα-methyl-D-glucoside in addition.

FIG. 2. — *Elution profile of* 11.8 *mg Con A-2 on a CM-cellulose column* (1.2 × 5 cm). The flow rate was 20 ml/hr. Fractions were pooled as indicated.

To see if Con A-2 contained more than one gonadotrophin, one batch was applied to a column of CM-cellulose. Figure 2 shows the results of this step. About 90 p.100 of the protein was not adsorbed and was eluted in the first fraction (CM1). When the buffer was changed to IM ammonium acetate a second, much smaller, peak emerged (CM2).

The gonadotrophic activity was split equally between CM1 and CM2, with the result that CM2 had a specific activity much greater than CM1 (1.04 and 0.08 × NIH-LH-S19, respectively, as measured by the ^{32}P-chick bioassay). When assayed on the same day in the isolated testicular cell assay, the same solutions gave activities of 1.00 and 0.06 × NIH-LH-S19 respectively. The biological activities of various dogfish gonadotrophic fractions, from the same purification, in both the ^{32}P-chick and isolated testicular cell bioassays, are shown in figure 3. In all cases NIH-LH-S19 was used

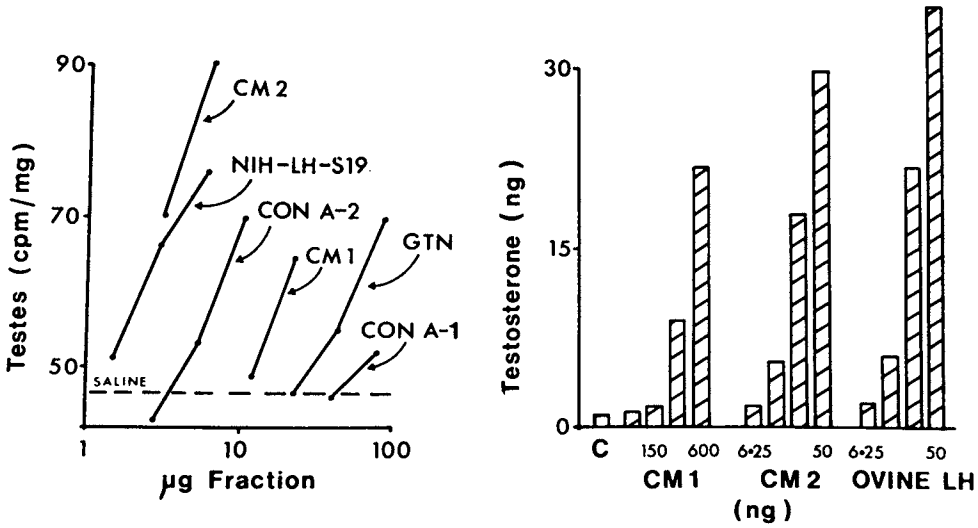

FIG. 3. — *The biological activities of various partially-purified dogfish gonadotrophic fractions in the 32p-chick* (left) *and isolated quail testicular cell* (right) *bioassays.*

TABLE 1

The weights and biological potencies of fractions from one purification
Gonadotrophic activity in μg NIH-LH-S19/mg ; Thyrotrophic activity in μg NIH-TSH-S8/mg ; with 95 p. 100 confidence limits

Fraction	Mg	GTH	TSH
GTN from 990 Ventral Lobes	365.9	29.7 (22.1-40.0)	2.59 (1.91-3.52)
Con A-Sepharose Chromatography			
Con A-1	115.0	40.0 (5.36-103.9)	
Con A-2	13.63	288.1 (186.3-433.6)	
Rechromatography of 80 mg Con A-1			
Con A-1	40.56	16.9 (0.7-29.3)	4.18 (3.13-5.58)
Con A-2	6.85	234.5 (143.6-248.2)	
Combined Con A-2	18.96	275.4 (211.8-358.1)	11.57 (9.07-14.75)
CM-Cellulose Chromatography			
CM1	6.6	80.5 (47.1-94.7)	
		54.5 in testicular cell assay	
CM2	0.70	1 041 (967.5-1 194)	
		1 000 in testicular cell assay	

as the common standard. All fractions gave dose-response curves parallel with the standard.

Table 1 shows that 700 μg of an active gonadotrophin (CM2) were obtained from 1 000 ventral lobes. TSH was purified along with the gonadotrophic activity, although no positive attempt was made to separate the two activities. When a saline extract of 400 ventral lobes was chromatographed on Sephadex G-100 (2.5 × 100 cm) all the biological activity was retarded and emerged at a point similar to that for rat FSH.

Discussion.

Glycoprotein extraction, followed by affinity chromatography on Con A-Sepharose, proved successful in concentrating the GTH and TSH activities while eliminating much protein. All the initial biological activity could be recovered in the Con A-2 fraction if conditions were chosen correctly. The behaviour of dogfish gonadotrophin in ammonium acetate : ethanol mixtures, and on Con A-Sepharose, indicates that it is a glycoprotein.

The results of chromatography on CM-cellulose, a classical method of separating LH from FSH, appear to suggest that the dogfish produces two gonadotrophins. However, bioassays do not support this view. The ^{32}P-uptake method measures both LH and FSH, at least in birds and mammals (Scanes and Follett, 1972), while the testicular cell assay measures only higher vertebrate LH. As the ratio of gonadotrophic activity in CM1 to that in CM2 is similar in both assays, it would appear that there is only one gonadotrophin with an LH-like activity. If a second FSH-like gonadotrophin had been present in CM1, one would have expected the ratio to have varied between the two bioassays. The reason for the existence of two peaks may simply be that CM-cellulose only adsorbs about 50 p. 100 of dogfish GTH. CM-cellulose is often a poor separator of LH and FSH.

Although the results suggest that the dogfish produces an LH-like gonadotrophin, no firm conclusion can be drawn since the intrinsic activities of gonadotrophins may change markedly when tested outside the same class of vertebrates (e. g. Light and Midgley, 1976). Also, a hormone may be potent in a bioassay specific for one of the two gonadotrophins, but be inactive in a different bioassay for that same gonadotrophin. This is illustrated by the *Tilapia* gonadiotrophin (Farmer and Papkoff, 1977), which was potent in a rat Leydig cell assay but not in stimulating ovulation in *Xenopus*. The studies reported here, because they were conducted on a batch of pituitaries from mature fish of both sexes, add nothing to the concept that a sexual difference may exist between male and female gonadotrophin, nor to the possibility that the gonadotrophin may change during ontogeny.

Many of the reports of the purification of fish gonadotrophic hormones have noted that they appear more fragile than mammalian gonadotrophins, and Breton *et al.* (1976) consider that spontaneous dissociation may occur in both trout and carp gonadotrophins. Losses of biological activity in the purified dogfish fractions have also been encountered, although this may be due to aggregation rather than disso-

ciation of the molecules. Storage in solution at — 70 °C, rather than in the dry form at — 20 °C, seems to have alleviated the problem.

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Résumé. Les gonadotrophines (GTH) de la roussette (*Scyliorhinus canicula* L.) ont été purifiées par préparation d'une fraction glycoprotéique séparée par chromatographie sur Con A-Sepharose et CM-cellulose. Les activités GTH et GTSH ont été testées sur poussin et caille. Bien que la chromatographie sur CM-cellulose produise deux pics d'activité gonadotrophique, leurs activités biologiques sont similaires, laissant penser que la roussette produit une gonadotrophine très proche de LH.

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Les cellules gonadotropes chez les salmonidés

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avec la collaboration technique de Jacqueline OLIVEREAU

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Summary. *The gonadotropic cells of* Salmonidae.

Pituitaries of *Salmo gairdneri* and *Salmo fario* were examined at several stages of sexual maturation in winter. Two forms of gonadotropic cells were observed. One, predominating in mature trout, had large granules and was labelled with antisera against HCG and bTSH. The other type had fine granules and reacted poorly with glycoprotein staining. In two 3-year old females with still immature ovaries, the cells of the latter were highly stimulated. Their significance is discussed.

Introduction.

Une seule hormone gonadotrope a été isolée jusqu'ici de l'hypophyse de quelques Téléostéens (Fontaine et Burzawa-Gérard, 1977). Cependant, chez plusieurs espèces, il est possible d'observer 2 formes de cellules gonadotropes (Olivereau, 1976). Le fait que l'une réagisse à l'accroissement de la photopériode et l'autre seule soit affectée par la castration chez *Lepomis*, semblerait indiquer l'existence de 2 catégories distinctes (Simon et Reinboth, 1974) ; de même, elles répondent différemment à l'apport de stéroïdes sexuels ou de gonadotrophines chez *Monopterus albus* (0 et Chan, 1974). La microscopie électronique permet aussi d'observer 2 types apparemment distincts, en particulier chez *Oncorhynchus* (Cook et Van Overbeeke, 1972) et l'Epinoche (Slijkhuis, 1977). Les résultats rapportés ici concernent *Salmo gairdneri* et *Salmo fario*.

Matériel et méthodes.

Salmo gairdneri et *S. fario* âgés de 3 ans, provenant d'une pisciculture, sont conservés dans de grands bacs (eau courante déchlorée, photopériode 12 L : 12D), alimentés avec des granulés pendant 2 mois et sacrifiés en décembre. *S. gairdneri* comprend 2 mâles mûrs (poids 1 060 et 1 230 g ; RGS 3,8 et 4) et 2 femelles (995 et 1 010 g) aux gonades réduites logées dans des masses lipidiques abondantes ; elles sont fixées au Bouin sans pesée. Ces Truites sont comparées à 10 Truites de 400-1 044 g, soit immatures (RGS ♂ 0,05-0,07 ; ♀ 0,26-0,36), soit mûres, et à 6 Truites de 2 à 6,5 kg

proches de la maturité sexuelle (RGS ♂ 2,25-2,8, ♀ 6-9) sacrifiées à la pisciculture en fin novembre et décembre. *S. fario* comprend un mâle (591 g) aux testicules infantiles (RGS 0,1), un mâle mûr (765 g) ayant déjà frayé (RGS 0,65) et une femelle (856 g) ayant partiellement pondu 15 j avant (RGS 6,5) dont l'ovaire contient encore des œufs commençant à dégénérer.

Les hypophyses sont fixées au Bouin-Hollande sublimé, coupées à 4 µm et colorées avec le tétrachrome d'Herlant, le PAS, le bleu alcian (BA), la fuchsine paraldéhyde (AF) et l'hématoxyline au plomb (HPb).

Résultats.

1° *Gonades.* — La gonade du mâle de *S. fario* immature contient des spermatogonies et quelques spermatocytes primaires ; l'activité mitotique est réduite. Les petits ovaires (12 et 15 mm de longueur, 2 mm de largeur) des 2 femelles contiennent quelques travées d'ovogonies et, par coupe longitudinale, 8-10 ovocytes (diamètre 100-150 µm) parfois altérés et 1 ou 2 ovocytes de 500 µm atteignant exceptionnellement le stade II ou III ; la masse principale est composée de kystes d'ovocytes au stade I avec de rares mitoses ; le cytoplasme est dépourvu de basophilie. Dans le noyau au caryoplasme clair, les chromosomes se répartissent à un pôle et le nucléole périphérique à l'autre pôle, structure similaire à celle décrite chez la Perche (Dimovska, 1976).

2° *Hypophyse.* — Aucune différence anatomique n'est décelable entre les hypophyses de ces deux espèces, mais chez *S. fario*, les cordons glandulaires sont plus souvent bordés par une couche homogène de cellules somatotropes (STH).

a) *Mâles et femelles immatures* : dans les 2 espèces, les cellules gonadotropes bien granulées font défaut ; les rares petites cellules situées au centre des cordons de cellules STH sont pauvres en glycoprotéines (GP) et dépourvues d'activité mitotique.

b) *Mâles mûrs* : la pars distalis proximale (PDP) contient de nombreuses cellules gonadotropes bien granulées, surtout situées au centre des travées glandulaires

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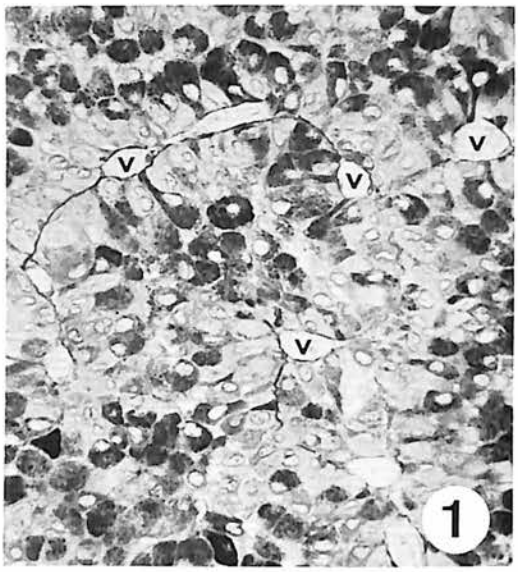
FIG. 1 et 2. — *S. gairdneri, mâle mûr.* Les cellules gonadotropes à gros granules riches en GP apparaissent en noir ; le pédicule cellulaire se termine sur les vaisseaux (v) ou le tissu nerveux et peut entourer une cellule gonadotrope non granulée (flèche). Figure 1 : tétrachrome × 370 ; figure 2 : BA-PAS × 925.

FIG. 3. — *S. fario, femelle après ponte partielle.* Bordure de cellules STH (s) entourant des cellules gonadotropes au cytoplasme peu granulé ou vacuolisé. Tétrachrome × 370.

FIG. 4, 5 et 6. — *S. gairdneri, femelle de 3 ans avec ovaires infantiles.* Hypertrophie cellulaire, nucléaire et nucléolaire. Figure 4, la vacuolisation cytoplasmique aboutit à la formation d'une cavité avec granules orangeophiles (tête de flèche), les cellules STH apparaissent en foncé. Neurohypophyse antérieure (n). Tétrachrome × 370. Figure 5, parmi les cellules gonadotropes non réactives, pauvres en GP, les cellules TSH riches en GP se détachent en foncé, les cellules à prolactine (p) en gris moyen. BA-PAS-Orange G × 370. Figure 6, Activité mitotique (m). Tétrachrome × 925.

FIG. 7. — *S. gairdneri, mâle mûr.* Marquage des cellules gonadotropes avec un IS anti-HCG et peroxydase. Infiltration dans la PDR et la paroi des follicules (f) de cellules à prolactine × 370.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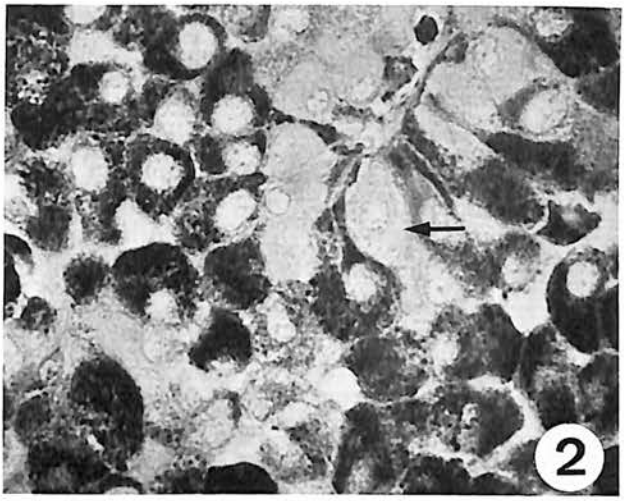
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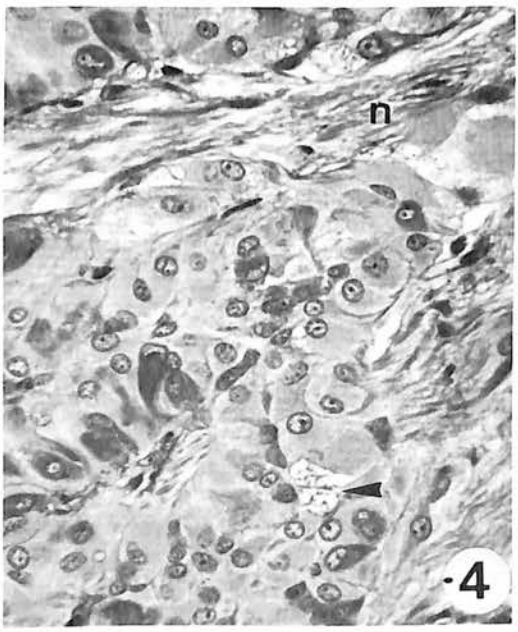
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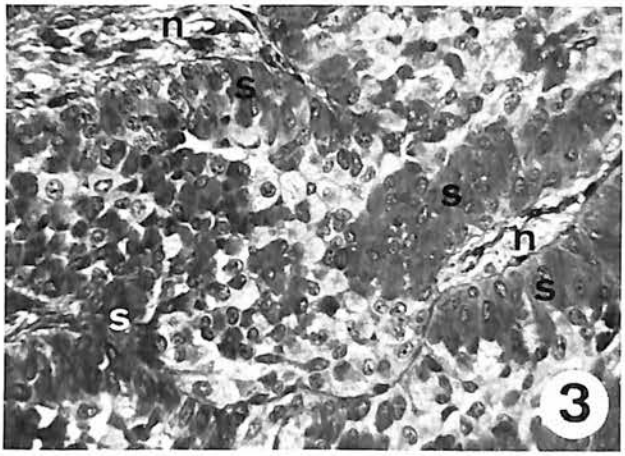
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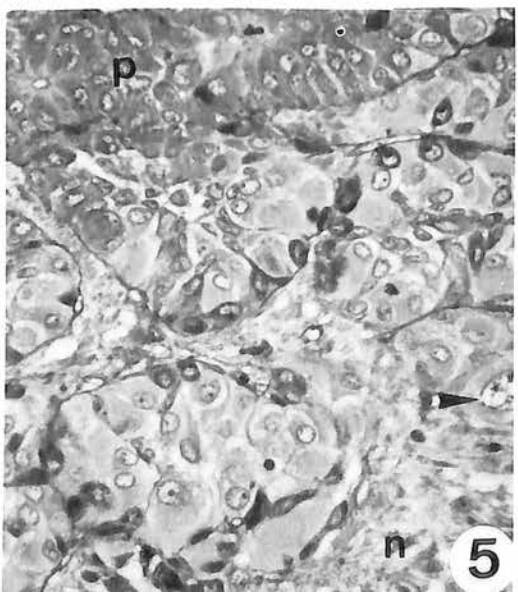
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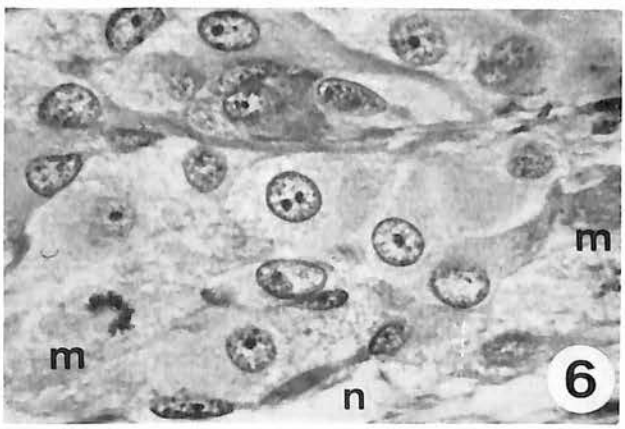
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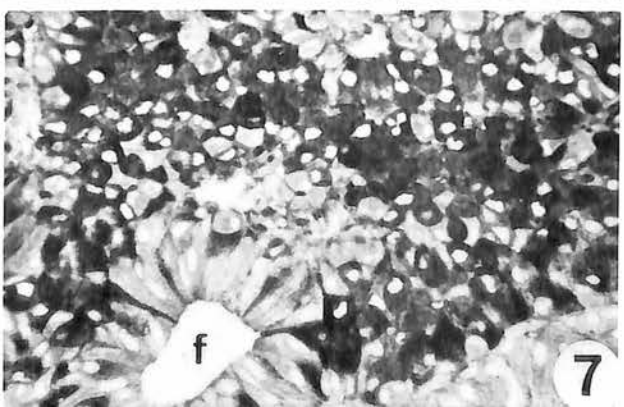
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(fig. 1). Leurs granulations denses, fortement colorées avec le BA, l'AF, le PAS, s'accumulent uniformément autour du noyau et dans le pédicule se terminant sur les espaces périvasculaires (fig. 2). Elles envahissent plus ou moins la pars distalis rostrale (PDR) formant des amas entre les follicules prolactiniques ou s'infiltrant dans leur paroi. Elles diffèrent des cellules thyréotropes (TSH) finement granulées. Elles s'observent plus rarement à la périphérie de la pars intermedia (PI).

D'autres cellules aux contours plus arrondis, aux granules peu discernables, sont situées en bordure des cordons surtout centraux ; elles pénètrent peu dans la PDR et pas dans la PI. Leur cytoplasme est parfois vacuolisé à l'apex, son affinité envers le BA, l'AF et le PAS est très réduite ou nulle. Même si la vacuolisation progresse avec accumulation de granules orangeophiles sur les parois du réseau vacuolaire, le noyau ne dégénère pas.

c) *Femelles mûres* : chez les femelles très proches de la maturité sexuelle, les 2 formes de cellules gonadotropes s'observent, la forme granulée riche en GP prédomine ; l'autre forme n'est pas vacuolisée et présente une activité mitotique occasionnelle, surtout dans la zone céphalique et centrale de l'hypophyse.

Chez les femelles mûres, les cellules de la 2e forme se vacuolisent plus nettement. Chez la femelle ayant pondu partiellement au laboratoire, les cellules à gros granules sont moins abondantes, beaucoup plus petites et en majeure partie dégranulées ; l'autre forme est aussi de taille réduite, souvent vacuolisée avec des granulations orangeophiles sur les trames du réseau vacuolaire (fig. 3). La teneur de l'hypophyse en GP est abaissée.

d) *Femelles de 3 ans sans indice de vitellogenèse* : les cellules gonadotropes fortement granulées font défaut. Les cordons glandulaires, surtout centraux, renferment des cellules très hypertrophiées atteignant 15-20 µm sur 30-40 µm, avec un nucléole et un noyau très volumineux (fig. 4, 5 et 6), et une faible activité mitotique. Le cytoplasme est très peu ou pas coloré avec le BA, l'AF ou le PAS ; la vacuolisation apicale est parfois marquée, mais les granulations orangeophiles restent rares. Situées en bordure des travées, elles offrent un large contact avec les espaces périvasculaires ou le tissu nerveux. A proximité de la PDR, les cellules TSH plus anguleuses, riches en GP, se distinguent aisément des cellules hypertrophiées.

3° *Essai de marquage avec un immunsérum (IS) anti-HCG et anti-TSH bovine.* — Les IS anti-HCG et anti-TSHb (inédit avec C. Bugnon) marquent fortement les cellules gonadotropes riches en GP de la PDP et la PDR (fig. 7) ; aucune réaction n'est décelée dans les cellules hypertrophiées des 2 femelles. Mais les différences de structure entre les gonadotrophines de Téléostéens et HCG ou TSHb ne permettent d'accorder qu'une valeur réduite à ce marquage qui révèle peut-être une sous-unité commune ou une séquence immunoréactive commune. L'anti-β LH ovine testée ne marque aucune cellule dans l'hypophyse de ces Salmonidés et d'*Oncorhynchus*.

Discussion.

L'hypophyse de ces 2 Salmonidés contient donc à 2 stades différents du développement sexuel 2 formes de cellules gonadotropes dont la taille, celle de leurs granula-

tions et les caractères histochimiques sont différents ; des formes intermédiaires typiques ne sont pas évidentes.

La présence d'ovaires infantiles chez des femelles de 3 ans pose un problème : est-elle liée à l'absence d'une hormone gonadotrope élaborée par les cellules riches en GP, seules marquées par les IS utilisés ? Ou bien ces gonades sont-elles réfractaires à la stimulation gonadotrope qui semble pourtant considérable d'après la cytologie hypophysaire ? L'absence de rétroaction des stéroïdes sexuels entretient-elle l'hyperactivité des cellules gonadotropes ? L'absence de marquage par les IS testés des cellules pauvres en GP résulte-t-elle simplement de l'absence de stockage de granulations ou traduit-elle une structure immuno-réactive différente ? Ces cellules sont-elles bien gonadotropes ?

Chez la Truite, se basant sur des expériences de hiérarchie sociale en fonction de l'activité interrénalienne, Boddingius (1975) décrit des cellules similaires, pauvres en GP, considérées comme corticotropes. Cette hypothèse ne peut être retenue : nous avons vérifié qu'un IS anti-$^{1-24}$ ACTH ou anti-$^{17-39}$ ACTH marque exclusivement les cellules HPb + de la PDR et la PI de ces mêmes Truites (Olivereau *et al.*, 1976).

Chez *Oncorhynchus kisutch* à la thyroïde hyperplasiée, la PDP contient de volumineuses cellules interprétées comme TSH (Sonstegard et Leatherland, 1976). Or chez les Salmonidés radiothyroïdectomisés, les cellules TSH surtout localisées au voisinage de la PDR n'envahissent pas toute la PDP (Olivereau, 1972). L'examen des thyroïdes de nos 2 Truites femelles révèle un épithélium très plat, une colloïde dense sans vacuolisation témoignant d'une faible activité. Le contraste entre cellules TSH et gonadotropes est d'ailleurs évident (fig. 5). Seule une fonction gonadotrope peut être attribuée à cette catégorie cellulaire pauvre en GP.

Cette hypothèse est étayée par les observations (inédites avec R. Billard et B. Breton) faites sur des Truites castrées avec ou sans régénération de la gonade, l'animal totalement castré comportant de nombreuses cellules gonadotropes de ce type évoluant vers l'aspect en chaton de bague.

La localisation périphérique des cellules gonadotropes très actives peut-elle s'expliquer par une stimulation plus efficace d'origine hypothalamique ? Nous ne savons pas si chez les Salmonidés un facteur de type LHRH atteint l'hypophyse par voie strictement nerveuse ou neuro-vasculaire, avec une action préférentielle sur les cellules les plus externes des massifs glandulaires. L'autre forme riche en GP présente un contact plus restreint avec le tissu neuro-hypophysaire. Comme chez le Saumon Atlantique ou Pacifique, elle prédomine largement lors de la reproduction et envahit alors la PDR.

Une étude ultrastructurale et le marquage avec des IS plus spécifiques aideraient à préciser s'il s'agit d'une même cellule à 2 stades fonctionnels très différents, l'une n'élaborant peut-être qu'une sous-unité, ou bien de 2 catégories distinctes d'éléments gonadotropes.

Conclusion.

Salmo gairdneri et *S. fario* possèdent 2 formes de cellules gonadotropes : la forme à gros granules, riche en GP, abondante chez les Truites mûres, est marquée par les IS

anti-HCG et anti-TSH bovine ; la forme à fins granules, pauvre en GP, non marquée par ces IS, est fortement stimulée chez 2 femelles de 3 ans aux ovaires restés infantiles ; sa signification fonctionnelle est discutée.

Symposium sur la Reproduction des Poissons Paimpont, France, 19-21 septembre 1977.

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The distribution and nature of gonadotropic cells in the rostral pars distalis of the Atlantic salmon, *Salmo salar*

par B. EKENGREN, J. PEUTE *, G. FRIDBERG

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Summary. Several techniques were applied to indentify the gonadotropic (GTH) cells in the rostral pars distalis (RPD) of the pituitary in the Atlantic salmon in phases around spawning. The double immuno method with rabbit anti-carp GTH as the first antibody revealed many cells in the RPD with a strong fluorescence. On consecutive sections stained with Herlant's tetrachrome these cells exhibited basophilic reactions. They are distributed all over the RPD, i.e., in small clusters at the periphery, as islands between the follicles. The cells coincide in fine structure with the « globular » cells demonstrated as GTH cells in teleosts. They contain secretory granules, dense core vesicles and globular inclusions but show great variations in size, morphology and fine structure. Only one GTH cell type could be identified in the RPD.

Introduction.

The gonadotropic (GTH) cells of teleost fishes are generally distributed in the proximal pars distalis. In the sockeye salmon the GTH cells also occur in the rostral pars distalis (RPD) as demonstrated by Cook and van Overbeeke (1972) and Nagahama (1973). In the Atlantic salmon, on the contrary, the GTH cells seem to be scarcer in this area of the pituitary (Olivereau, 1976). We have applied cytoimmunological, histocytological and electronmicroscopical methods to study the nature and the distribution of the GTH cells in the RPD of the Atlantic salmon in phases around spawning. In this species two GTH cell types have been described by Olivereau (1976) and this fact has been considered in view of the present discussion regarding the occurrence of one or two gonadotropins in teleost fishes (Schreibman *et al.*, 1973 ; Holmes and Ball, 1974 ; Doerr-Schott 1976).

Material and methods.

Specimens of both sexes of the Atlantic salmon were caught after migration into the Dalälven river. The fishes, belonging to a Baltic population, were killed 2 weeks before spawning, during spawning and 2 weeks later.

Electronmicroscopical procedures. — The pituitaries were fixed by immersion in 2 p. 100 OsO_4 in 0.15 M veronal-acetate buffer with 5 p. 100 sucrose, pH 7.2, for one hour at 0 °C. They were rinsed in the same buffer at 4 °C, and embedded in Epon 812. Semithin sections were stained with 1 p. 100 toluidin blue in a 1 p. 100 borax solution. A Philips 301 EM electron microscope was used.

Cytoimmunochemical procedures. — The pituitaries of four salmon were cut in half along the medial plane. One-half was fixed for electron microscopy ; the other half was fixed for light microscopy with Bouin-Hollande sublimate in phosphate-buffered saline (PBS) 0.01 M, pH 7.6, for 24 hrs. After rinsing in PBS 2 days the tissues were dehydrated over ethanol and embedded in paraplast. Sagittal sections of 5 μm were mounted on gelatin-coated glasses (consecutive sections on different glasses). Some sections were stained with Herlant's tetrachrome or azan. On parallel series the double immunofluorescence technique was applied using rabbit anti-carp GTH at a 1 : 20 concentration. Normal rabbit serum and PBS were used as controls. The first antiserum was treated with a homogenate of fresh salmon liver. The second antibody was swine anti-rabbit globulin labeled with FITC (Nordic, Bie and Berntsen, LTD, Denmark) used at a 1 : 100 concentration. After the second immunoreaction the sections were stained with Evan's blue (5 min.). The sections were mounted with buffered glycerin, pH 8.2, after rinsing in distilled water. A Zeiss microscope equipped with a HBO 200 UV lamp was used for the analysis.

Results.

The rabbit anti-carp GTH with swine anti-rabbit FITC as the second antibody, i.e., the double immuno method, resulted in a strong fluorescence in cells distributed all over the RPD (fig. 1). The cells occur in clusters close to the pituitary capsule or as islands between the follicles. Rather frequently fluorecent cells are also seen in the follicles. Basal to the follicles and at the border to the PPD the fluorescent cells are big and rounded. On consecutive sections stained with Herlant's tetrachrome the cells have a faint blue reaction.

Semithin sections stained with toluidin blue show that the fluorescent cells correspond to cells in different stages of vacuolization having blue cytoplasmic granules and droplets of varying sizes. Big rounded cells close to the PPD seem to be of different types. One cell type has vacuoles and inclusions as described and the other, never vacuolated, has small blue granules scattered all over the cytoplasm.

The fine structure of the cells which correspond to the fluorescent cells are the « globular » cells. Their significant features are globules, secretory granules and

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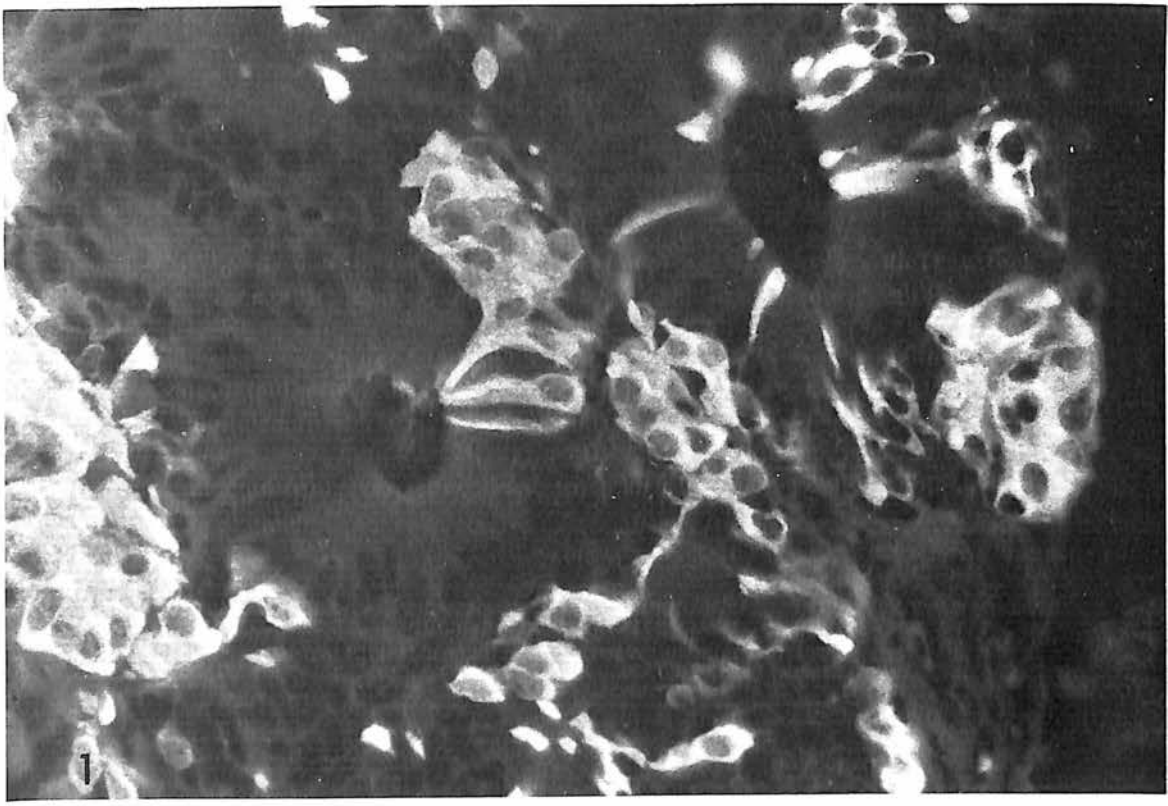
FIG. 1. — *An immunofluorescent section showing the distribution of GTH cells in the RPD.* × 500.

FIG. 2. — *GTH cells of the « globular » type in the RPD.* × 15 000.

FIG. 3. — *Apical part of a « globular » cell* (G) *in a follicle* (FL, follicular lumen). × 9 500.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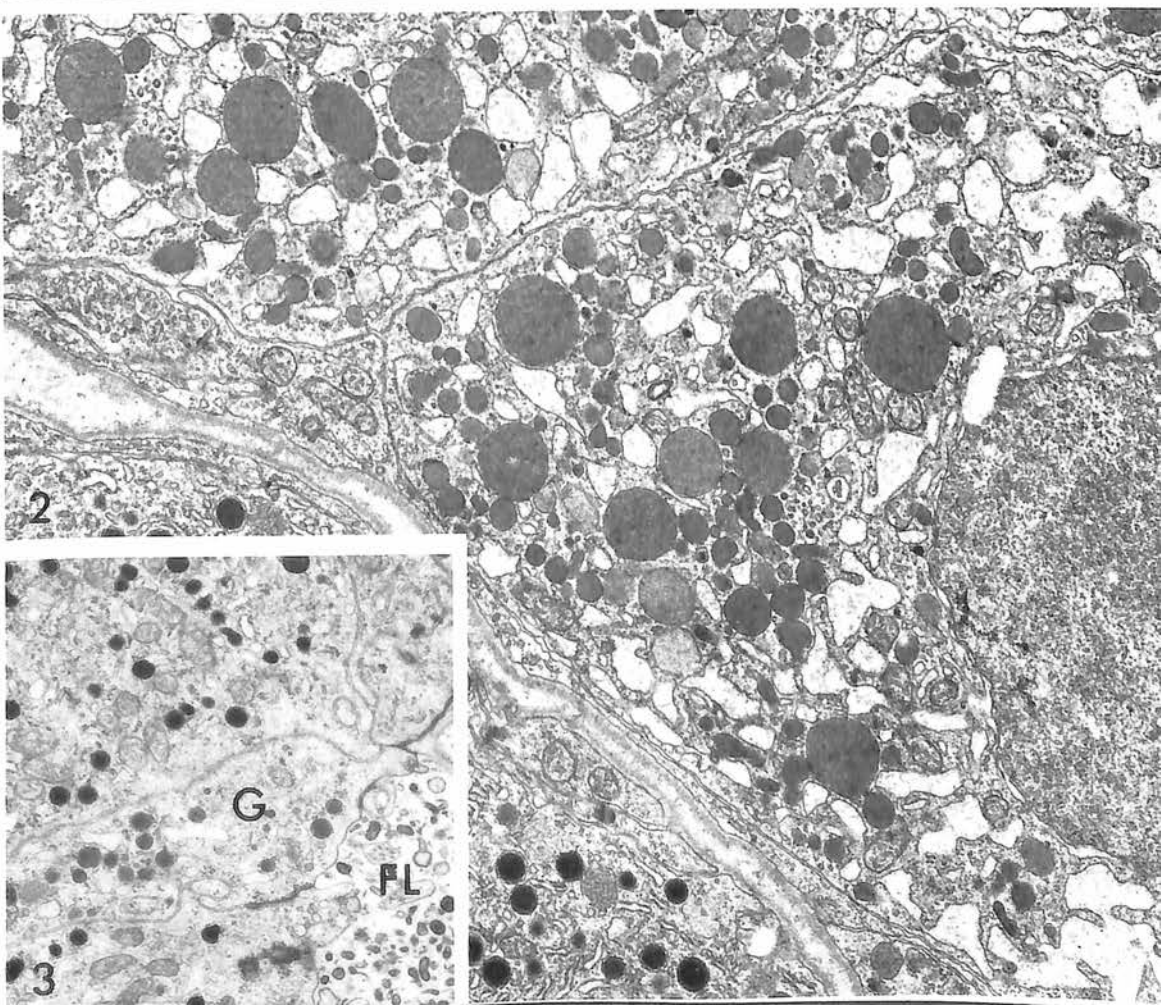
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dense core vesicles. The cisterns of the granulated reticulum is more or less dilated into a tubular system (fig. 2). This cell type could also, in accordance with the immunofluorescent results, be found in the follicles, where its apical part has a cilium similar to prolactin cells (fig. 3). The « globular » cell type is subject to considerable variations in size, morphology and fine structure. The cells may be small, just differentiated ; they are big and rounded when lying close to the basement membrane, and irregularly shaped when located in the endocrine parenchyma or elongated in the follicles. The « globular » cells show different activity levels around spawning, depending on the phase of the fish. In spawning fishes, however, there is a significant number of cells in which the cisterns have formed vacuoles that dominate the cells. This is combined with a depletion of the globules and granules.

The second cell type described as big and rounded on semithin section could not be established as fluorescent. It is never vacuolated and its electron-dense granules seem to be transformed into a translucent form (fig. 4). Occasionally this cell type can also be found as a component of the follicles (fig. 5). The cell shows no significant structural changes through the different phases of spawning, and in the same animal it may exhibit different levels of activity (fig. 4).

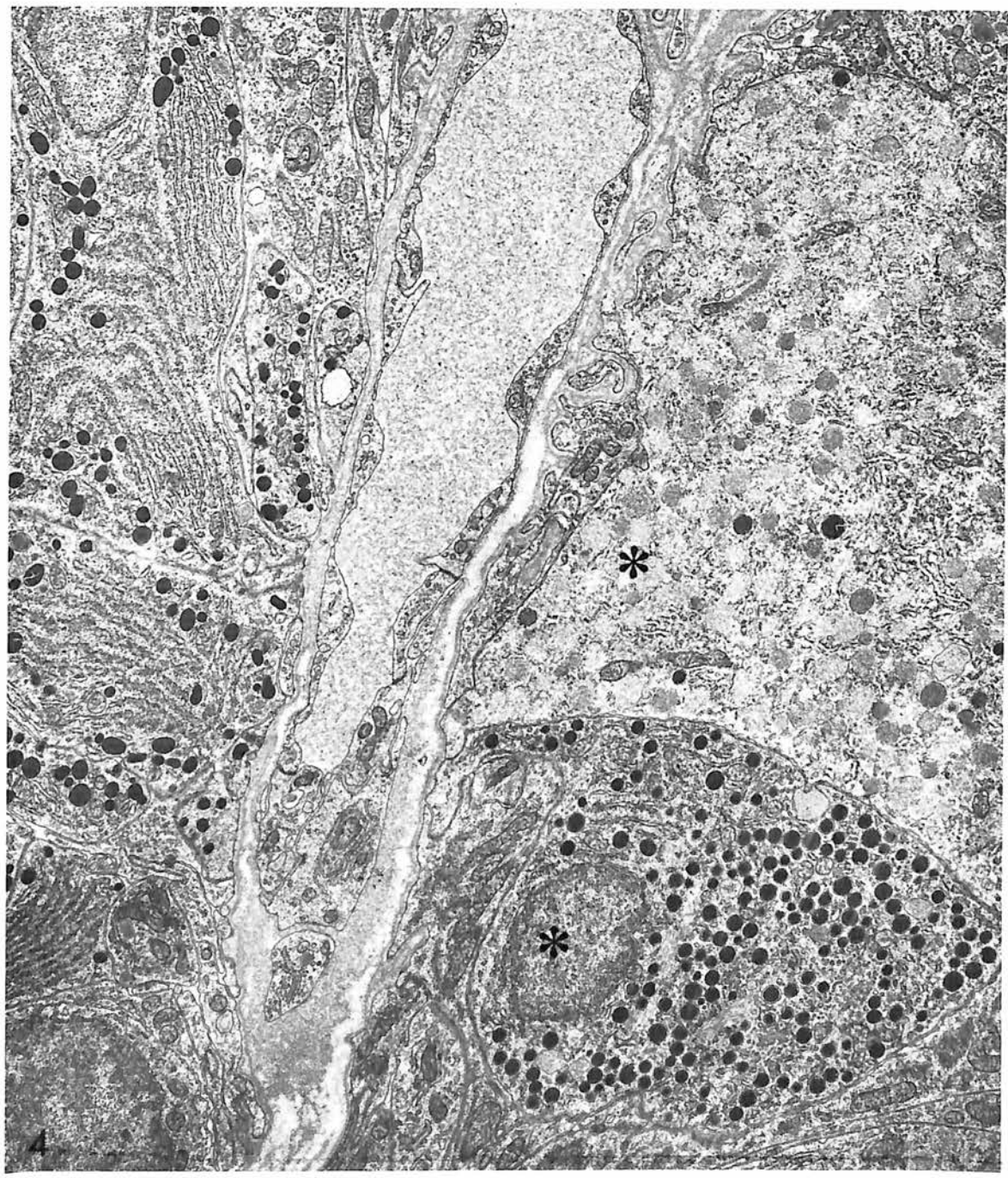
Discussion.

In the Atlantic salmon the rabbit anti-carp GTH method revealed a distribution of the GTH cells in the RPD which corresponds to that described with electron microscopy for the genus *Oncorhynchus* (Cook and van Overbeeke, 1972 ; Nagahama, 1973) and *Salmo irideus* (Follenius, 1963). It was concluded that « globular »-type GTH cells found in the Atlantic salmon are the only GTH cell type in *Oncorhynchus* (Nagahama, 1973) and in the rainbow trout (Follenius, 1963). The size as well as the morphology of the GTH cells varies within such limits that a general picture cannot be given. Their fine structure indicates different levels of activity of the cells in each specimen investigated. The presence of transitional cell forms, from those containing numerous globules and secretory granules to vesiculated and vacuolated cells depleted of secretion, indicates the occurrence of only one GTH cell type in the RPD of the salmon. In spawning fishes the vacuolated cells are numerous.

Another cell type which is never vacuolated may mimic the big rounded globular cells or occur in the follicles. It could not be established if this cell type cross-reacts with the antiserum used, but its fine structure shows no significant changes that imply its relation to spawning. Furthermore, it diverges from all known cell types of the adenohypophysis.

FIG. 4. — *Two cells (*) of unknown significance in the RPD mimicking the big « globular » cells in their gross morphology.* Note the differences in fine structure between the cells, implying different functional phases. × 11 000.

FIG. 5. — *A cell of the same type as in fig. 4 in a follicle* (FL, follicular lumen). × 7 000.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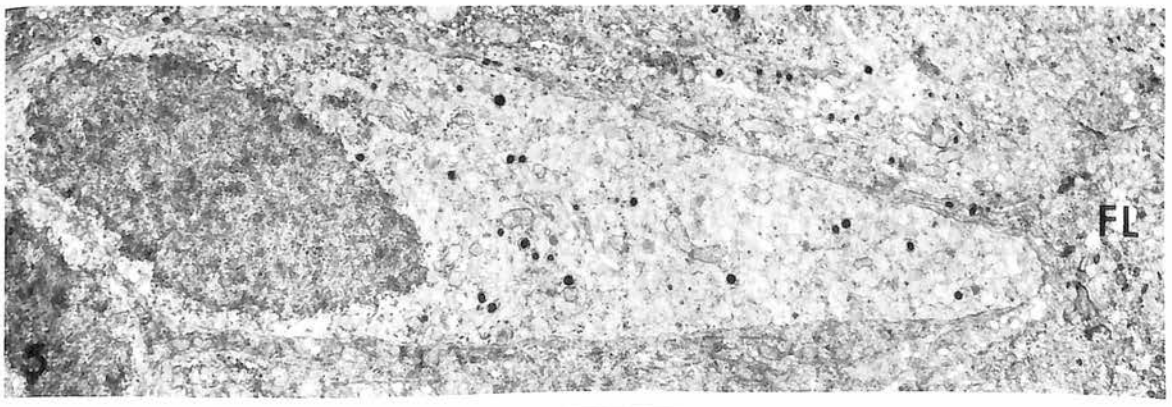
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Conclusions.

GTH cells are distributed all over the RPD and cross-react with rabbit anti-carp GTH. The cells vary greatly in size, morphology and fine structure. Only one GTH cell type (the « globular » type) could be identified.

Symposium sur la Reproduction des Poissons Paimpont, France, 19-21 septembre 1977.

Résumé. Plusieurs techniques ont été utilisées pour identifier les cellules gonadotropes (GTH) dans la pars distalis rostrale (RPD) de la pituitaire chez le saumon de l'Atlantique en période de fraie. La technique immunologique des doubles anti-corps, avec l'anti-corps de lapin anti-GTH de carpe a permis la mise en évidence de nombreuses cellules fortement fluorescentes dans la RPD. Sur coupes sériées colorées au tétrachrome d'Herlant les cellules présentent une réaction faible bleue. Elles sont distribuées dans toute la RPD, au sein des follicules et également à la périphérie et en petits massifs formant des îlots entre les follicules. Elles ont la même ultrastructure que les cellules « globulaires » démontrées comme étant cellules à GTH chez les Téléostéens. Elles contiennent des grcnules de sécrétion, des « dense core vesicles » et des inclusions globulaires mais sont très variables quant à leur taille, leur morphologie et leur ultrastructure. Seul un type de cellules à GTH a pu être identifié dans la RPD.

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Effet d'hormones gonadotropes, *in vitro*, sur la concentration de l'adénosine monophosphate cyclique dans l'ovaire de l'anguille (*Anguilla anguilla* L.)

par Elizabeth FONTAINE-BERTRAND, C. SALMON, Y. A. FONTAINE

avec la collaboration technique de Nadine DELERUE-LE-BELLEC et J. MARCHELIDON.

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Summary. *Cyclic adenosine monophosphate content in the ovary of the eel* (Anguilla anguilla *L.*). *Effect of gonadotropic hormones* in vitro.

Freshwater european eels never reach sexual maturity naturally but injecting carp gonadotropin (c-GTH) induced sexual development. *In vitro*, the incubation of pieces of silver eel ovary with c-GTH increased c-AMP levels. The size of the increase depended both on incubation time and hormone concentration (from 0.01 to 10 μg/ml) and the response could be used as a gonadotropin bioassay. Relative to c-GTH, the GTH from another Teleost, the indian catfish had the same potency in increasing c-AMP concentration in the eel ovary as in inducing spermiation in the frog. On the other hand, the two assays gave very different results in the case of the GTH isolated from a Chondrostean, the sturgeon. The ovaries from c-GTH-treated silver eels, which had undergone some development, showed a smaller *in vitro* response to gonadotropin than did ovaries from untreated eels. Yellow eel ovaries, which presented few macroscopical changes after *in vivo* treatment with c-GTH, were as sensitive to gonadotropin *in vitro* as silver eel ovaries.

Introduction.

Les ovaires de l'anguille européenne (*Anguilla anguilla* L.) restent infantiles pendant que ce poisson est dans nos cours d'eau et n'atteignent leur complet développement qu'au cours de la migration vers la mer des Sargasses. Malgré une notable augmentation du rapport gonadosomatique (RGS) lors de la transformation des anguilles

Abréviations. — AC : adénylase cyclase ; AMPc : 3', 5' adénosine monophosphate cyclique ; EDTA : acide éthylène diamine tétraacétique ; aci-GTH : gonadotropine d'esturgeon ; c-GTH : gonadotropine de carpe ; het-GTH : gonadotropine de poisson-chat indien ; o-LH : lutropine ovine ; o-FSH : follitropine ovine ; RGS : rapport gonadosomatique.

jaunes sédentaires en anguilles argentées migratrices, les ovocytes de ces dernières ne contiennent pas encore de vitellus. La gonadotropine de carpe (c-GTH) injectée aux anguilles argentées induit un développement des ovaires plus ou moins important selon la durée du traitement (Fontaine *et al.*, 1976).

L'action des gonadotropines, chez les poissons comme chez les mammifères, implique probablement une modification du métabolisme du 3'5' adénosine monophosphate cyclique (AMPc). En effet, la c-GTH stimule l'adénylate cyclase (AC) dans des homogénats d'ovaire de cyprin (*Carassius auratus*) (Fontaine *et al.*, 1970) et des extraits d'hypophyse de saumon (*Oncorhyncus keta*) augmentent la concentration de l'AMPc dans des fragments d'ovaire de truite (*Salmo gairdneri*), *in vitro* (Idler *et al.*, 1975). Dans le cas de l'anguille, le premier type d'expériences (AC) ayant donné des résultats négatifs (Fontaine *et al.*, 1976) nous avons utilisé la seconde approche. Une action de la c-GTH a été effectivement observée, *in vitro*, sur la concentration de l'AMPc dans l'ovaire d'anguille et divers caractères en ont été précisés.

De plus, nous rapporterons quelques résultats préliminaires concernant l'influence de l'état physiologique sur l'intensité de la réponse ovarienne à l'hormone, *in vitro*.

Matériel et méthodes.

Animaux. — Les anguilles, pesant de 130 à 330 g, proviennent d'étangs de Péronne (Somme) et sont gardées au laboratoire dans des bacs d'eau courante, à l'extérieur.

Hormones. — Nous avons utilisé les gonadotropines de carpe, *Cyprinus carpio*, (Burzawa-Gérard, 1974), de poisson-chat indien, *Heteropneustes fossilis*, het-GTH (Burzawa-Gérard et Sundararaj, en préparation) et d'esturgeon, *Acipenser stellatus*, aci-GTH (Burzawa-Gérard *et al.*, 1975*a*) ainsi que la o-LH et la o-FSH purifiées (Jutisz, CNRS). L'activité de la préparation de LH (M3) est 1,9 × NIH-LH S3 et celle de la préparation de FSH (P 28a) est 8,2 × NIH-FSH S3.

La poudre acétonique d'hypophyse de carpe a été fournie par les Stoller Fisheries (Spirit Lake, Iowa, USA).

Prélèvement et incubation. — Les anguilles sont sacrifiées par décapitation. Les ovaires sont prélevés en totalité et pesés pour le calcul du RGS : poids de l'ovaire (g) × 100/poids du corps (g). Des lots de 100 à 160 mg de tissu sont préparés, en plusieurs fragments pris dans la partie médiane, sauf dans le cas des anguilles jaunes où il a fallu utiliser la presque totalité de l'ovaire, leur RGS étant plus faible. Ils sont préincubés (20 mn, à 20 °C, sous carbogène 95 p. 100 O_2-5 p. 100 CO_2) dans 4 ml de solution de Krebs-Henseleit bicarbonate (1/2 en Ca) pH 7,4 contenant 1 mg/ml d'albumine, 1 mg/ml de glucose et de la théophylline 20 mM. L'incubation est faite dans 4 ml de tampon frais contenant éventuellement l'hormone (20 mn, à 20 °C, sous carbogène). Chaque essai est réalisé en double.

Extraction et dosage de l'AMPc. — A la fin de l'incubation, les fragments d'ovaire sont plongés dans 0,5 ml de tampon Tris-HCl 50 mM-EDTA 4 mM, pH 7,5, à 100 °C pendant 3 mn. L'AMPc de chaque échantillon est extrait de la manière suivante : homogénéisation au Potter à moteur dans les 0,5 ml de Tris-EDTA à 0 °C, suivie de

deux rinçages de l'appareil (2 × 0,25 ml) ; centrifugation à 3 500 g environ, 0 °C, 10 mn ; prélèvement de la couche intermédiaire entre le culot et les graisses qui surnagent ; puis rinçage du culot par 0,4 ml de Tris-EDTA, centrifugation et prélèvement de la couche intermédiaire qui est ajoutée à la première. Le volume total d'extrait (V) est mesuré et, après une nouvelle centrifugation, on prélève une fraction limpide dont des aliquots de 50 µl sont utilisés pour le dosage de l'AMPc. L'AMPc total présent dans l'échantillon est calculé connaissant V. L'AMPc est dosé par compétition entre le nucléotide stable inconnu et une quantité donnée d'AMPc tritié pour une protéine spécifique (Gilman, 1970 ; Tovey *et al.*, 1974). Les réactifs sont fournis par Amersham. Chaque extrait est dosé en double ; la moyenne des deux déterminations constitue le paramètre utilisé.

Variabilité et analyse statistique. — Le coefficient moyen de variation entre les deux déterminations d'AMPc sur le même échantillon de tissu est de 7 p. 100. Le coefficient moyen de variation entre les concentrations d'AMPc dans deux échantillons équivalents d'ovaire est de 10 p. 100.

Les activités de diverses préparations hormonales ont été comparées par analyse de covariance.

Résultats.

La gonadotropine de carpe augmente considérablement la concentration de l'AMPc dans des fragments d'ovaire. L'intensité de cet effet est indépendante du poids et du RGS de l'animal (tabl. 1).

TABLEAU 1

Mise en évidence de l'effet de la c-GTH sur la concentration de l'AMPc dans l'ovaire d'anguille argentée

Poids du corps (g)	RGS	AMPc * (pmoles/mg d'ovaire)	
		sans c-GTH	c-GTH (10 µg/ml)
127	1,65	0,12	2,59
160	1,40	0,16	2,08
216	0,98	0,14	2,84
228	1,84	0,11	1,76
290	1,67	0,09	2,77
320	2,16	0,20	2,18
334	1,75	0,17	4,05

* Chaque valeur est la moyenne de déterminations sur deux échantillons de tissus.

Pendant l'incubation (entre 0 et 20 mn), la concentration de l'AMPc des fragments de tissu ne varie pas notablement en absence d'hormone. Par contre, en présence de

c-GTH, le paramètre mesuré croît rapidement pendant 10 mn, puis l'accumulation du nucléotide cyclique est beaucoup plus lente (fig. 1).

Le taux d'AMPc est déjà doublé par 0,01 μg/ml de c-GTH et semble augmenter proportionnellement au logarithme de la concentration hormonale dans l'intervalle considéré (0,01-10 μg/ml) (fig. 2).

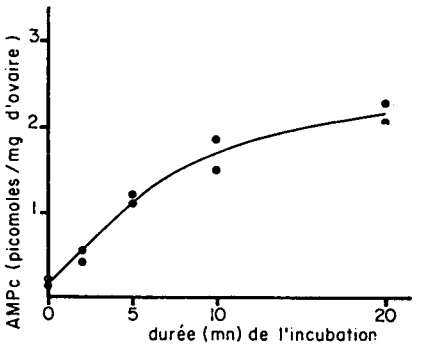

FIG. 1.

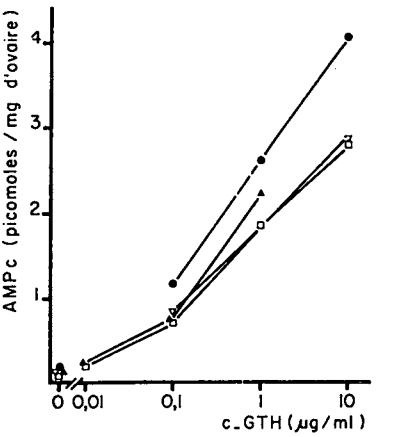

FIG. 2.

FIG. 1. — *Cinétique de l'augmentation de la concentration de l'AMPc dans l'ovaire d'anguille argentée sous l'influence de la c-GTH*, in vitro. L'anguille pesait 320 g et son RGS était égal à 2,16. La concentration de l'AMPc était 0,16 pmole/mg d'ovaire au temps 0 et 0,20 pmole/mg d'ovaire après une incubation de 20 mn sans hormone. La concentration hormonale utilisée était 10 μg/ml.

FIG. 2. — *Effet de la concentration de la c-GTH sur la concentration de l'AMPc dans l'ovaire d'anguille argentée.* Les animaux pesaient = □ : 290 g ; ● : 334 g ; ▽ : 216 g ; ▲ : 222 g. Les RGS étaient égaux à : □ : 167 ; ● : 1,75 ; ▽ : 0,98 ; ▲ : 1,81. Chaque point représente la moyenne des valeurs obtenues avec deux échantillons de tissu.

Deux autres gonadotropines de poissons se sont révélées actives dans notre système : celles du poisson-chat indien, het-GTH, et de l'esturgeon, aci-GTH. L'activité de la het-GTH est très voisine de celle de la c-GTH et les pentes correspondant aux deux régressions ne sont pas significativement différentes ($p < 0,05$). Le rapport d'activité, het-GTH/c-GTH est égal à 0,73 (limites pour $p = 0,05$: 0,35 et 1,52) et n'est pas significativement différent de la valeur mesurée par le test de spermiation de la grenouille, *Rana esculenta* (0,31 : limites : 0,18-0,56) (Burzawa-Gérard et Sundararaj, en préparation). Dans le cas de l'aci-GTH, la droite de régression n'est pas parallèle à celle obtenue avec la c-GTH (0,822 vs 0,997). Cette différence, significative étant donné la petitesse de la variance dans chaque groupe, est cependant faible. Nous avons donc calculé un rapport d'activité moyen qui est égal à 0,091 (limites : 0,080-0,105) tandis qu'il atteint 2,6 (limites : 1,7-3,6) sur la spermiation de la grenouille.

Enfin, les gonadotropines mammaliennes, o-FSH et o-LH, n'exercent qu'une action très faible pour des concentrations élevées ; 50 μg de o-LH ou de o-FSH par ml, bien qu'augmentant nettement (respectivement de 80 p. 100 et de 45 p. 100) la concentration d'AMPc sont considérablement moins efficaces que 0,1 μg de c-GTH.

Les gonadotropines mammaliennes sont au moins 500 fois moins actives que celle de carpe.

Le RGS de l'anguille argentée s'accroît au cours d'un traitement gonadotrope *in vivo* (cf légende du tableau 2) mais la réponse de l'AMPc à la c-GTH, *in vitro*, est bien moins importante que chez les témoins (tabl. 2).

Tous les résultats rapportés jusqu'ici ont été obtenus sur des anguilles argentées. Le traitement *in vivo* d'anguilles jaunes entraîne au bout de 50 jours une augmentation significative mais faible du RGS (témoins : 0,42 $\pm$ 0,06 ; traitées : 0,67 $\pm$ 0,04 ; dl : 10 ; t : 2,34 ; $p < 0,05$) ; cependant, *in vitro*, la c-GTH augmente la concentration ovarienne de l'AMPc de façon aussi intense chez l'anguille jaune témoin que chez l'anguille argentée (tabl. 2).

TABLEAU 2

Influence de l'état physiologique de l'ovaire sur l'augmentation par la c-GTH, in vitro, de la concentration ovarienne de l'AMPc

	Poids du corps (g)	RGS	AMPc		
			sans c-GTH	Accroissement (x) par c-GTH (µg/ml)	
				0,1	10
Anguilles argentées témoins	233 *** $\pm$ 23 (n = 9)	1,68 *** $\pm$ 0,11 (n = 9)	0,14 * $\pm$ 0,01 (n = 9)	6,40 * $\pm$ 0,46 (n = 4)	19,3 * $\pm$ 2,4 (n = 7)
Anguilles argentées traitées à l'hypophyse de carpe *in vivo*	181 245	4,01 17,8	0,29 ** 0,16 **	1,4 ** 1,5 **	5,7 ** 6,9 **
Anguilles jaunes témoins	152 *** $\pm$ 11 (n = 4)	0,48 *** $\pm$ 0,11 (n = 4)	0,17 * $\pm$ 0,02 (n = 4)	7,26 * $\pm$ 1,45 (n = 3)	24,2 * $\pm$ 2,4 (n = 4)

Les anguilles traitées reçoivent, par injection, 2,5 mg d'extrait hypophysaire de carpe trois fois par semaine. Le traitement a duré sept semaines pour l'anguille dont le RGS est 4,01 et seize semaines pour celle dont le RGS a atteint 17,8. Pendant ces périodes, les animaux étaient maintenus à 20 °C.

* Moyenne $\pm$ erreur standard des valeurs obtenues sur plusieurs anguilles (n = nombre d'anguilles). Chaque valeur est la moyenne d'une double détermination sur deux échantillons de tissu (sauf pour les anguilles jaunes).

** Moyenne des valeurs obtenues sur deux échantillons de tissu.

*** Moyenne $\pm$ erreur standard (n = nombre d'anguilles).

Discussion.

Il existe une contradiction apparente entre l'inactivité de la c-GTH sur l'AC dans des homogénats d'ovaire d'anguille (Fontaine *et al.*, 1976) et sa capacité d'augmenter considérablement la concentration en AMPc dans des fragments du même tissu. Diverses hypothèses pouvant en rendre compte ont été précédemment énoncées (Fontaine *et al.*, 1976) et ne seront pas discutées ici. En tout cas, la rapidité de l'effet

de la c-GTH sur la concentration en AMPc suggère qu'il s'agit bien d'une action primaire de l'hormone.

Le paramètre mesuré ici est très sensible puisqu'il augmente déjà significativement avec 0,01 µg/ml de c-GTH ; sa mesure peut constituer la base d'un dosage biologique de la gonadotropine étant donné qu'il semble augmenter, dans un large intervalle, proportionnellement au logarithme de la concentration hormonale ; Idler *et al.* (1975) ont utilisé dans ce but un système similaire. L'accroissement de la concentration en AMPc dans l'ovaire sous l'influence d'une stimulation gonadotrope est particulièrement important chez l'anguille (20 ×), plus élevé que ceux observés dans d'autres systèmes (environ 10 ×) (cf. Idler *et al.*, 1975).

Le rapport d'activité aci-GTH/c-GTH est très différent chez l'anguille et chez la grenouille comme ceci a déjà été noté (Burzawa-Gérard *et al.*, 1975*b* ; Goncharov *et al.*, 1975). Il s'agit donc de deux hormones nettement différentes, ce qui est probablement en rapport avec l'éloignement phylogénétique des deux poissons étudiés, un téléostéen et un chondrostéen. En effet, une telle variation n'est pas observée lorsqu'on compare les hormones de deux téléostéens, la carpe et le poisson-chat indien.

L'inactivité au moins relative des hormones de mammifères (LH et FSH) sur la concentration en AMPc de l'ovaire d'anguille est en accord avec de nombreuses données de la littérature (cf. Fontaine, 1975) obtenues également sur des poissons à ovaire peu développé, en particulier celles concernant la stimulation de l'AC de l'ovaire de cyprin (Fontaine *et al.*, 1970).

La diminution importante de l'action *in vitro* de la c-GTH provoquée par un traitement préalable *in vivo* peut être due au développement ovarien *per se*. Cependant les phénomènes de ce type décrits dans des ovaires de mammifères (Bockaert *et al.*, 1976 ; Hunzicker-Dunn et Birnbaumer, 1976 ; Conti *et al.*, 1976) ainsi que des résultats préliminaires obtenus au laboratoire suggèrent que nous observons plutôt l'induction d'un état réfractaire par la dernière injection de c-GTH qui a eu lieu 18 heures avant le sacrifice. Cet état peut correspondre à une saturation des récepteurs, ou, plus vraisemblablement, à une désensibilisation par la c-GTH du système récepteur-adénylcyclase.

Puisque, enfin, la c-GTH est aussi active *in vitro* sur la concentration de l'AMPc chez les anguilles jaunes que chez les anguilles argentées, la raison de la relative inefficacité de l'hormone *in vivo* chez les premières ne réside pas dans l'absence de récepteurs couplés à l'AC. Cette raison doit être recherchée dans des réactions parallèles (Rasmussen et Goodman, 1977) ou consécutives à la formation de l'AMPc.

Symposium sur la Reproduction des Poissons
Paimpont, France, 19-21 *septembre* 1977.

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Testicular feed back on the hypothalamo-pituitary axis in rainbow trout (*Salmo gairdneri* R.)

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Summary. A testicular feed back on the hypothalamo-pituitary system was studied in male rainbow trout (*Salmo gairdneri*) after bilateral castration and steroid or testicular supplementation. Experiments were carried out at various periods of the spermatogenetic cycle ; in March-April (resting stage), June (initiation of spermatogenesis), October (end of spermatogenesis and before spermiation), and December (running stage).

In March and April, plasma t-GTH is low in intact males (< 2 ng/ml) and castration induced a threefold rise of GTH. Testosterone (T) given by a silastic capsule inserted in the body cavity of the castrate did not change plasma t-GTH in comparison with the intact control. When T is given in the same manner in intact males GTH is significantly increased. In June, a significant t-GTH rise was observed after castration ($P < 0.05$), this rise was prevented by injection of T and estradiol (E_2). When E_2 and T were given to intact males plasma t-GTH was significantly decreased. In October plasma t-GTH was high (13 ng/ml) and was not modified by castration ; injected T or E_2 had no effects on plasma t-GTH either in intacts or castrates. In December, a sevenfold increase of t-GTH is observed after castration. This was partly suppressed by intraperitoneal injection of T and E_2 and declined after stereotaxic implantation of 11-ketotestosterone into the pituitary. It was not significantly suppressed by intraperitoneal injection of testicular extracts. It is suggested that at this period steroids are the main gonadal factors involved in the feed back at the hypothalamo-pituitary system level.

Introduction.

In male rainbow trout in France spermatogenesis is initiated early in summer and spermiation occurs in November. More precisely, in the population we are using in the laboratory, spermatogenesis commences in May-June with a slow phase of type A spermatogonia division followed in June-July by a rapid phase of multiplication of type B spermatogonia. Meiosis and spermiogenesis occur in August-September. Spermatogenesis is completed in October at which time the testes are then full of spermatozoa, and also contain spermatogonia (stem-cells) which remain in a resting stage until May when the next spermatogenetic cycle is initiated. Spermiation starts in November and continues until February. However, males in a running stage may be found late in March and even in April, but spermatozoa are of a poor quality

(low rate of motility) at this time and therefore February is considered as the end period of functional spermiation.

Previous experiments have shown that plasma GTH response to bilateral castration varies according to the stage of spermatogenesis (Billard *et al.*, 1977). In the present work, bilateral castration and steroid supplementation were performed at various stages of this reproductive cycle. In addition, in December (running stage) when castration-induced rise of GTH is maximum, castrates received testicular extracts or 11-ketotestosterone (11-KT) which is the main testicular androgen (Idler *et al.*, 1971).

Material and methods

Experiments were carried out in 1976 and 1977. Animal care and castration procedures are described in Billard *et al.* (1977).

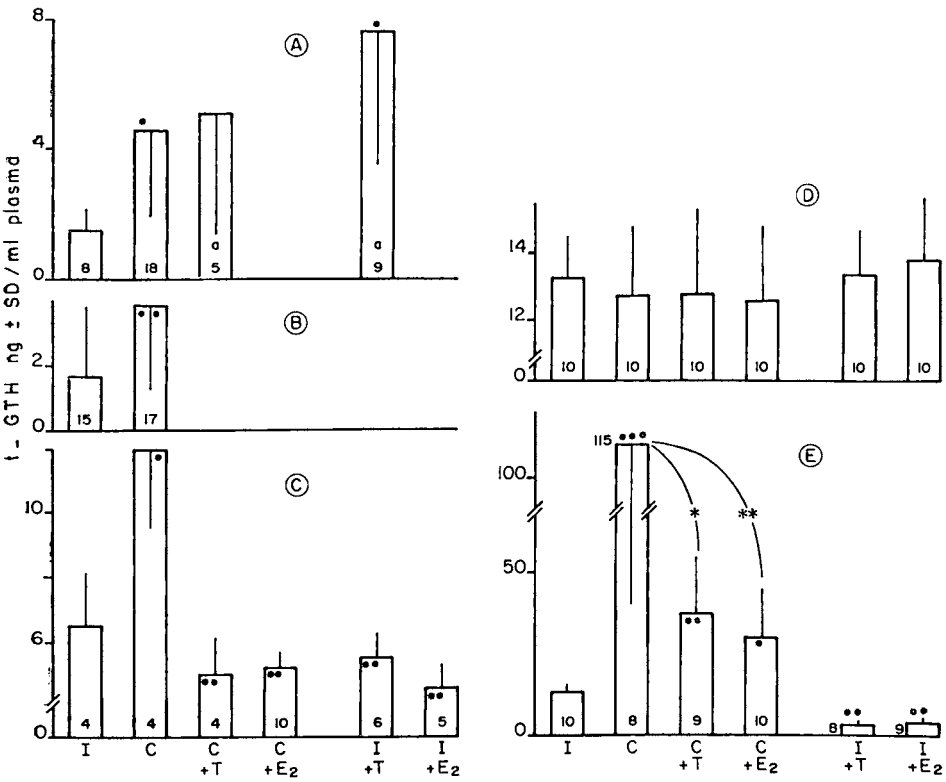

FIG. 1. — *Changes in immunoreactive t-GTH after castration and replacement therapy of T and E_2 in castrates (C) and intacts (I).*

A : Day of castration (Cast.) 29/3 ; *day of blood sampling (S)* 13/4 ; B : Cast. : 12/4 ; S : 31/5 ; C : Cast. : 18/6 ; S : 28/7 ; D : Cast. : 14/10 ; S : 30/10 ; E : Cast. : 3/12 ; S : 6/12.
Numbers correspond to the number of fish sampled at the end of the experiment ; a = T replacement therapy carried out with silastic capsule. t test = * : $P < 0.05$; ** : $P < 0.005$; *** : $P < 0.001$ (comparison with intacts). * : $P < 0.05$; ** : $P < 0.005$; (comparison between castrates and castrates + T or E_2).

Steroid supplementation in castrated and intact males. — Castration was performed at several stages of the reproductive cycle and blood was sampled for GTH measurements several days or weeks later at the time of expected maximum response in the castrates (see fig. 1 for time of castration and duration of the experiments). Steroid supplementation was started on the day of castration in intact or castrated animals and consisted of intraperitoneal administration of testosterone (T) or estradiol 17β (E_2) (Roussel). For injection the steroids were first dissolved in 90° ethanol (5 mg/ml) and diluted 5 times with saline before injection. Fish weighing between 150 to 250 g received one injection (2.5 μg/g body weight) per week. Controls, intact and castrate fish received only saline + 20 p. 100 ethanol. In March testosterone was supplied through silastic capsules inserted in the body cavity (tube was 1.6 mm external diameter, 0.6 mm wall thickness, 3 cm long and after a week released experimentally 13.6 μg/24 h/cm in water at 20 °C).

Implantation of 11 KT *in the pituitary and supplementation with testicular extracts.* — In December, when response to castration is maximal, implants of 11 KT (Ikapharm) were placed stereotaxically in the pituitary of castrates (Billard and Peter, unpublished) and pellets for implantation were prepared according to Billard and Peter (1977). After castration in December, testes were stored in liquid nitrogen and then homogenized. Two extractions of the testes were carried out ; one lead to a cytosol after 90 mm centrifugation at 105 000 g (extract n° 1) and the other corresponded to a steroid-free pellet after alcoholic precipitation (extract n° 2). A control extract was made from liver in a similar manner as for extract n° 2. Before injection the protein concentration was adjusted to 12.75 mg/ml (Folin) in all extracts. On the day of castration, a castrate control group ($n = 8$) received blank implantation in the pituitary and a castrate experimental group received 11 KT implants. Three other castrate groups received IP injection of testicular and control extracts on days 3, 5, 7 and 11 post castration (1 ml/male/injection). Blood was sampled the same days and at the end of the experiment (day 15). GTH was measured by RIA according to Breton and Billard (1977).

Results.

Effects of bilateral castration and steroid replacement therapy. — Castration carried out at the end of March when males have reached the end of the period of spermiation and spermatogenesis is at the resting stage, induces a significant rise ($P < 0.025$) of the circulating t-GTH (fig. 1 A). After replacement therapy by an intraperitoneal implant (silastic capsule) of T, plasma t-GTH was not significantly changed in castrates but it was significantly increased in intact fish.

A similar rise of plasma t-GTH ($P < 0.025$) was observed 49 days after castration carried out on April 12 (fig. 1 B). When castration is performed in mid-June, a rise of t-GTH is also observed at the end of July ($P < 0.05$) (fig. 1 C). At the same time replacement therapy by IP injection of T and E_2 induced a significant decrease of t-GTH ($P < 0.005$) in both intacts or castrates as compared to intact controls (fig. 1 C). In October, towards the end of spermatogenesis and before spermiation, no change was observed in the t-GTH level after castration or steroid replacement therapy

(fig. 1 D). On the contrary in December when males are at the running stage (full spermiation), the t-GTH response to castration is highly significant ($P < 0.005$) (fig. 1 E) Replacement therapy with both T and E_2 partly reduced this response ($P < 0.025$ for T and $P < 0.01$ for E_2), but t-GTH level stayed significantly higher than in the intact males ($P < 0.05$). t-GTH level was also reduced ($P < 0.005$) after replacement therapy with T and E_2 in intact males.

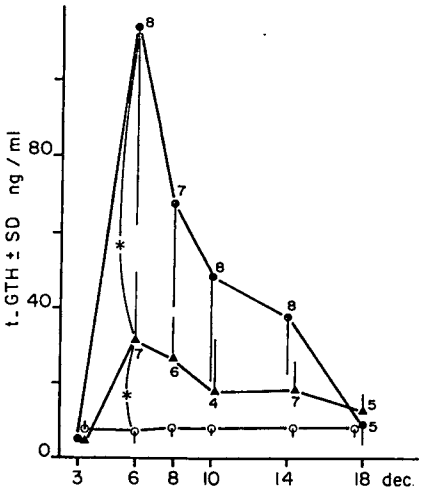

FIG. 2. — *Changes in immunoreactive t-GTH after castration and* 11 KT *implantation in the pituitary of male trout.* Day of castration and implantation : 3/12.
▲ : castrated + 11 KT ; o : intact males ; ● : castrated ; * : $P < 0.05$.

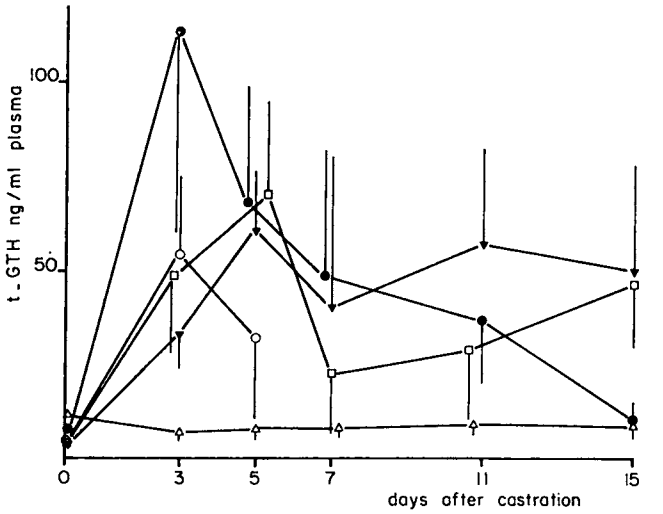

FIG. 3. — *Changes in immunoreactive t-GTH after castration and replacement therapy with testicular extracts* (TE).
● : castrated ; □ : castrated + TE n° 1 ; o : castrated + TE n° 2 ;
▼ : castrated + liver extract (control) : △ : intact male.
Day of castration : 3/12. Volume : 1 ml/injection.

Effects of implantation of 11 KT *in the pituitary and injection of testicular extracts in castrates in December.* — The high and short t-GTH rise which follows castration was significantly reduced by intrapituitary implants of 11 KT although it remained significantly higher than in the intact controls (fig. 2). Injection of testicular extracts slightly prevented the post-castration rise of t-GTH (fig. 3) but values are not significantly different. In addition liver extract had the same effect as TE n° 1.

Discussion.

Castration at most stages of the spermatogenetic cycle induced a significant rise of immunoreactive t-GTH as previously observed (Billard *et al.*, 1977) : in March and April (sexual rest), July (beginning of spermatogenesis) and December (full spermiation), but not in October when spermatogenesis is almost completed or before the onset of spermiation. In this case, the post-castration GTH rise may have occurred earlier than when blood samples were taken for GTH measurements.

The response to replacement therapy in castrates and supplementation in intact males also varied according to the stage of the spermatogenetic cycle. In March; T-increased plasma t-GTH in intacts, suggests a positive feed back action on the hypothalamo-pituitary axis. However, it should be pointed out that T was administered via silastic capsules releasing lower doses of T as compared to the injected males. These low doses may be responsible for the positive feed back as shown in rat (Bloch *et al.*, 1974). T and E_2 therapy was efficient in July and December but had no effect in October, suggesting a change in the gonadal control over the pituitary at this stage. In December, 11 KT is also shown to be involved in the negative feed back (fig. 2). However, T, E_2 and 11 KT independently did not completely prevent the rise of GTH ; they may act when they are present together. Other factors may also be responsible for the negative feed back. It does not seem that those factors were present in the testicular extract since the castration rise was not significantly prevented by the extracts. Clarification of the active substances requires further experimentation using more purified material.

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Résumé. L'existence d'une rétroaction testiculaire sur l'axe hypothalamo-hypophysaire a été étudiée chez la truite Arc-en-ciel après castration bilatérale et supplémentation par des stéroïdes et des extraits testiculaires. Les expériences ont été conduites à diverses périodes du cycle spermatogénétique : mars-avril (repos sexuel), juin (initiation de la spermatogenèse), octobre (fin de la spermatogenèse et avant spermiation), et en décembre (spermiation). La réponse au traitement est appréciée par les niveaux de t-GTH immunoréactive dans le plasma.

En mars-avril, le niveau de t-GTH plasmatique qui est faible (< 2 ng/ml) chez les mâles intacts se trouve multiplié par trois après castration ($P < 0.05$). Des implants intra-

abdominaux de testostérone en tube de silastique ne changent pas les niveaux de t-GTH des castrés, mais provoquent une augmentation significative ($P < 0.05$) chez les mâles intacts. En juin la castration est suivie d'une augmentation significative de t-GTH. Cette augmentation est supprimée après injection de testostérone (T) et estradiol (E_2). Chez les mâles intacts l'administration de T et E_2 provoque une baisse significative de t-GTH. En octobre, t-GTH atteint 13 ng/ml de plasma et n'est pas modifiée après castration et après traitement avec T et E_2 chez les castrés et les intacts. En décembre, les niveaux de t-GTH sont multipliés par 7 après castration. Cette augmentation est au moins partiellement diminuée par injection de T et E_2 et par implantation stéréotaxique de 11 KT dans l'hypophyse. Elle n'est pas significativement modifiée après injection d'extraits testiculaires. A cette période, les stéroïdes apparaissent être le facteur principal impliqué dans le feed back exercé par le testicule au niveau hypothalamo-hypophysaire.

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Hypothalamic lesions of goldfish : effects on gonadal recrudescence and gonadotropin secretion

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Summary. The effects of radiofrequency lesions of the nucleus lateral tuberis (NLT) and nucleus preopticus (NPO) on gonadal recrudescence and serum gonadotropin levels in male and female goldfish were investigated. Lesions destroying a substantial portion of the NLT caused a significant decrease in the gonadosomatic index in comparison to normal and sham-operated control groups. The ovary of NLT lesioned fish had a greater amount of atresia evident and recrudescence was frequently not as advanced as in the controls. Lesions in the NPO region also caused a decreased gonadosomatic index, but this was not found in other experiments. In the experiment in which NPO lesions were effective, ovarian recrudescence was generally at an earlier stage compared to controis. Serum gonadotropin levels in male or female NLT or NPO lesioned fish were not significantly different from the levels found in normal or sham control groups.

Introduction.

Lesions in the nucleus lateral tuberis (NLT) of goldfish cause a decreased gonadosomatic index (Peter, 1970). The specific region of the NLT found to be involved is the NLT pars posterior and the posterior NLT pars anterior. Lesions in other regions, including the nucleus preopticus (NPO), had no effects. These results were interpreted to demonstrate involvement of the NLT in regulation of gonadal activity, presumably via secretion of a gonadotropin (GtH) releasing factor (GRH). The presence of GRH activity in crude extracts of the hypothalamus of goldfish (Crim *et al.*, 1976) and other teleost species (for review see Crim *et al.*, 1976) supports the idea of a GRH center in the hypothalamus, although the origin of the activity was not localized in these studies. Contradictory to the idea of a hypothalamic origin for GRH, Goos and Murathanoglu (1977) have recently localized cells immunoreactive for luteinizing hormone-releasing hormone (LH-RH) in the telencephalon of rainbow trout. Since synthetic LH-RH has GRH activity in teleosts (for review see Crim *et al.*, 1976), the localization of immunoreactive LH-RH cells in the telencephalon in the trout suggests that area as the origin for GRH activity. In the present study the effects of lesions in the NLT and NPO regions on GtH secretion and gonadal activity of goldfish were examined.

Materials and methods.

Goldfish, common or comet variety, of 40 g average body weight were held in 96 l flow-through aquaria at 12-14 °C, with a 16 hr light-8 hr dark photoperiod, for the experiment. The fish were individually identified by a numbered tag attached to the operculum. Lesions were made with a radiofrequency current generator using 70-90 volts and 0 insect pins insulated to within 0.5-0.6 mm of the tip. The electrodes were stereotaxically positioned using techniques previously described (Peter, 1970 ; Peter and Gill, 1975).

For blood sampling, the fish were anesthetized with tricaine methanesulfonate and about 0.3 ml of blood taken by puncture of the caudal vasculature at between 2-4 hr after onset of the light period. Serum samples were collected and GtH concentrations determined by radioimmunoassay (Crim *et al.*, 1976). At the end of the experiment the fish were killed by decapitation, and the brain rapidly dissected free and immersed in Bouin's solution, The gonads were also dissected, weighed, the condition noted, and then a segment of one gonad fixed in Bouin's solution. For the analysis of results the experimental fish were grouped according to the final lesion location as determined by histological examination. The GtH values for the various groups within an experiment were compared by Student's « t » tests. The ovaries were histologically examined and the most advanced stage of oocyte development and the degree of atresia and/or ovulation noted, using the criteria of Yamazaki (1965) for the oocyte stages.

Table 1

Serum gonadotropin concentrations (ng/ml) and gonadosomatic index of female goldfish post-lesioning in the nucleus lateral tuberis (NLT) *region or the nucleus preopticus* (NPO) *region*

Group	Serum gonadotropin (ng/ml)			Gonadosomatic Index
	Days post-lesioning			
	7	21	42	
Normal (N = 9)	0.70 ± 0.16 (a)	0.85 ± 0.15	0.98 ± 0.18	13.97 ± 1.18 (a)
Sham (N = 7)	4.82 ± 3.21	1.20 ± 0.24	1.06 ± 0.21	11.28 ± 2.12
NLT (N = 9)	4.04 ± 2.66 NS (b)	1.75 ± 0.96 NS	0.61 ± 0.07 NS	5.70 ± 0.83 S (c)
NPO (N = 7)	9.07 ± 7.16 NS	1.78 ± 0.56 NS	1.50 ± 0.38 NS	5.02 ± 0.95 S

(a) mean ± SE.
(b) NS = not significant compared to normal or sham control.
(c) S = significant compared to normal and sham control.

Results.

Table 1 presents the data from female goldfish that were lesioned in the NLTa-NLTp region in February and maintained for 42 days postoperatively. The period of February through April is the period of time during which female goldfish have rapid oocyte growth, with at least some oocytes being developed to the tertiary yolk globule (or granule) stage, ready to undergo the final maturation processes and ovulation. The lesions in the NLT and NPO regions both caused a significant decrease in the GSI. There were no significant differences in serum GtH levels between the normal, sham and lesioned groups at either 7, 21 or 42 days postoperatively. Oocytes were developed to the tertiary yolk globule stage in the normal and sham control groups. In the NLT and NPO lesioned groups, only three fish out of nine, and four out of seven, respectively, had oocytes at the tertiary yolk globule stage ; other fish in these groups generally had oocytes only at the primary yolk globule stage. The NLT lesioned group had a greater prevalence of atretic oocytes.

Lesions in the NLT region of male goldfish also caused a significant decreased GSI and had no effects on serum GtH levels (table 2).

Table 2

Serum gonadotropin concentrations (ng/ml) and gonadosomatic index of male goldfish post-lesioning in the nucleus lateral tuberis (NLT) region

Group	Serum gonadotropin (ng/ml)			Gonadosomatic Index
	Days post-lesioning			
	7	21	42	
Normal (N = 11)	0.96 ± 0.20 (a)	0.88 ± 0.18	0.65 ± 0.11	3.40 ± 0.22 (a)
Sham (N = 11)	0.72 ± 0.17	0.66 ± 0.14	0.63 ± 0.10	3.67 ± 0.35
NLT (N=8)	1.99 ± 0.88 NS (b)	0.59 ± 0.15 NS	0.58 ± 0.13	2.57 ± 0.25 S (c)

(a) mean ± SE.
(b) NS = not significant compared to normal or sham control.
(c) S = significant compared to normal and sham control.

Discussion.

The lesions in the NLT region caused a decreased gonadosomatic index (GSI), blocked ovarian recrudescence and induced atresia. This confirms the earlier findings of Peter (1970) and supports an involvement of the NLT in secretion of GRH. However, no differences in serum GtH levels were found between the groups with lesions in the NLT region and the control groups. These results were confirmed in other experiments on females and males (Peter and Crim, unpublished results).

In the present experiment lesions in the NPO region also caused a decreased GSI of female goldfish, blocked ovarian recrudescence and induced some degree of atresia. No effects on GtH levels were found in the NPO lesioned animals. This was the only experiment in which lesions in the NPO region had an effect on ovarian or testicular recrudescence (Peter and Crim, unpublished results). These results are, therefore, suggestive of some involvement of the NPO region in GRF secretion, but the results require confirmation.

A basic hypothesis about the relationship between blood levels of GtH and gonadal recrudescence is that there are incremental amounts of GtH in the blood in correlation with, and presumably to stimulate, progression of gonadal recrudescence. There is evidence in support of this hypothesis from some species (Breton *et al.*, 1975 ; Crim *et al.*, 1975). In the present work there were no significant differences in serum GtH levels between the fish with brain lesions and the control groups, even though oocyte development was generally at an earlier stage and atresia was occurring in the lesioned fish. This obviously does not fit with the above hypothesis. In recent work on the daily cycles of serum GtH in goldfish under different temperature-photoperiod combinations and at different states of gonadal activity (Hontela and Peter, unpublished results), it was found that serum GtH levels were basal and similar in fish at different states of gonadal activity for most of the 24 hr period for fish held in cold water (12 °C) and on a long photoperiod (16L : 8D). The significant difference between fish with a regressed gonad and those undergoing recrudescence or with a mature gonad was that the latter each have a significant surge in GtH levels, indicating a surge in secretion, at between 8-12 hr after the onset of the light period. This may account for the lack of any differences in serum GtH between the lesioned fish and the controls in the present experiment since all blood samples were taken at between 2-4 hr after onset of the light period, a time when serum levels of GtH are basal. Preliminary results indicate that NLT lesions do alter the daily pattern of secretion of GtH in goldfish ; alteration of the daily pattern of secretion of GtH may be the basis for the observed effects of the NLT lesions on the gonads.

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Résumé. Nous avons étudié les effets de lésions du noyau latéral du tuber (NLT) et du noyau préoptique (NPO) sur le développement des gonades et le niveau de gonadotropine sérique chez le poisson rouge mâle et femelle. Les lésions qui détruisent une partie importante du NLT entraînent une diminution significative de l'index gonadosomatique par rapport aux animaux normaux et aux témoins qui ont subi une opération fantôme. L'atrésie est plus importante dans l'ovaire des animaux porteurs d'une lésion du NLT et le stade d'avancement de la gamétogenèse est souvent moins avancé que dans le groupe témoin. Les lésions dans la région du NPO entraînent également parfois une diminution de l'index gonadosomatique. Quand les lésions du NPO sont efficaces, la recrudescence ovarienne

est en général moins avancée que chez les témoins. Les lésions du NLT ou du NPO n'entraînent pas de modifications du niveau de gonadotropine dans le sérum chez le mâle et chez la femelle.

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Etude du contrôle hypothalamique de la fonction gonadotrope chez *Gambusia sp.* (poisson téléostéen) par la technique des greffes

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Summary. *Gonadotropic function in the teleost* Gambusia *sp. A study of hypothalamic control using grafts.*

In the viviparous poeciliid, *Gambusia sp.*, long-term (3 to 12 months) pituitary autografts permitted maintenance of the ovarian structure and normal functioning of vitellogenesis. It is likely that hypothalamic stimulation of gonadotropic cells of the graft was through a neuro-vascular tract.

Introduction.

Afin de préciser les modalités du contrôle hypothalamique de la fonction gonadotrope chez *Gambusia*, nous avons étudié l'activité de l'hypophyse autotransplantée, déconnectée de l'hypothalamus, mais, néanmoins, soumise à l'influence éventuelle des récepteurs. L'autogreffe d'hypophyse chez le Cyprin (Johansen, 1967) ou l'homogreffe chez *Poecilia* (Ball *et al.* 1965) entraîne en 1 ou 2 mois une régression des gonades et des cellules gonadotropes du greffon. Chez *Gambusia* (Chambolle, 1977), des autogreffes pratiquées en début de gestation ne permettent pas à la vitellogenèse de redémarrer après la parturition (1 mois après la greffe). Nous avons repris l'étude de l'influence d'une autotransplantation hypophysaire à long terme sur l'activité ovarienne.

Matériel et méthode.

Nous avons utilisé le *Poeciliidae* vivipare *Gambusia sp.* Sa période de reproduction s'étend de mai à septembre dans les conditions naturelles de notre région et 4 à 5 cycles de reproduction se succèdent sans interruption. Au moment de la parturition, l'ovaire ne contient aucun ovocyte en cours de vitellogenèse. Celle-ci débute immédiatement après. La période de repos sexuel s'étend d'octobre à avril, les ovaires ne contiennent alors que des ovocytes I et II.

Nous avons hypophysectomisé 50 femelles par voie buccale. Ces poissons considérés comme témoins des « greffés » sont sacrifiés chaque mois pendant un an pour l'étude histologique de l'ovaire. Témoins et opérés sont élevés à 25 °C avec une photopériode naturelle dans un mélange de 2/3 d'eau douce et de 1/3 d'eau de mer, permettant une survie parfaite (Chambolle, 1966).

Chez 90 animaux, nous avons pratiqué une autotransplantation de l'hypophyse, soit en la greffant dans la musculature caudale (chez 30 animaux) (fig. 1), soit en la réintroduisant dans la selle turcique (chez 60 animaux) (fig. 2). Chez ces animaux sacrifiés comme les témoins, nous avons fait l'étude histologique de l'ovaire et du greffon hypophysaire (coloration avec le Cleveland Wolfe). Nous avons toujours vérifié la réussite de l'hypophysectomie par un contrôle histologique. Les cas d'ablation partielle n'ont pas dépassé 2 p. 100 des animaux opérés et ont été éliminés.

Résultats.

Hypophysectomie : l'ablation pratiquée pendant la période de repos sexuel entraîne une réduction progressive du stroma ovarien qui, suivant les animaux, semble être maximale après 6 à 12 mois (fig. 3 et 4). Les ovocytes I et II ne dégénèrent pas. En bordure de la cavité ovarienne, l'épithélium est très réduit.

L'ablation pratiquée pendant la période d'activité sexuelle provoque, en outre, la dégénérescence de tous les ovocytes en vitellogenèse. Ces résultats sont en accord avec de nombreux travaux antérieurs montrant que l'hypophyse est indispensable au déclenchement et au maintien de la vitellogenèse. L'hypophyse est aussi indispensable pour conserver une structure normale du stroma et de l'épithélium ovarien chez *Gambusia*.

Greffe d'hypophyse : chez des animaux opérés au cours de la période de repos sexuel, la vitellogenèse se déclenche en même temps que celle des animaux normaux non opérés, lorsque le greffon a été placé dans la selle turcique et à condition, toutefois, que la greffe ait été pratiquée au minimum deux mois avant le début de la période de reproduction (résultats sur 25 animaux). Lorsque le greffon est placé dans la musculature caudale, le même phénomène s'observe (résultats sur 10 animaux) mais avec un retard plus ou moins prononcé suivant les animaux (1 mois en moyenne). Quel que soit le type de greffe pratiqué, la vitellogenèse se déroule normalement (fig. 5). Chez certains animaux conservés suffisamment longtemps (plus de 3 mois), nous avons pu obtenir une gestation et une parturition normales. L'histologie des

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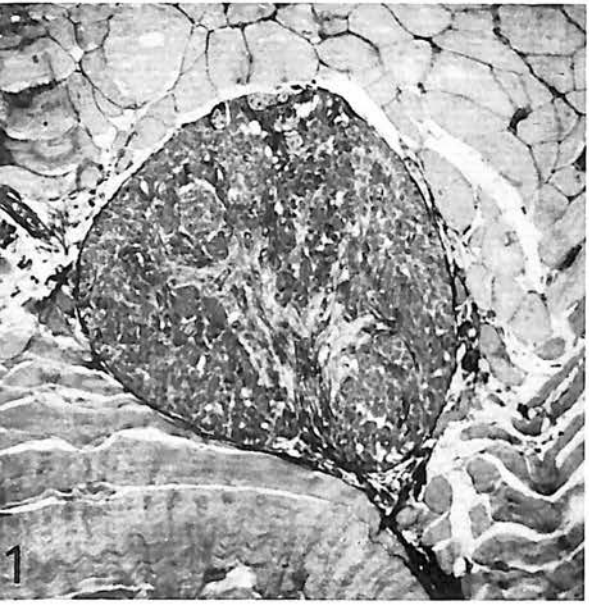
FIG. 1. — *Greffon d'hypophyse dans la musculature caudale, 12 mois après la greffe* (G = 140).

FIG. 2. — *Greffon d'hypophyse dans la selle turcique, 5 mois après la greffe* (G = 168).

FIG. 3. — *Ovaire 10 mois après hypophysectomie* (comparez avec la fig. 4) (G = 108).

FIG. 4. — *Ovaire d'un animal témoin pendant la période de repos sexuel* (G = 62).

FIG. 5 et 6. — *Ovaire après autogreffe d'hypophyse dans la musculature caudale 12 mois après la greffe* (G = 148) *et cellules gonadotropes du greffon du même animal* (G = 1 530).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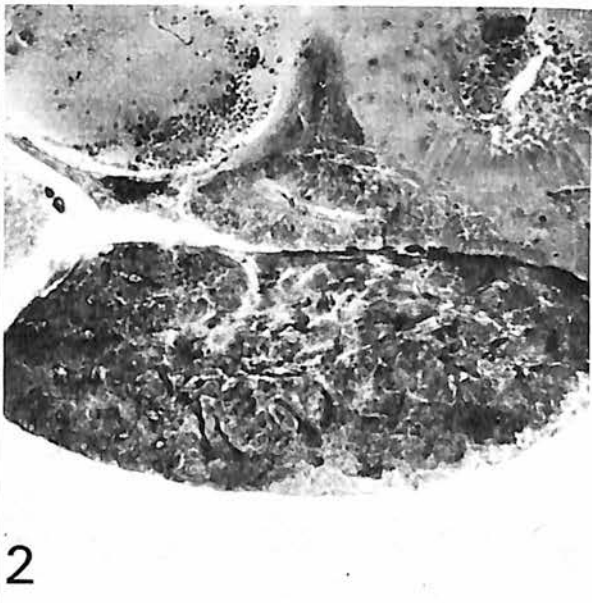
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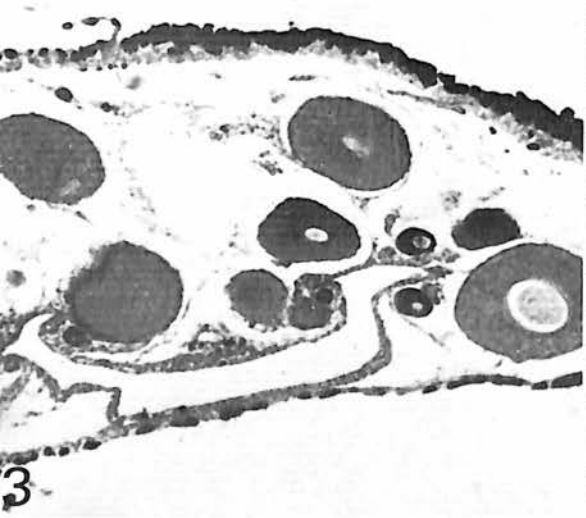
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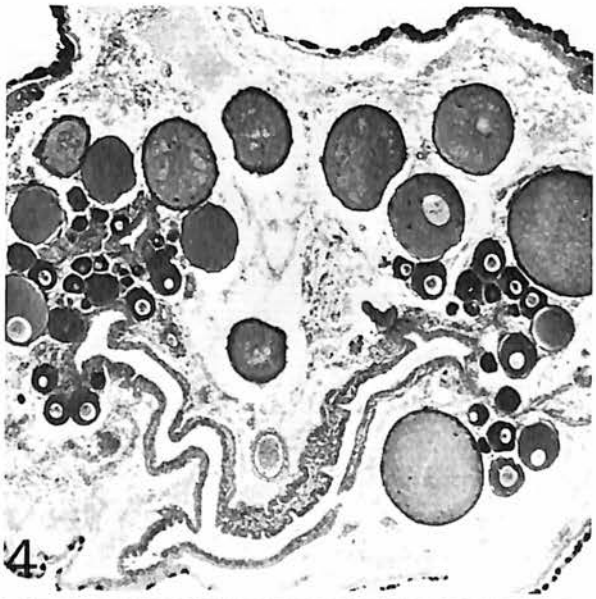
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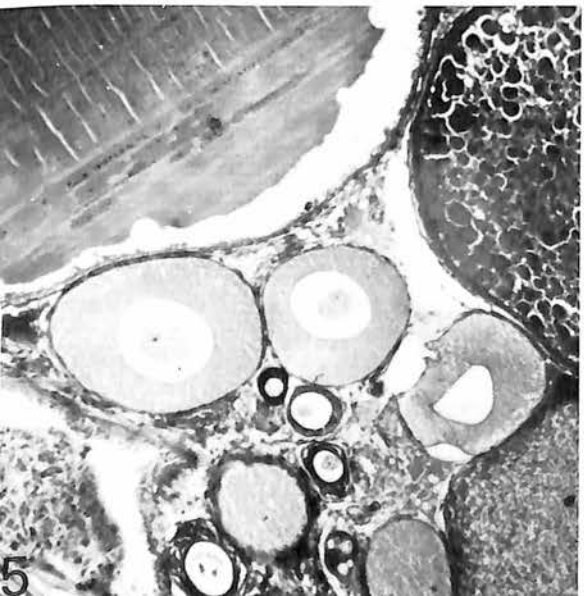
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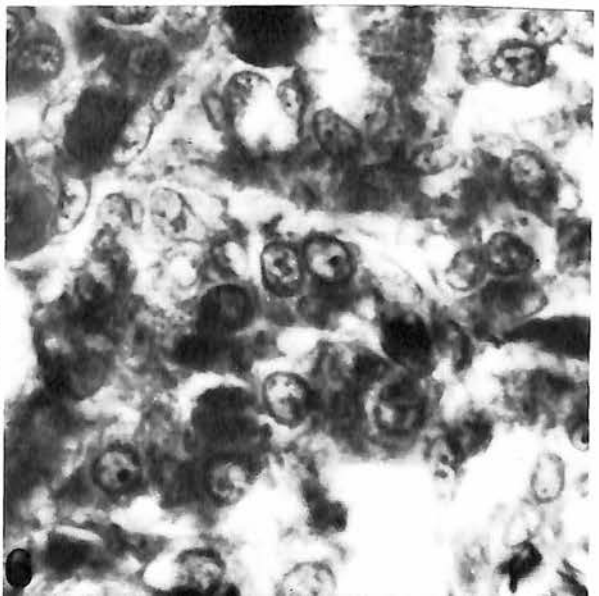
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greffons nous a permis d'identifier toutes les catégories cellulaires et, en particulier, les cellules gonadotropes qui paraissent très actives avec un noyau arrondi à nucléole bien visible et un cytoplasme bien granulé (fig. 6).

Chez des animaux opérés durant la période de reproduction, les résultats sont plus difficiles à interpréter. Dans un premier temps, l'ablation a pour conséquence l'atrésie des ovocytes en vitellogenèse. Dans un deuxième temps, on peut observer une reprise de la vitellogenèse qui se fait au début de la période de reproduction suivante, soit 7 à 8 mois après l'opération. Il est fréquent d'observer dans l'ovaire la présence d'ovocytes dont l'atrésie n'est pas terminée à côté d'ovocytes en vitellogenèse.

Les résultats les plus démonstratifs et les plus facilement interprétables nous paraissent être ceux obtenus après ablation au cours de la période de repos sexuel. Dans ce cas lors de l'opération, les cellules gonadotropes et l'ovaire sont au repos et il apparaît clairement que, au moment de la reprise normale de l'activité sexuelle chez cette espèce, les cellules gonadotropes, quelle que soit la localisation du greffon, sont capables de stimuler l'activité ovarienne, en particulier la vitellogenèse.

Discussion.

Les résultats obtenus chez *Gambusia* diffèrent de ceux signalés chez *Poecilia* (Ball *et al.*, 1965). Les différences observées semblent dues à la longue durée de nos expériences et aux nombreux essais pratiqués à des époques différentes de l'année. Jusqu'à présent, nous n'avons pu obtenir la reprise de la vitellogenèse après autogreffe d'hypophyse dans la musculature caudale, qu'au moment de la reprise de l'activité sexuelle de l'espèce, au début du printemps. Il semble qu'à cette époque, les cellules gonadotropes sont plus sensibles à l'action d'un facteur hypothalamique dont le taux serait alors maximal. Cette hypothèse est en accord avec les travaux de Weil *et al.* (1975), chez la Carpe.

Nos résultats sont à rapprocher de ceux obtenus chez le Rat. Dans le cas de greffes multiples d'hypophyse, les greffons peuvent sécréter de petites quantités de LH et FSH (Lu *et al.*, 1977). La présence d'une LH-RH, bien démontrée chez les Vertébrés supérieurs, semble vérifiée chez la Carpe (Breton et Weil, 1973). Mais son mode d'action est peu connu. Des cellules à LH-RH ont été mises en évidence dans le télencéphale de la Truite, mais le trajet exact des axones n'a pu être précisé (Goos et Murathanoglu, 1977). Le transport de cette hormone pourrait se faire par voie neuroglandulaire (Zambrano, 1972 ; Ekengren, 1975). Deux types de fibres nerveuses (A et B) établissent des contacts synaptiques avec les cellules gonadotropes de *Poecilia* (Peute *et al.*, 1976). Les fibres B proviendraient du noyau latéral de tuber qui paraît être impliqué dans le contrôle de la reproduction (Peter, 1970 ; Zambrano, 1972). Cette zone est en outre très sensible à la rétroaction des œstrogènes sur l'activité gonadotrope chez le Cyprin (Billard et Peter, 1977). L'existence d'une voie neurovasculaire a cependant été envisagée par Sathyanesan (1970, 1971), Fridberg et Ekengren (1977).

S'il existe une stimulation hypothalamique des cellules gonadotropes par voie nerveuse chez *Gambusia*, dans nos expériences de greffe, le transport de la LH-RH par voie strictement nerveuse n'est plus envisageable. Il faut donc admettre la possibilité d'un transport par voie neuro-vasculaire, le chaînon vasculaire devenant alors le plus important.

Une autre hypothèse pour expliquer nos résultats ne peut être écartée : celle d'une sécrétion autonome de gonadotrophine s'établissant après greffe de longue durée similaire à la sécrétion de FSH observée lors de culture d'hypophyse de Rat pendant 18 semaines à 3 ans (Sheridan et Pasteels, 1977).

En conclusion, chez *Gambusia* des autogreffes d'hypophyse permettent le maintien de la structure ovarienne et le déroulement normal de la vitellogenèse. Il est probable que la stimulation hypothalamique des cellules gonadotropes du greffon se fait par voie neuro-vasculaire.

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Neuroanatomical substrates of reproductive behavior in male sunfish (Genus *Lepomis*)

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Summary. Electrical stimulation of the brain has been carried out in both free-swimming (chronic preparation) and anesthetized (acute preparation) sunfish. Nestbuilding has been evoked from the area dorsalis telencephali pars centralis (ADTPC) of the telencephalic lobe. This response has also been triggered by stimulation in the preoptic area (POA), as has courtship circling, spawning maneuvers and sperm release in chronic preparations and sperm release in acute preparations. The latter response has been elicited in acute preparations by stimulation of a locus of points extending from the POA, through the lateral hypothalamus, lateral tegmentum of the midbrain, basal medulla and initial portion of the spinal cord. The primary motor neurons controlling sperm release are located in the rostral spinal cord since the response was triggered by stimulation of an isolated segment in this area. Recent autoradiographic studies using H^3-testosterone have demonstrated that the parvocellular region of the preoptic area and parts of both the tuberal nuclear complex and anterior pituitary contain testosterone-concentrating neurons. It is suggested that androgens may influence the sperm release system and perhaps other reproductive mechanisms via some of these specialized cells. This is consistent with findings that certain reproductive responses in male sunfish are androgen dependent. A tentative model of some aspects of the neural control of reproductive behavior in male sunfish can be suggested. The ADTPC mediates its effects by influencing the POA.This idea is supported by electrophysiological experiments. The POA is involved in the integration of hormonal, olfactory and probably other information relevant to reproductive behavior. It influences sexual activities through systems such as the one described for sperm release.

Introduction.

Relatively little is known of the neural mechanisms controlling reproductive behavior in fishes. In response to this lack of information, our laboratory has been engaged in a series of experiments concerned with the anatomical mapping of brain areas involved in male sexual responses in North American sunfish (genus *Lepomis*). Two general methods have been used. The first consists of electrical stimulation of small areas of the brain in free-swimming (Demski and Knigge, 1971) as well as anesthetized, partially immobilized animals (Demski *et al.*, 1975). The second method consists of autoradiography using labeled testosterone to identify and map androgen-concentrating neurons in the fish brain (Pfaff, Morrell, Kelley and Demski, in prepara-

tion). The purpose of this paper is to review our results and assimilate these data into a working hypothesis concerning the neural pathways and mechanisms controlling male sexual behavior in sunfish.

Materials and methods.

Electrical stimulation of the brain.

Details of the techniques for implantation and testing of stimulation electrodes in free-swimming sunfish have been previously published (Demski and Knigge, 1971 ; Demski and Picker, 1973). One of the drawbacks of this method is the limitation on the number of brain sites that can be tested in each animal. Anatomical mapping can be greatly facilitated if studies can be carried out in fishes with their heads immobilized such that the cranial cavity can be opened and held above the surface of the water in which the rest of the animal is immersed. With this preparation many electrode tracks can be run in each fish by using a standard micromanipulator to advance the electrode. The major drawback of this preparation is that the test animal, which in our procedure is anesthetized, can not perform the normal sequences of reproductive behavior. However, the finding that sperm release could be elicited under these conditions permitted a thorough mapping of the major areas of the brain from which at least part of the male reproductive sequence can be evoked. This method also allows the lowest threshold point along each electrode track to be identified and marked for histological verification. Futher details of this procedure are beyond the scope of this paper (see Demski *et al.*, 1975).

Autoradiography.

The following procedures were carried out in collaboration with Drs. Pfaff, Morrell and Kelley at Rockefeller University. Methodological details are essentially the same as reported by Pfaff and Keiner (1973). Six mature male green sunfish (*Lepomis cyanellus*) were surgically castrated, and within 72 hrs, four of them were given intraperitoneal injections of tritiated-testoterone (200 Ci/100 g body weight). All fish (four experimentals and two controls) were sacrificed between 1 to 3 hrs following the injection time and their brains removed and frozen using dry ice. Tissue was blocked and sectioned on a freezing microtome. Parasagittal and transverse sections were placed on glass slides, coated with Kodak NTB-3 nuclear emulsion and placed in the dark for a period up to 14 months. Slides were developed and counterstained with cresyl violet. Only cells with grain counts of at least five times the mean background level (an adjacent cell-sized area of neuropil) were considered as labeled. Areas with labeled cells were plotted on a series of representative sections (fig. 1).

Results and conclusions.

Several reproductive patterns have been elicited by electrical stimulation of the brain in unanesthetized, free-swimming sunfish. Sweeping or nestbuilding has been evoked in bluegills (*Lepomis macrochirus*) of both sexes (Demski and Knigge, 1971).

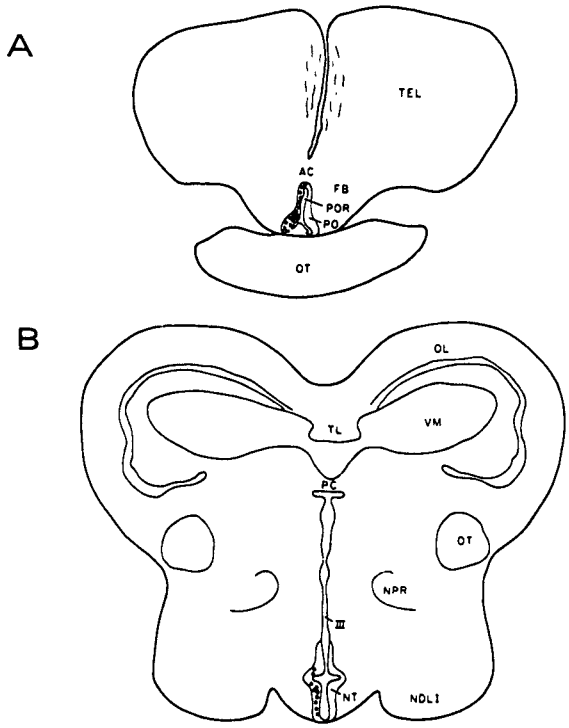

FIG. 1. — A) *Transverse section through the brain (telencephalon) of the green sunfish,* Lepomis cyanellus *showing labelled cells in the preoptic area following injection of* H^3*-testosterone.* The left side of the figure shows the precise positions of labelled cells in this section, each black dot indicating one labelled cell. The right side of the figure shows anatomical structures indicated with abbreviations. Nomenclature is from Demski *et al.*, 1975.

B) *Transverse section through the diencephalon of the green sunfish, at the level of the anterior portion of nucleus tuberalis, showing testosterone-concentrating cells in nucleus tuberalis.* Conventions as for section A of this figure. AC, anterior commissure ; FB, forebrain bundles ; NDLI, nucleus diffusus lobi inferiors; NPR, nucleus prerotundus ; NT, nucleus tuberalis ; OL, optic lobe ; OT, optic tract ; PC, posterior commissure ; PO, preoptic area ; POR, preoptic recess of third ventricle ; TEL, telencephalon ; TH, thalamus ; TL, torus longitudinalis ; VM, ventricle of the midbrain ; III, third ventricle.

Nestbuilding in sunfish appears to be androgen dependent (Smith, 1969 ; 1970). This may be the reason that under natural conditions only males have been observed to perform this activity (Miller, 1963). Sweeping responses have resulted from stimulation in the area dorsalis telencephali pars centralis (ADTPC) and the preoptic area (POA) in several bluegills (Demski and Knigge, 1971 ; Bauer and Demski, in preparation). An interesting feature of this response is that it was evoked most readily by low frequency stimulation (2-10 Hz). This may be correlated with neurophysiological findings that similar frequencies of telencephalic lobe stimulation are most effective in triggering repetitive discharges from parvocellular neurons in the sunfish POA (Hallowitz *et al.*, 1971). It can be suggested that the low frequency telence-

phalic stimulation in free-swimming sunfish results in nestbuilding by selectively activating the POA and that stimulation in the POA results in sweeping through direct activation of the same units.

Courtship, defined as circling another fish with attempts to lead it toward a nesting area, has only been evoked by stimulation of the POA in mature males including both bluegills (Demski and Knigge, 1971) and green sunfish (Demski, unpublished observations). One of the green sunfish was stimulated in the presence of a preserved female, held on a wire and maneuvered by the experimenter. The test animal not only approached, circled and attempted to lead the stimulus fish but also tried to spawn, i.e. placed its ventral aspect against the dead female and released semen.

As mentioned above, the use of anesthetized-immobilized animals can greatly facilitate brain mapping. In a series of pilot experiments using anesthetized green sunfish, it was determined that stimulus bound semen discharge with active spermatozoa could be evoked from brain sites ranging from the POA to the spinal cord. Later more detailed mapping studies (Demski *et al.*, 1975) revealed that the lowest threshold sperm release sites formed a locus running from the POA through the dorsal hypothalamus, lateral tegmentum of the midbrain' basal medulla and rostral spinal cord.

Combined stimulation-ablation experiments indicated that sperm release could be evoked by stimulation of a small isolated segment of rostral spinal cord and thus, that the lower motor system controlling the response originated at this level (see details in Demski *et al.*, 1975). Recent experiments have demonstrated that sperm release responses can also be evoked by brain stimulation in bluegills (Bauer and Demski, in preparation).

With regard to autoradiographic mapping, brains from four experimental green sunfish have been studied (Pfaff, Morrell, Kelley and Demski, in preparation). So far, testosterone-concentrating neurons have been located in the POA (parvocellular neurons only), the tuberal nuclear complex and specific segments of the anterior pituitary (fig. 1). The androgen-concentrating cells in the POA are found in the same region from which sperm release and courtship have been electrically evoked in green sunfish (Demski *et al.*, 1975). Testosterone uptake by the tuberal area and pituitary most likely relates to feedback mechanisms controlling gonadotropin release (Peter, 1970 ; 1973). Testosterone-concentrating neurons were not found in other areas of the sunfish brain, including the ADTPC and the midbrain and medullary portions of the sperm release system.

The following tentative model of the neural control of reproductive behavior in male sunfish is put forward in an attempt to assimilate and summarize the results discussed above. Telencephalic lobe areas (especially the ADTPC) seem to be involved in nestbuilding and perhaps other aspects of reproductive behavior in male sunfish. These areas probably are not directly affected by androgenic hormones. Based on electrophysiological evidence, higher forebrain influences on reproductive behavior may well be mediated by activation of small neurons in the POA. The POA is the only region from which more than one reproductive response has been electrically evoked and thus seems to be an important area for the control of sexual behavior in sunfish. Some of its small neurons concentrate testosterone and presumably their electrical activity is influenced by variations in circulating androgen levels. This may be one

of the key factors regulating seasonal breeding activities. Small preoptic neurons in sunfish may also be influenced by olfactory stimuli since they can readily be activated by olfactory tract stimulation (Hallowitz *et al.*, 1971). This observation gains special importance in light of evidence that male sunfish may use olfactory cues in setting up nesting colonies (Gerald, 1970). Thus, the POA may be involved in the integration of hormonal, olfactory and probably other types of information relevant to reproductive behavior. On the output side, the POA appears to have functional connections that permit artificial electrical activation of complex behavioral sequences such as courtship as well as more elemental patterns like sperm release. It seems reasonable to suggest that under natural conditions the POA influences reproductive behavior, at least in part, through systems such as the one proposed for sperm release.

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Résumé. La stimulation électrique du cerveau a été pratiquée sur des poissons (gen. *Lepomis*) nageant librement et sur des poissons anesthésiés et immobilisés. La stimulation de l'area dorsalis telencephali pars centralis (ADTPC) du lobe télencéphalique a stimulé la nidification. Cette réaction, ainsi que plusieurs phases du frai et la libération de spermatozoïdes, a également été déclenchée par la stimulation de l'aire préoptique (POA) chez les poissons non anesthésiés, nageant librement. Chez les poissons anesthésiés et immobilisés, la libération de spermatozoïdes a été obtenue par la stimulation d'un ensemble de points allant du POA à travers l'hypothalamus latéral, le tegmentum latéral du mésencéphale, la medulla ventrale et la partie supérieure de la moelle épinière. Les neurones moteurs primaires qui contrôlent la libération des spermatozoïdes sont situés dans la moelle épinière rostrale puisque la réaction a été obtenue par la stimulation d'un segment isolé de cette région.

Des études radioautographiques récentes avec H^3-testostérone ont démontré que la région préoptique parvocellulaire ainsi qu'une partie du nucleus tuberalis et du lobe antérieur de l'hypophyse contiennent des neurones qui concentrent de la testostérone. Ceci semble indiquer que les hormones androgènes peuvent influencer le système de la libération de spermatozoïdes et peut-être d'autres mécanismes de reproduction via certaines de ces cellules spécialisées. Ceci est en accord avec les résultats établissant que certaines réactions de reproduction chez les poissons mâles dépendent des hormones androgènes. On peut suggérer un modèle préliminaire de certains aspects du contrôle neural du comportement reproductif chez les poissons (gen. *Lepomis*). L'ADTPC agit en influençant la POA. Ceci est appuyé par des expériences électrophysiologiques. La POA est impliquée dans l'intégration d'information hormonale, olfactive et probablement autre, liée au comportement de reproduction. Elle influence les activités sexuelles par des systèmes tels que celui décrit pour la libération des spermatozoïdes.

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Hypothalamic centers and innervation of the hypophysis in the Atlantic salmon (*Salmo salar*) and the rainbow trout (*Salmo gairdneri*)

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Summary. The nucleus preopticus (NPO), the nucleus lateralis tuberis (NLT) and the paraventricular organ (PVO), presumed to produce adenohypophysiotropic factors and to be involved in reproductive processes, have been studied in two salmonids. The cell bodies and especially their axonal contacts with the hypophysis were thoroughly examined.

The Falck-Hillarp method revealed a high amount of aminergic fibers in all parts of the neurohypophysis. There were only small differences between different areas and between the two species. The fibers may originate in the PVO. The pseudoisocyanin method showed that all parts of the neurohypophysis had NPO-terminations, most abundant in the neuro-intermediate lobe. Fluorescing, possibly aminergic, cells were found in the trout hypophysis. The NPO had a high input of aminergic fibers, possibly originating in the PVO. Cells of the medial part of the NLT received many aminergic fibers ; NPO-fibers also passed very close to these cells. This pattern implied a rich double innervation of the NLT cells. The results lead to the suggestion of a functional relationship among the three hypothalamic nuclei.

Introduction.

In the hypothalamus of teleost fishes three nuclei have received special attention. They are the nucleus preopticus (NPO), the nucleus lateralis tuberis (NLT), and the complex of the nucleus recessus lateralis (NRL) and posterioris (NRP) comprising the paraventricular organ (PVO) of teleosts (NPO : Holmes and Ball, 1974 ; Simon and Reinboth, 1974 ; NLT : Ekengren, 1973 ; PVO : Ekengren, 1975*b*). These nuclei have received attention because they have been suggested to influence the hypophysis by nerve fibers innervating the hypophysis (Simon and Reinboth, 1974 ; Holmes and Ball, 1974 ; Baumgarten and Braak, 1967 ; Fremberg and Meurling, 1975). Contacts among the three nuclei have also been reported (Baumgarten and Braak, 1967 ; Weiss, 1970, 1976 ; Ekengren, 1973, 1975*b*). The present study deals with the distribution of cells of the NPO, NLT and PVO (NRL/NRP) and especially their relation to the hypophysis and to each other.

Materials and methods.

Animals. — Rainbow trout (*Salmo gairdneri*) and salmon (*Salmo salar*) 1-3 years old, most of them sexually mature, were supplied from hatcheries in Vaasen, Holland and Älvkarleby, Sweden, respectively. They were kept in aquaria some days before being killed.

Histological techniques. — The brains of 14 trout and 12 salmon were excised and fixed in Bouin-Hollande. After embedding, transverse and sagittal 5-7 μm sections were cut. They were stained with Gomori's chromalum-haematoxylin-phloxin, Bock's p-rosanilin-crotonaldehyde, N-N'-diethylpseudoisocyanin chloride or Herlant's tetrachrome.

12 trout and 10 salmon were used for the Falck-Hillarp technique. Some fishes of both species received an injection of nialamide intraperitoneally (25-100 mg/kg) 3 hrs before killing. The brains, with hypophyses attached, were excised, frozen in liquid propane and freeze-dried in a Pearse or a coldfinger freezedryer. The material was then treated with gaseous paraformaldehyde for 1-3 hrs, embedded in paraffin and cut into transverse, sagittal and horizontal sections of 5-7 μm. These were examined in a Zeiss fluorescence microscope.

Results.

Hypothalamic nuclei. — The localization and structure of the NPO in the two salmonids is similar to that described for other teleosts ; i.e. a pars parvocellularis and a pars magnocellularis with small and large cells, respectively, many of them liquor-contacting.

The NLT starts at the level of the horizontal commissure and extends caudally to the lateral recesses. The most prominent part is the pars lateralis with many rows of large cells. In the salmon the pars medialis is much more extensive than in the trout.

FIG. 1. — *F — H + fibers in the rostral neurohypophysis* (↗ RNH). Note some fluorescent cells. × 375.

FIG. 2. — *F — H + beaded axons and terminations in the RNH*, Prolactin follicle (P.). × 375.

FIG. 3. — *F — H + fibers in the neuro-intermediate lobe* (NIL), *with a concentration at the neuro-adeno interface* (↙). × 240.

FIG. 4. — *PIC + fibers in the RNH.* × 200.

FIG. 5. — *F — H + fibers in the NIL.* × 375.

FIG. 6. — *PIC + fibers in the NIL.* × 375.

FIG. 7. — *F — H + fibers running through the NLT.* × 150.

FIG. 8. — *PIC + fibers from the NPO passing throogh the NLT* (↙). × 150.

FIG. 1-3,5,7 : - - - - - - - Falck-Hillarp reaction (F-H) ; FIG. 4,6,8 : - - - - - - - - Pseudoïsocyanin reaction (PIC).

FIG. 1 is from trout and the others are from salmon.


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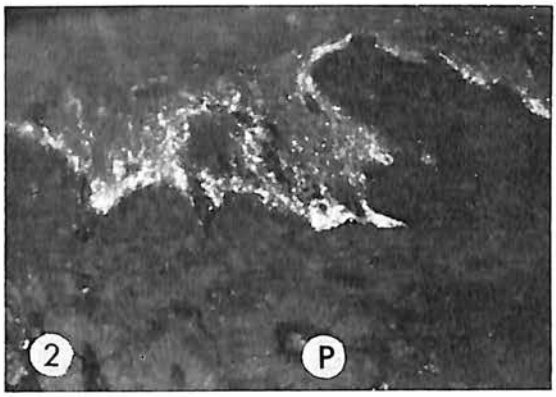
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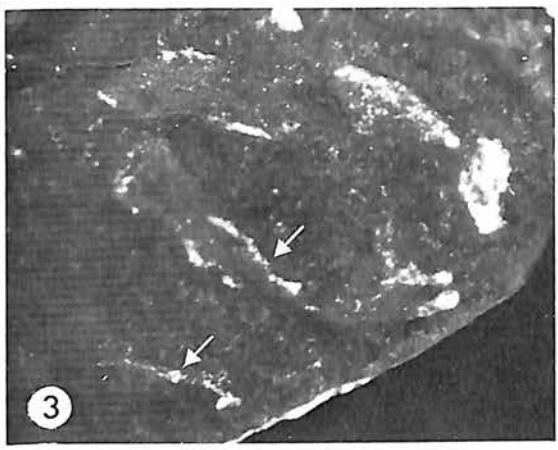
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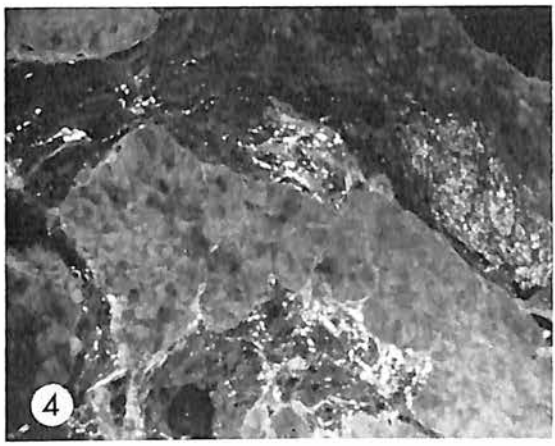
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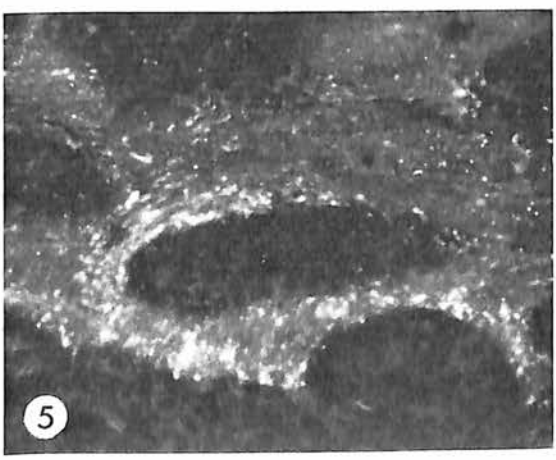
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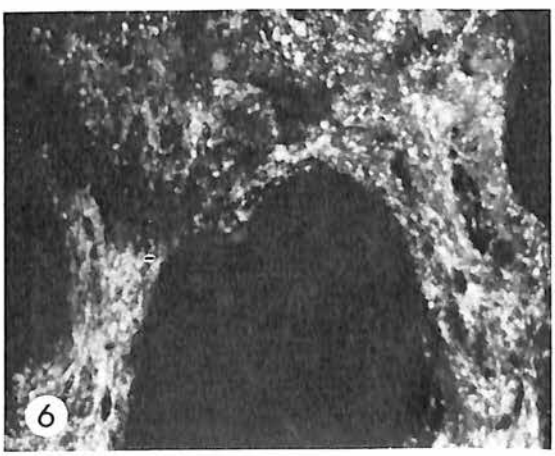
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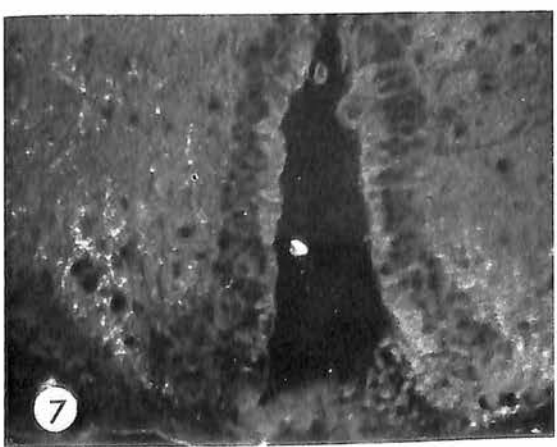
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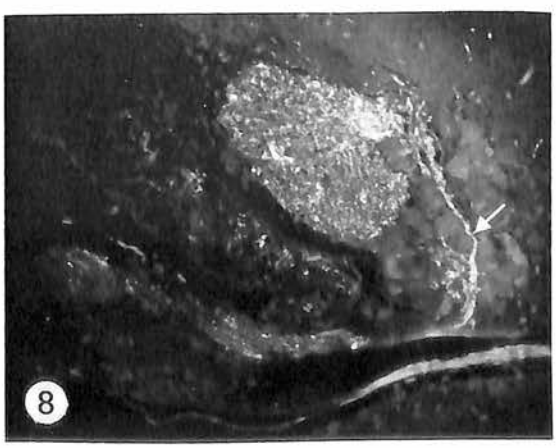
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The Falck-Hillarp technique revealed fluorescent cells on both sides of the ventricular system comprising the complex of the NRL and NRP. The localization is in agreement with that described for other teleosts.

Innervation of hypophysis. — After treatment according to the Falck-Hillarp method, fluorescent fibers were seen in all parts of the neurohypophysis (fig. 1, 2, 3, 5). The distribution was the same for both species, but the intensity was much lower in the trout (fig. 1). Fluorescent cells (fig. 1) were only found in the trout. Pseudoisocyanin-positive (PIC +) fibers were found in both the rostral neurohypophysis (fig. 4) and in the neuro-intermediate lobes (NIL) (fig. 6). The distribution of aminergic and PIC+ fibers in the NIL are strikingly similar (fig. 5, 6).

Innervation of hypothalamic nuclei. — The NPO was found to have a high input of fluorescent fibers after the Falck-Hillarp reaction. They very probably originate in the NRL/NRP. PIC + fibers from the NPO pass between and close to the cells of the NLT pars lateralis (fig. 8) and contacts were indicated. A few Falck-Hillarp-positive fibers rostrally also invade this part of the NLT (fig. 7), but most fibers are close to cells of the medial part of the nucleus.

Discussion.

The general composition of the NPO coincides well with descriptions of other teleosts (Holmes and Ball, 1974 ; Simon and Reinboth, 1974). This is also true of the NLT (Ekrengren, 1973), except that the medial part is more extensive in the salmon. The general outline of the PVO is also in agreement with other descriptions (Ekengren, 1975*b*).

As in most species the majority of the NPO fibers terminate in the neuro-intermediate lobe (Simon and Reinboth, 1974), but as in a few species some fibers end in the rostral neurohypophysis (Båge *et al.*, 1974*a*, *b*). The entire neurohypophysis receives a strong aminergic innervation, which has been described for a few species (Ekengren, 1975*a* ; Fremberg and Meurling, 1975). Fluorescent cells were only present in the trout hypophysis, a feature described for just a few species (Weiss, 1970 ; Båge *et al.*, 1974*b*). It is for the moment uncertain which cell type is reacting, but more work is planned.

The presence of aminergic fibers in close contact with NPO cells points to an aminergic innervation of the nucleus. This is in accordance with results from other teleost fishes (Baumgarten and Braak, 1967 ; Weiss, 1970 ; Ekengren, 1973). To trace the origin of these fibers lesion experiments will be carried out.

An interesting result of this study was the high amount of both NPO and aminergic fibers passing between the cells of the NLT complex. A similar pattern has been described for the brook trout (*Salmo trutta fario*) by Weiss (1976), and for the roach (*Leuciscus rutilus*) (Ekengren, 1973). These findings must be considered when lesions are made in this area.

The contact among the three hypothalamic nuclei as well as their contact with the hypophysis suggests interesting functional interactions.

Conclusions.

All parts of the neurohypophysis have a more or less rich innervation from the NPO, and some aminergic nucleus, possibly the PVO. Aminergic cells are possibly present in the pituitary of trout. The NPO receives many aminergic fibers, very probably from the PVO. The NLT has a high input of NPO and aminergic fibers, the latter possibly originating in the PVO. All these contacts indicate a functional relationship among the three hypothalamic nuclei and also with the hypophysis.

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Résumé. Les noyaux pré-optiques (NPO), latéral du tuber (NLT), et l'organe paraventriculaire (PVO), supposés responsables de la production des facteurs adénohypophysiotropes et concernés dans la fonction de reproduction, ont été étudiés chez 2 salmonidés. Nous nous sommes intéressés aux corps cellulaires des neurones et particulièrement à leurs contacts axonaux avec l'hypophyse.

La technique de Falck et Hillarp permet la mise en évidence d'une grande quantité de fibres aminergiques dans toute la neurohypophyse. Seules de petites différences entre les différentes parties de la neurohypophyse et entre les 2 espèces ont pu être notées. Ces fibres semblent prendre naissance dans l'organe paraventriculaire. La technique à la pseudoisocyanine permet de montrer qu'il existe à tous les niveaux de la neurohypophyse des terminaisons de fibres issues du noyau NPO, la plus grande densité s'observant au niveau du lobe nerveux-intermédiaire. Des cellules fluorescentes pouvant être aminergiques ont été trouvées chez la truite. Le NPO reçoit un grand nombre de fibres aminergiques pouvant provenir de PVO. Les cellules de la partie médiane du NLT reçoivent de nombreuses fibres aminergiques ; de plus des fibres originaires du NPO passent très près de ces cellules. Cela indique l'existence d'une riche double innervation des cellules du NLT. Ces résultats suggèrent donc une relation fonctionnelle entre les 3 noyaux hypothalamiques, et également à l'hypophyse.

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Use of immunofluorescence for localization of somatostatin-like antigen in the rainbow trout (*Salmo gairdneri*). Comparative distribution of LH-RF and neurophysin

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Summary. A somatostatin-like antigen has been localized in the brain and the digestive tract of the rainbow trout. In the brain, SRIF perikarya were observed scattered throughout the hypothalamus or present in definite hypothalamic nuclei (i) nearly all the Gomori negative perikarya of the NPP (Peter and Gill, 1975) appeared to react with anti-SRIF but not at all with anti-neurophysin (ii) a small unidentified nucleus was present in the dorsomedial hypothalamus and showed a few SRIF-containing perikarya (iii) many SRIF cells were scattered in the rostral and median peri-infundibular areas of the NLT (iv) in the wall of the 3rd ventricle, hypendymocytes next to the upper part of the NPO did not react uniformly to anti-SRIF. Axonal endings containing SRIF, LH-RF and neurophysin followed the digitations of the pars nervosa that enter the adenohypophysis. LH-RF and SRIF fibers followed the same routes and terminated only in the mesoadenohypophysis ; neurophysin fibers end only in the meta-adenohypophysis. At the periphery, SRIF cells were observed in Langerhans islets of the endocrine pancreas and in the mucosa of the gastro-duodenal duct.

Abbreviations used : LH-RF : Luteinizing Hormone-Releasing Factor ; SRIF : Somathormone Release Inhibiting Factor ; TSH-RF : Thyroid Stimulating Hormone-Releasing Factor ; NPO : Nucleus Preopticus ; NLT : Nucleus Lateral Tuberis ; NPP : Nucleus Preopticus Periventricularis.

Introduction.

If somathormone release inhibiting factor (SRIF) does not directly control the gonadotropic hormones release in mammals (Besser *et al.*, 1974 ; Wale *et al.*, 1975), it interferes in the physiology of their reproduction by the way of prolactin (Davis, 1975 ; Davis and Anfinson, 1975), this hormone operating on the LH-RF release as reviewed by Leonardelli (1977).

In this prospect, we tried to determine in this report immunofluorescent localization of hypothalamic centers the cells of which synthesize SRIF. Results on the rainbow trout are presented.

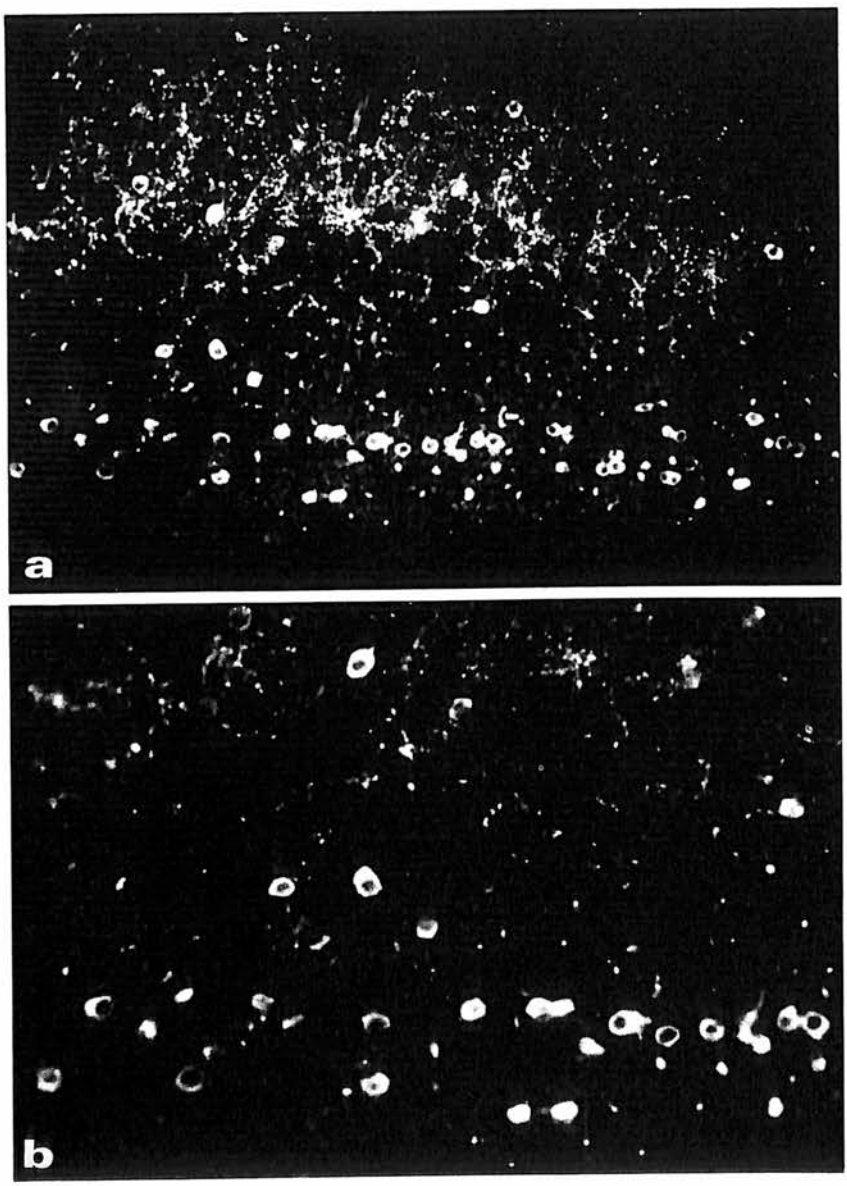

FIG. 1. — *Trout brain. Parasagittal section close to the symmetric plane of the brain showing the median peri-infundibular area in the hypothalamus. a*) SRIF immunoreactive cells scattered among non-reacting parvocellular perikarya of the NLT. At its periphery, a dense network of SRIF fibers surrounds the periventricular grey substance. × 175. *b*) The same field showing immunoreactive perikarya at a higher magnification. × 175.

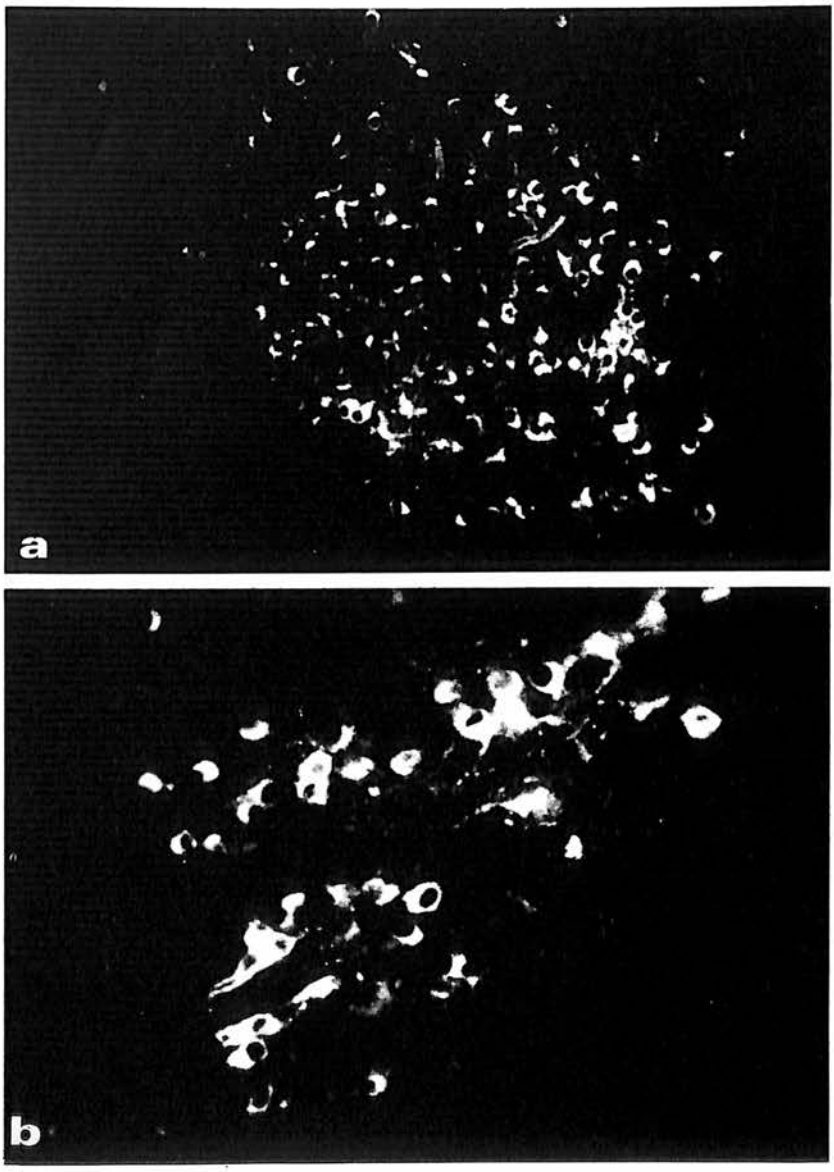

FIG. 2. — *Trout brain. More lateral parasagittal section than the former. Hypothalamic suprachiasmatic area.* NPP (Peter and Gill, 1975). *a*) Many immunofluorescent perikarya reacting with SRIF seen at a low magnification. × 175. *b*) Detail of a field at the periphery of the NPP seen at a higher magnification. × 300.

Material and methods.

Brain and other tissue samples were obtained from adult male or female rainbow trouts killed at different times of the year. They were fixed with Bouin-Holland and embedded in paraffin. Rehydrated sections of 5 μ thickness were studied by immunofluorescence using the indirect method and a counter stain of 0.01 p. 100 Evans Blue. The preparation and specificity of rabbit anti-sera used (anti-SRIF, anti-LH-RF, anti-bovine neurophysin) have been reported previouly (Dubois, 1975 ; Dubois, 1976*a* ; 1976*b* et 1978 ; Leonardelli *et al.*, 1973 ; Dubois and Barry, 1974).

Results.

Encephalic localization of SRIF immunoreactive perikarya and axonal endings.

Perikarya. — Perikarya appear isolated and scattered throughout the hypothalamus, grouped in well-individualized hypothalamic nuclei, or scattered over the periventricular grey nucleus lateral tuberis (NLT) substance. They are small uni- or bipolar neurons ; their nucleus (10 to 12 μ ∅) is surrounded by a thin halo of immunoreactive cytoplasm.

In the suprachiasmatic area in front of the hypothalamus, two well-condensed symmetric nuclei surround the supra optic recess of the 3rd ventricle at the periphery of the rostral end of each nucleus preopticus (NPO) (fig. 2). This is well demonstrated using an anti-bovine neurophysin antiserum. The nuclei are well separated from the NPO, and do not react with anti-neurophysin. According to the nomenclature of Peter and Gill (1975), they would correspond to the Gomori negative nucleus preopticus periventricularis (NPP), distinct from Gomori positive NPO.

Two symmetric cell groups containing a few cells appear in the dorsomedial hypothalamus at a distance from the wall of the 3rd ventricle. They have not been related to well-defined nuclei.

Many immunoreactive cells are scattered in the NLT, close to the ventricular cavity in the rostral and the median peri-infundibular areas (fig. 1). A dense network of SRIF immunoreactive fibers surrounds the periventricular grey substance when their perikarya are present.

Many SRIF immunoreactive hypendymocytes may be seen at times next to the upper part of the NPO.

Axonal endings. — The axons end in the pituitary. Their dense network is observed in the digitations of the pars nervosa only distributed in the mesoadenohypophysis (fig. 3). The LH-RF immunoreactive fibers follow the same route (fig. 4) ; they are very scarce, while axonal endings reacting with anti-neurophysin are only seen in the ramifications of the pars nervosa distributed in the meta-adenohypophysis.

Peripheric localization of SRIF cells.

As seen in other classes of vertebrates, SRIF cells are observed in the Langerhans islets of the endocrine pancreas ; they are already numerous in the 1-day old hatched

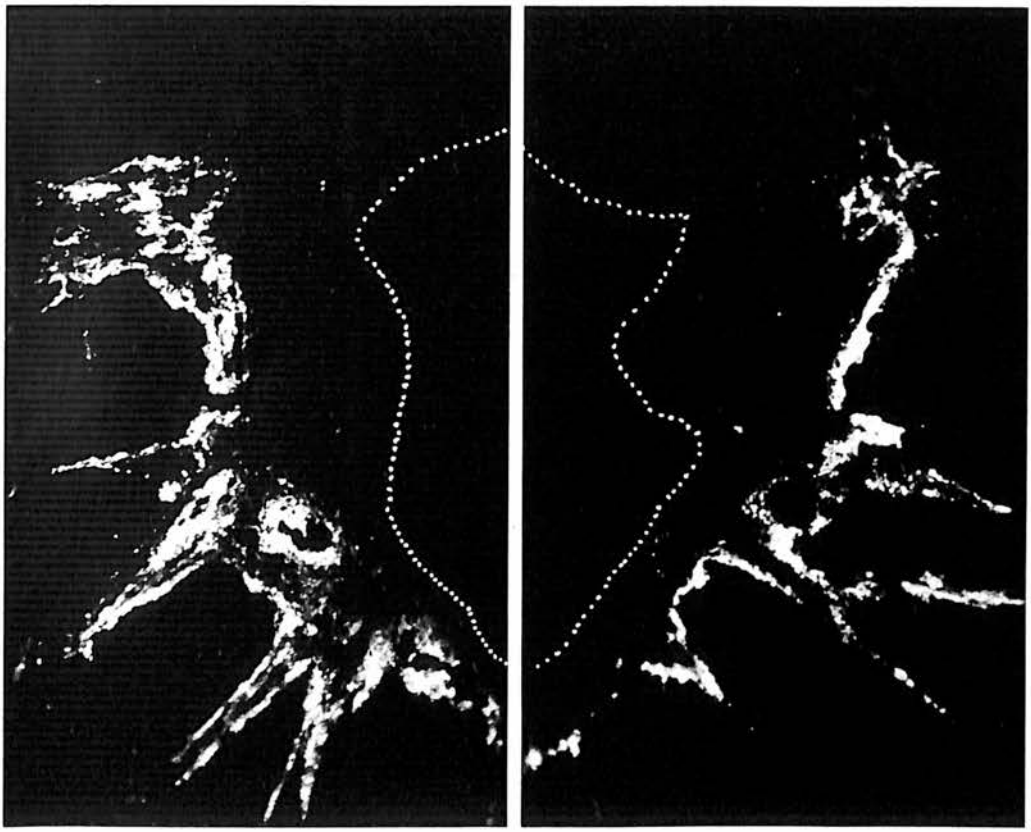

FIG. 3. — *Trout brain. Cross-section in a plane perpendicular to the sagittal axis of the brain.* Infundibulum and pituitary (pars nervosa). SRIF immunoreactive fibers are distributed only in the symmetric ramifications of the pars nervosa entering the mesoadenohypophysis. Dotted line shows the limit of the infundibulum. × 175.

trout. These cells are also present in the anterior part of the gastro-intestinal duct where they are scattered in the digestive mucosa.

Discussion.

The immunocytological pattern of the hypothalamic centers systematized above appears to differ, depending on the various physiological conditions of the individuals investigated at different times of the year. Therefore, to obtain a representative picture of SRIF cell distribution, we had to include all the observations made during the year, not taking into account the physiological state of the individuals. Particular aspects of these different centers in relation to definite physiological or experimental situations will be reported later.

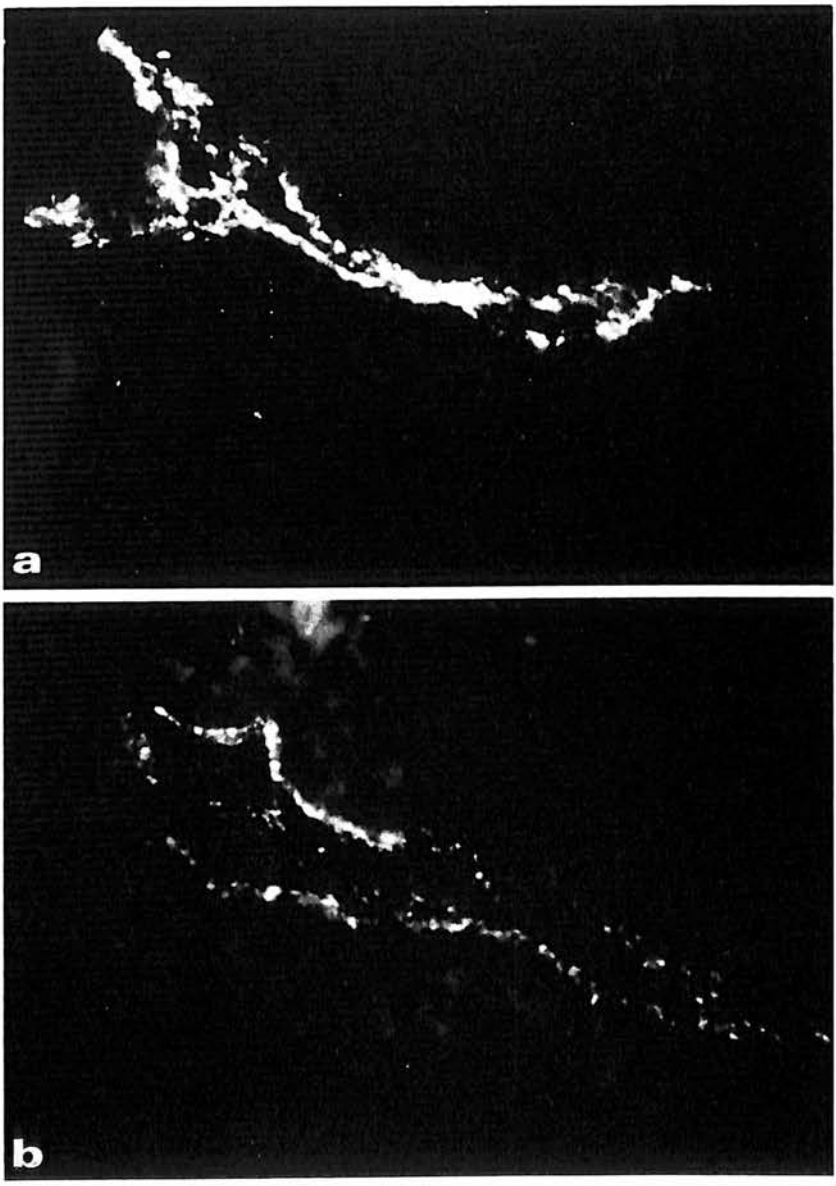

FIG. 4. — *Trout brain. Pituitary.* Comparative distribution of SRIF and LH-RF immunoreactive axons in the ramifications of the pars nervosa that enter the mesoadenohypophysis showing SRIF and LH-RF fibers following the same route. *a*) SRIF fibers, *b*) LH-RF fibers. × 450.

The number of SRIF cells, the intensity of their immunofluorescent reaction, and the size of given immunoreactive cell groups appear to vary considerably depending on the physiological state of the individuals. These variations are unrelated to the intensity of the reaction observed in axonal endings. This discrepancy confirms the hypothesis postulating the rarity (or absence) of immunoreactive neurosecretory cells observed in vertebrates in various physiological states. This paucity might be essentially due to a too low concentration of the neuropeptide in those cells, which would be the result of either a high axonic flow, low-level SRIF synthesis, or both these factors (Dubois, in press).

Peter and Gill (1975) separated the NPP from the NPO according to the difference observed with paraldehyde Fuchsin staining. This distinction agrees with the presence of neurophysin in the NPO and its absence in the NPP.

In the adult trout, hypendymal cells reacting with anti-SRIF are observed in undefined conditions. Such hypendymocytes have been continuously seen in mammalian fetus (Dubois, 1976*a* ; 1976*b*, 1978).

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Résumé. Un antigène « somatostatine-like » a été localisé dans le cerveau et le tractus digestif de la truite Arc-en-ciel. Dans le cerveau, les péricaryons SRIF+ sont épars dans l'hypothalamus ou localisés dans des noyaux hypothalamiques bien définis : i) presque tous les péricaryons Gomori négatifs du NPP réagissent avec l'anti-SRIF ; ii) un petit noyau non identifié dans l'hypothalamus dorso-median présente quelques péricaryons contenant du SRIF ; iii) de nombreuses cellules à SRIF+ apparaissent éparses dans les aires péri-infundibulaires médianes et rostrales du NLT ; iv) dans la paroi du 3e ventricule, les hypendymocytes près de la partie supérieure du NPO ne réagissent pas uniformément à l'anti-SRIF. Les terminaisons axonales contenant du SRIF, du LH-RF et de la neurophysine suivent les digitations de la pars nervosa qui pénètrent dans l'adénohypophyse. Les fibres LH-RH et SRIF+ se terminent seulement dans la mésoadénohypophyse, tandis que les fibres à neurophysine se terminent seulement dans la métaadénohypophyse. A la périphérie, des cellules SRIF+ sont observées dans les îlots de Langerhans du pancréas endocrine et dans la muqueuse du conduit gastro-duodénal.

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Scanning cytophotometry : A method for studying the amount of neurosecretory material in the preoptic nucleus of the trout (*Salmo gairdneri*)

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Summary. In order to study the activity of the preoptic nucleus (NPO) during the annual cycle, the amount of neurosecretory material was measured by means of scanning cytophotometry. This objective method implies standardization of the histological method and computerized procedures for the analysis of the data. Preliminary results of such studies on the NPO of sexually mature male rainbow trout, together with cytological information, point to cyclical changes in the activity of the nucleus. An active period was indicated from the beginning of June to January and an inactive period from February to June.

Introduction.

The hypothalamic regulation of the pituitary gland in salmonids is controlled by at least 3 types of neurosecretory systems, i. e. the peptidergic AF-positive *nucleus preopticus* (NPO), the peptidergic AF-negative *nucleus lateralis tuberis* (NLT) and the monoaminergic *nucleus recessus lateralis* and *nucleus recessus posterioris*. Fibers are projected from these centers onto the hypophysis. The NPO fibers mostly terminate in the caudal neurohypophysis, whereas only a few of them seem to end in the rostral neurohypophysis close to the *pars distalis*. Although the NLT fibres are difficult to trace, they are assumed to end primarily at the rostral neuro-adenohypophysial interface. In the Atlantic salmon and the rainbow trout, monoaminergic fibres have also been demonstrated at the neuro-adenohypophysial interface. The NLT is assumed to control the activity of the gonadotropic cells in the hypophysis. This is based on correlative changes in the activity of the NLT during the reproductive cycle, and on the effect of castration and lesion experiments (Peter, 1970 ; Zambrano, 1971 ; Holmes and Ball, 1974). However, changes in the amount of neurosecretory material (NSM) in the NPO, which can be correlated to phases in the reproductive cycle are indications that the NPO is involved as well (Ball and Baker, 1969 ; Holmes and Ball, 1974). An indirect role of the NPO via the NLT was strongly advocated by Weiss (1976) and Ekengren and Terlou (1978), because NPO fibres running towards the pituitary are in direct contact with cell bodies of the lateral part of the NLT. For these

reasons, it seemed worthwhile to include the NPO in studies on hypothalamic control of pituitary function in relation to reproductive processes. In the present study the amount of NSM is used as a criterium to quantify the activity of the NPO during the annual cycle.

As visual estimates are rather irregular and subjective, the measurements have been carried out by scanning cytophotometry. The regions of the NPO were measured separately for differential analytical purposes.

Materials and methods.

Three-year old sexually mature male trout (*Salmo gairdneri*) were obtained from a hatchery in Vaassen (The Netherlands). Before decapitation the animals were anesthetized with MS 222 (0.035 p. 100) or carbon dioxide gas. The brain was dissected out, fixed in Bouin-Hollande for 2-3 days, dehydrated, and embedded in paraffin wax. Bieniarz (1974) described a circadian rhythm in the amount of NSM in the NPO, and for that reason the animals were fixed between 13.30 and 15.00 hrs. A total of 10 specimens was used throughout the annual cycle (see table 1). Transverse sections of 6 microns were cut and every 11th section was mounted on an object glass. To demonstrate the presence of NSM, the sections were stained with p-rosanilin-crotonaldehyde according to Bock and Ockenfels (1970 ; p-rosanilin instead of Diaminobenzophenon). For quantification studies this method has the advantage of not requiring a differentiation step. In order to obtain mutually comparable material and to minimize changes due to histological procedures, the following precautions were taken : (1) sectioning was carried out with one and the same microtome, (2) after mounting the sections from all animals, oxydation was carried out before staining (oxydation time : 2 min.) ; the oxydation step was standardized by refreshing one-third of the Gomori mixture for every subsequent rack of 10 slides, (3) staining was carried out with one batch of dye (staining time : 1 min.).

NSM measurement. — The light transmission in tissue containing the NPO was determined by means of a Zeiss scanning cytophotometer and with monochromatic light of 620 nm. This wavelength produces the highest absorption of p-rosanilin-stained tissue components and prevents interference of the phloxin counterstain. Scanning was carried out in meander mode, measuring adjacent (not overlapping) square areas of 36 μm^2. The background transmission value was set at 100 p. 100 for a representative and homogeneously structured area lateral to the NPO. In order to analyse the amount of NSM in different parts of the NPO, areas with different morphological cell types were measured separately. The papertape with cytophotometer data was fed into a computer. The first computer program included the selection of values (all values higher than 96 p. 100 transmission were rejected), frequency distribution over 20 transmission classes (ranging from 0-100 p. 100), and the conversion of transmission into extinction values. The extinction is linearly proportional to the concentration of NSM. All procedures are carried out per area. The results were printed and punched on cards, which in the next program were combined per left/right half of the section, because most of the transverse sections were not perfectly symmetrical. The results per left/right section were printed and plotted in 3 graphs : 1) total extinc-

tion per left/right section versus longitudinal axis of the brain (= NPO), giving the distribution of the amount of NSM over the whole NPO ; 2) mean extinction versus longitudinal axis of the brain ; 3) cumulative percentages of the size of the 20 transmission classes versus the longitudinal axis of the brain (fig.). Graphs 2 and 3 show the density distribution of NSM in the whole NPO.

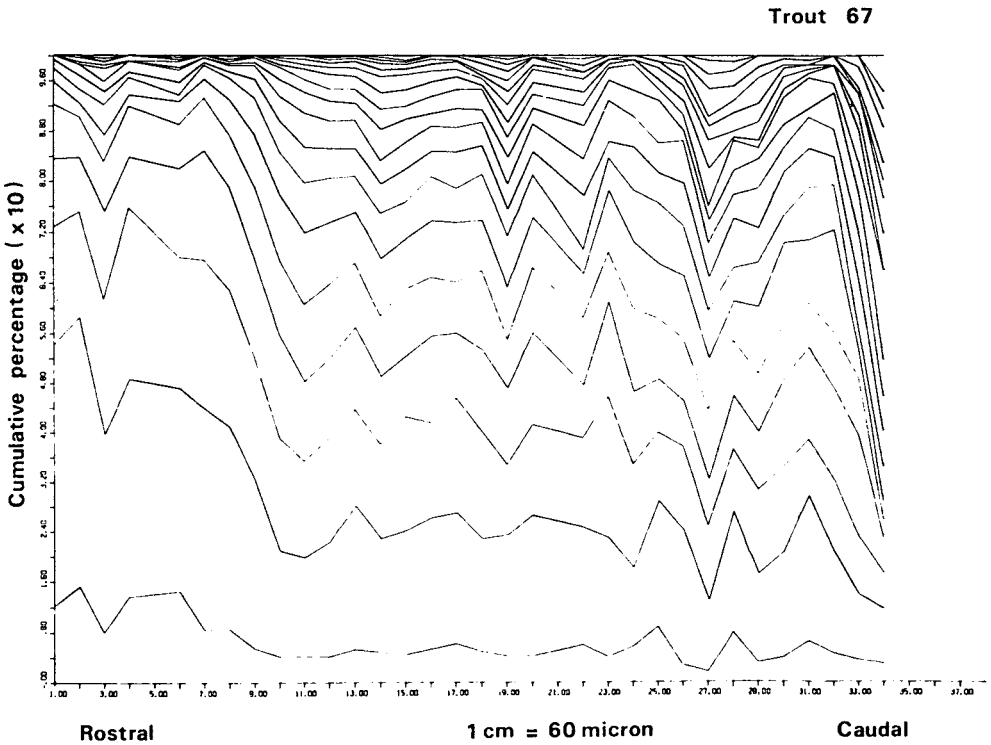

FIG.

Results.

The amount of NSM of the whole NPO, as represented by the total extinction, varies during the annual cycle just as the mean extinction (table 1). The NSM was high at the beginning of June, decreased during autumn (except in trout 21) and increased during January and February (spawning period). After February, the NSM decreased again, and increased in June. The mean extinction reveals a similar pattern.

Visual examination of the sections showed that the preoptico-hypophysial tract originating from the NPO was filled with NSM from June to November. In this period the NPO cells contained active, swollen nuclei, with evenly distributed chromatin ; the nuclei contained a few (2-8) small nucleoli. In November NSM in the tract had diminished, and this was accompanied by a reduced activity of the nuclei in the NPO

cells. In January, the activity of the cells increased and more NSM was observed in the tract. From February to June, the NPO-cells were distinctly less active. The nuclei contained one or two large nucleoli, and the chromatin was irregularly distributed.

TABLE 1

Changes in the amount of neurosecretory material measured by scanning cytophotometry at various periods of the reproductive cycle of male rainbow trout

Trout	Date	Total extinction	Mean ext.
5	3-6-75	34 270	0.128
15	7-8-75	16 339	0.083
16	7-8-75	18 164	0.077
21	24-9-76	51 856	0.145
31	19-11-75	14 945	0.069
40	7-1-76	40 329	0.093
46	25-2-76	42 928	0.115
53	25-2-76	26 104	0.075
61	5-5-76	13 081	0.055
67	9-6-76	44 043	0.153

The tracts did not contain any noticeable NSM. In June, the tracts were filled again and the cells reactivated. The analysis of the different parts of the NPO has not been finished yet. Differences in the quality of the NSM, however, have already been determined. As an example, the figure shows the cumulative percentages of consecutive classes of the frequency distribution versus the longitudinal axis of the brain of trout 67. The class with the lowest density is situated near the X-axis and the class with the highest density at the top of the graph. Evaluation proved the NPO to be composed of 3 regions : 1) section nr. 1-10 : NSM is present in low densities ; in section nr. 7 and the following sections NSM is found in progressively higher density classes, 2) section nr. 11-18 : NSM in higher densities, rather evenly distributed in the density scale, 3) section nr. 18-34 : NSM is characterized by a changing composition ; most of the highest densities are found in this part of the NPO. This distribution corresponds to the regional distribution of cell types : rostrally the *pars parvocellularis* (with medium-sized magnocellular cells in the middle part) and dorso-caudally the *pars magnocellularis* with the largest cells of the NPO.

Discussion.

Scanning cytophotometry has proved to be an accurate and objective method for determining the amount of NSM in the NPO. The present study, based on experiments with a limited number of test animals, points to cyclic changes in the activity of the NPO during the year. As a result of synthesis and release, the amount of NSM together with cytological information (nucleus size, chromatin distribution and nucleolus morphology) indicates an active period from the beginning of June to January, with a possible decrease in November, and an inactive period from February to June. Addi-

tional work is in progress to complete and confirm these results, which do not wholly agree with those of Sterba and Weiss (1968). A visual estimate of the amount of pseudoisocyanin-positive NSM in the NPO of the brook trout indicates an annual cycle with a maximum in November, December and January (spawning period), and a gradual leveling off until August, followed by a rise until November. Sterba and Weiss did not observe a marked increase in June. The presently observed maximum during the spawning period seems to be a common feature which was noticed also in other salmonid species by Arvy *et al.* (1959) and Barannikova (1961, 1963). In addition, those authors reported a correlation between changes in the amount of NSM and changes in salinity of the water and in thyroid activity, which together suggest a multifunctional role of the NPO. Results of the present study do not contradict this hypothesis. Based on a qualitative analysis of NSM, three different areas can be distinguished in the NPO, each containing a morphologically different cell type. It is known that arginin, vasotocin and isotocin are formed in the NPO (see Perks, 1969). Immunofluorescence indicates these hormones to be produced in different cell types, each with a preferential localization in the NPO (Dierickx and Goossens, pers. commun.). Whether other hormones are synthesized in the NPO, e. g. somatostatin (see, Doerr-Schott and Dubois, 1977), must be investigated. The morphological relation of the NPO directly in the pituitary, or indirectly via other hypothalamic nuclei (e. g. NLT), finally determines the potential of the nucleus to regulate different hypophysial functions. The relations between NPO activity and various endocrine processes should be experimentally investigated.

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Résumé. Afin d'étudier l'activité du nucleus préoptique (NPO) de truites Arc-en-ciel mâles pendant le cycle annuel, la quantité de matériel neurosécrétoire a été mesurée au moyen de cytophotométrie à balayage. Cette méthode objective a demandé la standardisation du procédé histologique et l'intervention d'un ordinateur pour l'analyse des valeurs obtenues. Des résultats préliminaires de telles études du NPO, liés avec des données cytologiques, indiquent des changements cycliques dans l'activité du NPO. Une période active a été trouvée dès le début du mois de juin jusqu'à janvier et une période inactive de février à juin.

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Immunohistochemical cross-reaction of anti-mammalian LH-RH in lower vertebrates

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Summary. The double antibody immunofluorescence method has been applied to demonstrate LH-RH in several amphibian and teleost species. In all amphibians tested, perikarya and axons running towards the median eminence could be visualized using anti-mammalian-LH-RH. In the teleost species investigated so far, only the trout *Salmo gairdneri* showed a positive reaction. It is argued that this is caused by lack of cross-reactivity in the other species rather than by insufficient sensitivity of the method.

Introduction.

In fish and amphibians the gonadotropic function of the pituitary is controlled by the hypothalamus. In teleosts this hypothesis is mainly based on observations of correlative changes in environmental factors, hypothalamic nuclei and adenohypophysial activity (Peter, 1970 ; Ball *et al.*, 1972). In addition, neurosecretory fibres, originating from the hypothalamus, are known to terminate on glandular cells of the adenohypophysis, on neighbouring capillaries, and on membranes of intracellular spaces (Zambrano, 1972 ; Peute *et al.*, 1976). These observations and the results of lesioning experiments (Peter, 1970) focussed attention on the *nucleis lateralis tuberis* (NLT) as the source of a gonadotropin releasing hormone (GRH) in teleosts. Similar results were obtained with amphibians. By isolating the ventro-caudal region and the adjacent pituitary from the rest of the hypothalamus in *Rana temporaria*, Dierickx (1966, 1967) proved that the ventral *tuber cinerium hypothalami* is involved in the central regulation of the pituitary. That area contains the peptidergic, Gomori-negative *nucleus infundibularis ventralis* (*NIV*). In *Rana esculenta*, it is one of the cell types in this nucleus which after castration or testosterone treatment shows correlative changes with the gonadotropic activity of the adenohypophysis (Peute and Mey, 1973). Consequently, this cell type may produce GRH. Definite conclusions about the cellular origin of GRH cannot be drawn until this hormone is demonstrated directly within the perikarya and in the axons ending in the neurohypophysis. For this purpose immunohistochemical methods can be applied, provided that pure antigen is available.

Amphibian and fish GRH has not been isolated and purified yet. The only GRH available at present is mammalian LH-RH. A number of experimental data indicate

that mammalian LH-RH is biologically active in amphibians and fish. Some of the results (see discussion) already make it doubtful that mammalian LH-RH is identical to the GRH of lower vertebrates ; this is supported by the results of radioimmunoassay of fish hypothalamic extracts and by the present results.

The aim of the present study was to ascertain whether antimammalian LH-RH shows histo-immunological cross-reaction with material in the brain of amphibian and teleosts species.

Material and methods.

Experimental animals. — Sexually mature specimens have been used.

Amphibians : male and female green frogs (*Rana esculenta*). We examined no other amphibians, but compared our results with published information on other anurans.

Teleosts : male and female trout (*Salmo gairdneri*) ; barbels (*Barbus conchonius*) ; head-and-tail-lights (*Hemmigrammus caudovitatis*) and the goldfish (*Carassius auratus*).

Preparation of the tissues. — All tissues were fixed in buffered Bouin's fluid without acetic acid.

Antibodies. — Antibodies were raised against synthetic LH-RH (*Bochem-California*) conjugated to bovine serum albumine according to the method of Jeffcoate *et al.* (1973). The presence of antibodies against LH-RH was tested by means of radioimmunoassay and agar diffusion.

Immunofluorescence. — The double antibody technique was used, the first antibody being the anti-LH-RH preparation, the second a commercial sheep anti-rabbit globulin, labelled with FITC (NBC or Nordick). If unspecific fluorescence was observed, both the first and the second antiserum were treated with liver homogenates from the species tested to avoid further unspecific binding of antibody to non-LH-RH tissue components.

For a more detailed description of material and methods, see Goos *et al.* (1976) and Goos and Murathanoglu (1977).

Observations.

In *Rana esculenta*, perikarya reacting with anti-LH-RH were found in an unpaired nucleus, situated immediatly in front of the preoptic recess. Axons from these cells form a narrow single tract, which passes underneath the preoptic recess. Behind the recess the tract divides into two bundles running between the preoptic nucleus and the optic chiasma, in a caudal direction. Just before entering the median eminence this tract splits up into numerous individual fibres apparently ending near the capillaries in the outer zone (Goos *et al.*, 1976). In *Hemigrammus caudovitatus*, *Barbus conchonius* and *Carassius auratus* no positive reaction has been observed until now in any part of the brain. In *Salmo gairdneri*, however, numerous immunoreactive fibres are running diffusely in the lateral walls of the diencephalon and ending dorsally of the pituitary

stalk. Small oval or round perikarya with immunoreactive cytoplasm and a protrusion, which is apparently the beginning of an axon, were found in the *area dorsalis partis medialis* of the telencephalon (Goos and Murathanoglu, 1977).

Discussion.

Mammalian LH-RH is known to be biologically active in a number of fish species. Deery (1973) and Deery and Jones (1975) proved that synthetic LH-RH causes an activation of the adenylcyclate system in the gonadotropin-containing ventral lobe of the pituitary of the dogfish, *Scyliorhinus canicula*. Gonadotropin secretion was obtained in the carp, *Cyprinus carpio* (Breton and Weil, 1973) and in the brown trout, *Salmo trutta* (Crim and Cluett, 1974) ; ovulation was induced in the ayu, *Plecoglossus altivelis* (Hirose and Ishida, 1974) and in the goldfish, *Carassius auratus* (Lam *et al.*, 1975) ; depletion of small electron-dense granules from the gonadotropic cells, together with ovulation was reported by Lam *et al.* (1976) for the goldfish.

There are, however, several arguments for considering teleost GRH to be different from mammalian LH-RH. Breton and Weil (1973) found that carp hypothalamic extract stimulates gonadotropin secretion in the carp with a different time sequence, when compared to synthetic LH-RH. Extracts of the hypothalamus of the goldfish did not appear to have any immunological cross-reaction with synthetic LH-RH when tested by radioimmunoassay (Deery, 1974).

The present results show that from the fish tested only the trout, *Salmo gairdneri*, has a substance that is immunoreactive with antimammalian LH-RH. It might be possible that the other species indeed contain this substance but in minute amounts ; the failure to demonstrate it than could due to lack of sufficient sensitivity of the applied method. However, since not a single immunoreactive axon, nerve ending or perikaryon has been observed this failure is more likely caused by lack of immunological cross-reaction. On the other hand, it can not be excluded that if these teleost species had been checked during different phases of their reproductive cycle, or after castration or hypophysectomy, more positive results could have been obtained.

In the frog, *Rana pipiens*, mammalian LH-RH has been demonstrated to be biologically active (Thornton and Geschwind, 1974). In all species tested so far, antimammalian LH-RH has an immunological binding to perikarya, axons and nerve endings in the forebrain and neurohypophysis. This applies to *Rana pipiens* and *Rana catesbiana* (Alpert *et al.*, 1976) ; *Xenopus laevis* (Doerr-Schott and Dubois, 1976) ; *Bufo vulgaris* (Doerr-Schott and Dubois, 1975) ; *Rana esculenta* (Goos *et al.*, 1976). Moreover, Deery (1974) demonstrated that in radioimmunoassay with anti-mammalian LH-RH, hypothalamic extracts of *Xenopus laevis* did have an immunological binding.

The available information indicates that a GRH-like system must be present in lower vertebrates, i. e. fish and amphibians. There is no conclusive evidence for mammalian LH-RH being similar to such a substance in lower vertebrates, but amphibian GRH seems to have a closer resemblance to mammalian LH-RH than to the corresponding hormone in fish. Given the many failures in demonstrating GRH in fish by using anti-mammalian LH-RH, it is obvious that isolation, purification and synthe-

tizing of fish GRH is a prerequisite for studying the hypothalamic regulation of GTH production in these vertebrates.

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Résumé. La technique d'immunofluorescence par double anticorps a été utilisée pour mettre en évidence la LH-RH dans plusieurs espèces d'Amphibiens et de Téléostéens. Chez les Amphibiens étudiés, les périkaryons et les axones allant vers l'éminence médiane peuvent être visualisés en utilisant un anti LH-RH mammalien. Parmi les Téléostéens étudiés, seule la Truite *Salmo gairdneri* a montré une réaction positive. Il est probable que ce n'est pas la sensibilité de la méthode qui est en cause, mais l'absence d'immunoréactivité vis-à-vis de l'anti LH-RH des facteurs hypothalamiques des autres espèces étudiées, d'origine mammalienne.

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Pituitary response to LH-RH at different stages of gametogenesis in the rainbow trout (*Salmo gairdneri*)

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Summary. Pituitary response to LH-RH has been studied in the male and female rainbow trout (*Salmo gairdneri*) at different stages of gametogenesis. The male was hardly sensitive at the onset of spermatogenesis, but a response occured at the spermatid stage and continued during spermatogenesis and spermiation. We particularly studied the end of the female cycle. Response was low in immature fishes and in the early stages of oocyte maturation, but became stronger at vitelline maturation. The relationships of these responses to plasma and pituitary t-GTH levels are discussed.

Introduction.

Hypothalamic control of gonad activity (Peter, 1970), and especially of gonadotropin secretion *in vitro* (Breton *et al.*, 1971*b*), has been demonstrated in teleost cyprinid fish. Synthetic LH-RH causes gonadotropin hormone release *in vivo* in the carp (Breton et Weil, 1973), trout (Crim and Cluett, 1974) and goldfish (Crim, Peter and Billard, 1976). However, the pituitary response *in vivo* to LH-RH differs, and depends on the time of the reproductive cycle in carp (Weil, Breton and Reinaud, 1975).

As it has been demonstrated that trout plasma gonadotropin levels vary in relation to the state of the gonads (Breton *et al.*, 1975 ; Crim, Watts and Evans, 1975), we wished to find out if pituitary response to LH-RH varied with the stage of gameogenesis i n the male and female trout. In the female, we have particularly studied the final stage of gametogenesis.

Material and methods.

Trials were conducted in 1976 on adult 2-year old males during their second reproductive cycle. The animals came from the Etrun fish harm in the Pas-de-Calais department. They were kept in rearing tanks under natural photoperiod and tempe-

rature until the experimental dates. The 4 lots studied corresponded to 4 different stages of gametogenesis according to the criteria of Billard and Escaffre (1975). The various stages were determined by microscopic examination.

Lot 1, stage II : onset of gametogenesis ;

Lot 2, stage III : spermatocytes present and beginning of spermatid stage ;

Lot 3, stage V : all spermatogenetic stages present but no spermiation ;

Lot 4, stages VI and VII : onset of spermiation.

Experiments were carried out on the females in February 1977 using fishes from Denmark. The animals were 2-years old and in their first reproductive cycle. Five lots were studied :

Lot 1 : immature animals (I) having oocytes of less than 100 μ diameter ;

Lots 2-5 : animals presenting oocytes in different stages of maturation as defined by Jalabert *et al.* (1976). The stage was determined after examination of several oocytes obtained by abdominal massage.

Lot 2, stages (i) and (o) : end of vitellogenesis (i) and oocyte with migrating germinal vesicle (GV) (stage o) ;

Lot 3, stage 1 : oocyte with peripheral GV ;

Lot 4, stages 2 and 3 : maturing oocyte before GV breakdown (GVBD) (2) and after GVBD (3) ;

Lot 5, stage 4 : mature and ovulated oocytes.

The animals of the different lots were anesthetized with 0.5 ml/l phenoxyethanol and then kept under anesthesia by perfusion (0.3 ml/l) in the gills during the experiment. 1 μg/kg LH-RH (Hoechst, Batch Op. R6) was injected into each experimental animal by intracardiac catheter ; a buffer was injected by the same method into controls. Blood samples were taken with a heparinized syringe before (basic level) and 2, 4, 6, 8, 15 and 20 min. after the LH-RH or buffer injection. t-GTH level in the samples was determined by radioimmunological assay (RIA) as previously described for c-GTH by Breton *et al.* (1971*a*). Antibody was raised against a pure t-GTH and used at a dilution of $1/2.10^{-5}$. The animals were killed 20 min. later and the pituitary excised and ground to determine t-GTH content by RIA. A piece of testis was fixed for quantitative analysis of germ cells.

LH-RH response was studied by characterizing the gonadotropin secretion of each fish by a single overall value, obtained by adding the t-GTH levels from 2 to min. after the basic level had been subtracted from each value. All fish having an overall value higher than that of the corresponding control lot were considered as responsive to LH-RH. We term this overall value « t-GTH secretion during 20 min. » in order to simplify.

Statistical methods. — Mean confidence intervals ($P < 0.05$) were computed only when there were more than 5 animals in a lot. Samples were compared either by variance analysis or by the U-test of Mann-Whitney (non-parametric method).

Results.

Pituitary and plasma t-GTH levels (table 1).

Males. — Basic plasma t-GTH level was low at the onset of gametogenesis (stage II, lot 1) and increased when spermatocytes were present (stage III, lot 2 : $P < 0.001$). It then remained constantly high until spermiation. Pituitary t-GTH level was low in the first stages (II and III, lots 1 and 2) and then increased to a maximum at spermiation (lot 4).

TABLE 1

Plasma (basic level) and pituitary t-GTH levels

		Lots					
		1	2	3	4	5	
♂	Plasma ng/ml	(1) 1.3 ± 0.2 (24)	← *** → 4.1 ± 0.5 (24)	← NS → 3.3 ± 0.3 (24)	← NS → 3.6 ± 0.3 (20)		(a)
	Pituitary µg/mg	0.6 ± 0.1 (7)	← NS → 0.7 ± 0.2 (8)	← * → 1.6 ± 0.3 (8)	← *** → 7.6 ± 1.1 (7)		(b)
♀	Plasma ng/ml	3.2 ± 0.6 (16)	← NS → 3.8 ± 0.6 (18)	← NS → 5.1 ± 1.1 (12)	← ** → 10.3 ± 1.4 (21)	← NS → 10.1 ± 2.3 (15)	(a)
	Pituitary µg/mg	0.55 ± 0.4 (8)	← *** → 2.2 ± 0.4 (9)	← NS → 2.5 ± 1.6 (4)	← NS → 3.0 ± 0.8 (6)	← NS → 2.7 ± 0.9 (8)	(b)

(1) : mean ± s.e.
(a) : variance analysis.
(b) : Mann-Whitney U-test.
NS : $P > 0.05$; * : $P < 0.05$; ** : $P < 0.025$; *** : $P < 0.001$.
( ) : Number of animals. The mean basic level of t-GTH was computed using data from control and experimental animals. Mean pituitary t-GTH content was computed from data on control animals.

Females. — Basic plasma t-GTH, level was low in immature fish and at the end of vitellogenesis (lots 1 and 2), but increased progressively during oocyte maturation. The lots 3 and 4 (« oocyte with peripheral GV » and « maturing oocyte ») showed a significant difference ($P < 0.025$). At ovulation (lot 5), the level was still high. Pituitary t-GTH was constantly high in lots 2 to 5 (« end on vitellogenesis » until « ovulation ») as compared to immature fish (lot 1 : $P < 0.001$).

LH-RH response.

Males (fig. 1*a*). — At onset of gametogenesis (stage II), 1 animal out of 8 showed a very low response. LH-RH sensitivity increased when spermatocytes were present

(stage III). At that stage, 2 animals out of 8 responded to LH-RH ; one of these showed a very high peak (90 ng/ml). When all the spermatogenetic stages were present (stage V), 3 out of 4 animals responded as compared to the controls. However, maximum t-GTH was about 6 ng/ml. At spermiation (stages VI and VII), 2 animals out of 5 showed sensitivity ; high t-GTH values were found at that stage in some control subjects. Due to the heterogeneity of response in control and experimental animals beginning at stage III, a significant difference ($P < 0.05$) between the overall mean value of treated animals and controls only appeared at stage V.

Females (fig. 1*b*). — Because of the small number of animals studied, our results can only be considered as preliminary. Immature animals in the early stages of oocyte maturation showed a weak LH-RH response. Two out of 5 subjects responded at immature stage (I) ; 3 out of 5 at stages (i) and (o), and 1 out of 2 at stage (1). The maximum value found was 8 ng/ml. Large amounts of t-GTH were only secreted at the matured oocyte stage (lots 2 and 3) ; 4 subjects out of 7 responded and 2 of them had t-GTH peaks of 25 ng/ml and 13 ng/ml, respectively. On the other hand, there was no response at ovulation, and LH-RH even seems to have a depressive effect on t-GTH level.

We only found a significant difference in the overall mean between control and experimental animals at the matured oocyte stage ($P < 0.037$) and the ovulated stage ($P < 0.028$).

Discussion.

Pituitary response to LH-RH varies during gametogenesis ; there is none in the early stages of gametogenesis, but it increases later. This confirms the results of Crim and Cluett (1974) who found that LH-RH didnot release t-GTH in seasonally imma-

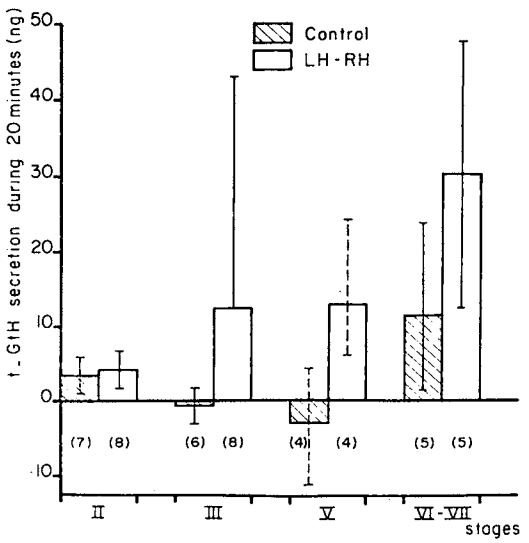

FIG. 1*a*.

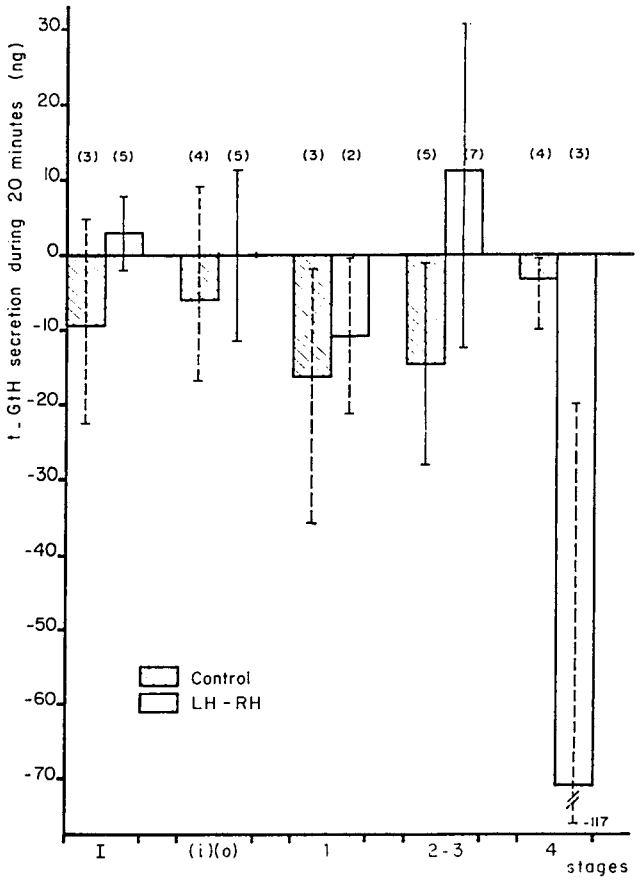

FIG. 1b.

FIG. 1a and 1b. — *Response to LH-RH at differents stages of gametogenesis.*

I mean and confidence limit at a threshold of $P < 0.05$ when $n \geqslant 5$;

T
I mean and true high and low values when $n < 5$;
I

( ) number of animals.

ture animals. The appearance at stage III of pituitary sensitivity to LH-RH corresponds to an increase of plasma t-GTH.

In females, basic level of plasma t-GTH increases progressively during oocyte maturation. It should be noted that pituitary response to LH-RH varies in the same way until maturation when it is maximal. After ovulation, there is none. As pituitary t-GTH levels are identical from immature oocyte stages to ovulation, this difference in response may be due to the steroid environment. It is known now that estradiol 17β level declines progressively until ovulation (Fostier *et al.*, 1978). Perhaps the ratio

between estradiol 17β and 17α-hydroxy-20β-dihydroprogesterone, the most potent steroid-inducer of trout oocyte maturation (Fostier, Jalabert and Terqui, 1973), modifies pituitary sensitivity to LH-RH.

After an LH-RH injection, the amounts of gonadotropin hormone released in trout plasma at reproductive time are low as compared to the carp (Weil *et al.*, 1975). The maximum values observed vary between 30 and 100 ng/ml in carp, and between 6 and 25 ng/ml in trout. At that stage, pituitary gonadotropin hormone content is 15 times higher in the carp than in the trout female and 7 times higher than in the trout male. The only elevated level in trout was observed in a male in mid spermatogenesis (beginning of spermatids).

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Résumé. La sensibilité hypophysaire au LH-RH a été étudiée chez la truite Arc-en-ciel mâle et femelle à différents stades de la gamétogenèse. Chez le mâle, elle est presque nulle au début de la spermatogenèse ; elle apparaît lorsque le stade spermatide est atteint, puis se maintient au cours de la spermatogenèse et de la spermiation. Chez la femelle, la fin du cycle a été plus particulièrement étudiée. La réponse est faible chez les poissons immatures et aux premiers stades de la maturation ovocytaire ; elle est la plus forte au moment de la maturation vitelline. Les relations de ces réponses avec les teneurs plasmatiques et hypophysaires en t-GTH sont discutées.

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Early sex differentiation in *Mugil* (*Liza*) *auratus* Risso, 1810 (Teleost Mugilidae). An ultrastructural study

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Summary. Using electron microscopy, 5 types of early germ cells (primordial germ cells or PGC, spermatogonia and primary spermatocytes, oogonia and primary oocytes) were identified by their morphological and cytological characteristics. The various stages in gonadal organization (PGC colonization, ovarian and testicular differentiation) were determined. These data have been compared to those of other fish and vertebrates and the germinal potentialities of both sexes evaluated.

Introduction.

Grey-mullet are heterosexual fish and gonochorism is the general rule. Cytological cnalysis of sex-differentiation was studied by light microscopy (Stenger, 1959 ; Leray, 1968 ; Thong, 1969 ; Cassifour, 1975), but germ-cell characteristics were not sufficiently defined at this level and no distinction could be made between primordial germ-cells, oogonia and spermatogonia.

Electron microscopy has been used to accurately describe most fish, but fine structure during early sex-differentiation was chiefly investigated on freshwater species such as *Oryzias* (Satoh, 1974), *Poecilia* (Grier, 1975) and *Carassius* (Yamamoto and Onozato, 1965). There are few data in the literature on the germ-cells of marine species and no information concerning grey-mullet.

We used electron microscopy for cytological identification of early germ-cells in juvenile and young golden-grey mullet *Mugil* (*Liza*) *auratus*.

Material and methods.

The subjects were caught by electric fishing in a mediterranean lagoon (Leucate) and the gonads were fixed in 2 p. 100 glutaraldehyde buffered with 0.12 M sodium cacodylate and postfixed in 2 p. 100 osmium tetroxide. Ultrathin sections were stained with uranyl-acetate and lead-citrate solutions (Reynolds, 1963).

Results.

A) *Different stages of differentiating juvenile and young gonads* (see fig. 1).

1. Undifferentiated gonads including scattered PGC, are observed in fish from 10 to 16.5 cm total body length (TL) ;

2. Ovarian differentiation is recognized (PGC, oogonia and oocytes) from 14 cm TL ;

3. Testicular differentiation occurs (PGC, spermatogonia and spermatocytes) from 20 cm TL.

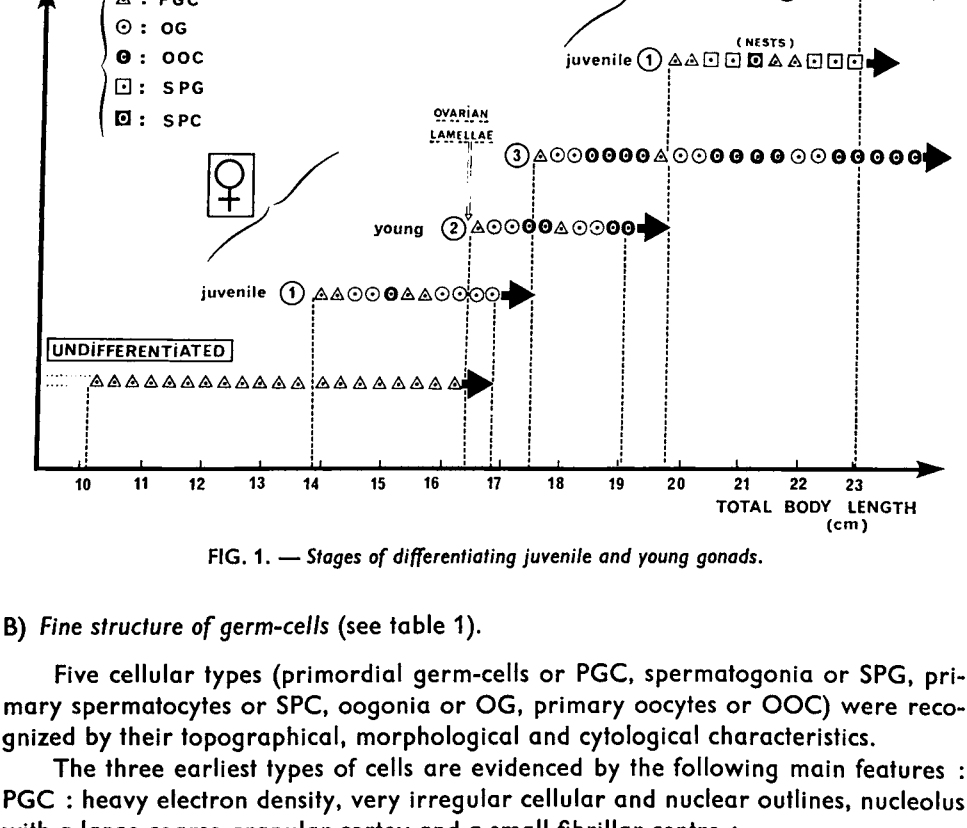

FIG. 1. — *Stages of differentiating juvenile and young gonads.*

B) *Fine structure of germ-cells* (see table 1).

Five cellular types (primordial germ-cells or PGC, spermatogonia or SPG, primary spermatocytes or SPC, oogonia or OG, primary oocytes or OOC) were recognized by their topographical, morphological and cytological characteristics.

The three earliest types of cells are evidenced by the following main features :
PGC : heavy electron density, very irregular cellular and nuclear outlines, nucleolus with a large coarse granular cortex and a small fibrillar centre ;
SPG : low electron density, slightly irregular cellular and nuclear outlines, nucleus eccentrically located, nucleolus with thin granular cortex and a large fibrillar centre ;
OG : low electron density, cellular outlines almost regular, nucleus ovoid or roughly spherical, almost centrally located, with regular outlines, nucleolus similar to spermatogonium.

TABLE 1

Characteristics	Cellular types				
	Undifferent.	♂		♀	
	Primordial germ-cells	Spermatogonia	Spermatocytes	Oogonia	Oocytes
Topography	isolated or clustered	in nests or cysts	in cysts	isolated or in nests	in nests
— Cytoplasmic bridges	0	0	++	0	++
Morphology					
— Shape	oval	oval	oval	oval	oval
— Size L	13.5 ± 1.8 µm	14.2 ± 3.3 µm	11.5 ± 2 µm	14.3 ± 2.2 µm	11.8 ± 2 µm
— Size l	9.5 ± 1.7 µm	10.5 ± 2.5 µm	7 ± 1.6µm	11.3 ± 3.3 µm	7.5 ± 1.3 µm
— Outlines	very irregular	slightly irregular	almost regular	almost regular	almost regular
Cytology					
a) Nuclear structures :					
— Size L	8 ± 1.6 µm	8.3 ± 1.9 µm	7.3 ± 1.8 µm	8.1 ± 1.5 µm	7.1 ± 1 µm
— Size l	5.5 ± 1.6 µm	7 ± 1.8 µm	5.8 ± 1.3 µm	7.1 ± 1.4 µm	5.9 ± 1.2 µm
— Shape	oval	oval	oval or nearly round	nearly round	nearly round
— N/C	0.38 ± 0.07	0.40 ± 0.06	0.54 ± 0.08	0.41 ± 0.08	0.55 ± 0.07
— Location	eccentric	eccentric	eccentric	almost central	slightly eccentric
— Nucleoplasm	dense	less dense	less dense	less dense	less dense
— Nucleolus	single eccentric, 2 µm	single, eccentric	single, 1.5 µm (1)	single, ⩾ 2 µm	single (1)
center	small fibrillar	larger fibrillar		fibrillar	
cortex	large granular	denser granular		granular	
— Chromatin or chromosomes	finely granular and widely dispersed	rather granular, with clumps	synaptonemal complexes rather regular	dispersed	synaptonemal complexes
— Nuclear envelope	irregular outlines	slightly irregular	outlines	regular outlines	regular outlines
b) Cytoplasmic structures :					
— Organelles polarity	0	0	+	0	+
— Ribosomes	+++	++	+	++	+
— Endoplasmic reticulum	++	++	scarce	+	scarce
— « Whorl »	few	+	0	+	0
— Golgi complexes	+	+	<, near bridges	+	<, near bridges
— Mitochondria	round-shaped, 0.5-1 µm randomly distributed	round-shaped, 0.5-1 µm crescent-shaped distrib.	round or elongated, 0.3-0.5 µm, few numerous	round, 0.6-1 µm	round or elongated, 0.2-0.6 µm, denser matrix
— Dense substance					
« nuage »	++	++	∞	++	∞
« ciment »	++	++	+	++	+
— Mitochondrial groupings	++	+	+	+	+
— Inclusions	0	0	0	0	0

(1) Disappears at pachytene stage.

Discussion and conclusion.

Our data reveal that the colonization of somatic tissues by PGC is late in *Mugil auratus*. No germ-cell was evident in the gonad until a TL of 9-10 cm was reached, when the fish were about eight months old. In other species of grey-mullet, Stenger (1959), Leray (1968), Thong (1969) asserted that the sex-colonization of gonadal primordia was earlier.

The onset of sex-differentiation in females is earlier than in males as shown in other fish ; the ovary organized from 13-14 cm while the testis organizes from 19-20 cm total length. This onset of sex-differentiation occurs at the end of the first year for the females and at the beginning of the second year for the males and therefore earlier than observed by Ezzat (1965), Cassifour (1975) and Chervinski (1976) in *Mugil auratus*.

Some authors (Thong, 1969 ; Cassifour, 1975 ; Remacle *et al.*, 1977) have concluded that all early germ-cells present a similar morphological appearance. Our data reveal clear cytological differences between PGC and gonia and between oogonia and spermatogonia ; these results do not agree with the findings of Satoh (1974).

Similarity in ultrastructural features in male and female PGC suggests that these undifferentiated cells are bipotential. PGC are present in undifferentiated gonads and, in low numbers in young and even submature gonads; they constitute a permanent stock in the two sexes. It is probable that PGC are responsible for the sexual lability which characterized most fish and especially grey-mullet.

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Paimpont, France, 19-21 *septembre* 1977.

Résumé. Une étude des gonades juvéniles du Muge doré, effectuée en microscopie électronique, a permis d'identifier à l'aide de critères morphologiques et cytologiques cinq catégories de cellules germinales précoces (cellules germinales primordiales, spermatogonies et spermatocytes I, ovogonies et ovocytes I). Il a été possible de distinguer différents stades dans l'organisation gonadique : la colonisation par les cellules germinales primordiales, la différenciation ovarienne, la différenciation testiculaire, et de juger des potentialités germinales.

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The gonadal cycle of the captivity-reared hermaphroditic teleost *Sparus aurata* (L.) during the first two years of life

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Summary. The gonadal cycle of the hermaphroditic teleost *Sparus aurata* reared in captivity was studied during the first two years of life. Until the age of 8 months, the dorsal ovarian part of the bisexual gonad was dominant. Towards the first breeding season at the end of the first year of life, the ventral testicular part of the gonad proliferated, forming a mature testis. At the end of the first breeding season, all the fish began to undergo sex reversal. From the age of 17 months, development proceeded in two directions : in about 80 p. 100 of the population the sex reversal process was completed and the individuals developed into females ; in the rest of the population the sex reversal process was not completed and the individuals developed into males.

Introduction.

Normal hermaphroditism and sex reversal are well-known phenomena in Teleostei (Atz, 1964 ; Reinboth, 1970). Pasquali (1941) and d'Ancona (1941,1949), studying the gonadal cycle of *Sparus aurata* caught in the Adriatic Sea, demonstrated protandrous hermaphroditism in this species. These authors found that the entire population functions as males at the end of the second year of life, then changes sex and performs as females from the end of the third year.

The goal of the present research was to carry out an histological study of the gonadal cycle of *S. aurata*, based on captivity-reared fish of known age.

Materials and methods.

Fry of *S. aurata* about 20 mm long were collected in their natural habitat in the Mediterranean sea once a year at the end of March. They were stocked at densities up to 5 kg/m^3 in outdoor experimental tanks according to 2 age classes, i. e. 4 months

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to 1 year and 1 year or older. An open circulation system supplied the tanks with sea water from the Gulf of Eilat. The fish were exposed to the natural photoperiod at water temperatures of 21 °C ± 2 all the year round ; water salinity was 40 p. 100. Fish were fed once a day *ad libitum* on artificial pellets. From April 1975 though June 1976, monthly gonad samples were taken from 5 to 10 individuals in each of the 2 age classes. A total of 245 fish were sampled.

Immediately after decapitation, gonads were removed from the fish. The tissues used for histological studies were fixed in either Bouin or Karnovsky (1965) solution. Tissue processed in Bouin solution was embedded in paraffin, while that treated in Karnovsky solution was post-fixed with 1 p. 100 OsO_4 and embedded in Epon 812. Staining procedures included Azan, hematoxylin and eosin, methylene blue and toluidine blue.

Results.

4 to 8-month old fish.

During the period from 4 to 8 months of age, the early development of the bisexual gonad can be followed. At the age of 4 months, the gonad is undifferentiated, and protogonial nests are unevenly spread throughout the gonad, concentrated mainly in the vicinity of the central cavity which is dorsally located.

A month later, a topographical differentiation becomes evident in the gonadal cross-section. The gonad is divided into dorsal and ventral regions. The dorsal region, which contains the gonadal central cavity, constitutes about 90 p. 100 of the gonadal cross-section area. This is the future ovarian part of the gonad. The ventral region, not invaded by the central cavity, is the future testicular part of the gonad.

At the age of 8 months (fig. 1), the dorsal part of the gonad has the structure of a young ovary. It consists of lamellar folds densely packed with oogonia. At this stage, the oogonia begin to degenerate ; the process begins in the centrum and spreads out centrifugally, affecting all the oogonia except for a rim of oogonia near the juxtacavicular part of the ovigerous folds. Between these remaining oogonia and the central cavity there is a visible layer of squamosial epithelium cells. The ventral part of the gonad is populated by nests containing spermatogonia.

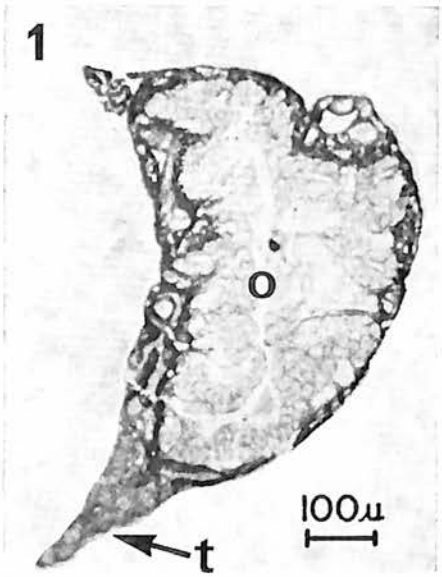
9 to 12-month old fish.

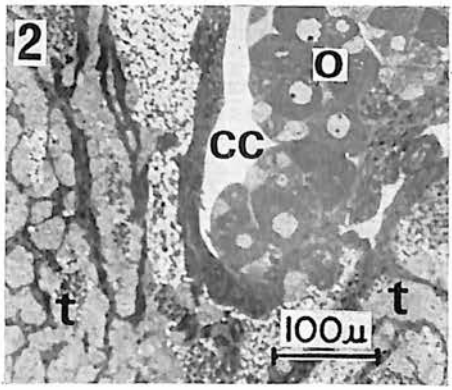
During the period from 9 to 12 months of age, the ovarian part of the gonad regresses while the testicular one develops. At 9 months, the dorsal part of the gonad is still dominant. Its oogonial degeneration process is finished ; all the lamellae, which a month earlier were densely populated by oogonia, appear now to be empty except for the above-mentioned oogonia near the central cavity.

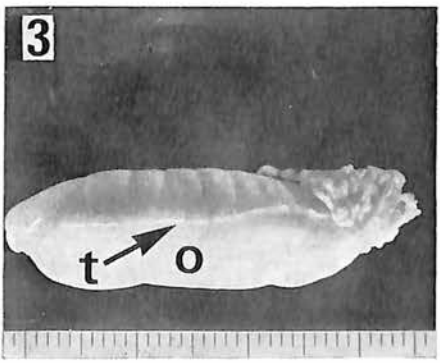
In 10 to 11-month old fish, active spermatogenesis takes place in the ventral part of the gonad. The testicular part increases in size, engulfing the ovarian part in which the lamellar cavities have disappeared (fig. 2). At the end of the first year of life, in the first breeding season, the ventral part of the gonad forms a functional testis. In the ovarian component, the oogonial rim near the central cavity is still visible.

13 *to* 16-*month old fish.*

The period from 13 to 16 months of age is characterized by the beginning of a sex reversal process. The testicular part of the gonad is spent and remains latent. Throughout this period, it is densely packed with spermatogonia. The ovarian part of the







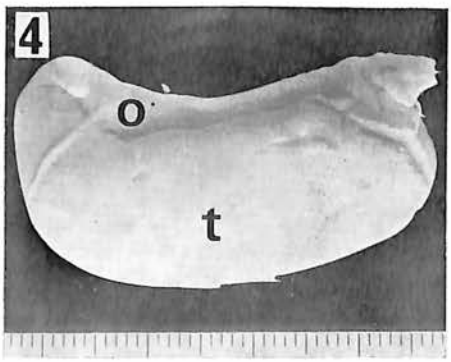


FIG. 1. — *Transverse section in the gonad of an* 8-*month old fish.*
The gonad is divided into a dominant dorsal ovarian part (0) and a ventral testicular part (t).

FIG. 2. — The testicular part of the gonad (t) envelops the ovarian one (0) in which oogonia and primary oocytes line the central cavity (CC) in *a* 10-*month old specimen.*

FIG. 3. — *Gonad of a* 24-*month old mature female.* Degenerated testis (t) is ventrally attached to the fully developed ovary (0). The millimetric scale is shown.

FIG. 4. — *Gonad of a* 24-*month old mature male.*
Functional testis (t) envelops the remains of the ovary (0). The millimetric scale is shown.

gonad undergoes intensive growth and the oogonia start to undergo oogenesis. As a result, ovigerous lamellae develop and the ovarian part of the gonad constitutes 85 p. 100 of the gonadal cross-section area.

17 to 24-month old fish.

During the period from 17 to 24 months of age, two opposite patterns develop. In about 80 p. 100 of the individuals sampled, the dorsal ovarian part of the gonad continues to develop ; the oocytes finish their previtellogenetic growth and undergo vitellogenesis. Simultaneously, the ventral testicular part of the gonad progressively degenerates and its spermatogonia undergo rapid necrosis. At 23 to 24 months the second breeding season, the gonad of these fish consists of a dorsal mature ovary and a ventral degenerated rudimentary testis (fig. 3). In the remaining 20 p. 100 of the sampled population, the reverse happens. Spermatogenesis starts in the ventral testicular component of the gonad which increases in size. The ovarian component of the gonad undergoes degeneration and the previtellogenetic oocytes populating it become atretic. At 23 to 24 months, the ventral part of the gonad is a functional testis, enveloping almost completely the remains of the dorsal ovary (fig. 4).

Discussion.

As previously shown by Pasquali (1941), D'Ancona (1941, 1949) and Reinboth (1962), this study demonstrates that *S. aurata* is a protandrous hermaphroditic species. Nevertheless, previous to the functional male stage we followed a phase of oogonial proliferation which results in an ephemeral ovary at the age of seven to eight months. In addition, our data is some what different as compared to earlier studies : 1) Two phases, not previously noted, are described here : *a*) the enlargement of the dorsal part of the gonad into the form of a young ovary in 7 to 8-month old fish and *b*) the subsequent degeneration of the oogonia in 8 or 9-month old fish. The decrease in the protogonia frequency mentioned by Pasquali (1941) and D'Ancona (1941) corresponds to an earlier stage in the gonadal development, previous to central cavity formation and is not described in the present study. 2) According to Pasquali (1941) and D'Ancona (1941), *S. aurata* reaches first complete sexual maturity at the end of the second year of life when all the fish function as males. The present study shows that by the end of the first year of life, all the fish function as males. 3) According to Pasquali (1941) and D'Ancona (1941), during the third year of life all the fish undergo sex reversal and from the third breeding season on, they function as females. Our study demonstrated that at the end of the first breeding season all the fish start to change sex. In the second half of the second year, a split occurs in the gonadal development, resulting in 80 p. 100 females and 20 p. 100 males in the second breeding season.

The discrepancies between our study and those of Pasquali (1941) and D'Ancona (1941) could be explained by one or more of the following : 1) There may be differences in detail of the gonadal cycle between fish growing in their natural habitat, as mainly used by Pasquali (1941) and by D'Ancona (1941), and our fish reared in captivity. Some environmental conditions under which the fish were maintained in our study, i. e., relatively high and constant water temperature throughout the year

and daily feeding, may explain their accelerated gonadal development and precocious sexual maturation as compared to the fish studied by Pasquali (1941) and D'Ancona (1941). 2) There may be differences in detail of the gonadal cycle between fish originating in the Adriatic sea as used by Pasquali (1941) and by D'Ancona (1941) and our fish originating in the eastern Mediterranean. 3) The exact age of some of the fish sampled by Pasquali (1941) and by D'Ancona (1941) was perhaps uncertain, in contrast to the exact age determination in the present study. 4) Pasquali (1941) and D'Ancona (1941), not being able to capture successive fish samples throughout the year, possibly lacked a few phases of the gonadal development ; however in the present study a routine monthly sampling supplied details of the entire process.

The present study demonstrates the lability of the sex reversal process. Although in all the fish over one-year old, which at the age of twelve months were functional males, the testicular tissue begins to regress and the ovary to develop, the sex reversal process is not completed in all of them. Some of the fish redevelop the testis and become functional males at the age of two years. This fluctuation between masculinity and femininity may be the result of a social control of the sex reversal process, a mechanism which ensures the appropriate male-to-female ratio in the population.

Through an inhibitory effect caused by fish with a more developed ovary, the control mechanism may act on the further development of the gonadal ovarian part in fish in which this part is less developed. Hence, in the inhibited individuals, the ovary starts to degenerate while the latent testis develops. In the other individuals, the ovary matures while the testis degenerates. A similar control of the sex reversal process was studied in a few protogynous hermaphroditic teleosts (Robertson, 1972 ; Fishelson, 1970, 1975 ; Warner, Robertson and Leigh, 1975). A detailed experimental study is needed to confirm this hypothesis.

Symposium sur la Reproduction des Poissons Paimpont, France, 19-21 *septembre* 1977.

Résumé. L'étude du cycle sexuel du poisson téléostéen hermaphrodite *Sparus aurata* a été réalisée jusqu'à l'âge de 2 ans sur des animaux élevés en captivité. Jusqu'à 8 mois, c'est la composante ovarienne, en position dorsale, qui est prépondérante dans la gonade bisexuée A l'approche de la première saison de reproduction (fin de la première année), la composante testiculaire, en position ventrale, prolifère et forme un testicule mature. En fin de première saison de reproduction, une inversion sexuelle s'ébauche chez tous les individus. A 17 mois, deux évolutions sont possibles : chez environ 80 p. 100 des individus le processus d'inversion sexuelle s'achève et donne des femelles ; chez les individus restants, l'inversion demeure incomplète, et le développement se fait à nouveau dans le sens mâle.

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Postovulatory changes in the theca folliculi of the trout

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Summary. At time of ovulation the cells composing the *theca folliculi* of trout are morphologically smooth muscle like.The physiological behaviour of this tissue confirms its smooth muscle nature. Following ovulation a further cellular transformation and differentiation takes place : within 12-24 hrs after ovulation the theca cells start phagocytozing the adjacent collagen. Bundles of collagen are partially surrounded at first by cellular processes of the thecal cells while finally small bundles of collagen are interiorized. The number of collagen fibers per phagocytotic vesicle varies from one to many. The bundles can be recognized morphologically until 72 hrs after ovulation after which time they become indistinct, indicating a possible hydrolysis. Tests with the « Api-zim » test system demonstrate the presence of a number of lytic enzymes in freeze-thawed post-ovulatory ovarian tissue, in support of the morphological observations.

The contraction of the cellular components of the theca results in the expulsion of the oocyte, that is ovulation (Jalabert and Szöllösi, 1975). The *theca folliculi* behaves physiologically like smooth muscle whose contraction can be evoked *in vitro* in isolated follicles by the addition of prostaglandin $F_2\alpha$. The presence of smooth muscle (or smooth muscle-like cells) as an important component of the theca has been reported for several vertebrate classes (mammals-see Amsterdam *et al.*, 1976 ; fish-Szöllösi and Jalabert, 1974 ; amphibian-Larsen *et al.*, 1977). The post-ovulatory structure of the ovary however, was not studied thus far systematically in lower vertebrates. We can report here that the smooth muscle cells become phagocytotically active few hours after ovulation and that these cells incorporate and break down the collagen fiber framework of the theca.

Materials and methods.

Female trout, *Salmo gairdneri*, of reproductive age (3-4 years old) were controlled for ovulation daily during the spawning period, from November to February. When ovulation was observed females were stripped to remove all free oocytes. The ovaries were then either removed and spent follicles isolated and fixed immediately or only 12-148 hrs after ovulation. Individual follicles were also fixed after *in vitro* induction of ovulation (Jalabert and Szöllösi, 1975). The fixative employed was a 2.5 p. 100

glutaraldehyde, 0.5 p. 100 paraformaldehyde solution in 0.15 M cacodylate buffer at pH 7.2 containing 0.1 p. 100 potassium ferricyanid (Elbers *et al.*, 1965).

The presence of some lytic enzymes in the coelomic fluid recovered within 24 hrs after ovulation was investigated by the « Api-zym » test system (Monget, 1975). The collagenase activity in the post-ovulatory ovarian tissue was also controlled (we are very thankful to Dr. Wahl, NIH who kindly analysed our material for collagenase activity according to Nagai *et al.*, 1966). For these tests the ovaries were perfused by trout balanced salt solution (Jalabert, 1978) immediately after sacrifying the animal to remove the potentially present collagenase inhibitor often found in blood plasma.The ovaries were then frozen thawed three times. Ovarian fragments were removed by filtration through three layers of cheezecloth. The so produced turbid solution was centrifuged at 1 200 r.p.m. to remove cellular debries. The supernatant was slightly yellow and clear, transparent solution ; it was dialysed against several changes of 30 μM Tris-HCl (pH 7.5) buffer for three days at 4 °C and deep froozen until lyophilisation. The powder obtained was stored until analysis.

Excised post-ovulatory follicles were studied for proteolytic activity on fixed gelatin or collagen membranes (Gaddum and Blandau, 1970 ; Owers and Blandau, 1971). The follicles were blotted dry and placed on the protein membranes and moistened either in a saline-citrate buffer pH 4.2 or in saline-veronal acetate buffer pH 7.2. Coverslips were placed on such preparations and sealed by mineral oil to protect them from drying. These preparations were incubated at 25 °C for 4 hrs. At both pH levels 0.001 M EDTA was added to some slides.

Results.

The *theca ovarii* of the pre-ovulatory trout ovary is constituted of alternating layers of flattened smooth muscle cells and collagen fibers (fig. 1, 2). The basal lamina towards the follicular cells is very thick, measuring 90-100 nm and represents a convenient marker. The outline of the cells composing the theca is usually smooth with several pinotcytotic vesicles. The cytoplasmic microfilaments of 70-80 Å in diameter represents the most characteristic structural component of these cells (fig. 2). Occasional dense bodies are also seen.

Because the females were tested only once dayly for ovulation the samples representing the earliest times (2-4 hrs) after ovulation have been taken following *in vitro* induction of ovulation. The images are identical under the two conditions and no further mention will be made later of the conditions from which the follicles were taken. The intracellular filaments, the most stricking cellular marker of thecal cells,

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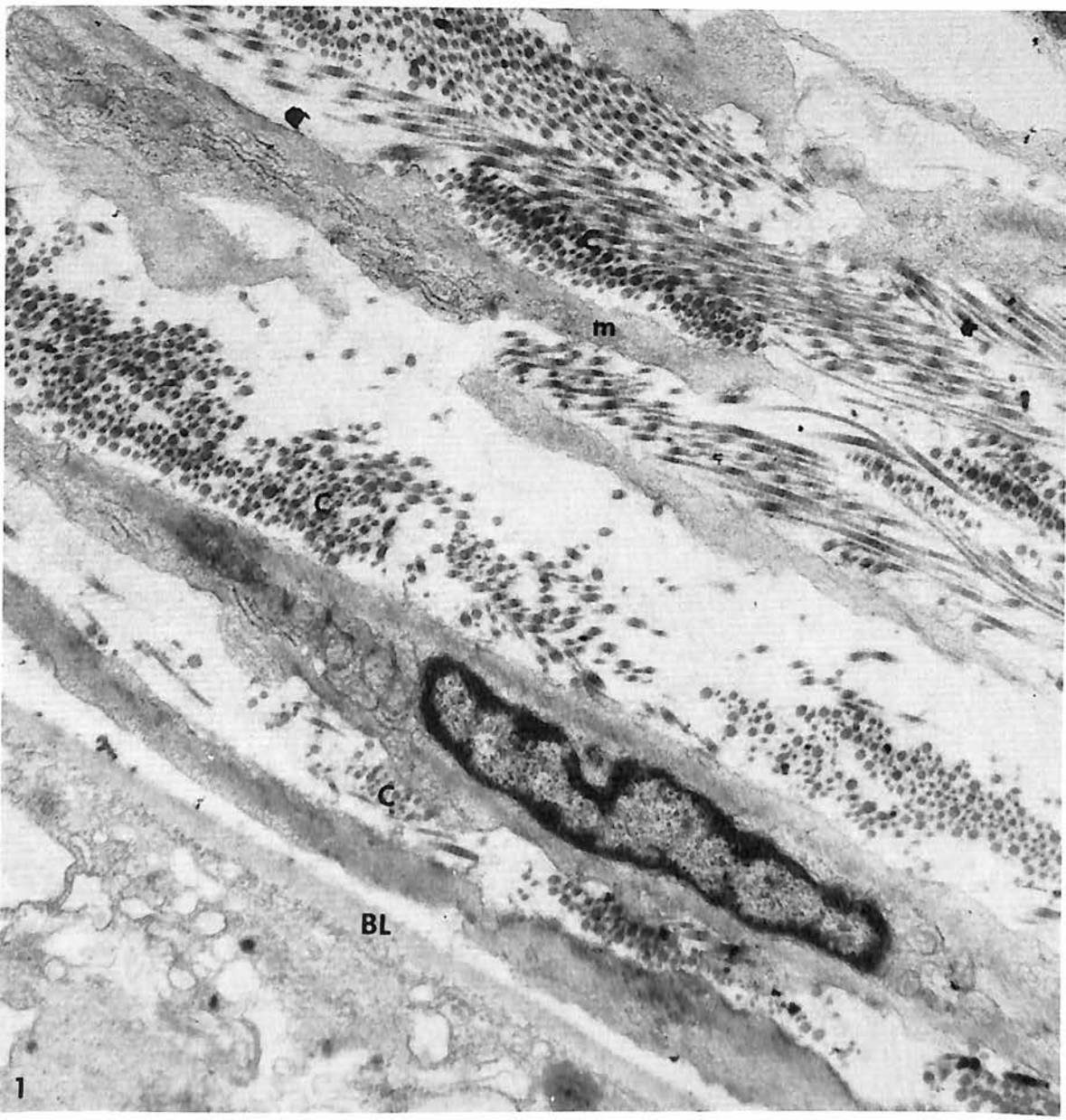
FIG. 1. — *Adjacent to the thick basal lamina,* (BL) *layers of the smooth muscle-like cells* (m) *of the theca alternate with layers of collagen* (c). Cell organelles are limited to a small cytoplasmic islet at the nuclear poles. The rest of the cytoplasm is occupied by thin filaments. × 15 000.

FIG. 2. — *Thin filaments, measuring 7-8 nm in diameter occupy the cytoplasm of the smooth muscle-like cells.* Numerous pinocytotic vesicles are close and in apparent association with the plasma membrane. × 60 000.



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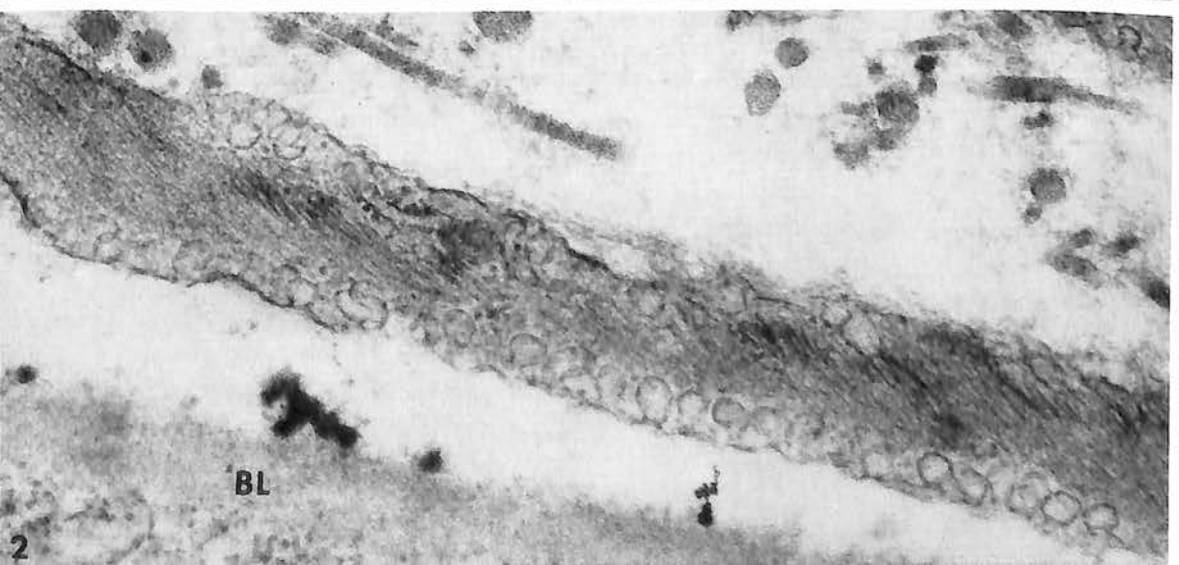

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are unaltered after ovulation. The basal lamina towards the follicular cells becomes very tortous and on many places the cell to basal lamina contact is not retained ; buckles of the basal lamina are formed. No firm attachment must thus exist between it and the cells on either side (fig. 3). The cell shape and outline has drastically changed, however. The cells become stellate with many, slender cell processes penetrating the surrounding connective tissue space (fig. 4). The cell processes seem to surround and isolate bundles of collagen fibers consisting of varrying but always large numbers. In the earliest samples taken after ovulation only occasional phagocytotic vesicles are seen containing a few collagen fibers (fig. 5). In specimen taken 24 hrs after ovulation the number of phagocytic vesicles per cell has greatly increased ; the size of the vesicles may be very uniform in which one to six collagen fibers are isolated, but larger vesicles may be found next to the small ones. In the cytoplasm many smooth walled, clear, small vesicles are seen, a component usually observed in smooth muscle cells. These vesicles are often in close proximity of the phagocytotic vesicles and occasionally even fusion between the two can be seen (fig. 6) ; the small vesicles thus may be primary lysosomes. The Golgi element is not well developed. It is always small, localized in small cytoplasmic islands close to the nuclear poles free of the small filaments occupying the major portion of the cytoplasm. In most sections the incorporated collagen fibers are obliquely or cross sectioned. In a few, rare cases a longitudinally cut fiber can be followed for several microns ; the phagocytotic vesicles are thus very long.

When longer time interval was permitted to ellapse after ovulation the cell appears to be riddled by phagocytitic vesicles which make nearly contact with each other, leaving only a small cytoplasmic strand between the individual vesicles (fig. 7). The thin cyto-filaments can be recognized in such places.

From the cell surface many processes project which surround either partially or totally bundles of collagen fibers ; the cells thus are still active phagocytotically.

Phagocytosis occurs by every cell composing the theca. But phagocytosis of collagen appears to be a generally occuring process in the post-ovulatory ovarian connective tissue compartment. Cells in the interstitium and even in the theca composed of a single cell layer around small oocytes, which appear to be fibroblasts, phagocytise collagen.

The collagen becomes morphologically indistinct within phagocytotic vesicles 3-7 days after ovulation ; there is a nearly uniform electron dense content recognisable (fig. 8). These images may be due to partial of full breakdown of the collagen fibers.

Digestion of india ink colored gelatin or collagene membranes occurs only at pH 4.2. At pH 7.2 there was no sign of any digestion around the follicle and the appea-

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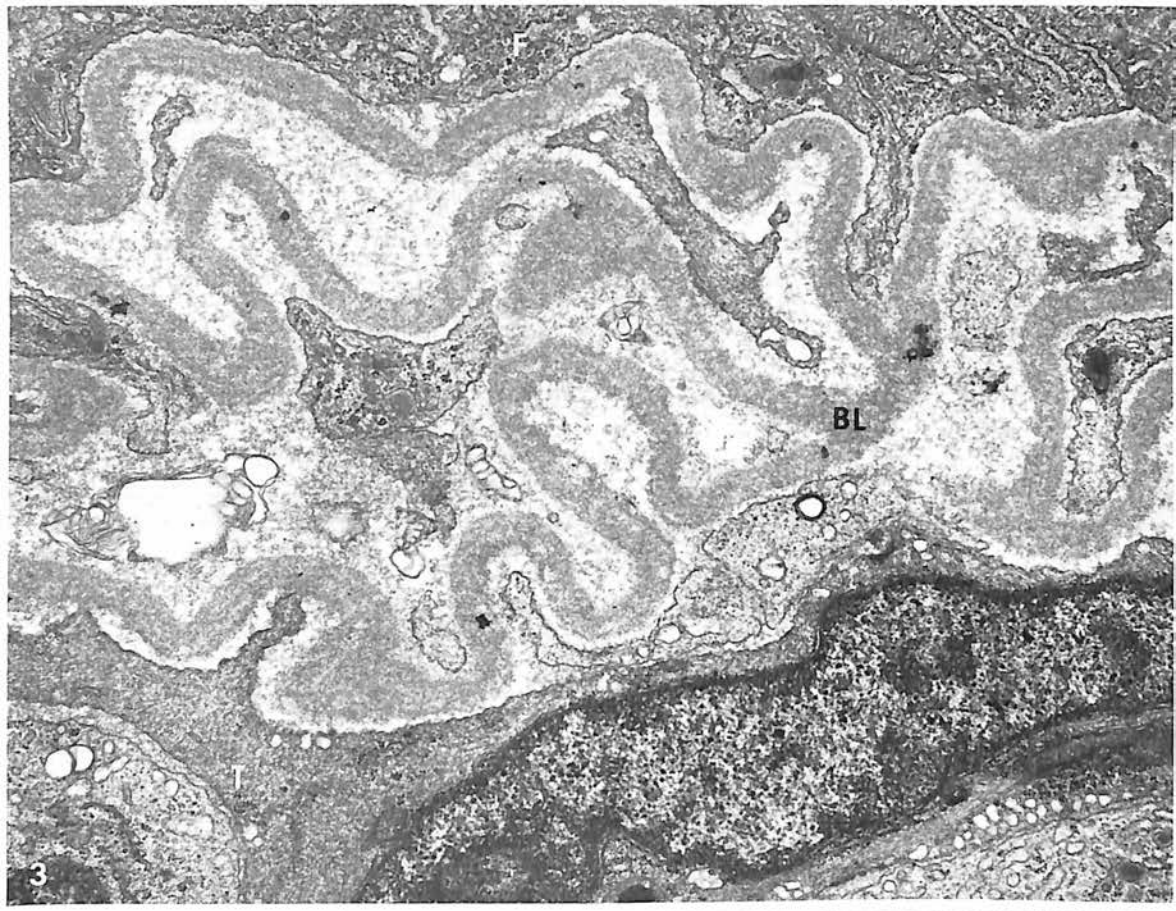
FIG. 3. — *The thick basal lamina* (BL) *between the follicle cells* (in the center) *and the theca is extensively folded.* × 14 000.

FIG. 4. — *The post-ovulatory thecal cells become stellate, forming several foot-processes.* These processes isolate large bundles of collagen fibrils. Two, small, internalized, isolated phagocytotic vesicles containing few collagen fibrils are seen. × 30 000.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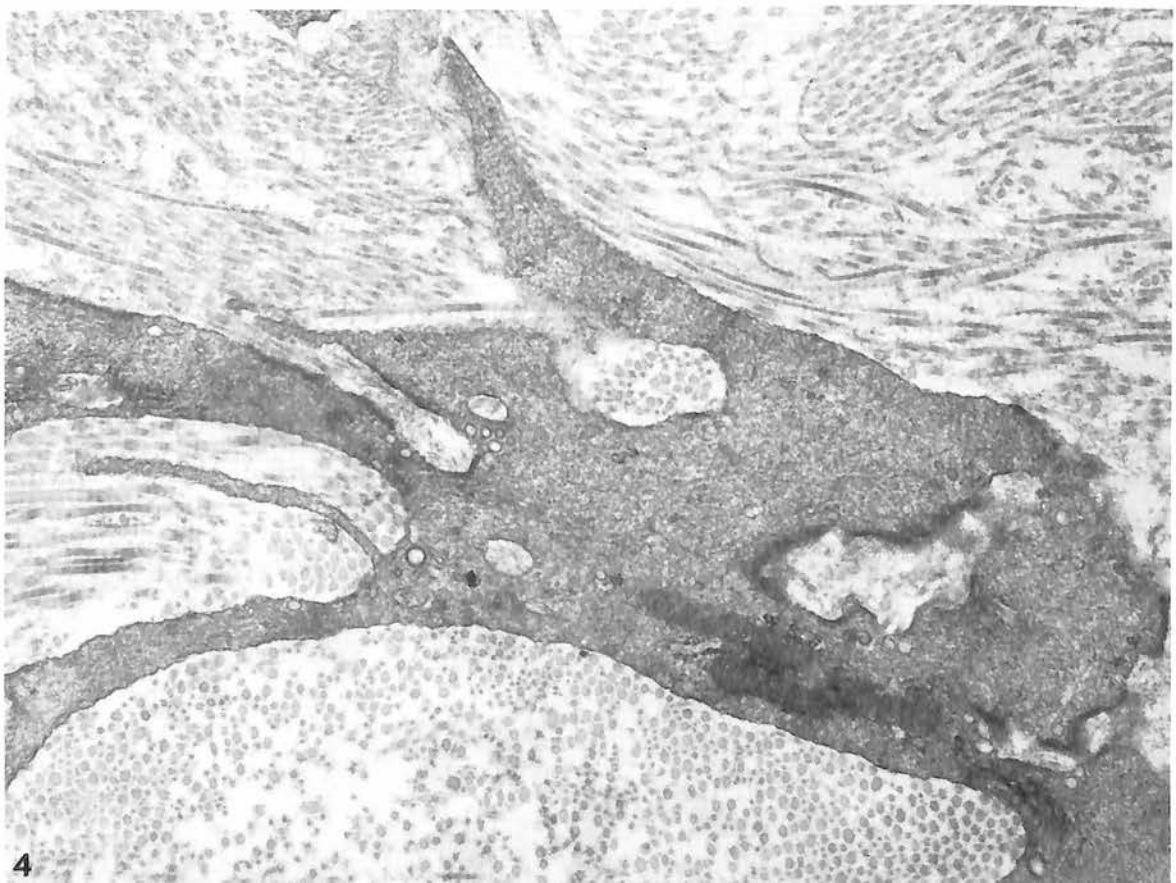
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rance remained identical to that of controles. At pH 4.2 the digestive activity is not inhibited by the addition of EDTA.

The « Api-Zym » enzyme test system disclosed the presence of leucine and valine aminopeptidases and chymotripsin, all three giving a medium strong color reaction being evaluated as 2 on a scale of 0-4. Trypsin was negative. Acid and alkaline phosphatase were also present. The tests were positive for various esterases, such as butyric acid and caprilic acid esterases, phosphoaminidase, α and β galactosidase, β glucoronidase. Collagenase could not be demonstrated. Collagen breakdown does not occur exclusively intracellularly. In ovaries removed 17 days after ovulation near the cell surfaces many small fibrils can be found in the intercellular spaces, not to be seen at all in preovulatory follicles. One may therefore imply that some extra cellular collagen breakdown also occurs.

Discussion.

After expulsion of the mature oocytes from the trout ovary at time of ovulation ts size is greatly reduced. The size of the ovary is further reduced within the following days subsequent to the resorbtion of the spent follicles ; the size of the ovary of an adult will increase only in the middle of the next reproductive cycle after the induction of vitellogenesis.

Even though only the initial phases of the post ovulatory fate of the trout ovarian follicle was studied it can be stated that the phagocytosis and breakdown of the collagen framework may be responsible at least in part for the second ovarian size reduction.

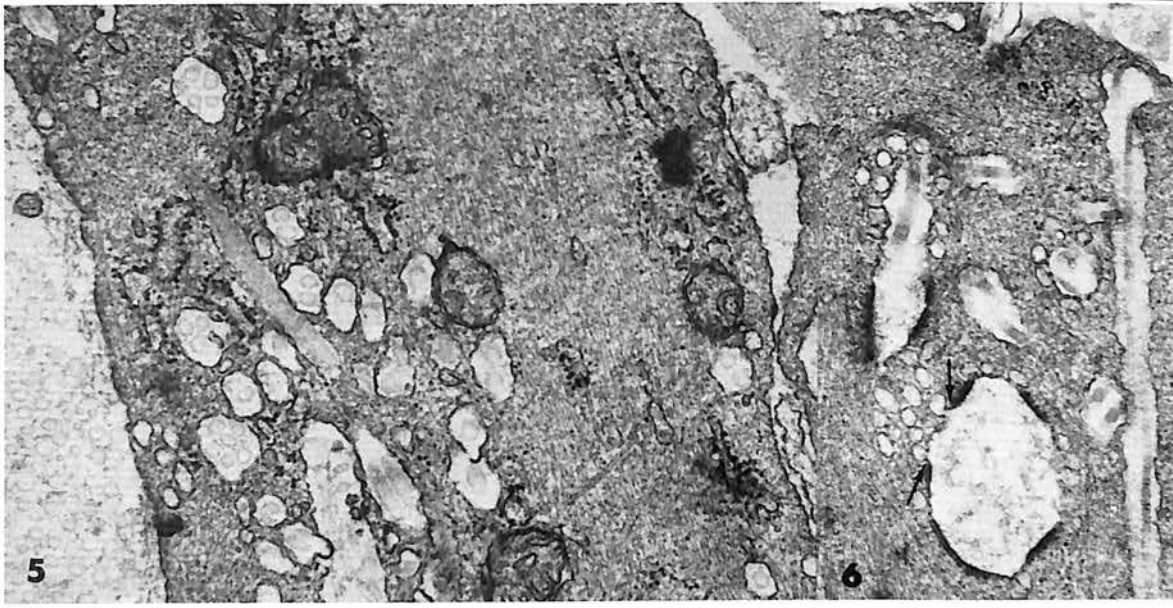
Phagocytosis appears to be a process induced in general by fibroblasts throughout the ovarian tissue. It was observed, as described here in greatest detail, in the theca of spent follicles, but also in the theca of small follicles and by cells on the interstitium. It is possible thus, that a substance is produced or released at time of ovulation to which fibroblasts and their derivatives respond in general by phagocytosis. A tetrapeptide, referred to as Tuftsin, recently identified and isolated (Satoh *et al.*, 1972) is known to induce phagocytosis specifically by white blood cells. Certainly in the case described here such a phagocytosis inducing material remains at best hypothetical, but a secuding possibility.

FIG. 5. — *4-5 hrs after ovulation the number of collagen containing phagocytotic vesicles increases.* In this case the fibrils are transveresely or obliquely sectioned. Note the large numbers of cytoplasmic fibrils of 7-8 nm in dimater. × 32 000.

FIG. 6. — *Small vesicles are seen close to the phagocytotic vesicles ; in a few cases they fuse* (arrows) *with the phagosomes.* × 24 000.

FIG. 7. — *Very large numbers of phagocytotic vesicles are found in the smooth muscle cells of the theca 4 days after ovulation.* At the periphery large collagen bundles are either fully or partially surrounded by cellular footprocesses. × 24 000.

FIG. 8. — *The outline and contrast of the collagen fibers (arrow-head) becomes less distinct in some vacuoles 5 days after ovulation.* × 65 000.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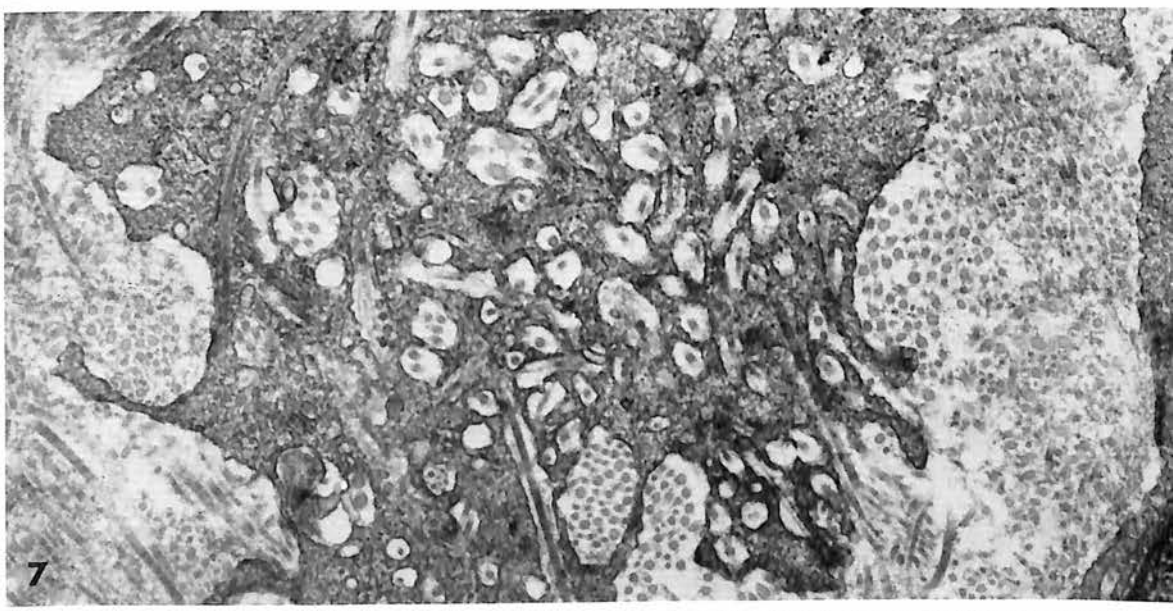
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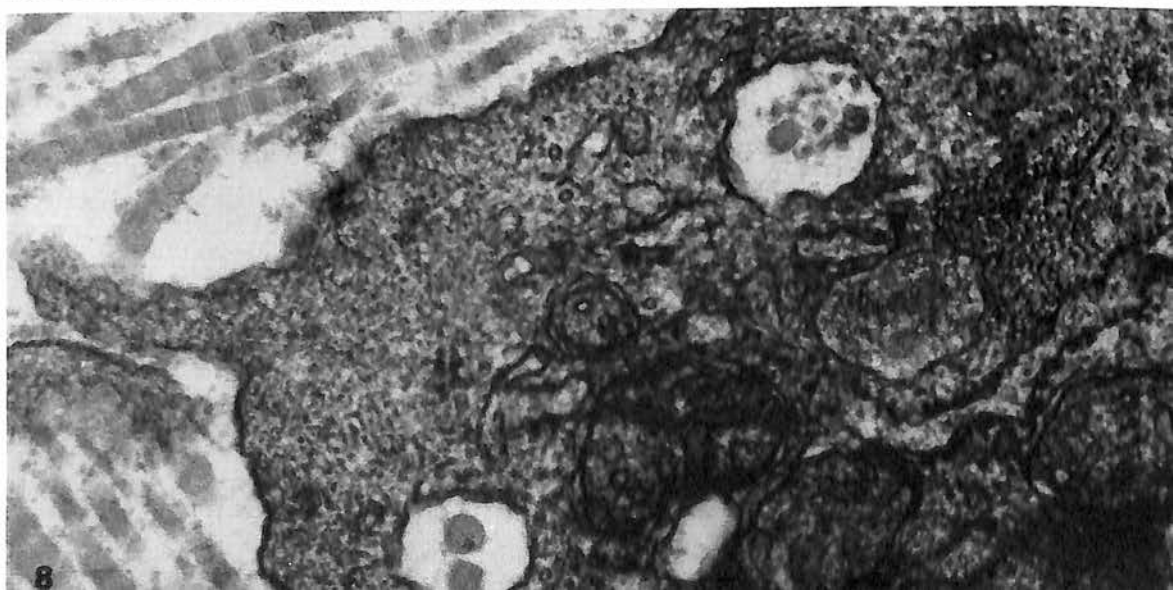
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The collagen breakdown apparently is not due to collagenase activity because the assay was negative ; however, the presence of a collagenase inhibitor can not be excluded inspite of perfusion of the ovary with trout balanced salt solution and removal of most of the plasma. The experiments with the fixed gelatin and collagen membrane digestion tend to confirm the finding that it is not collagenase that is responsible for the breakdown of the interiorized collagen. Digestion was observed only at pH 4.2 but not at pH 7.2 ; the latter is the optimal pH for collagenase. Furthermore, EDTA which is known to inhibit collagenase activity, did not stop digestion of the films at pH 4.2. It must be recognized, however, that the serosa and follicular cells were also present in these preparations and it can not be stated that the lytic enzymes originate uniquely from the theca. The demonstration of the presence of chymotrypsin, and other peptidases with the « Api-Zym » test system points to their potential role at least in part of the breakdown of the structural framework of the theca. The participation of other lytic enzymes is certainly possible but was not tested for.

The smooth muscle cells become phagocytotically active within a few hours after ovulation. The factors necessary for the induction of this new cell activity are not understood. It is clear, however, that the cell surface becomes activated because of the extensive and rapid shape changes that follow ovulation. The smooth muscle cells which have a smooth outline and are flattened cells before ovulation transform within a few hours into stellate cells with many cellular or amoeboid processes. The collagen bundles originally isolated by the cellular micropodia are large but the phagocytosed bundles interiorized into the cytoplasm are small. In cross sectional profiles most frequently 1 to 6 collagen fibers are found per vesicle even though vesicles with larger numbers are also found. A two step process may be involved in phagocytosis ; at first, the isolation of a relatively large fiber bundle by amoeboid processes and secondarily the incorporation of a smaller number of bundles within true phagocytotic vesicles. The longitudinally sectioned vesicles indicate that very long fiber portions are interiorised into the cells. The breakdown of collagen proceeds later on within the vesicles, possible by a lysosomal system. Enzyme histochemical analysis was not yet performed but the proximity of tiny, smooth walled vesicles which occasionally fuse with the long, collagen containing vesicles suggests that in the smooth muscle cells primary lysosomes are formed which are secondarily responsible for the degradation of the collagen.

It has been known for a long time that smooth muscle cells can synthesize collagen and elastic fibers but to the knowledge of the authors this report is the first one which demonstrates a further cellular differentiation or modulation which leads to the elimination of the extracellular structural framework manufactured by the same cell.

Symposium sur la Reproduction des Poissons
Paimpont, France, 19-21 *septembre* 1977.

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Résumé. Au moment de l'ovulation, les cellules constituant la *theca folliculi* de la truite sont morphologiquement très semblables à des muscles lisses. Le comportement physiologique de ce tissu confirme sa nature de muscle lisse. Une transformation et une différenciation nouvelle apparaissent dans les quelques heures qui suivent l'ovulation : les cellules de

la thèque commencent à phagocyter les fibres de collagène adjacentes. Des faisceaux de fibres de collagène sont d'abord partiellement entourés par des extensions cellulaires des cellules de la thèque puis de petits faisceaux sont finalement totalement englobés. Le nombre de fibres de collagène par vésicule varie de une à plusieurs. L'activité phagocytotique peut être détectée morphologiquement jusqu'à 17 jours après l'ovulation. Après ce temps, les fibres deviennent indistinctes, ce qui indique probablement une hydrolyse. Des tests effectués avec le système « Api-zim » et démontrant la présence de nombreuses enzymes lytiques dans les tissus ovariens post-ovulatoires sont à l'appui de ces observations morphologiques. La trypsine et la collagénase n'ont cependant pas été détectées.

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The cellular sources of sex steroids in teleost gonads

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Summary. Histochemical and ultrastructural studies of gonads from goldfish *Carassius auratus* and several species of salmon (genus *Oncorhynchus*) indicate that the Leydig or interstitial cells of the testis and special cells of the theca interna of the ovary are the major sources of gonadal steroids. Lobule boundary cells are considered homologous with the Sertoli cells of the testis and the granulosa cells of the ovary. In our preparations, lobule boundary, Sertoli and granulosa cells do not show convincing evidence of steroidogenesis.

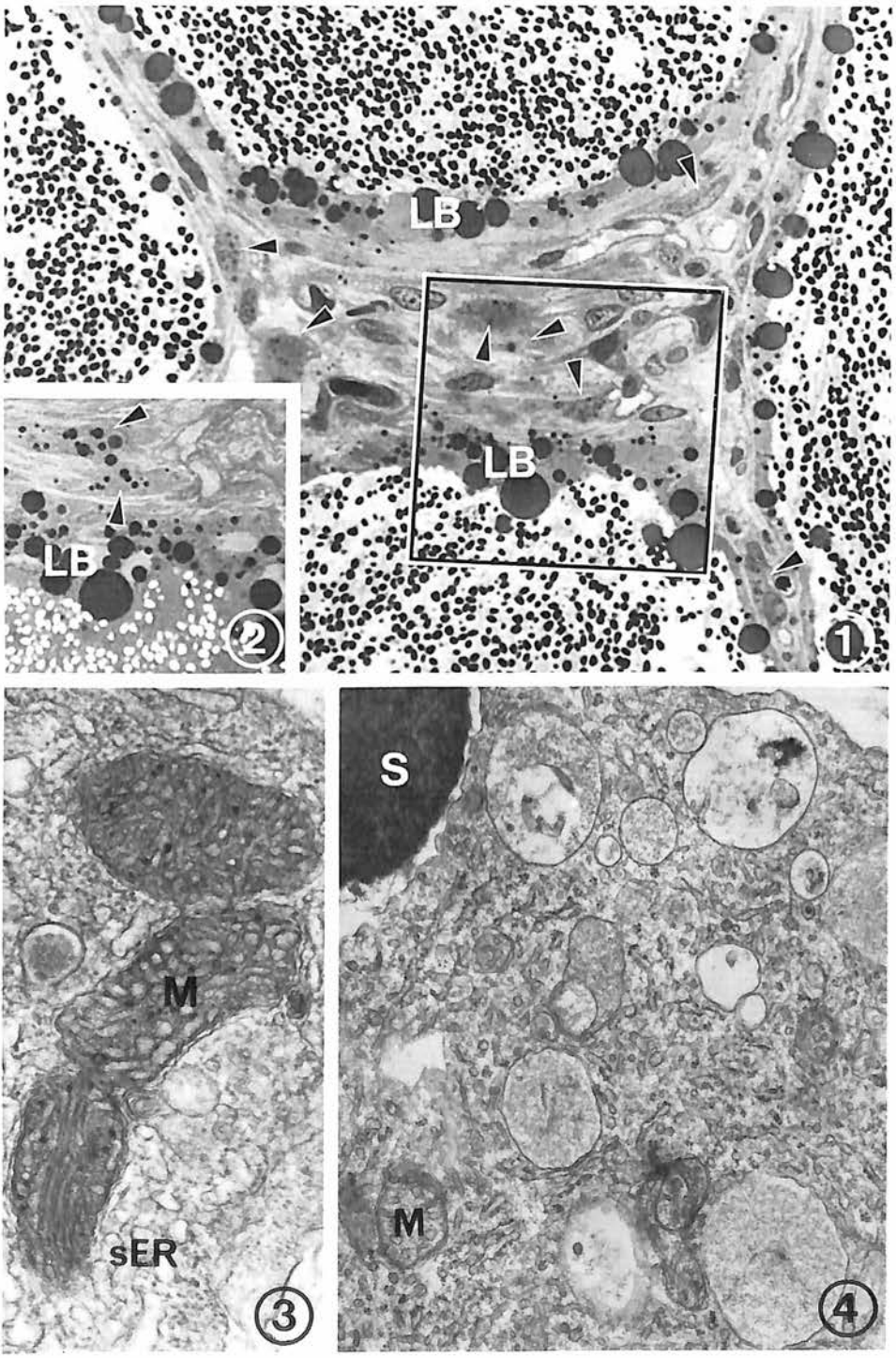
Although biochemical studies have shown that teleost fishes — like other vertebrates — produce gonadal steroids, the cellular sources of these hormones remain uncertain. Relevant literature is summarized in recent reviews (Lofts and Bern, 1972 ; Guraya, 1976*a*, *b*) and in the papers cited below. Electron microscopy and improved methods of histochemistry now offer possibilities for localizing the sources of these hormones. To this end, gonads of goldfish *Carassius auratus* and salmon (especially the coho *Oncorhynchus kisutch* and pink *O. gorbuscha*) have been examined at different times during the sexual cycle.

Leydig or Interstitial cells.

Our findings confirm those of most workers and assign to these cells the primary role in the synthesis of androgens. These large polygonal cells are usually located within the triangular interlobular spaces (figs 1 and 2), and are characterized by smooth endoplasmic reticula and mitochondria with tubular cristae (fig. 3) ; they contain many lipid droplets (fig. 2) and react positively to tests for enzymes concerned with androgen synthesis. Furthermore, gonadotropin treatment caused marked stimulation of the interstitial cells in immature goldfish (unpublished). These findings confirm several recent studies of Leydig cells using similar techniques (Nicholls and Graham, 1972 ; Gresik *et al.*, 1973 ; Van den Hurk *et al.*, 1974).

Lobule Boundary cells or Sertoli cells.

Lobule boundary cells, described in several teleosts, have been homologized with Leydig cells by some workers (O'Halloran and Idler, 1970) and with the Sertoli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cells by others (Nicholls and Graham, 1972 ; Billard *et al.*, 1972 ; Van den Hurk *et al.*, 1974 ; Grier, 1976). In our preparations, lobule boundary cells lack the ultrastructural characteristics of Leydig cells and are clearly homologous with the Sertoli cells. Although they contain many large lipid droplets (figs 1 and 2), they react negatively in histochemical tests for steroidogenesis. They occur in close proximity to spermatids and developing sperm, and possess some ultrastructural features suggesting phagocytosis and an involvement in transporting metabolites (fig. 4). Although Sertoli cells may synthesize steroids in some vertebrates, we found no evidence for this in salmon or goldfish.

Granulosa and Theca Interna.

Ovarian steroidogenesis has usually been attributed to the granulosa — or its postovulatory derivatives (Lambert and van Oordt, 1974 and reviews cited). However, granulosa cells of goldfish and salmon do not possess ultrastructural features typical of steroid-synthesizing cells but contain organelles suggestive of protein synthesis. Moreover, histochemical tests proved negative except for a very brief period following ovulation in the goldfish (Nagahama *et al.*, 1976). The evidence is equivocal. Although a transient period of steroidogenesis may occur in the postovulatory granulosa of the goldfish, this layer does not appear to be a major centre for steroidogenesis at any time in the ovarian cycles of the goldfish or salmon. We conclude that granulosa cells are concerned with oogenesis (formation of zona pellucida and yolk) and not hormone synthesis.

Ovarian hormones are apparently synthesized in special cells of the theca interna. These cells occur singly or in small nests and are cytologically similar to Leydig cells (Nagahama *et al.*, 1976) with which they appear to be homologous. Moreover, the special cells seem to retain their steroidogenic capacities for considerable time after

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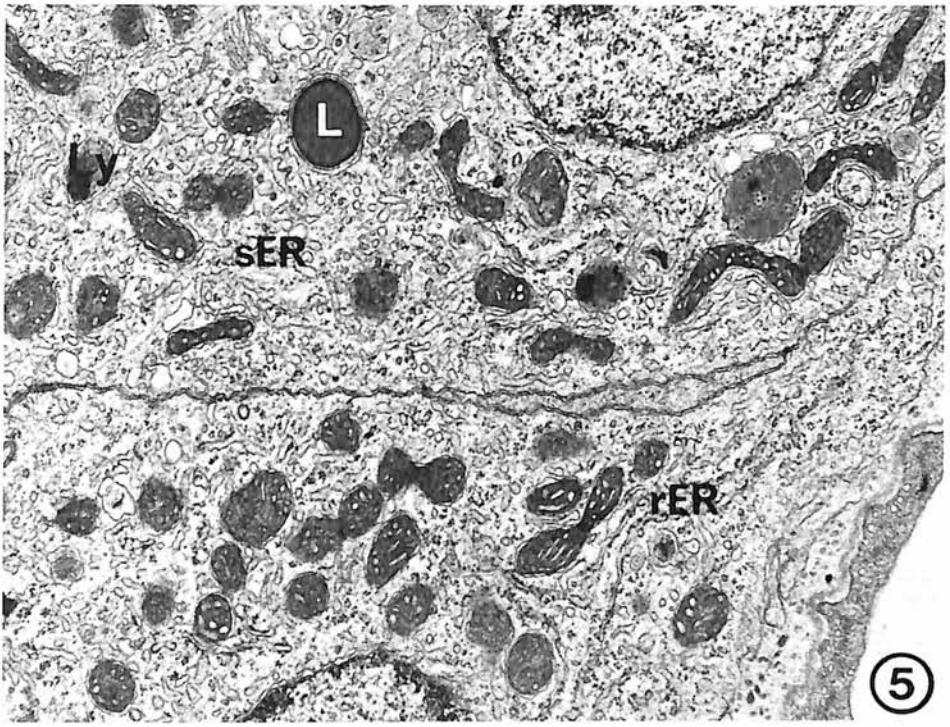
FIG. 1. — *Epon section* (1 μ) *of a portion of the testis of a mature pink salmon.* Interstitial cells (arrows) are seen in the interlobular space. Lobule boundary cells (LB) have many large lipid droplets. Methylene blue-azur II. × 700.

FIG. 2. — *Sudan black B stained-epon section* (1 μm) *of square area outlined in figure* 1. Note darkly stained lipid droplets in interstitial cells (arrows) and lobule boundary cells (LB). × 700.

FIG. 3. — *Electron micrograph of a portion of the interstitial cell of a mature coho.* Note large mitochondria (M) containing tubular cristae and intramitochondrial granules, and well-developed smooth endoplasmic reticulum (sER). × 40 000.

FIG. 4. — *Electron micrograph of a portion of the lobule boundary cell of a mature coho.* A sperm head (S) is found in close association with a lobule boundary cell. M, mitochondrion. × 20 000.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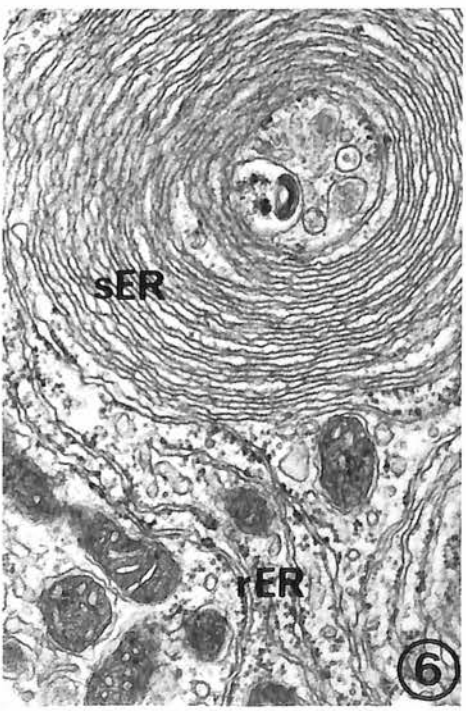
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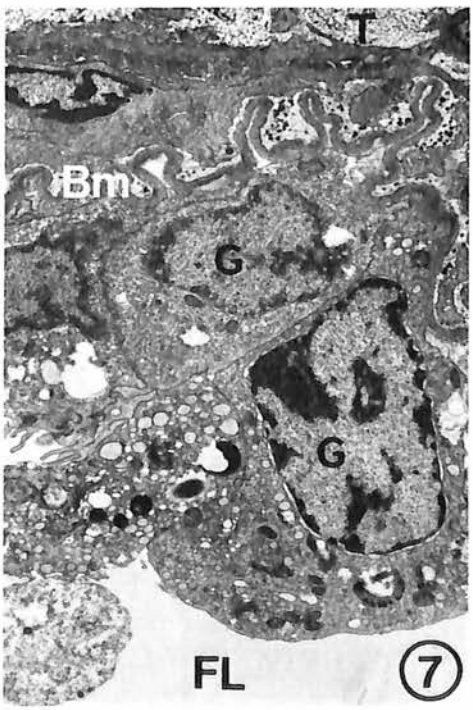
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ovulation (figs 5 and 6), while granulosa cells degenerate soon after ovulation (fig. 7). Several recent studies have reached similar conclusions in other teleosts (Nicholls and Maple, 1972 ; Saidapur and Nadkarni, 1976).

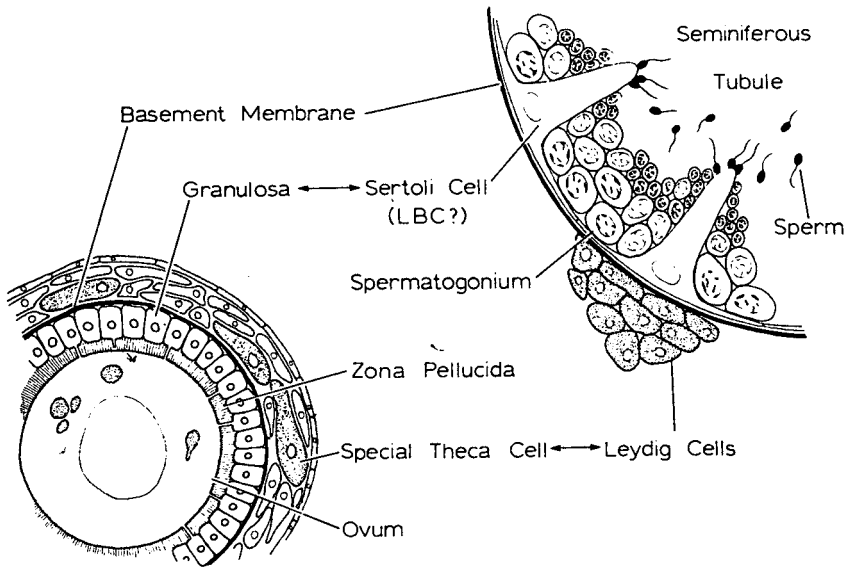

FIG. 8. — *Diagram showing steroid synthesizing cells in teleost gonads.* LBC, lobule boundary cell.

Figure 8 summarizes the probably sources of sex steroids in teleost gonads, based on several recent studies and our examination of goldfish and salmon. The interstitial cells in the testis and the special theca cells in the ovary are considered the main sources.

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FIG. 5. — *Electron micrograph of portions of two special theca cells of the post-ovulatory follicle of a coho.* Note many mitochondria with tubular cristae, and well-developed smooth (sER) and rough endoplasmic reticulum (rER). L, lipid droplet ; Ly, lysosome. × 15 000.

FIG. 6. — *Electron micrograph of a portion of a special theca cell of the post-ovulatory follicle of a coho.* Note extensive smooth endoplasmic reticulum (sER) arranged in the form of concentric whorls. × 30 000.

FIG. 7. — *Electron micrograph of a portion of the post-ovulatory follicle of a coho.* Note degenerated granulosa (G) cells. Bm, basement membrane ; FL, follicular lumen ; T, theca layer. × 4 000.

Résumé. L'étude histochimique et ultrastructurale des gonades du poisson rouge, *Carassius auratus*, et de plusieurs espèces de saumon (genre *Oncorhynchus*) montre que les cellules de Leydig ou cellules interstitielles du testicule et les cellules spéciales de la thèque interne de l'ovaire sont les principales sources des stéroïdes gonadiques. Les « lobules boundary cells » peuvent être assimilées aux cellules de Sertoli du testicule et aux cellules de la granulosa de l'ovaire de mammifère. Les « lobules boundary cells », les Sertoli et les cellules de granulosa ne montrent pas de signes nets d'activité stéroïdogène.

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Cyclic changes in the testis and vas deferens of the rainbow trout (*Salmo gairdneri*) with special reference to sites of steroidogenesis

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Summary. Enzyme cytochemical and electron microscopical data indicate Leydig cells as the main source of steroids in the testis of *Salmo gairdneri*. Steroidogenesis in Leydig cells is found during the annual testis cycle, with a peak in activity from January to June, i. e. the period when the testes are mature and new primary spermatogonia are formed. In June, Leydig cells are mainly found around seminiferous tubules containing secondary spermatogonia. Enzymes involved in steroidogenesis are also present in Sertoli cells of November animals during spermiation. Steroid formation has furthermore been demonstrated in stroma cells distributed in the connective tissue around the epithelium of the vas deferens. Steroidogenic activity in these stroma cells is stronger in the period when sperm cells are stored in the lumen of the vas deferens.

Introduction.

Several papers have dealt with the morphology of the testis of the rainbow trout *Salmo gairdneri*. The anatomy of the urogenital system of the trout was described by Henderson (1967), and the histology of the testis by Weisel (1943), Oota *et al.* (1965) and Boddingius (1975).

Several authors have discussed possible sites of gonadal hormone production. Robertson (1958) considered the Leydig cells as endocrine structures, but this was not confirmed by Boddingius (1975). Oota and Yamamoto (1966) presented ultrastructural evidence of steroidogenesis in Leydig cells. In some teleosts a steroidogenic function is ascribed to lobule boundary cells or Sertoli cells (Lofts and Bern, 1972) ; but this role was not substantiated in rainbow trout by ultrastructural data (Billard *et al.*, 1972).

The present study deals with the localization of steroid formation and the estimation of steroid synthesizing capacity in the testis and vas deferens of *Salmo gairdneri* using enzyme cytochemical methods. Electron microscopy was carried out to detect possible sites of steroidogenesis in the testis. A brief histological description of the annual spermatogenetic cycle is presented as a basis for these studies.

Material and methods.

Thirty male specimens of three-year old rainbow trout, *Salmo gairdneri* Richardson, were obtained from a trout hatchery in Vaassen (the Netherlands), in 1975 on June 3, August 7, September 24, and November 4, and in 1976 on January 7, February 25 and May 5. The animals were anesthetized with 0.035 p. 100 MS 222 or carbogen gas, followed by decapitation and removal of the testes and vasa deferentia. Histological studies were carried out on 4 μm sections of tissue fixed in Smith's formaldehyde-bichromate. Cytochemical tests were performed with 10 μm sections of tissues frozen at — 20 °C with CO_2. For histology and cytochemistry, sections were cut from various parts of the testis and rostral part of the vas deferens. The following enzyme reactions were carried out : 3β and 3α-hydroxy steroid dehydrogenase (3β- and 3α-HSD) using the method of Baillie *et al.* modified by van den Hurk (1973), and glucose-6-phosphate dehydrogenase (G6PD) using the method of Rudolph and Klein as modified by van den Hurk (1973). Intensity of the enzyme reactions was graded in arbitrary units, as follows : no reaction, (—) ; moderate, (++) ; and strong, (+++).

For electron microscopy, medial and distal parts of the testis were fixed according to Peute *et al.* (1976). The tissues were then dehydrated in graded ethanol and propyleneoxide, and subsequently embedded in Epon. Thin sections were examined with a Philips EM 300.

Results.

The testis of the rainbow trout is tubular (van den Hurk, 1973) with a network of efferent ducts situated on the mesorchial side. The seminiferous tubules are connected with a vas deferens by the efferent duct system. On the basis of histology, three periods can be distinguished in the spermatogenetic cycle :

1. The multiplication period from June to September is characterized by the formation of increasing numbers of cysts containing secondary spermatogonia. In June these cysts are present mainly at the mesorchial side of the testis, but in August they can be observed in all seminiferous tubules.

2. During the maturation period from September to January, primary spermatocytes are formed, and maturation divisions and spermiogenesis are in progress. From November, the testis tubules and vasa deferentia are filled with masses of ripe sperm.

3. The period of functional maturity commences when spermatogenesis is complete in January and lasts until June. In the Dutch hatcheries, rainbow trout do not spawn spontaneously. Between February and June, the sperm cells in the testis tubules and the efferent duct system are resorbed by Sertoli cells. To that end Sertoli cells form protrusions into the lumen of the tubule and subsequently become detached from the tubule wall. Desintegrating sperm cells remain visible in the efferent ducts until September. The third period may also be referred to as the period of accumulation of primary spermatogonia. These primary spermatogonia are situated in the peri-

phery of the testis tubules and are surrounded by Sertoli cells. In January, primordial germ cells invade the interstitium and change into primary spermatogonia when surrounded by Sertoli cells in the wall of the testis tubules. The greatest increase in the number of primordial germ cells and primary spermatogonia is in February. The number of primary spermatogonia continues to increase until June, whereas the number of primordial germ cells decreases after February.

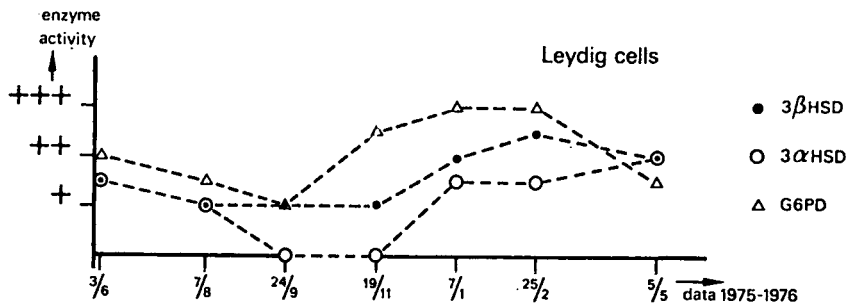

FIG. 1. — 3β-HSD, 3α-HSD *and* G6PD *activity in Leydig cells during the annual cycle.*

The activity of steroidogenic enzymes — 3β-HSD, 3α-HSD and G6PD — is almost entirely confined to the interstitial Leydig cells. A moderate to strong activity of these three enzymes occurs during the period of functional maturity and accumulation of primary spermatogonia (figs. 1 and 2). At the beginning of the multiplication period, large numbers of steroid-synthesizing Leydig cells are centered around the tubules containing cell nests with primary and secondary spermatogonia. Later during this period, the enzyme reactions become weaker. In September, no activity of 3α-HSD could be demonstrated. During the maturation period, 3β- and 3α-HSD activity of the Leydig cells remains unchanged while the G6PD activity increases. In November, however, a distinct though slight 3β-HSD (fig. 3), 3α-HSD and G6PD activity could be demonstrated in Sertoli cells lining post-spermatogonial cysts. At other times of the year these cells show a weak G6PD activity only.

The ultrastructure of the Leydig cells (fig. 4) is characterized by smooth endoplasmic reticulum (SER) and numerous mitochondria with tubular cristae and a dense matrix (MTC). This combination of cell organelles is characteristic of steroidogenesis. The Leydig cell cytoplasm also contains rough endoplasmic reticulum, free ribosomes, polysomes and Golgi-cisternae. Sertoli cells have a SER and a few round to tubular mitochondria with lamellar cristae (fig. 5). Lysosomes are characteristic of Sertoli cells lining seminiferous tubules containing residual sperm cells. Studies of November testes did not contribute to a solution of the structure of the mitochondria in the Sertoli cells. Throughout the annual cycle, 3β-HSD-positive cells are found in the connective tissue surrounding the epithelium of the vas deferens (fig. 6). These stroma cells also show 3α-HSD and G6PD activities. The fluctuation in enzyme activities of the stroma cells parallels the activity of the Leydig cells, with the strongest activity from January untill June, when sperm cells are stored in the lumen of the vas deferens.

Discussion.

Leydig cells are the main source of testis steroids in teleosts (Lofts and Bern, 1972 ; van den Hurk, 1973). In rainbow trout, Leydig cells were considered as possible endocrine structures by Robertson (1958) and Oota and Yamamoto (1966). Boddingius (1975), among others, suggested an endocrine role for the lobule boundary cells of this species. EM-studies by Billard *et al.* (1972), however, demonstrated the improbability of steroidogenesis in these cells ; these latter workers found lobule boundary cells and Sertoli cells to be identical.

In our experiments, the presence of 3β-HSD, 3α-HSD and G6PD activities, SER and MTC are indicative of steroidogenesis in Leydig cells of rainbow trout. Steroidogenic activity in Leydig cells appear to be strong in January, February and May, when ripe sperm cells are stored in the lumen of the testis tubules, indicating that steroids are involved in these processes. In June, the presence of steroid-synthesizing Leydig cells at sites where secondary spermatogonia are formed suggests a functional relationship. The localization of Leydig cells around cysts with spermatogonia and the epithelium of the efferent duct system in *Poecilia latipinna*, was considered to be indicative of intratesticular effects of androgens (van den Hurk, 1975). As compared to these findings, the distribution of steroid-synthesizing stroma cells around the epithelium of the vas deferens of the trout may indicate that steroids are involved in the functioning of the sperm duct. Although G6PD activity was found in the Sertoli cells throughout the annual cycle, 3β- and 3α-HSD activities in these cells were observed in November only. This may indicate steroidogenesis in the Sertoli cells during this limited period, when the production of steroids in Leydig cells is comparatively low. As the presence of steroidogenic enzymes coincides with spermiation, steroid hormones possibly produced in the Sertoli cells might stimulate the transport of sperm cells from the testis tubules to the efferent duct system. This is in support of the findings of Yamazaki and Donaldson (1969) who in studies on the goldfish, *Carassius auratus*, were the

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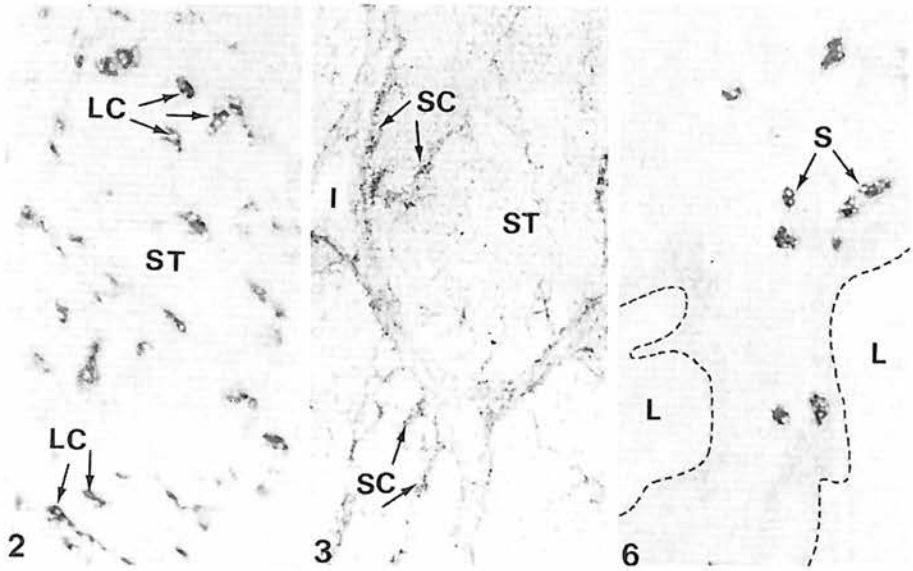
FIG. 2. — 3β-HSD *activity in Leydig cells* (LC). ST = seminiferous tubule. × 140.

FIG. 3. — 3β-HSD *activity in Sertoli cells* (SC) from November. I = interstitium. × 140.

FIG. 4. — *Ultrastructure of a Leydig cell.* Solid arrows indicate SER, dotted arrow indicates RER. MTC = mitochondrium with tubular cristae ; R = ribosomes. × 28 000.

FIG. 5. — *Ultrastructure of a Sertoli cell* (May). Solid arrows indicate SER, dotted arrow indicates RER. MLC = mitochondrium with lamellar cristae ; N = nucleus ; L = lumen of a seminiferous tubule ; SP = sperm cell ; I = interstitial tissue. × 13 000.

FIG. 6. — 3β-HSD *activity in stroma cells* (S) *of the vas deferens.* L = lumen. × 140.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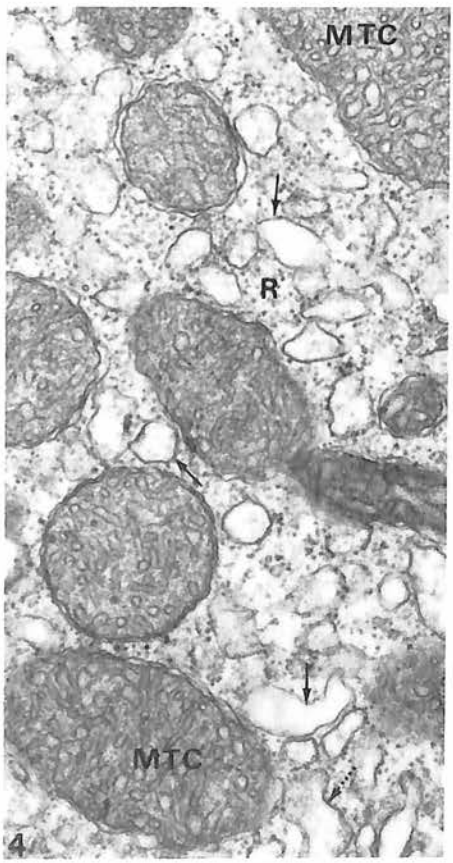
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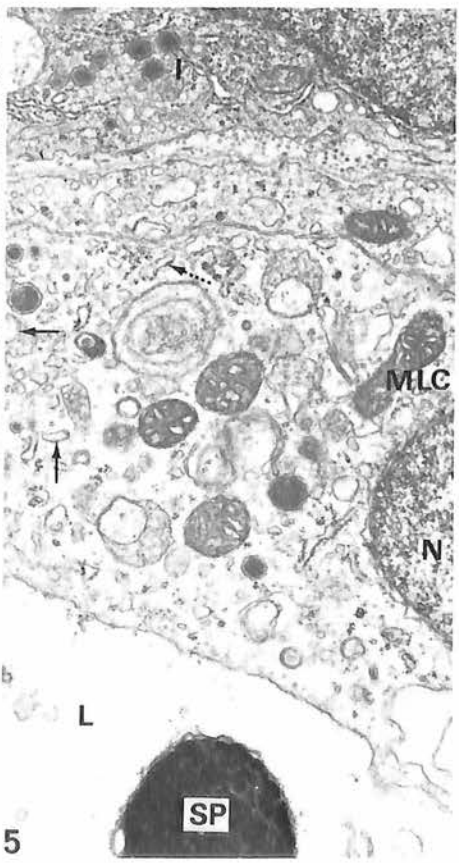
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first to demonstrate a regulation of spermiation by androgens. In the present studies however, steroidogenesis in the Sertoli cells has not been proved by ultrastructural verification of the cytochemical results.

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Résumé. L'histoenzymologie et la microscopie électronique montrent que les cellules de Leydig sont la source principale de stéroïdes dans le testicule de *Salmo gairdneri*. L'activité stéroïdogène de ces cellules est trouvée tout au long du cycle reproducteur, avec un pic d'activité de janvier à juin, quand les testicules sont matures et que de nouvelles spermatogonies se forment. En juin, les cellules de Leydig sont principalement localisées à la périphérie des tubules séminifères contenant des spermatogonies secondaires. Les enzymes de la stéroïdogenèse sont également présentes dans les cellules de Sertoli en novembre, pendant la spermiation. La synthèse de stéroïde a de plus été mise en évidence dans les cellules du stroma distribuées dans le tissu conjonctif autour de l'épithélium du vas deferens. L'activité stéroïdogène dans ces cellules du stroma est plus intense durant la période de stockage des spermatozoïdes dans la lumière du vas deferens que pendant d'autres phases du cycle reproducteur.

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Gonadotropic cells of the rainbow trout pituitary during the annual cycle. Ultrastructure and hormone content.

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Summary. The GTH cells in the rainbow trout *Salmo gairdneri* appeared in a « globular » or in a « cisternal » stage with intermediates. Most GTH cells in females were in the globular stage up to June, after which predominately cisternal ones were observed for about 2 months. A similar shift was seen in male GTH cells in September. Correlation between RIA and ultrastructural studies of the pituitary suggested the storage of GTH in secretory vesicles and globules. The drop in pituitary hormone content was concomitant with the onset of exogenous vitellogenesis in June/July and with the acceleration of spermiogenesis in September. Both processes appeared to be GTH-dependent.

Introduction.

The salmonid pituitary produces a gonadotropic hormone (GTH) inducing spermatogenesis and vitellogenesis in goldfish (Yamazaki and Donaldson, 1968). The plasma concentration of this hormone increases during sexual maturation (Crim *et al.*, 1975). The pituitary cells producing GTH simultaneously increase in number and secretory activity (Olivereau and Ridgway, 1962 ; Van Overbeeke and McBride, 1967). It is assumed that quantitative changes in plasma and pituitary GTH correspond with changes in the ultrastructure of the GTH cells. The validity of this assumption has been tested by studying the hormone content and the ultrastucture of rainbow trout GTH cells at various stages of the reproductive cycle. The gonadotropic nature of these cells was determined by means of histophysiological and immunohistochemical studies by Boddingius (1975) and Goos (unpublished results), respectively. As in other salmonids such as *Salmo salar* (Ekengren *et al.*, 1978), the gonadotropic cells in the rainbow trout pituitary were basophils, situated in the rostral as well as in the proximal *pars distalis* ; depending on the physiological state of the animal, the cells contained both secretory vesicles and globules.

Material and methods.

Animals. — At intervals of one or two months, pituitaries were collected from three male and three female *Salmo gairdneri*, reared in a Dutch hatchery. The ani-

mals together cover one annual cycle (1975/1976). Prior to decapitation the animals were anaesthetized with Ms222 or CO_2.

Electron microscopy. — The method of Peute *et al.* (1976) was used.

Radioimmunoassay. — Pituitaries were extracted in 200 µl of phosphate buffered saline (0.01 M, pH 7.2). A heterologous radioimmunoassay system was used for measuring pituitary GTH content. With this system, all data from the assay have to be considered as relative values. Therefore, the amount of GTH detected in pituitary extracts is expressed in assay units (AU) instead of weight units.

Antiserum was raised against salmon gonadotropin (SG-G100 from Dr. E. Donaldson-Vancouver) and used at a working dilution of 1 : 32 000, which means in this assay a final dilution of 1 : 180 000. Maximal B/F ratio with this dilution was 0.2.

According to the chloramine-T method ^{125}iodine-labeled pure carp gonadotropin (from Dr. E. Burzawa-Gérard-Paris) was used as label (500 cpm/tube. The same carp-GTH has been applied as standard hormone in a range of 12.5 pg/tube to 0.5 ng/tube. Assay sensitivity was defined as the lowest detectable amount of hormone (about 1.4 AU/ml).

Observations.

Electron microscopy.

During the greater part of the year, most GTH cells in males and females are characterized by the combined presence of globules, measuring 4 000-8 000 Å in diameter, and round to oval or somewhat elongate secretory vesicles, 1 000-3 000 Å in diameter. The cells contain small, rounded cisternae of rough endoplasmic reticulum (RER, fig. 1). These represent the so-called « globular » stage, and are mainly situated in the proximal *pars distalis*. In addition, GTH-cells can also be observed between the follicles of the rostral *pars distalis* and among the prolactin cells as part of the follicles (fig. 2). During some months, however, the majority of GTH-cells are in the so-called « cisternal » stage. Globules and secretory vesicles have almost completely disappeared and the cytoplasm is mainly composed of numerous more or less round and dilated cisternae, the membranes of which are studded with a few ribosomes (fig. 3). The cisternae contain material of moderate electron density. Intermediate stages have also been noticed, i. e. cisternal cells with few or no globules, but with active Golgi systems budding secretory vesicles. Depending on the time of the year,

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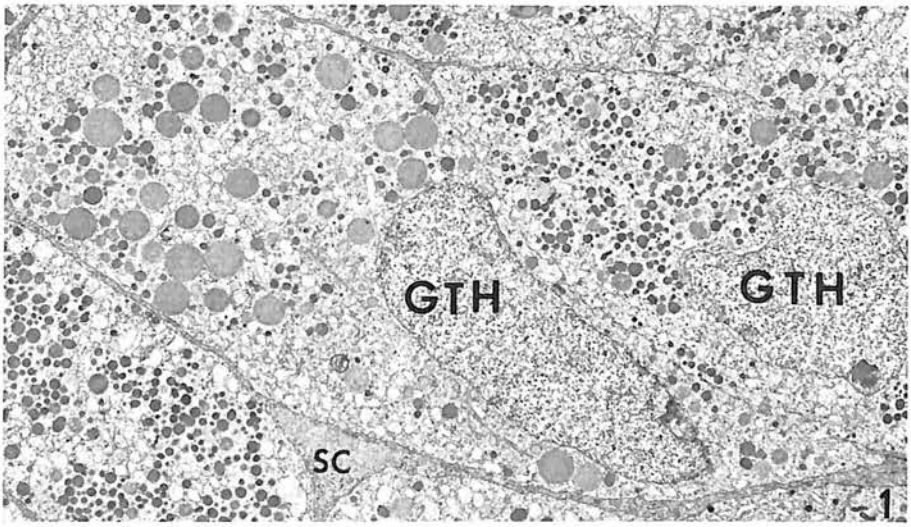
FIG. 1. — *Proximal* pars distalis ; *GTH cells in « globular » stage.* SC : stellate cell (× 5 000).

FIG. 2. — *Rostral* pars distalis ; *« globular » GTH cell in a follicle of prolactin cells.* L : lumen (× 5 000).

FIG. 3. — *Proximal* pars distalis ; *GTH cells in « cisternal » stage.* GA : Golgi area ; SC : stellate cell, STH somatotrop (× 5 000).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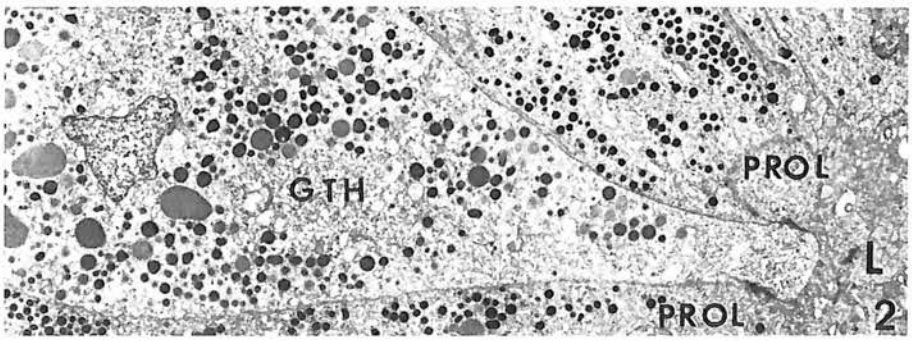
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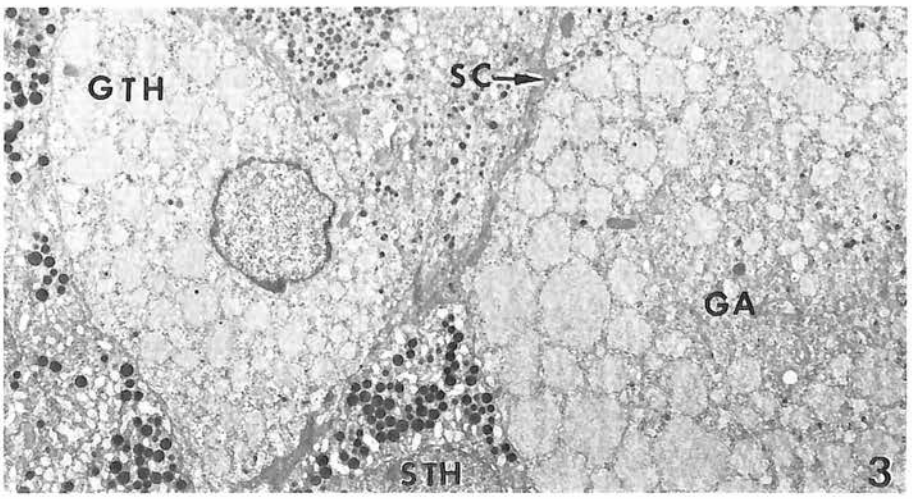
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both the globular and the cisternal stages as well as intermediate forms may be found within one and the same pituitary. In female trout a predominantly cisternal stage is reached in June and lasts about until October. Then a gradual regranulation takes place, and in December more cells containing secretory vesicles and globules can be observed. This shift to the globular stage continues from January to March ; later, GTH-cells are hardly present in the cisternal stage.

In males the cisternal stage was much shorter, i. e. from September to November. Then a gradual regranulation of many GTH-cells was observed, leading to a heterogeneous population of GTH-cells in which the globular stage finally predominates. In this period some GTH-cells contain one or two large cisternae, which almost completely fill up the cytoplasm. Such cells have no globules and only a few secretory vesicles. Between March and May the cisternal stage gradually disappears and the globular stage predominates up to September.

Radioimmunoassay.

The pituitary of females collected during June and July contains an average of 2 000 AU. This increased to 20 000 AU in August, to 30 000 AU in November and to 100 000 AU in December. The GTH content remained high up to April-May.

In pituitaries of males, low values (4 000 AU) were not found until September ; thereafter the values increased again to 100 000 AU, and in some animals even to 200 000 AU by December. Such high values continued to be present up to August. In August the GTH content dropped to about 20 000 AU per pituitary, and this decrease continued until September.

Discussion.

From a comparison of the present ultrastructural observations with the results of the RIA method, it may be concluded that there is generally a correlation between the presence of the globular stage of the GTH-cells and a high titer of GTH assay units. Low levels of GTH coincide with predominantly cisternal GTH-cells. This means that both the secretory vesicles and the globules probably represent the storage organelles of the gonadotropin principle (see also Nagahama and Yamamoto, 1969).

This would make it unlikely that in *Salmo gairdneri* the globules of the GTH-cells represent lysosomes, as suggested for other species by Olivereau (1967), Ball and Baker (1969) and others. Indeed, it has not been possible to detect the important lysosomic enzyme, acid phosphatase, in the globules of trout GTH-cells. However, in addition to the globules, round organelles resembling lysosomes have occasionally been observed ultrastructurally (Peute, unpublished results). The drop in GTH content, and the disappearance of secretory vesicles and globules in the GTH-cells of the females during June and July, coincide with an augmentation in steroidogenic capacity of the ovary and also of exogenous vitellogenesis in the oocytes (Lambert *et al.*, 1978). A similar correlation can be made in male trout during September-October, when germ cell maturation and spermiogenesis are accelerated (Van den Hurk *et al.*, 1978). Thus in *Salmo gairdneri* both vitellogenesis and the formation of sperm cells can be correlated with a decreased pituitary GTH content.

In other salmonids these gonadal processes were accompanied by an increased blood GTH level (Crim *et al.*, 1975).

On the other hand, the high steroidogenic activity in the post-ovulatory follicles (Lambert *et al.*, 1978) and in the testicular Leydig cells (Van den Hurk *et al.*, 1978) during January-February is not concomitant with a decrease in GTH storage in the pituitary. This does not exclude a marked GTH release during that part of the year. Indeed, Goos (unpublished) recorded a high GTH level in the blood of female *Salmo gairdneri* in January-February. In the same period, GTH-cells with extremely large cisternae have been observed in the pituitary of male trouts. Such large cisternae have been interpreted by Kaul and Vollrath, (1974) and by Lam *et al.* (1976) as the resul of a high secretory activity.

The present results indicate that changes in storage of secretory vesicles and globules can be related to changes in the pituitary GTH content, but do not necessarily reflect variations in GTH release. In the present study it is demonstrated that during the annual cycle the GTH-cells pass through different phases of activity, which are not synchronous in all GTH-cells. The result is a mixed population, its composition depending on the sex of the animal and the time of the year.

These data do not justify the assumption that there is more than one type of GTH-cells in the pituitary of *Salmo gairdneri*. Similarly, Nagahama (1973) and Ekengren *et al.* (1978) found only one GTH-cell type in salmonids, whereas Cook and Van Overbeeke (1972) and Olivereau (1976) defended the presence of two GTH-cell types in the pituitary of this. group of teleosts.

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Résumé. Les cellules gonadotropes chez la Truite *Salmo gairdneri* peuvent apparaître sous deux états « globulaire » ou « citernal » (avec des intermédiaires). Chez la femelle, la plupart des cellules gonadotropes sont de type « globulaire » jusqu'en juin, puis « citernal » pendant environ 2 mois. Le même changement est observé chez le mâle en septembre. La comparaison de ces résultats avec ceux obtenus par dosage de la gonadotropine (GTH) dans l'hypophyse et études de l'ultrastructure hypophysaire suggère que la GTH est stockée dans des vésicules sécrétoires et des globules. Une corrélation a également été mise en évidence entre la chute de la teneur hypophysaire en GTH et le début de la vitellogenèse exogène (juin/juillet) et l'accélération de la spermiogenèse (septembre). Les deux processus semblent sous la dépendance de la GTH.

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Evolution des cellules gonadotropes de *Boops salpa* L. (Téléostéen Sparidae) au cours de la spermatogenèse

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Summary. *Gonadotropic cell evolution in the teleost sparid,* Boops salpa *L., during spermatogenesis.*

The glycoprotein gonadotropic cells of *Boops salpa*, located in the proximal *pars distalis*, presented two cell forms clearly discernable by their distinctive staining reactions, localization and secretory activity cycle. The first cell type, mainly localized ventrally, appeared in the juvenile *Boops* when the germ cells reached the gonads ; it predominated and maximally developed during the spawning season. This cell type contained coarse granules, strongly stained with alcian or aniline blue and PAS, and some vacuoles. It degranulated slightly and some pyknotic nuclei appeared at the end of the spawing period. The second cell type, appearing in adult specimens and localized somewhat dorsally, was more voluminous and slightly PAS-positive and MacConaill hematoxylin-positive. At maturity, the number and size of these elements increased while progressive degranulation and intense vacuolization occurred in most of these cells which then contained a few coarse acidophilic granules at the spawning season.

Introduction.

Chez les Téléostéens, les cellules gonadotropes glycoprotéiques sont localisées, en majeure partie, dans la pars distalis proximale, comme l'a confirmé l'immunofluorescence (Billard *et al.*, 1971 ; Goos *et al.*, 1976 ; Peute *et al.*, 1976). Selon les espèces, une ou deux catégories ont été décrites, en microscopie photonique et électronique (Olivereau, 1976). Chez la Saupe, deux types cellulaires ont déjà été signalés (Michele, 1973).

Comme les autres Sparidae, *Boops salpa* présente une gonade bisexuée. De nombreux travaux portant sur des saupes de diverses régions méditerranéennes ont suggéré la coexistence d'un hermaphrodisme protandrique et d'un hermaphrodisme potentiel (Malo-Michele, 1977). C'est-à-dire qu'un individu à gonade juvénile bisexuée peut donner soit un hermaphrodite potentiel mâle ou femelle, soit un protandre typique plus ou moins tardivement mâle fonctionnel, se transformant par la suite en femelle au cours de l'inversion sexuelle. La principale caractéristique des gonades de ce Sparidae est leur grande variabilité d'un individu à l'autre, puisque l'on peut constater des spermatogenèses précoces dès la fin de la troisième année, tandis que des cas

d'inversion sexuelle tardive s'observent encore au-delà de la septième année, pour une taille corporelle de 46 cm.

Les nombreuses captures (550) échelonnées sur 7 ans et effectuées jusqu'ici dans la région niçoise et de Biarritz n'ayant pas procuré de femelles complètement matures, la fin de la vitellogenèse n'a pas encore pu être observée ; c'est pourquoi cette note histologique concerne uniquement l'évolution simultanée des cellules gonadotropes et du testicule, au cours de son développement, de la maturité et du frai, chez l'hermaphrodite potentiel mâle ou le protandre.

Matériel et méthodes.

Sur 112 individus capturés de Cannes à Monaco, n'ayant pas séjourné en aquarium, de taille variant de 17 à 33 cm, et un de 46 cm provenant de Biarritz (an 2 + à an 8 +), 34 présentent des cystes en spermatogenèse ou en spermiogenèse dans le testicule. Les sacrifices ont lieu régulièrement pendant l'année, surtout en août, où la spermatogenèse s'amorce, et en septembre-octobre, période de la reproduction.

Hypophyse et gonades sont prélevées sur ces 112 individus sacrifiés par section de la moelle. Les hypophyses sont fixées au Bouin-Hollande sublimé, coupées à 5 microns et colorées au trichrome de Cleveland-Wolfe, au tétrachrome de Herlant, au Bleu alcian (BA)-PAS-orange G (OG) et au PAS suivi de l'hématoxyline au plomb de MacConaill (HPb). Les gonades sont fixées au Bouin, coupées à 5 et 7 microns et colorées à l'hématoxyline de Harris-éosine et au trichrome de Mallory.

Résultats.

a) *Gonade.* — Mis à part des phénomènes préspermatogénétiques très localisés, observés chez de rares exemplaires d'octobre 2 + à octobre 3 + (19 à 24 cm), la spermatogenèse débute, pour un certain nombre d'individus, en août 3 + et s'accomplit en septembre 3 + et octobre 4 + (26 à 28 cm). En août 4 +, la spermatogenèse concerne un plus grand nombre de saupes gonochoriques secondaires mâles ou protandres : les gonies sont encore prédominantes, mais de nombreux cystes de spermatocytes I et II et quelques autres de spermatides s'observent dans les tubules séminifères. Dès la

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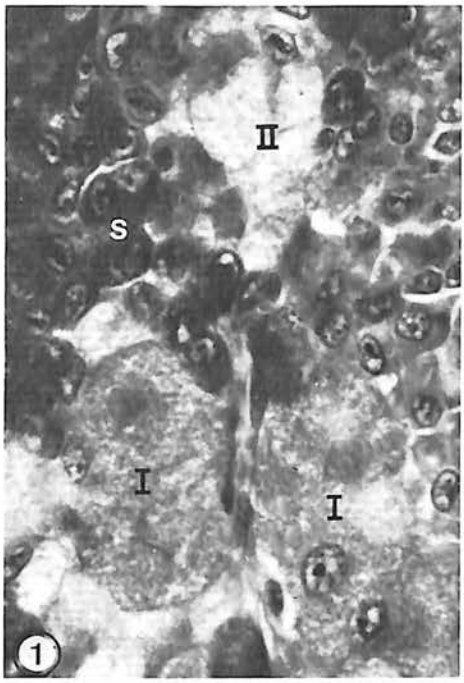
FIG. 1. — *Distinction des deux catégories de cellules gonadotropes au milieu des cellules somatropes(s) qui apparaissent en noir* : le type I à gros granules gris et le type II très clair en cours de dégranulation et début de vacuolisation.

FIG. 2. — *Idem* : une des cellules du type II (flèche) présente de gros granules répartis dans toute la cellule alors que les autres sont déjà complètement vacuolisées et ne contiennent plus que le noyau et quelques gros granules.

FIG. 3. — *Entre les cellules somatotropes sombres, s'insinuent les cellules gonadotropes du type II, hautes et claires, vers les ramifications neurohypophysaires.*

FIG. 4. — *Vacuolisation intense du type II.* Images de « bague à chaton » (flèches).

FIG. 1, 2 et 3, trichrome de Cleveland et Wolfe ; fig. 4, PAS-HPb. × 1250. Filtre vert.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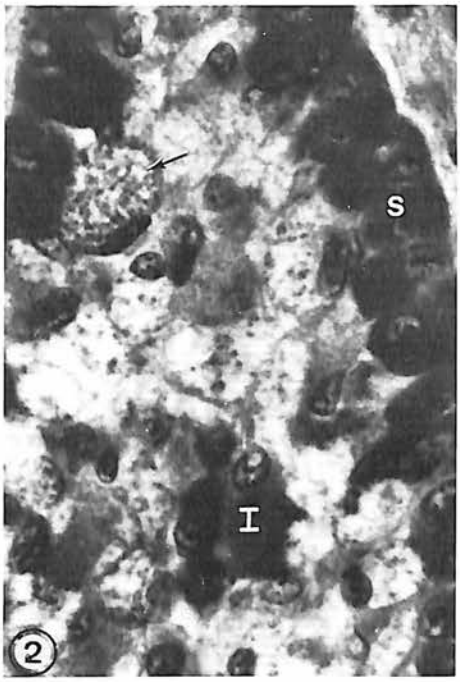
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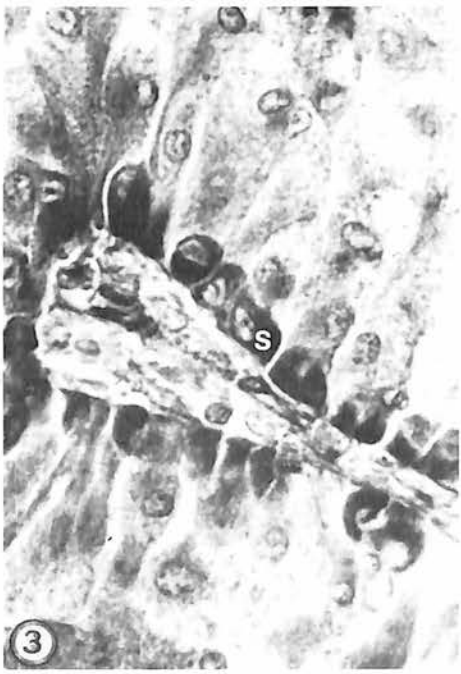
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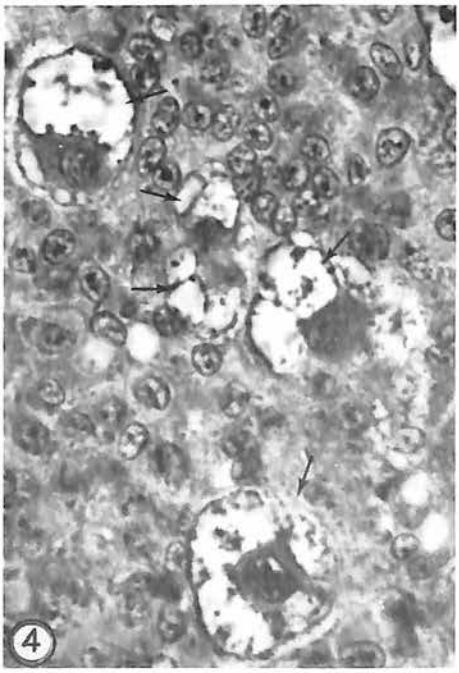
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fin septembre et pendant le mois d'octobre, dans les lobules très développés qui s'anastomosent et convergent vers le hile, plus de 90 p. 100 des éléments germinatifs sont des spermatides ou des spermatozoïdes ; le sperme fluent est stocké dans la zone centrale et le *vas deferens* qui fait saillie contre la cavité ovarienne ainsi réduite à une mince fente.

b) *Hypophyse.* — Chez les très jeunes saupes de 5 cm, l'ébauche de la gonade contient peu ou pas de cellules germinales et les cellules gonadotropes sont rares. Au-dessus de 10 cm, leur nombre augmente surtout ventralement et postérieurement pour former une couche autour de la pars intermedia. A l'approche de la maturité, dans les cas relativement rares où elle est très précoce et le frai souvent partiel, l'expansion de la zone gonadotrope est modérée. Mais, chez les individus qui parviennent à la maturité à la fin de l'an 4 + ou au-delà, la zone gonadotrope est considérablement modifiée. Son expansion, surtout par augmentation du nombre et de la taille des cellules ventrales, repousse vers l'avant les cellules à prolactine, et vers l'arrière enveloppe la pars intermedia. Deux types de cellules gonadotropes se distinguent alors nettement.

Le type I (fig. 1 et 2), prédominant et ventral, contient d'épaisses granulations intensément colorées par le bleu alcian, le bleu d'aniline et le PAS, masquant les limites cellulaires. Son développement maximal se situe pendant le frais. Il comporte quelques petites vacuoles et tend à se dégranuler et, à la fin du frai, à présenter des noyaux pycnotiques. Puis la taille cellulaire et la chromophilie diminuent progressivement.

Le type II, dorsal et mauve-rosé après BA-PAS-OG ou trichrome de Cleveland-Wolfe, faiblement HPb-positif, n'existe pas chez les jeunes saupes. Chez l'adulte hors de la période de frai, ces cellules plus volumineuses que celles du type I, ovalaires, sont souvent isolées ou en petits groupes entre les cordons des cellules somatropes. A la maturité, elles s'intercalent entre celles-ci pour former de hautes palissades de cellules hypertrophiées contre la neurohypophyse (fig. 3) ; elles tendent à se dégranuler (fig. 1 et 3) et à se vacuoliser presque complètement : les images de « Chaton de bague » (fig. 2 et 4), avec de gros granules amphophiles, ayant une affinité particulière pour l'OG, sont nombreuses. Après le frai, ces cellules perdent leur polarité vers les ramifications neurohypophysaires.

Discussion.

Le testicule est plus sensible que l'ovaire à de petites quantités de gonadotropines (Ball *et all.*, 1965 ; Crim *et al.*, 1975). Chez la Saupe, la précocité de la spermatogenèse par rapport à l'ovogenèse entraîne, entre autres, un dimorphisme sexuel net entre les femelles et les hermaphrodites potentiels mâles, puisque le taux de croissance des mâles décroît sitôt la maturité sexuelle accomplie, sous l'effet des hormones androgènes (Schreibman *et al.*, 1973). Un moyen d'identification des hermaphrodites potentiels mâles consiste à noter la taille testiculaire croissante avec l'âge, pour une taille corporelle stationnaire (30-32 cm).

Boops salpa présente deux formes de cellules gonadotropes distinctes chez l'adulte par leurs affinités tinctoriales, leur localisation et leur cycle d'activité sécrétoire,

comme c'est le cas pour de nombreuses espèces dont la revue a déjà été faite (Olivereau, 1976). Le type I prédominant, très fortement BA-positif et PAS-positif, a son point d'activité culminant pendant le frai, alors qu'à cette période, le type II, plus volumineux mais moins abondant et faiblement PAS-positif, se vacuolise fortement. Au contraire, d'autres espèces ne présentent qu'une seule catégorie de cellules gonadotropes (Olivereau, 1976) ; c'est aussi le cas de *Poecilia latipinna* étudié en microscopie électronique (Batten *et al.*, 1975) et en immunofluorescence (Peute *et al.*, 1976 ; Goos *et al.*, 1976). Le problème de l'unicité ou de la dualité des cellules gonadotropes et celui des hormones gonatrodopes n'est pas résolu, bien que jusqu'ici une seule hormone ait été isolée ; l'hypothèse d'Haider et Blum (1977) selon laquelle il existerait plusieurs schémas endocriniens de la reproduction pourrait expliquer la variabilité notée chez les Téléostéens.

Conclusions.

Boops salpa présente deux formes de cellules gonadotropes nettement distinctes chez l'adulte par leurs affinités tinctoriales, leur localisation et leur cycle d'activité sécrétoire.

Symposium sur la Reproduction des Poissons
Paimpont, France, 19-21 *septembre* 1977.

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The annual reproduction cycle in adult carp in Poland : ovarian state and serum gonadotropin level

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Summary. In environmental conditions in Poland, new vitellogenesis began in carp just after the spawning season. This phase was correlated with the maintenance of a high serum, gonadotropin level, which decreased and remained low when the temperature was also low ; oogenesis remained blocked at the stage attained before the drop in temperature and gonadotropin level. The rise of temperature in May increased GTH and ovarian responsiveness to hypophysation treatment. Although pituitary function seemed normal in some fish, there was no spawning even after hypophysation treatment. Resorbing oocytes were present during the whole year, and a minimum rate of GTH secretion would be necessary to prevent massive resorption. Animals were ready for a new reproduction cycle right after spawning ; this could not be achieved with the environmental conditions in Poland, namely the temperature. Existence of a limit temperature is discussed.

Introduction.

Most female carp in Poland reach sexual maturity in the fifth year of life, but some may attain it in the fourth year. Spawning occurs once a year in late May when the temperature reaches 18 °C. The female ovaries regenerate a few weeks after spawning and are ready for the next spawning (Bieniarz *et al.*, 1977). All stages of oogenesis are present in the ovary during spawning (Bieniarz *et al.*, 1977 ; Brzuska, 1977). Few data are available in the literature on gonadotropin regulation of vitellogenesis and the final stages of oogenesis, but the increase of pituitary responsiveness to hypothalamic hormones at the final maturation stage of the ovary has been correlated with temperature by Weil *et al.* (1975).

In the present report, we investigated the evolution of ovarian development in correlation with serum GTH concentration.

Material and methods.

The experiment was carried out between July 1975 and July 1976 on 5-year old female carp weighing 3 to 4 kg and kept in a conventional pond under normal rearing conditions. At the end of the experiment the animals were divided into 4 groups

according to the time and the number of animals spawning : 1. Spawning in June 1975 and June 1976 (5 animals) ; 2. Spawning in June 1975 but not in 1976 (10 animals) ; 3. Spawning in June 1976 but not in 1975 (11 animals) ; 4. No spawning in either 1975 or 1976.

Group 2 females had spawned naturally in 1975. In the other groups all spawnings were induced by hypophysation (4 mg pituitary powder per kg body weight). Blood was sampled monthly from the caudal vein at 11 a. m. After coagulation and centrifugation sera were kept frozen at — 20 °C until gonadotropin (GTH) determination by radioimmunoassay according to Breton *et al.* (1971).

Ovarian samples were taken *in vivo* by means of intraabdominal puncture using the technique described earlier (Bieniarz and Epler, 1976). Young oocytes (< 0.9 mm diameter) were estimated to permanently represent 50 p. 100 of the total number of oocytes. Large oocytes (0.9 to 1.3 mm) with completed vitellogenesis were classified as follows : Stage 1 : nucleus in the center of the oocyte ; Stage 2 : nucleus shifted to oocyte periphery ; Stage 3 : resorbing oocytes characterized by a disorganized inner structure.

The results were statistically analyzed by the t-test.

Results.

No statistical differences were found between the serum gonadotropin levels in the first 3 groups (fig. 1). High GTH levels were observed from July to September, which then decreased and remained low up to May. Between May 19 and June 7, serum GTH secretion rose from 0.83 ng/ml to about 2 ng/ml. The serum GTH concentration was lower one month after than in the days preceding spawning (0.24 ng/ml against 1.44 ng/ml). In group 4 where no spawning had occurred, the same type of secretion curve was noted, but a significant final increase of serum GTH was observed

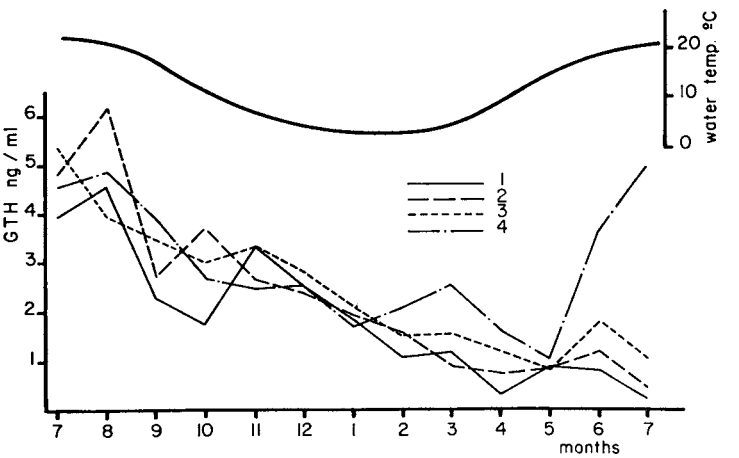

FIG. 1. — Top : *annual change in water temperature.* Bottom : *changes in blood gonadotropin level of females carp during the reproductive cycle.* 1 : group 1 females which spawned in 1975 and 1976 ; 2 : group 2 females which spawned only in 1975 ; 3 : group 3 females which spawned only in 1976 ; 4 : group 4 females which did not spawn.

in comparison with other groups (3.54 ng/ml). The GTH level continued to increase up to 4.92 ng/ml after the presumed spawning period.

In the first 3 groups, there were no statistical differences in the percentages of the different oocyte stages during the dates cited above ; figure 2 shows the curve of evolution of these parameters. Stage 1 represents about 50 p. 100 of the oocytes from July to October. From this period their decrease in number correlated with a rise in the percentage of type 2 oocytes. The number of stage 2 oocytes remained high until the spawning season, except for a small decrease occurring in May at the same time as a peak of resorbing oocytes. After spawning, the percentage of stage 2 oocytes declined to 15 p. 100, corresponding to the generation of new oocytes in stage 1. In these 3 groups resorbing oocytes always represent 20 to 30 p. 100 of the ovarian germ cells.

Groups 4 differs from the others only by the fact that resorbing oocytes continued to increase after the normal spawning season.

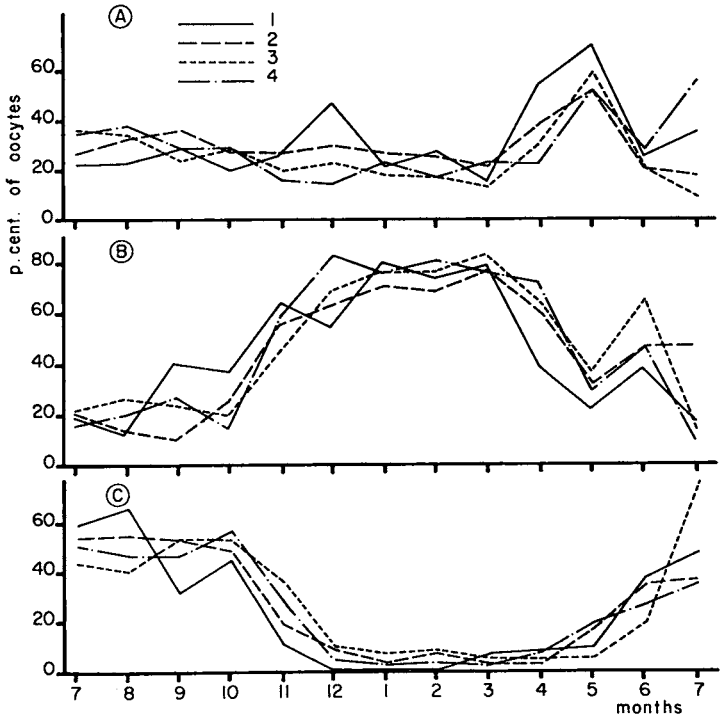

FIG. 2. — *Changes in the percentage of resorbing oocytes* (A), *oocytes with the nucleus in position* « 2 » (B), *or oocytes with the nucleus in position* « 1 » (C) *during an annual reproductive cycle in female carp.* 1, 2, 3, 4 : experimental groups, see figure 1.

Discussion.

GTH levels remained high in all groups from July to September and did not decrease after the spawning season. In other studies, Bieniarz and Epler (1976) have demonstrated a new vitellogenesis occurring in carp within 2 months after spawning.

The present results are in agreement with those findings. The high serum GTH levels observed from July to September could be correlated with vitellogenesis stimulation, as already demonstrated in trout and tench (Breton *et al.*, 1975). Afterwards, GTH levels remain low until the onset of the spawning season ; they begin to rise slowly in May and June in the first 3 groups when the temperature reaches about 14 °C. Therefore, GTH levels are higher before spawning than after, indicating that spawning may be under gonadotropin control. A peak of serum GTH secretion is not observed during this period, possibly because it would have been necessary to take samples more than once a month to detect such a rise. An alternative explanation may be that in the present experiment females usually did not spawn spontaneously and ovulation had to be induced by hypophysation. In this hypothesis, no blood GTH surge is expected and only an elevation of the GTH base line level is obtained.

It must also be emphasized that an increase of resorbing oocytes occurs in April and May when the serum GTH level reaches minimum values. Thus, in carp it seems that a limit value of GTH secretion is necessary to prevent massive oocyte resorption. It is interesting to note that the resorption peak appears at the end of the coldest period and at the spring rise in temperature.

There is no spawning in group 4, even after hypophysation although GTH level is higher than in the other groups. It may be hypothesized that in these animals gonadotropin secretion occurs normally, but there is lack of ovarian GTH responsiveness leading to resorption.

As early as October, the number of oocytes in stage 2 increases and reaches a maximum in December. Thus, immediately after spawning the animals are ready for a new reproduction cycle which cannot be achieved in the natural environmental conditions in Poland, mainly due to the temperature.

Temperature seems to the most important factor in the carp since maintenance at high temperature (23 °C) accelerates ovarian recrudescence and spawning (Gupta, 1975). It is difficult to determine the limit temperature under which reproduction cannot take place in carp, but it may around 14 °C. For example, Pandey and Hoar (1972) were able to induce vitellogenesis and spawning by clomiphene in goldfish reared at 14 °C. It is also possible to induce spawning in carp using a sequential treatment (pituitary extract and 17α-hydroxy-20β-dihydroprogesterone) at 14 °C (Jalabert *et al.*, 1977), but this cannot be done at 10 °C (Breton *et al.*, unpublished data). This temperature also corresponds either to the decrease of GTH secretion in September or to the spring rise of GTH in this experiment.

Conclusion.

In environmental conditions in Poland, the new oogenetic carp cycle (oocyte maturation and ovulation) starts right after spawning and does not end in the same year, probably because of the drop in ambiant temperature. During the coldest period stage 2 oocytes (with peripheric nucleus) remain in a quiescent stage until a rise in temperature the following spring leads to a rise of gonadotropin secretion and oocyte maturation.

Symposium sur la Reproduction des Poissons
Paimpont, France, 19-21 septembre 1977.

Résumé. Chez la Carpe, dans les conditions polonaises d'élevage, une nouvelle vitellogenèse démarre aussitôt après la fraie. Cette phase est corrélée avec le maintien d'un haut niveau de sécrétion de gonadotropine. Le niveau décroît et reste faible lorsque la température est elle-même basse, la vitellogenèse restant bloquée au stade atteint avant la décroissance de la température, et du niveau sérique de GTH. L'élévation de la température au printemps conduit à une augmentation de la GTH sérique et à l'apparition de la réceptivité ovarienne au traitement d'hypophysation. Dans certains cas, bien que le fonctionnement gonadotrope hypophysaire apparaisse normal, l'ovaire n'est pas réceptif au traitement d'hypophysation. Des ovocytes en résorption sont présents tout le long de l'année ; un seuil minimum de GTH pourrait être nécessaire pour prévenir une résorption massive. En conclusion, juste après la fraie les animaux sont prêts à engager un nouveau cycle de reproduction, qui ne peut s'achever en raison de conditions d'environnement défavorables, principalement la température. L'existence d'une température limite est discutée.

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Annual cycle of plasma oestradiol-17β in the female trout *Salmo gairdneri*

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Summary. Levels of oestradiol in plasma of adult female rainbow trout *Salmo gairdneri* were determined during the annual reproductive cycle using a radioimmunoassay. From January to June, during previtellogenesis and the period of endogenous vitellogenesis plasma levels were low (1,3 ± 0,2 ng/ml). At the beginning of the period of exogenous vitellogenesis, the œstradiol level rose and a maximum (16,9 ± 0,2 ng/ml) was reached at the end of vitellogenesis in November, some weeks before spawning. A positive correlation ($r = 0,58$) was established between plasma œstradiol levels and the gonadosomatic index. From enzymatic cytochemical studies it was concluded that the granulosa cells, as well as the interstitial cells in the ovary, are involved in œstradiol production.

Introduction.

In oviparous vertebrates it is suggested that oestradiol-17β may trigger the liver to synthesize yolk proteins (for review : Clemens, 1974 ; Tata 1976). To test this hypothesis in *Salmo gairdneri*, we have studied the relation between oestradiol-17β levels in plasma during the annual cycle and the development of the oocytes ; we have also investigated the location of the 3β-hydroxysteroid dehydrogenase enzyme in the ovary.

Materials and methods.

Animals.

Adult female specimens of the rainbow trout were obtained from a hatchery in Vaassen (the Netherlands). After anaesthesia with MS 222, blood was taken from the *ductus Cuvieri* with a heparinized vacuum syringe, then centrifuged and the plasma stored at — 20 °C. The ovaries were removed and prepared for histological and enzyme-cytochemical examination as described by van den Hurk *et al.* (1978).

Assay method for plasma oestradiol-17β.

The organic solvents were redistilled once just before use. (2, 4, 6, 7(n)-^{3}H) Oestradiol-17β (Spec. act. 109 Ci/mmol) was obtained from the Radiochemical Centre (Amersham) and purified on TLC. The radioactivity was measured with a Nuclear Chicago Mark I scintillation counter with a scintillator of PPO (5.4 g) and POPOP (100 mg) in toluene-triton X 100 (2 : 1) mixture (1 L). Radioactive areas on the TLC plates were located by means of a Berthold thinlayer chromatogram scanner. Oestradiol-17β (E_2) was measured by radioimmunoassay. Antiserum (anti-E_2-6-CMO-BSA) was obtained from Dr. R. J. Scaramuzzi (MRC unit for Reproductive Biology, Edinburgh, Scotland, U. K.) through Dr. S. J. Dieleman (Clinic for Veterinary Obstetrics, Gynaecology and A. I., Utrecht, the Netherlands). The antiserum had a high specificity ; cross reactions with oestrone and oestradiol-17α were 3 p. 100 and 1.2 p. 100 respectively. For the assay of plasma E_2, 1 ml of plasma was added to an extraction tube together with 2 000 dpm of ^{3}H-E_2. After one hour at room temperature, extraction was performed with peroxide-free diethylether (2 × 5 ml). The combined ether fraction was evaporated under nitrogen at 40 °C and the residue dissolved in 2 ml of buffer containing Na_2HPO_4-$2H_2O$ (10.78 g), NaH_2PO_4-H_2O (5.4 g), NaN_3 (1 g), NaCl (9 g), gelatine (1 g) per litre aqua dest. From the sample 400 μl was counted for recovery determinations and 100 μl and 200 μl aliquots (both in duplo) were transferred to assay tubes containing 15 000 dpm ^{3}H-E_2. In all tubes the volume was raised, if necessary, to 200 μl with buffer. A standard curve was set up by adding 15 000 dpm ^{3}H-E_2 to a series of assay tubes containing 0, 25, 50, 100, 150, 200, 300 and 400 pg unlabeled E_2. After adding 200 μl of antiserum (1 : 60 000) and vortex mixing, the tubes were incubated overnight at 4 °C. To separate the free and bound E_2, all assay tubes were closed with plastic caps containing 200 μl of dextran-coated charcoal in buffer. Following incubation, the assay samples were simultaneously mixed with charcoal by shaking the tubes. After a contact time of 5 min. the charcoal was centrifuged and 100 μl of the supernatant, containing the bound E_2, was measured. The binding percentage was calculated and after adjustment for recovery (70-90 p. 100), values were expressed as ng/ml plasma. For determining the unspecific binding, a blank of 1 ml of buffer was run through the entire procedure. The value of the blanks was 0.017 ng/ml. Intra-assay precision was determined by a tenfold analysis in the optimal range from 25 to 200 pg. The coefficients of variation for analysis of 25, 50, 100, 150 and 200 pg were 10.7, 4.1, 2.1, 1.2 and 0.7 p. 100 respectively.

Results.

Histological studies showed annual changes in the growth and ripening of ovarian follicles. In the cycle, three periods could be distinguished. 1. The period of previtellogenesis, from January to June, characterized by the presence of young follicles (max. diameter 0.3 mm) and the differentiation of the follicular layer into granulosa and theca. 2. The period of endogenous vitellogenesis, which is restricted to May and June. In this period, the oocytes reach a diameter of 0.6 mm, as a result of endogenous yolk formation. Part of this process is the appearance of cytoplasmic vacuoles with chromophobic contents. 3. The period of exogenous vitellogenesis, during which

the oocytes take up yolk proteins and grow to 4.5 mm. This period lasts from July to December. In December or January, ovulation may take place, leading to the formation of postovulatory follicles. The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) has been demonstrated in the granulosa cells of growing follicles during the first half of the period of exogenous vitellogenesis (fig. 1), in interstitial cells during the entire cycle (peak in January), and in the postovulatory follicles. In comparison with the strong activity of the surrounding interstitial cells, the 3β-HSD activity in the granulosa cells of the postovulatory follicles is rather weak (fig. 2). The plasma E_2 levels were determined at irregular intervals for 1 1/2 year. The individual E_2 levels are presented in table 1 and figure 3. Both in 1975 and 1976, they clearly followed a seasonal pattern.

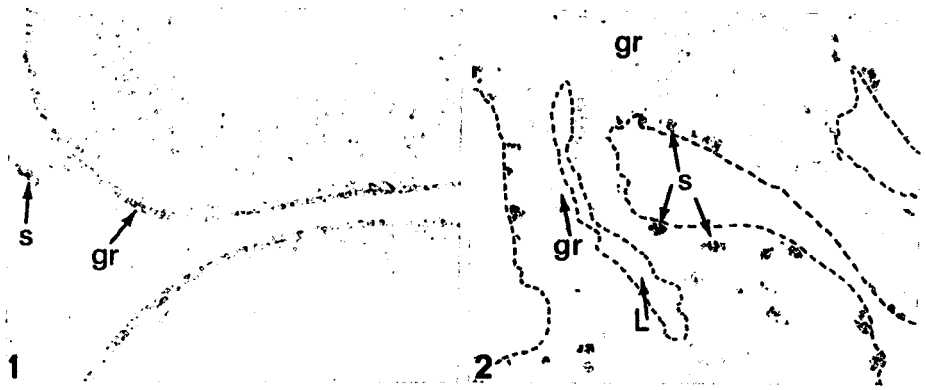

FIG. 1 and 2. — 3β-*HSD activity in the ovary of* Salmo gairdneri.
1. Detail of two growing follicles.
2. Detail of a corpus luteum :
gr : granulosa cells ; L : lumen of the corpus luteum ; s : stromal or interstitial cells.

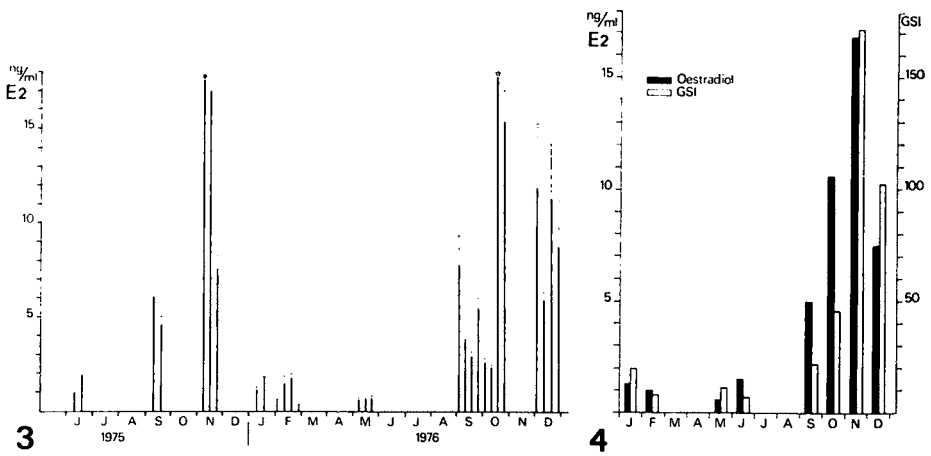

FIG. 3 and 4. — *Seasonal changes in the concentrations of plasma oestradiol-17β in female* Salmo gairdneri.
3. The individual E_2 levels (ng/ml) during 1975 and 1976.
4. The mean values of E_2 levels and gonado-somatic index (GSI p. 1 000) during an annual cycle.

The cyclical changes have been summarized in figure 4. A rise in E_2 level obviously starts before September, probably in July or August. After a maximum in November (16.9 $\pm$ 0.2 ng/ml), the values drop to 1.3 $\pm$ 0.2 ng/ml in January. Such low values last to June. Comparison of the plasma E_2 levels with the gonadosomatic index (GSI) of individual animals (table 1) indicate a positive correlation (correlation coefficient after Ferguson, 1966 : 0.58) (fig. 4).

TABLE 1

The oestradiol-17β (E2) levels in plasma of Salmo gairdneri. GSI : gonadosomatic index, (n) : number of assays

Date	GSI (p. 1 000)	E2 ng/ml (n)
3-6-1975	4.9	1.0 $\pm$ 0.1 (11)
	8.8	1.9 $\pm$ 0.2 (13)
24-9-1975	30.1	6.0 $\pm$ 1.1 (7)
	18.8	4.5 $\pm$ 0.5 (7)
19-11-1975	164.4	26.2 $\pm$ 0.6 (2)
	247.5	17.0 $\pm$ 0.9 (4)
	86.7	7.5 $\pm$ 1.3 (3)
7-1-1976	10.4	1.1 $\pm$ 0.2 (14)
	38.5	1.5 $\pm$ 0.3 (11)
25-2-1976	6.8	0.6 $\pm$ 0.1 (7)
	6.6	0.4 $\pm$ 0.1 (5)
	—	1.7 $\pm$ 0.2 (13)
5-5-1976	10.6	0.6 $\pm$ 0.1 (7)
	6.1	0.6 $\pm$ 0.1 (9)
	14.6	0.5 $\pm$ 0.1 (15)
23-9-1976	28.9	7.7 $\pm$ 1.6 (12)
	20.4	3.8 $\pm$ 0.4 (15)
	18.6	2.9 $\pm$ 0.4 (12)
	26.1	5.4 $\pm$ 1.9 (9)
21-10-1976	14.1	2.5 $\pm$ 0.4 (7)
	38.7	2.3 $\pm$ 0.2 (8)
	47.8	22.1 $\pm$ 3.6 (6)
	80.9	15.4 $\pm$ 1.6 (10)
2-12-1976	251.8	5.8 $\pm$ 0.4 (4)
	80.1	11.2 $\pm$ 3.1 (4)
	78.0	8.7 $\pm$ 2.5 (3)
	—	11.8 $\pm$ 3.4 (5)

Discussion.

Comparing radioimmunoassay and histological results, it appears that plasma E_2 levels are low during endogenous vitellogenesis and increase with the beginning of exogenous vitellogenesis. This increase continues throughout the period of exogenous yolk formation, and a maximum is reached some weeks before spawning. Schreck *et al.*, (1973) found 4.4 ng E_2 per ml plasma in adult female *Salmo gairdneri* in October. Judging from this value, the blood samples must have been collected before the E_2 levels reached maximum value. The correlation between the change in plasma E_2 level and exogenous vitellogenesis corresponds to the observations of Eleftheriou *et al.*, (1966), Schreck and Hopwood (1974) and Wingfield and Grimm (1977) in other

teleosts, and also with the hypothesis that E_2 triggers the synthesis of yolk proteins in the liver (Clemens, 1974 ; Tata, 1976). The continuous presence of E_2 in the blood corresponds to the uninterrupted activity of 3β-HSD in the interstitial cells of the ovary. The increase in plasma E_2 levels coincides with the development of 3β-HSD activity in the granulosa cells of the follicles. These correlations seem to indicate that E_2 is formed both in the interstitium and in the granulosa cells of growing follicles. During October and November, the plasma E_2 level continues to rise, but this is not reflected by an increased 3β-HSD activity in the ovary. On the contrary, during this period, 3β-HSD activity could not be demonstrated in the granulosa cells. This might be attributed to the fact that the oocytes become very large and cause stretching of the granulosa cells. A slight 3β-HSD activity is again noticeable in the granulosa cells after ovulation and collapse of the follicle. The presence of some 3β-HSD activity in the postovulatory follicles coincides with a maximum 3β-HSD activity in the interstitial tissue, which is not reflected by a rise in plasma E_2. This means that the 3β-HSD activity cannot be used as parameter for E_2 production, and that next to E_2 other steroids are being synthesized by the ovary during the annual cycle. On the other hand, the positive correlation in *Salmo gairdneri* between plasma E_2 level and GSI seems to indicate that the latter can be used for estimating E_2 production.

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Résumé. Les niveaux d'estradiol dans le plasma de femelles truites adultes ont été déterminés durant le cycle reproducteur annuel en utilisant un dosage radioimmunologique. De janvier à juin, pendant la prévitellogenèse et la période de vitellogenèse endogène, les niveaux plasmatiques sont faibles (1,3 $\pm$ 0,2 ng/ml). Au début de la période de vitellogenèse exogène les niveaux d'estradiol augmentent et un maximum (16,9 $\pm$ 0,2 ng/ml) est atteint à la fin de la vitellogenèse en novembre, quelques semaines avant la fraie. Une corrélation positive $r = 0{,}58$ a été calculée entre l'estradiol plasmatique et le rapport gonado-somatique. A partir d'études cytoenzymologiques on conclut que les cellules de la granulosa comme les cellules interstitielles de l'ovaire sont impliquées dans la production d'estradiol.

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Plasma estradiol-17β and gonadotropin during ovulation in rainbow trout (*Salmo gairdneri* R.)

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Summary. A radioimmunoassay (RIA) for estradiol-17β (E2-17β) with a chromatographic step has been tested on rainbow trout plasma. Overloading and dilution tests gave satisfactory results. Intracardiac injection of E2-17β (10 μg/kg) in July gave a peak in the first 30 min after injection ; return to the basic level occurred within the next 24 hrs. Using RIA, trout-gonadotropin (t-GTH) and E2-17β were followed in trout plasma during the very last stages of the reproductive cycle. Oocyte maturity was determined simultaneously. t-GTH increase during maturation and ovulation was confirmed while E2-17β dropped before oocyte maturation to reach a low level when t-GTH was high.

Introduction.

Since egg yolk precursor synthesis is known to be stimulated in fish, like in other lower vertebrates, by estrogens (Chester Jones *et al.*, 1972) those have been almost only related to vitellogenesis. While this latter process is associated with high levels of plasma estrogen, low values have been found during the spawning season in salmonid females (Cedard, Fontaine and Nomura, 1961 ; Breton *et al.*, 1975*a* ; Whitehead, Bromage and Forster, 1977) ; similar results have been reported in other species (Eleftheriou, Norman and Summerfelt, 1968 ; Wingfield and Grimm, 1977). The highest levels of plasma gonadotropin have been detected in the same period, even in ovulated fish (Crim, Meyer and Donaldson, 1973 ; Crim, Watts and Evans, 1975 ; Breton *et al.*, 1975*a* ; Jalabert *et al.*, 1976) but none of these studies have correlated estrogen and gonadotropin levels with accurate maturation stages at the end of the reproductive cycle. In the present work, we used frequent blood sampling for estradiol-17β (E2) and trout gonadotropin (t-GTH) determination combined with oocyte biopsy at different stages.

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Material and methods.

Blood and oocyte samples. — Two-year old females weighing 500 to 800 g were kept in a recirculating water system (Petit, 1974) at 12 °C during December 1976 and submitted to natural photoperiod ; they were anesthetized with phenoxyethanol solution before sampling (Sehdev, McBride and Fagerlund, 1963). Blood was withdrawn from a caudal vessel using a syringe rinsed with sodium heparinate at 700 IU/ml. The sample was kept on crushed ice until centrifugation at 3 000 g for 15 min., the plasma was stored at — 20 °C.

Maturity stages were determined under binocular on fresh oocytes collected by abdominal stripping (Jalabert *et al.*, 1976).

Estradiol-17β assay. — Except for cyclohexane, analytical grade solvents were distilled before use ; glass and polypropylene tubes by LKB were silanized (water soluble, Silicad Clay-Adams). E2-17β-2, 4, 6, 7 (n)-^{3}H was obtained from the Radiochemical Center (Amersham) ; purity was checked on thin-layer chromatography in chloroform-ethyl acetate system (80:20) with cold steroid as a reference (Steraloids). After adding 2 000 dpm E2-^{3}H as recovery tracer to each sample, 100 µl of plasma were extracted twice with 2 ml cyclohexane-ethyl acetate (50 : 50 v/v). After evaporation, the dry residue was dissolved in 200 µl of benzene-methanol (85 : 15), transferred on a small Sephadex LH 20 column (80 mm × 5 mm), then rinsed with an additional 100 µl. Elution was performed in the same solvent system ; the first 1.8 ml « estrone fraction » was discarded, and the next 2.4 ml « estradiol fraction » collected (adapted from Camerun and Jones, 1972). After evaporation, these fractions were

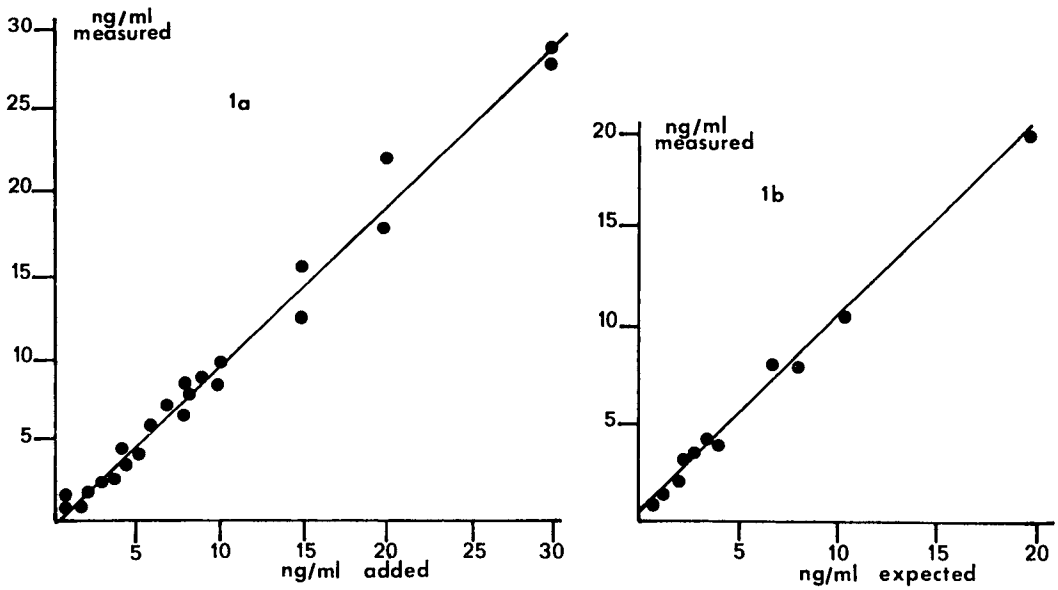

FIG. 1. — a) *Overloading test* : Y = 0.95 × — 0.14 ρ = 0.993 ;
b) *Dilution test* : Y = 0.97 × — 0.49 ρ = 0.995.

dissolved in 500 µl or 1 ml of phosphate buffer. Two aliquots of 100 µl were used for the radioimmunoassay (RIA) ; 200 µl or 600 µl were counted for recovery. Double antibody RIA was then performed according to Terqui, Dray and Cotta (1973) using a rabbit anti-E2-17β-6-CMO-BSA (Dray *et al.*, 1971). After precipitation and centrifugation, the precipitate was counted according to Saumande and André (1975).

Trout-gonadotropin assay. — Gonadotropin was measured as described by Breton *et al.* (1971) using a pure trout gonadotropin as a standard against guinea-pig anti-SG-G-100.

Results.

Assessment of E2 RIA for trout plasma.

Overloading tests. — Increasing quantities of cold E2 (1 to 30 ng/ml) were added to different plasmas and measured for E2 (fig. 1*a*). The regression line slope between added and measured quantities does not differ from 1 and the zero intercept does not differ from 0 (variance analysis : $p > 0.05$). Cold E2 was injected in the heart of 5 fish at 10 µg/kg of body weight. In the first sample, a peak of E2 was detected in 3 fish after 10 min ; it was detected after 30 min. in the rest of the fish in the second sample. Following a regular decrease, the basic level was reached within 24 hrs (fig. 2).

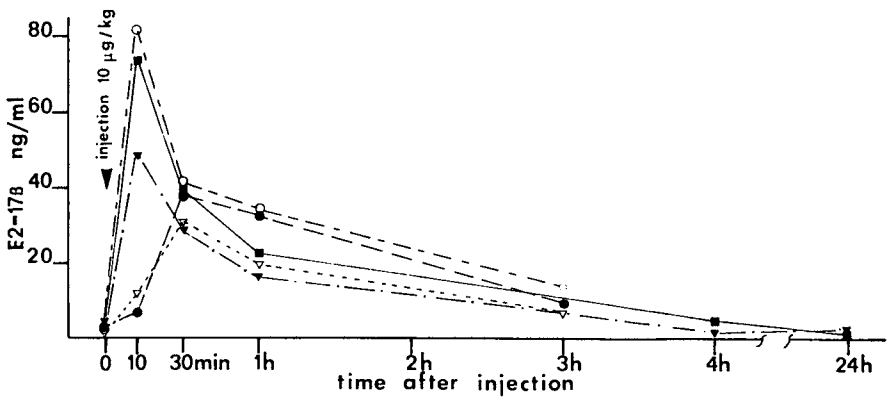

FIG. 2. — *Profile of E2-17β in plasma after intracardiac injection of E2-17β (10 µg/kg) in 5 females in July.*

Dilution test. — Different plasmas were diluted (1/2, 1/3, 1/4, 1/6, 1/8) and each dilution measured for E2. The regression line slope between the expected and the measured quantities does not differ from 1 and the zero intercept does not differ from 0 (variance analysis : $p > 0.05$). No difference is found when the slope of logit versus log dose curves, drawn with known quantities of cold E2 (standard curve) or serial plasma dilution, are compared (covariance analysis : $p > 0.05$). There is therefore no immunological difference between pure E2 and measured E2.

E2 ant t-GTH during ovulation.

In a first experiment, 64 fish were divided into 7 groups according to maturity stage, and sampled once. In fish not ready to spawn, hormone levels were low (E2-17β = 2.8 ± 0.2 ng/ml ; t-GTH = 2.6 ± 0.2 ng/ml). In the other groups, E2-17β decreased before meiotic maturation while t-GTH rose during the last stages (fig. 3). Unequal variance analysis shows a very highly significant difference ($p < 0.005$) for the two hormones let ween the subpopulations « end of vitellogenesis + migrating germinal vesicle » (E2 = 8.82 ± 1.8 ng/ml ; t-GTH = 4.0 ± 0.7 ng/ml), and « maturation without germinal vesicle breakdown (GVBD) + maturation with GVBD » (E2 = 2.6 ± 0,3 ng/ml ; t-GTH = 11.1 ± 1.6 ng/ml).

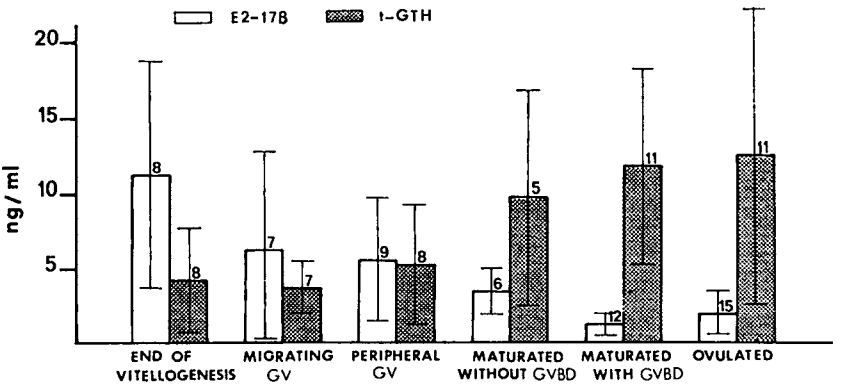

FIG. 3. — *Plasma* E2-17β (clear) *and t-GTH* (shadowed) *in female trouts at different maturity stages* (mean ± SD).

In a second experiment, 10 fish with migrating GV oocytes were isolated and sampled every 2 or 3 days (fig. 4). When ovulation was detected, the same hormonal profiles as in the first experiment were observed, except for one fish (N° 8), showing a higher E2-17β level with a low t-GTH level. E2-17β remained high with low t-GTH levels in non-ovulating fish (N° 9 and 10).

Discussion.

RIA specificity is a difficult problem in new species where an unknown metabolite may cross-react with the antiserum. However, the LH-20 chromatographic step increases specificity, and measurement of diluted plasma shows that the substance being measured is immunologically E2. E2 values, which are high as compared to mammals, confirm those described by other authors studying salmonides (Cedard, Fontaine and Nomura, 1961 ; Schreck, Lackey and Hopwood 1973 ; Whitehead, Bromage and Forster, 1977).

No ovulatory t-GTH peak has been detected in trout. This level increases slowly before ovulation and remains high afterwards. Such late high levels have been found in different salmonid species by Crim, Watts and Evans (1975) who suggest the role of

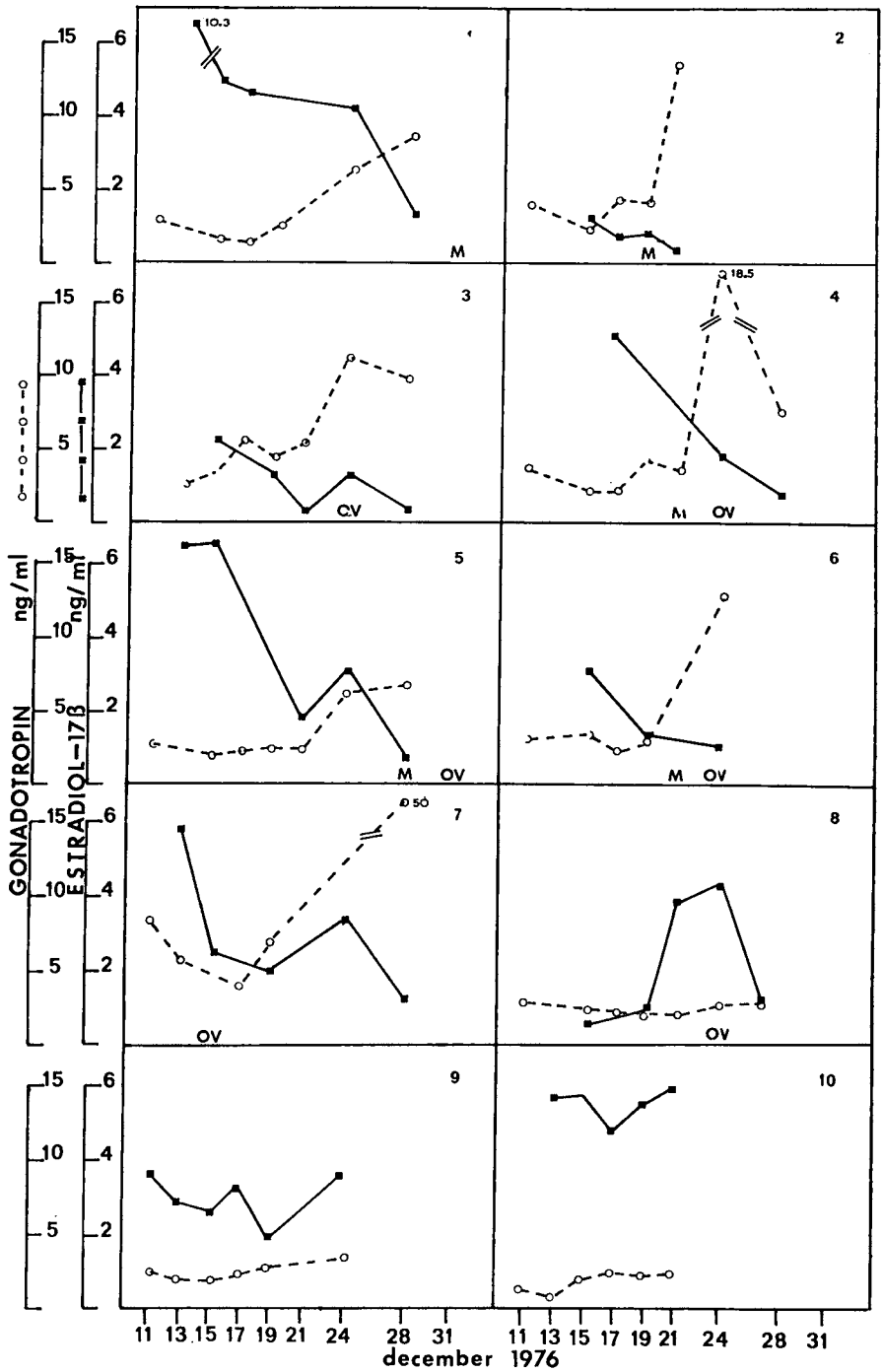

FIG. 4. — *Individual plasma profiles of E2-17β (■—■—■) and t-GTH (o—o—o) in trout during ovulation.* (M : maturation ; OV : ovulation).

gonadotropin in spawning behavior. We cannot eliminate the possibility of pulsative discharges for both hormones, undetectable with the sampling frequency, and we must find an answer to the need for numerous samples during a very short time in fish. In any case, E2 and t-GTH patterns at the end of the cycle are different from those in mammals (Pelletier and Thimonier, 1975).

A negative feedback has been seen on ovary development in fish (De Vlaming, 1974) and an anti-estrogen, clomiphene citrate, stimulates carp gonadotropin secretion (Breton *et al.*, 1975*b*) and ovulation in goldfish (Pandey and Hoar, 1972) and loach (Ueda and Takahashi, 1976).

There is direct action on the ovary besides these indirect effects. In trout, estrogen depresses *in vitro* the gonadotropic effect on oocyte maturation (Jalabert, 1975) ; similar results were obtained with amphibian oocytes (Wright, 1961 ; Schuetz, 1972). No studies in fish permit us to generalize on the role of estrogen in cytoplasmic maturation, as has been suggested in mammals (Thibault, 1977).

TABLE 1

Assessment of E2-17β *RIA in trout plasma*

Antibody specificity (1)		Estradiol 17β	100
		Estrone	11
		16 ketoestradiol 17β	9
		16 epiestriol	8
		Estriol	< 0.5
		Estradiol-17α	< 0.5
		Testosterone	< 0.5
		11 ketotestosterone	< 0.5
		17α-hydroxy-20β-dihydro Pg	< 0.5
Recovery for the chromatographic step (2)		E2 = 89 ± 9 p. 100 (n = 105)	
(LH 20 benzene-methanol 85/15)		E1 = 8.5 ± 0.4 p. 100 (n = 30)	
Total recovery (3)		E2 = 73 ± 7 p. 100 (n = 323)	
Sensitivity of the standard curve		5 pg/tube	
Usuable limits		20-800 pg/tube	
Blank (buffer)		< 5 pg/tube (n = 30)	
Variability (4)	Intra assay n = 5	X = 11.6 ng/ml	SD = 0.5 CV = 0.04
		X = 7.7 ng/ml	SD = 0.3 CV = 0.04
		X = 4.6 ng/ml	SD = 0.2 CV = 0.05
	Inter assay n = 6	X = 3.1 ng/ml	SD = 0.5 CV = 0.15

(1) Defined as 100 x/y, where X is the weight of the unlabelled E2-17β and y the weight of the heterologous steroid required to produce 50 p. 100 inhibition of the binding of ^{3}H-E2-17β tracer.
(2) Estimated by chromatography of 8 000 dpm E2-17β-^{3}H.
(3) Estimated from samples.
(4) Means, standard deviations and coefficient of variation for 4 blood samples.

The regulation of gonadotropin secretion during spawning season in trout appears original. If E2-17β in laying hens has no effect on LH surge (Laguë, VanTienhoven and Cunningham, 1975), other steroids, and especially progesterone, can stimulate it (Wilson and Sharp, 1976). However, in trout, 17-hydroxy 20β-dihydro progesterone induces *in vivo* maturation and ovulation without raising t-GTH (Jalabert *et al.*, 1976). The drop in E2 may be a signal.

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Résumé. Un dosage radioimmunologique de l'œstradiol-17β comprenant une étape de purification chromatographique a été éprouvé sur le plasma de truite Arc-en-ciel. Les tests de surcharge et de dilution plasmatique ont donné des résultats satisfaisants. Une injection intracardiaque d'E2-17β (10 μg/kg) réalisée au mois de juillet provoque l'apparition d'un pic d'E2-17β dans les premières 30 mn après l'injection, puis le niveau de base est retrouvé au cours des 24 h suivantes. La gonadotropine de truite et l'EA-17β, dosées par radioimmunologie, ont été suivies dans le plasma de femelles pendant les tous derniers stades du cycle de reproduction. Simultanément, l'état de maturité des ovocytes a été déterminé. L'augmentation du niveau de t-GTH pendant la maturation et l'ovulation est confirmée, tandis que l'E2-17β chute avant la maturation méiotique pour atteindre un minimum quand la t-GTH est élevée.

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Emergence of ovarian 11-deoxycorticosteroid biosynthesis at ovulation time in the sea bass, *Dicentrarchus labrax* L.

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Summary. Steroidogenesis in the ovary of the teleost *Dicentrarchus labrax* has been studied *in vitro* at five different stages : 1) previtellogenic ; 2) postvitellogenic ; 3) postovulatory, one day after HCG-induced spawning ; 4) postovulatory, 9 days after induced spawning ; 5) atretic. Ovarian tissues were incubated with [4-^{14}C] pregnenolone and [4-^{14}C] progesterone for 6 hr at 15-16 °C. The following metabolites were formed after ovulation : progesterone (from pregnenolone), 11-deoxycorticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone, androstenedione, testosterone and estradiol-17β. Percentages of conversion were high for 11-deoxycorticosteroids and androgens and low for estradiol-17β. Before ovulation, yields of androgens were high at stage 2 but negligible at stage 1. Estradiol-17β and 11-deoxycorticosterone were obtained in small amounts at maturity but could not be detected during quiescence. Atretic ovarian tissue converted pregnenolone to progesterone but it went no further in steroid hormone biosynthesis.

Introduction.

The biosynthesis of 11-deoxycorticosterone (DOC) and/or 11-deoxycortisol (S), through 21-hydroxylation of progesterone and 17α-hydroxyprogesterone, respectively (Orta-Flores *et al.*, 1976), has been shown to occur in the ovaries of 6 teleost species (Colombo *et al.*, 1973 ; Colombo and Colombo Belvedere, 1977), two urodeles (Colombo *et al.*, 1977*a*, *b*) and 2 reptiles (Colombo *et al.*, 1974 ; Colombo and Yaron, 1976). The information available, however, was insufficient to establish whether 11-deoxycorticosteroid production was in alternative to that of estrogens by competing, for instance, for C_{21} intermediates, or whether 11-deoxycorticosteroids alternated with estrogens through a shift in ovarian steroidogenesis at a certain stage of the oogenic cycle.

The observation that the teleost aromatizing system was more apparent in the vitellogenic ovary of *Gobius jozo* than in pre- and post-ovulatory ovaries of *Diplodus annularis* and *Solea impar*, while the reverse was true for steroid 21-hydroxylase activity (Colombo and Colombo Belvedere, 1977), could reflect either a sequence of distinct steroidogenic phases or simply a species specificity in ovarian sterold patterns. To avoid

the latter complication, we decided to use the sea bass, a commercially reared species, to study *in vitro* the course of ovarian steroid hormone biosynthesis during the reproductive cycle.

Materials and methods.

Sixteen females of *Dicentrarchus labrax* (mean standard length : 38.0 cm $\pm$ 2.9 SD ; mean body weight : 994 g $\pm$ 225 SD) were used. Mature or spawned specimens were obtained from the SIRAP Marine Fish Hatchery at Pellestrina whereas nonbreeding animals were captured in coastal lagoons near Venice. Both ovaries were dissected out following pithing of the fish. Five ovarian stages were investigated : 1) quiescent ovary with only previtellogenic oocytes obtained from females caught outside the breeding period in April ; 2) mature or postvitellogenic ovary packed with yolky oocytes and some growing follicles ; 3) ovary one day after spawning, induced in mature females by injecting 1 000 IU/kg body weight of human chorionic gonadotropin (HCG) ; 4) ovary nine days after induced spawning ; 5) ovary with extensive follicular atresia as found in females confined to low salinity (5-10 p. 1000) brackish water during ovarian maturation.

Portions of ovarian tissue from animals at the same stage were pooled in ice-cold physiologic medium of the following composition : 187.3 mM NaCl, 3.34 mM KCl, 2.30 mM $CaCl_2$, 3.98 mM $MgCl_2$, 0.37 mM $MgSO_4$, 1.61 mM Na_2HPO_4, 0.36 mM KH_2PO_4, 20.0 mM $NaHCO_3$, 5.55 mM glucose, phenol red 2 mg/liter. 2-g samples of tissue were then incubated in 50-ml Erlenmeyer flasks containing 1 μCi of either [4-^{14}C] pregnenolone (specific activity (SA) : 52.8 mCi/mmole) or [4-^{14}C] progesterone (SA : 52.8 mCi/mmole) previously dissolved in 200 μl of propylene glycol, plus 20 ml of medium. Incubations were carried out in a Dubnoff metabolic shaker for 6 hr at 15-16 °C. Aliquots of 2 ml were withdrawn from the incubation medium after 7.5, 15, 30, 60, 120, 240 and 360 min. and replaced by an equal volume of fresh medium without precursor. At the end of incubation, the medium was separated from the tissue. Metabolism was terminated by mixing thoroughly with 3 volumes of ethanol.

Before extraction, suitable amounts of carrier and tritiated steroids were added to samples. Metabolites were then extracted, chromatographed bidimensionally on thin-layer plates, autoradiographed, eluted and identified by their isopolarity and isomorphicity with authentic steroids, as previously described (Colombo and Colombo Belvedere, 1977).

Results.

The following compounds were formed from both precursors by ovulated ovarian tissues (stages 3 and 4) : progesterone (from pregnenolone), 11-deoxycorticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone, androstenedione, testosterone and estradiol-17β. The steroidogenic pattern was not greatly affected by the time interval elapsed from spawning (figs. 3 and 4), despite the fact that the involution of non-ovulated oocytes was more advanced after 9 days. The release of the above metabolites in the medium during incubation with either precursor was measured by the integrated

values of their yield vs time curves : it was high for 11-deoxycorticosteroids (S > DOC) and androgens (testosterone > androstenedione) but low for estradiol-17β.

Before ovulation, accumulation of androgens in the incubation medium was high at stage 2 but negligible at stage 1. Estradiol-17β and DOC (but not S) were obtained in small amounts at maturity but could not be detected during quiescence (figs. 1 and 2). On the other hand, the production of 17α-hydroxyprogesterone was always conspicuous from stages 1 to 4, especially with progesterone as a precursor. Metabolite yields in the incubated tissues were correlated according to the same patterns outlined for the

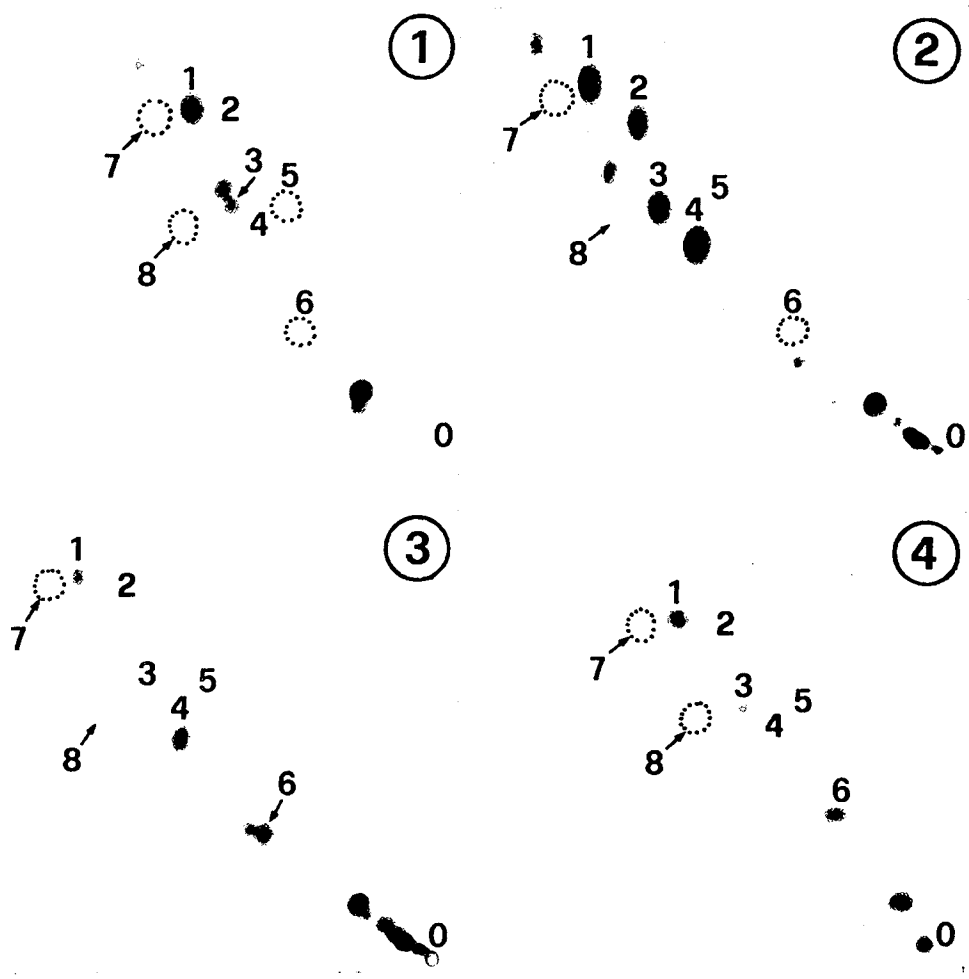

FIG. 1-4. — *Autoradiographs of thin-layer chromatographic profiles of metabolites released in the medium after* 6-hr *incubation of sea bass ovarian tissue at quiescence* (fig. 1), *at maturity* (fig. 2), *one day after deposition* (fig. 3) *and 9 days after deposition* (fig. 4) *with* [4-^{14}C] *progesterone.* (0) chromatographic origin ; (1) progesterone ; (2) androstenedione ; (3) 17α-hydroxyprogesterone ; (4) testosterone ; (5) 11-deoxycorticosterone ; (6) 11-deoxycortisol ; (7) estrone ; (8) estradiol-17β. Dotted circles indicate the position of carrier steroids associated with very little or no [^{14}C] label.

media. Tissues, however, showed greater yields of unidentified compounds of low and intermediate polarity.

At stage 5, atretic ovarian tissue converted pregnenolone to progesterone but was unable to perform further steps for steroid hormone biosynthesis. Radioactivity was instead channelled along other transformation routes but their products could not be characterized.

Discussion.

The present work demonstrates that the following steroid-converting enzymes may contribute to the ovarian steroid profiles of *D. labrax* : 3β- and 17β-hydroxysteroid dehydrogenases, 5-ene-ketosteroid isomerase, 17α, 20-C_{21}-desmolase, steroid 17α- and 21-hydroxylases and aromatizing system. Interestingly, the testicular tissue in the same species was devoid of the last two enzymatic activities (Colombo *et al.*, 1978a). The ovarian location of steroid 21-hydroxylase in the sea bass, a member of the family Serranidae, confirms similar findings in 3 other families of the order Perciformes (Colombo *et al.*, 1973 ; Colombo and Colombo-Belvedere, 1977).

The changing patterns of steroid metabolism from ovarian quiescence to the post-ovulatory stage suggests that steroidogenic shifts occur in the ovary of *D. labrax* during the oogenic cycle making androgen intermediates more available for estrogen biosynthesis during follicular growth and causing a rise in 11-deoxycorticosteroid formation around the time of ovulation.

The former change confirms the role of estrogens in the promotion of hepatic synthesis and release of vitellogenin, as documented in fish by recent reports (Plack *et al.*, 1971 ; Campbell and Idler, 1976). On the other hand, the induction of steroid 21-hydroxylase activity concomitantly with spawning fits very well with the fact that in a wide spectrum of steroid compounds, DOC and S were the most potent inducers *in vitro* of meiotic maturation in goldfish (Jalabert *et al.*, 1973) and in catfish (Goswami and Sundararaj, 1974). These observations cannot be reconciled, however, with the model proposed by Indian workers (Goswami *et al.*, 1974) in which 11-deoxycorticosteroids are produced in the interrenal under gonadotropin stimulation and act on the ovary.

A last comment should be made on the extensive atresia likely to result from prolonged gonadotropin deprivation. In this case, suppression of ovarian endocrine activity seems to parallel the involution of gametic elements and the phagocytic conversion of follicular cells.

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Résumé. La stéroïdogenèse de l'ovaire du téléostéen *Dicentrarchus labrax* a été étudiée *in vitro* à 5 stades différents : 1) prévitellogenèse, 2) postvitellogenèse, 3) post-ovulation, 1 jour après la fraie induite par HCG, 4) post-ovulation, 9 jours après la fraie induite, 5)

atrésie. Les tissus ovariens sont incubés avec [4-^{14}C] prégnenolone et [4-^{14}C] progestérone pendant 6 h à 15-16 °C. Les métabolites suivants se forment après ovulation : progestérone (à partir de prégnenolone), 11-desoxycorticostérone, 11-desoxycortisol, 17α-hydroxyprogestérone, androsténedione, testostérone et œstradiol-17β. Avant l'ovulation, les productions d'androgènes sont élevées au stade 2, mais négligeables au stade 1. L'œstradiol-17β et la 11-désoxycorticostérone sont obtenus en faible quantité à maturité, mais ne peuvent être détectés durant la phase de repos sexuel. Le tissu ovarien atrétique convertit la prégnenolone en progestérone mais la biosynthèse des hormones stéroïdes ne va pas au-delà.

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The spermiation period in the rainbow trout (*Salmo gairdneri*). Plasma gonadotropin and androgen levels, sperm production and biochemical changes in the seminal fluid

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Summary. Plasma immunoreactive t-GTH and androgens, sperm production and biochemical changes in seminal fluid were studied during the spermiation period in rainbow trout. The volume of sperm collected by hand-stripping was very low (< 0.1 ml) at the onset of spermiation, increased slowly during the next 4 weeks and sharply thereafter. Plasma t-GTH was high (6 ng/ml) at the onset and then decreased ; the sharp rise in sperm production started when circulating androgens had reached maximum values. In a second phase (6-12 weeks), the elevation in sperm production was strongly correlated wth plasma t-GTH, while plasma androgens fluctuated. Spermatocrit did not vary significantly during the period studied so that increased sperm production corresponded to an elevation in spermatozoal production. During spermiation Na^+ ionic concentration in the seminal fluid increased sharply and that of K^+ only slightly. Total protein in the seminal fluid decreased significantly after 8 weeks.

Introduction.

There is little information in the literature on spermiation in fish, and especially on hormonal control as related to the biochemical composition and quantitative production of sperm. In the present work, we have studied variations of plasma gonadotropin (GTH) and androgens as well as differences in some seminal fluid parameters, such as spermatocrit and protein and mineral concentration, which could serve as sperm quality criteria during spermiation in rainbow trout.

Material and methods.

Experimental procedure. — Three-year old males weighing 875 $\pm$ 75 g were purchased from a commercial hatchery and kept in a recycled, bacteria-filtered water system from September until February under seasonal photoperiod and temperature ranging between 8 and 12 °C. After a 1-month acclimation to laboratory conditions, the animals were weighed and their blood sampled on 15 October. From this date,

the fishes were hand-stripped every week to detect the onset of spermiation. Once it was detected, blood and sperm samples were taken on anesthetized fishes (propoxate R7464, Janssen Pharmaceutica, 2 mg/l) every 2 weeks for a period of 12 weeks.

Measurements. — Plasma gonadotropin (GTH) levels were measured by a double specific radioimmunoassay (RIA) (Breton *et al.*, 1975). Plasma androgen levels were determined by a double antibody RIA without chromatography and with a non-specific antibody (gift of Mr. Terqui), mainly binding testosterone (100 p. 100) and 11 Ketotestosterone (110 p. 100) but less androstene-dione (57 p. 100) and 5α-DHT (37 p. 100) ; adrenosterone, 17α-hydroxy-20α-dihydroprogesterone and 17β-estradiol were not bound (< 0.5 p. 100).

The volume of sperm released was determined by milt-stripping every time the animals were sampled. The spermatocrit was measured only when the volume of stripped sperm was at least 0.5 ml. Total protein concentration in the seminal fluid, obtained after 20 min. of centrifugation at 1 500 g, was determined using Lowry's

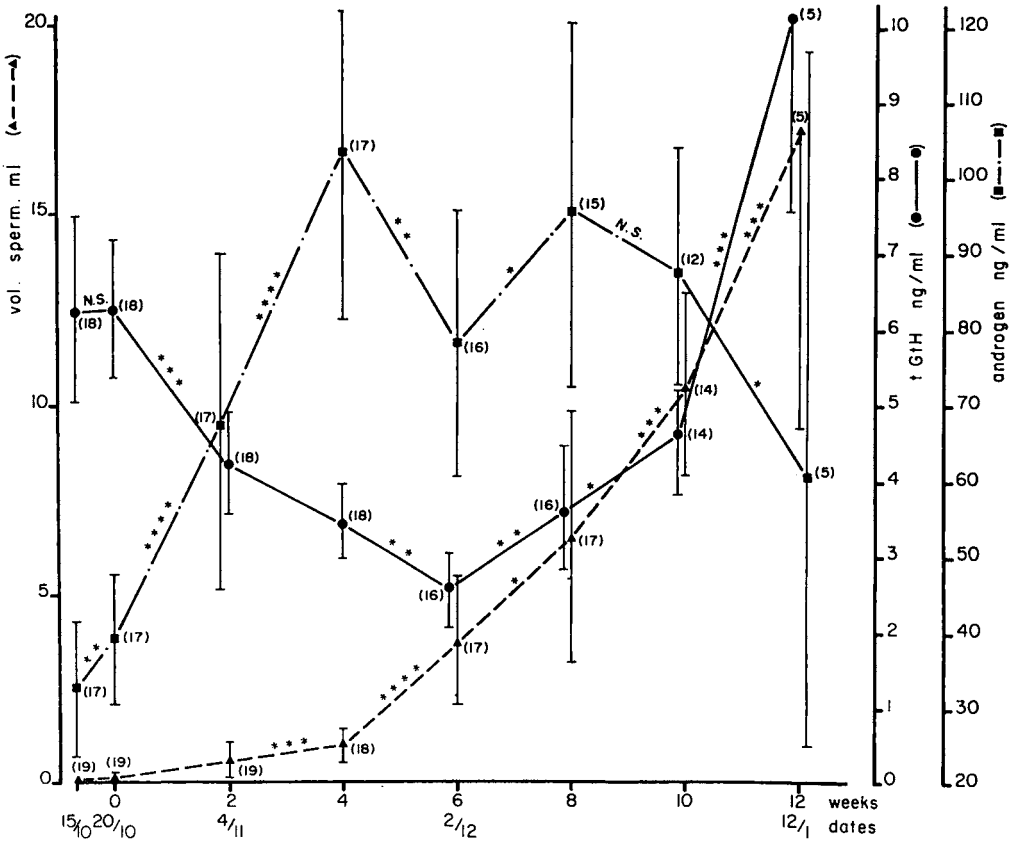

FIG. 1. — *Changes in plasma immunoreactive t-GTH, total androgen levels and sperm production during the 12 weeks after the onset of spermiation in rainbow trout.*

Graphs show the mean value and standard deviation for each parameter. Difference between values are expressed as : N.S. : non-significant ; * : P < 0.1 ; ** : P < 0.05 ; ***: P < 0.02 ; **** : P < 0.01 ; (n) : number of fishes.

method modified by Hartree (1972) ; sodium and potassium were measured by flame spectrophotometry (Eppendorf model).

The statistical methods used were variance analysis and t-tests ; the data were compared with Couple's method.

Results.

Plasma t-GTH and androgen levels (fig. 1).

Plasma GTH was high at the onset of spermiation (6.3 ng/ml), decreased during the following 6 weeks, then augmented between weeks 6 and 12, reaching a maximum value at 12 weeks (10 μg/ml). Androgen evolution was the reverse, increasing during 4 weeks from 34 to 104 ng/ml while t-GTH decreased. Total androgen levels then fluctuated, showing a slight decrease.

Sperm analysis.

The volume of milt produced between 2 samplings increased significantly during the period studied (fig. 1). Sperm production was very low at the onset of spermiation

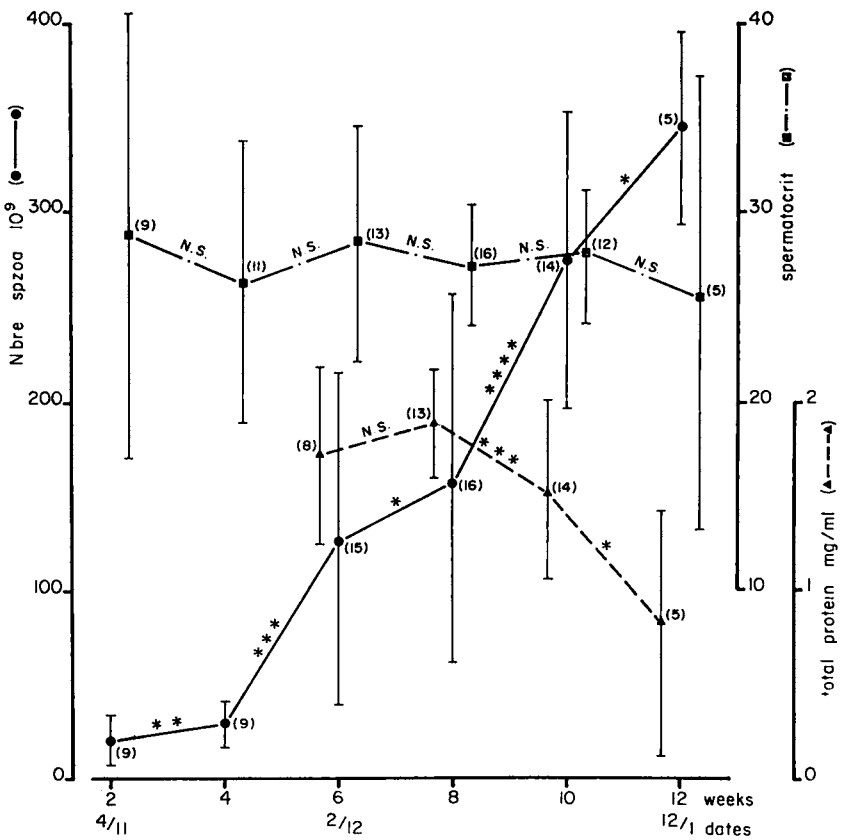

FIG. 2. — *Evolution of spermatocrit, number of spermatozoa collected at 2-week intervals, and total protein* (ref. albumin) *in the seminal fluid during spermiation from weeks 2 to 12.*

(0.1 ml) and rose steadily to reach a maximal value of 17.16 ml at 12 weeks. Spermatocrit did not vary significantly during this period, fluctuating between 28.3 p. 100 at the beginning and 25.4 p. 100 at the end (fig. 2). Spermatozoal production (fig. 2) therefore followed that of sperm (fig. 1).

Total protein. — Seminal fluid protein concentration was at its highest level (1.74 to 1.89 mg/ml) at the onset of spermiation. It decreased significantly afterwards and reached a concentration of 0.8 mg/ml at week 12 (fig. 2).

Sodium and potassium (fig. 3.) — Na^+ concentration increased steadily from 1.400 mg/ml at 6 weeks, when first measured, to 2.034 mg/ml at 12 weeks. Variations in K^+ concentration during the same period were different; K^+ increased from 784.6 mg ml at 6 weeks to 1 156 at 8 weeks ($P < 0.02$), but decreased to 853.9 mg/ml at 10 weeks ($P < 0.01$) to increase again at 12 weeks to 1 113.6 mg/ml ($P < 0.02$). The relation Na^+/K^+ varied also ; its minimal value was 1.79 and its maximal value 2.36.

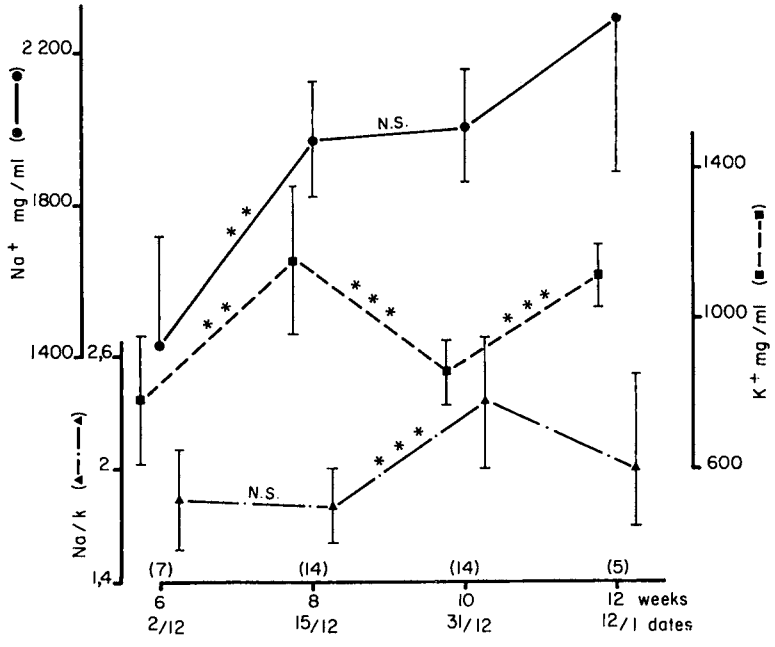

FIG. 3. — *Changes in Na^+ and K^+ and ratio in the seminal fluid during spermiation in male rainbow trout.*

Discussion.

These data elucidate hormonal changes, sperm production and variation in seminal fluid composition during the 12 weeks following the onset of spermiation. When sperm release is first detected, low amounts of sperm are produced and high levels of t-GTH are recorded in the plasma. Significant sperm production only occurred several weeks later when t-GTH decreased and androgens had reached a maximum level (fig. 1). At the onset of spermiation, the effect of GTH and androgen on sperm

production were not clear ; GTH might be involved in the initiation of spermiation and the rise of circulating androgens which, in turn, could reduce the t-GTH secretion, thus suggesting a negative androgen feedback on t-GTH. Later, between weeks 6 and 12, sperm production was more closely correlated with the plasma GTH level. This agrees with data in the literature stating that gonadotropin is involved in sperm release (Clemens and Grant, 1965 ; Yamazaki and Donaldson, 1968). A high level of GTH has also been observed at the end of gametogenesis and during the spawning season in salmonids (Crim, Watts and Evans, 1975; Breton *et al.*, unpublished data). Plasma androgens reach a maximum value at the same period (Schreck, Lackey and Hopwood, 1972 ; Idler, Horne and Sangalang, 1971). Androgens could also be involved in spermiation, and high levels may be required to stimulate sperm release (fig. 1). Whether only one specific active androgen is involved, or whether there are several, cannot be determined since the antibody used was not specific for any androgen. From the present data two stages are determined during spermiation, (1) initiation and preparatory stage lasting several weeks followed by (2) an active stage of sperm production when androgens are high and GTH is rising. Exogenous androgens alone stimulate spermatogenesis *in vivo* only when used in very large doses of 100 or 200 μg/g (Billard, 1974). This observation may also apply to spermiation. The spermatocrit stability already reported in this species (Chemayel, 1975) shows that stimulation of sperm production between weeks 6 and 12 is consequently a true stimulation of spermatozoal production.

The drop in seminal fluid protein concentration corresponds to a sharp increase of sperm production, and may suggest that protein synthesis capacity is limited and cannot deal with the copious seminal fluid secretion.

Ionic concentration of the seminal fluid is very high and increases mainly in Na^+ during the period studied and to a lesser extent in K^+. The rise in Na^+ concentration is strongly correlated with elevated sperm production and plasma t-GTH, suggesting hormonal regulation.

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Résumé. Une étude réalisée durant la période de spermiation chez la truite Arc-en-ciel a porté sur les taux plasmatiques de t-GTH et d'androgènes immunoréactifs sur la production de sperme et les changements dans la composition du liquide séminal. Le volume de sperme recueilli après massage abdominal est très faible au début (0,1 μl), augmente très légèrement au cours des quatre premières semaines et très fortement ensuite. Les teneurs plasmatiques en t-GTH sont élevées lorsque débute la spermiation et diminuent ensuite. La forte augmentation de production de sperme débute lorsque les teneurs en androgènes circulants ont atteint leur valeur maximum. Dans une seconde phase (6-12 semaines) l'augmentation de production de sperme est fortement corrélée avec les teneurs en t-GTH tandis que les niveaux d'androgènes fluctuent (fig. 1). La concentration du sperme en spermatozoïdes (spermatocrite) ne varie pas significativement pendant la période d'observation, de sorte que l'augmentation de production de sperme correspond à une augmentation de la production de spermatozoïdes (fig. 2). Pendant la période de

spermiation la teneur du plasma séminal augmente fortement dans le cas de Na^+ et légèrement dans le cas de K^+. Par contre la teneur en protéines totales diminue significativement après la 8e semaine.

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Circadian responses of teleostean oocytes to gonadotropins and prostaglandins determined by cyclic AMP concentration

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Summary. The work describes the circadian rhythm of teleostean oocyte responses to exogenous gonadotropins and prostaglandins as determined by the level of cyclic AMP accumulation, and the influence of photoperiodicity.

Mature females, *Mugil cephalus*, were divided into two groups and acclimated under a constant photoperiod regime of equal phases (12 L/12 D) at 21 $\pm$ 1 °C for 45 days. Two photoperiod conditions were used. One timed the onset of the light phase at 6 a. m. and the other at 6 p. m. The oocyte responses to NIH-LH, NIH-FSH, SG-G 100, prostaglandins E_2 and F_{2a} were examined at 3-hr intervals. The concentrations of cyclic AMP, assayed by the protein binding technique, were used to indicate the sensitivity of the oocytes to the hormones.

The circadian rhythm of oocyte response to gonadotropins and prostaglandin E_2 appeared to be « timed » by the onset of the light phase. Distinct increases of cyclic AMP accumulation generally occurred at 3 and 12 hr after exposure to light. In the dark phase, synchronized increases were not observed. The circadian rhythm of the endogenous cyclic AMP level was suggested as a reflection of a temporal relationship between the oocyte response and gonadotropin-surge in the plasma. An additive effect of LH and SG-G 100 in activating adenyl cyclase system was further indicated.

Introduction.

A diurnal rhythm of pituitary gonadotropin synthesis and release has been demonstrated in the teleosts *Salvelinus fontinalis*, *Salmo gairdneri* and *Notemigonus crysoleucas* (O'Connor, 1972 ; De Vlaming and Vodicnik, 1977). The physiological significance of this diurnal rhythm is not understood. However, temporal variation in gonadal response to gonadotropins, related to the diurnal cycle of pituitary gonadotropin content, was demonstrated in *N. crysoleucas*, and a maximal stimulatory effect of gonadotropins for inducing ovulation *in vitro* was reported in the late period of light phase in *Oryzias latipes* (Hirose and Donaldson, 1972 ; Hirose and Hirose, 1972).

Interaction of gonadotropins at the ovarian level with a highly specific membrane-bound receptor which triggers enzymatic production of cyclic AMP has been established by Ahren *et al.* (1969), Mason *et al.* (1973) and others. Both carp and salmon gonadotropins have been shown to activate the adenyl cyclase system in goldfish

and salmon (Fontaine *et al.*, 1970 ; Menon and Smith, 1971 ; Fontaine *et al.*, 1972). Prostaglandins, particularly those of the E series, have been shown to mimic the stimulatory action of LH *in vitro* on the formation of cyclic AMP by intact ovaries of the mouse and rat (Kuehl *et al.*, 1970 ; Lamprecht *et al.*, 1973). The cyclic AMP then acts as an intracellular second messenger and mediates biological actions common to LH and PGE_2 on the ovaries. These include the induction of ovum maturation (Tsafriri *et al.*, 1972*a*, *b*), luteinization (Channing, 1970 ; Kolena and Channing, 1971), ovarian steroidogenesis (Pharriss *et al.*, 1968 ; Speroff and Ramwell, 1970 ; and others), and ovarian protein kinase activity (Lamprecht *et al.*, 1973).

Biological rhythms are either endogenous to the organisms or driven by environmental cues. Synchronization depends upon external entraining agents. Light and temperature have been considered two critical variables. The stimulatory effects of different amounts of different hormones could therefore be determined by the temporal relationship between hormones, receptors, and cyclic AMP production.

This report describes the circadian rhythm of oocyte responses to exogenous gonadotropins and prostaglandins *in vitro*, as determined by the level of cyclic AMP accumulation, and the influence of photoperiodicity.

Materials and Methods.

Six mature female mullet, *Mugil cephalus* L., with oocytes at the tertiary yolk globule stage and larger than 500 μ, were divided into two groups and maintained in a constant photoperiod regime of equal phases (12L/12D) at 21 $\pm$ 1 °C for a period of 45 days. Two photoperiod conditions were used. One timed the onset of the light phase at 6 a.m., and therefore simulated natural conditions (normal light cycle) ; the other timed the onset of the light phase at 6 p.m., and therefore reversed the conditions (reversed light cycle). At the end of the acclimation period, the oocytes developed beyond a mean diameter of 600 μ, at which stage they are known to be effectively responsive to SG-G100 (Kuo *et al.*, 1974). Samples of oocytes were withdrawn through a polyethylene cannula from every fish at 3-hr time intervals throughout a 24-hr period.

The samples of oocytes were preincubated for 30 min in Tris buffer solution (pH 7.4), and then incubated in duplicate subsamples in Tris buffer containing theophylline and 2-Mercaptoethanol together with one of the following hormones :

(i) NIH-LH-B10, sp. potency 1.06 $\times$ NIH-LH-S1 : 5 μg/ml ;
(ii) NIH-FSH-B1, sp. potency 0.49 $\times$ NIH-FSH-S1 : 5 μg/ml ;
(iii) SG-G100, sp. potency 0.11 $\times$ NIH-LH-S1 : 5 μg/ml ;
(iv) Prostaglandin E_2(PGE_2) : 0.5 μg/ml ;
(v) Prostaglandin F_{2a}(PGF_{2a}) : 0.5 μg/ml.

At the end of the 5-min incubation period, the subsamples of oocytes were homogenized in 6 p. 100 Trichloroacetic acid solution. The proteins were quantified by the method described by Lowry *et al.* (1951). The levels of cyclic AMP in each sample were measured by the protein binding assay method described by Wombacher and Korber (1971) and Tsang *et al.* (1972).

Results.

The diurnal variations in the endogenous cyclic AMP levels in the mature oocytes are illustrated in figure 1. Levels of cyclic AMP varied between 1.89 and 2.65 pMole/mg protein for those fish exposed to the normal light phase, and between 2.34 and 2.69 pMole/mg protein for those in reversed conditions. Although variations in the levels of endogenous cyclic AMP concentration were recorded, they were not different statistically at 5 p. 100 significant level by student-t test and analysis of variance throughout the 24-hr cycle. There were also no differences statistically in the endogenous cyclic AMP levels between fish exposed to the two different light conditions.

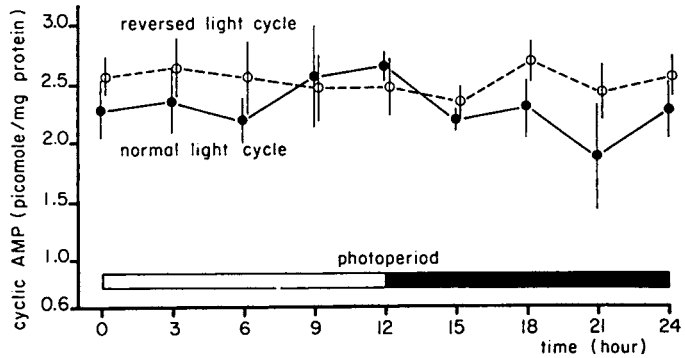

FIG. 1. — *Endogenous cyclic AMP levels in the mature oocytes of* Mugil cephalus (mean pMole/mg protein ± S. E. M.). (———) females exposed to normal light cycle, and (- - - -) for those exposed to reversed light cycle. The time represents hours after the onset of the light phase.

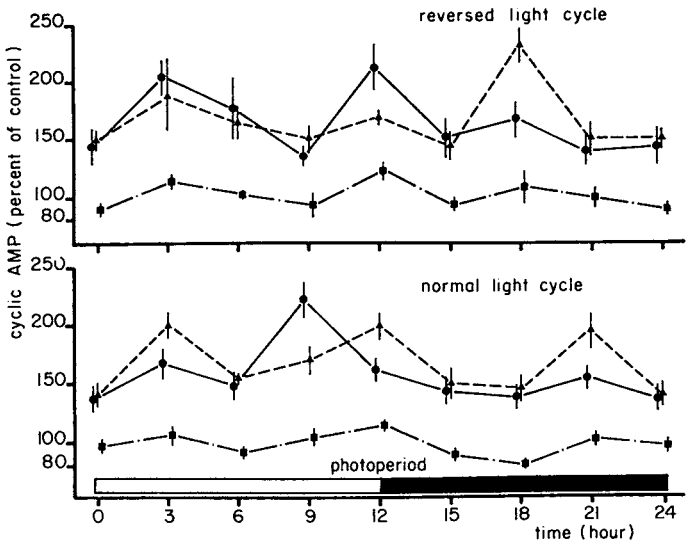

FIG. 2. — *Diurnal rhythm of cyclic AMP accumulation* (mean ± S. E. M.) *in mature oocytes exposed to LH* (———), *FSH* (— · — ·), *and SG-G* 100 (- - -). The time represents hours after the onset of the light phase.

The diurnal rhythms of mature oocyte response to each of the three gonadotropins was similar in form (fig. 2). In the light phase of both normal and reversed light conditions, distinct increases of cyclic AMP accumulation resulted from gonadotropin stimulation *in vitro*. This generally occurred at 3 and 12 hr after exposure to light. Both LH and SG-G100 appeared to be equally effective in stimulating cyclic AMP synthesis. In the dark phase, synchronized increases were not observed. Response to the gonadotropins increased 9 hr after the onset of darkness in normal conditions, and 6 hr after darkness in the reversed conditions. It also appeared that SG-G100 was more effective than LH.

The gonadotropin FSH was much less effective than either LH or SG-G100 in activating the adenyl cyclase system. The levels of cyclic AMP accumulation under FSH stimulation varied between 82-115 p. 100 of the endogenous level in normal conditions, and between 90-122 p. 100 in the reversed conditions.

Response of the oocytes to the two prostaglandins varied. The rhythmic response to PGE_2 was similar to that effected by the LH stimulation for both phases and light conditions (fig. 3). The effects of PGF_{2a} were greatest at 9 and 12 hr after exposure to light in normal conditions, and 6 hr before and after darkness in the reversed conditions. Although PGF_{2a} was less effective in stimulating cyclic AMP synthesis in the oocytes, the response of the oocytes to it was similar to that stimulated by LH and PGE_2 in the light phase under normal conditions. However, the rhythmic trend was directly opposite from these two hormones under reversed conditions.

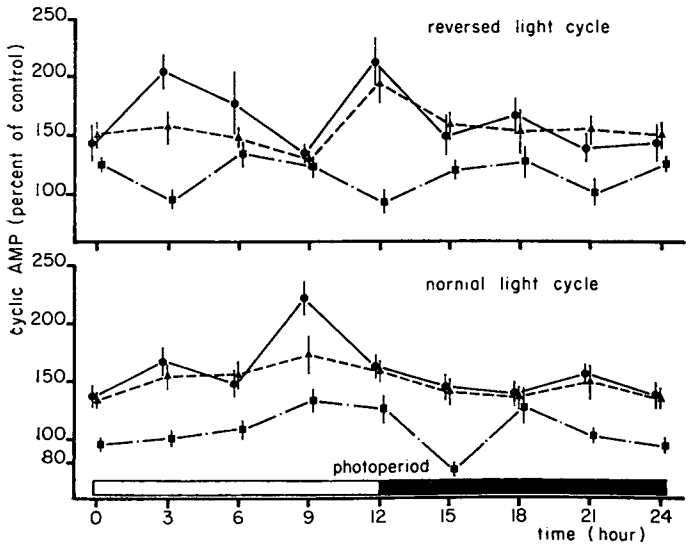

FIG. 3. — *Diurnal rhythm of cyclic AMP accumulation* (mean ± S. E. M.) *in mature oocytes exposed to LH* (———), PGE_2 (- - -), PGF_2 (—·—·). The time represents hours after the onset of the light phase.

The diurnal rhythm of the cyclic AMP levels in oocytes exposed to PGF_{2a} are illustrated in figure 4. There is the suggestion that the diurnal variation of oocyte response to PGF_{2a} is not related to this particular photoperiod regime, but might reflect

either an endogenous physiological rhythm or another unknown environmental influence.

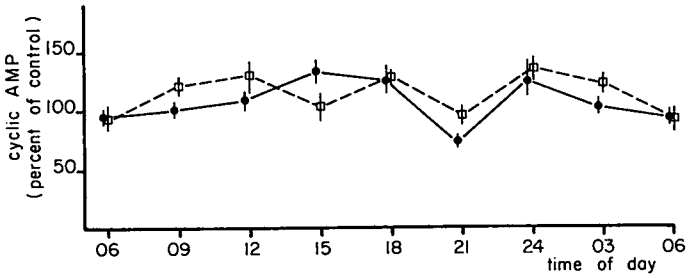

FIG. 4. — *levels of cyclic AMP accumulation in the mature oocytes under prostaglandin* $F_{2\alpha}$ *stimulation.* (———) represents the females exposed to normal light cycle and (- - -) represents those exposed to reversed light cycle.

Discussion.

Evidence by several workers (Koch *et al.*, 1974) suggests that the effect of hormones on different tissues through the activation of the adenyl cyclase system is determined largely by the capacity of the tissues to respond and the amount of hormone present. Continuous presence of the hormone at the binding site of the plasma membrane is necessary to activate the system, but only fractional presence at the receptor site is necessary to induce maximum production of cyclic AMP by the target tissues. In addition, some of the biological effects of LH and HCG on ovarian tissues are reported to require only brief exposure to the hormone (Perklev *et al.*, 1971 ; Lindner *et al.*, 1973), which then triggers an irreversible biochemical reaction.

The maximum accumulation of cyclic AMP in the mature oocytes of grey mullet was recorded for total levels of 15 μg LH or SG-G100. This level was comparable to the pituitary contents reported in *N. crysoleucas*, which ranged between 6.1-34.0 μg of SG-G100 during the 24-hr cycle (de Vlaming and Vodicnik, 1977).

In the experiments, responses to LH and SG-G100 were more pronounced than that of FSH at concentrations of 5 μg/ml. Similar results were reported in rat ovaries (Mason *et al.*, 1973), although Marsh *et al.* (1972) showed that stimulation of cyclic AMP synthesis in rabbit Graafian follicles by FSH was due to LH contamination. Several workers have indicated that LH or HCG, and SG-G100, are effective in inducing ovulation in teleosts, but not FSH (reviewed by de Vlaming, 1974). The types of pituitary gonadotropin cells present in the teleostean fishes are still the subject of conflicting arguments. Results with FSH in this study indicated that there was little response to this gonadotropin as shown by the cyclic AMP accumulation in the 24-hr cycle.

SG-G100 and LH, at a concentration of 5 μg/ml, are equally effective in stimulating adenyl cyclase activity in the mature oocytes of mullet, although the biological potency of SG-G100 is about one-tenth that of LH preparation as indicated by the chick testis radiophosphate uptake assay. Because of the specificity and lack of physiological cross-reaction of pituitary gonadotropins between heterologous species of vertebrates, the assayed potency of the hormones from heterologous species is not comparable if

the assay system of phylogenetically different species is used. Evidence suggests that mammalian gonadotropins are active in nonmammalian species, whereas pituitaries of nonmammalian species, particularly poikilotherms, tend to be devoid of activity when tested in mammals (Channing *et al.*, 1974). Scanes *et al.* (1972) reported a poor cross-reaction of poikilotherm gonadotropins in the chicken LH radioimmunoassay system. In contrast, piscine pituitary gonadotropins were shown to be effective in teleost systems, although they showed a specificity among teleosts (Breton *et al.*, 1973). Species specificity within teleosts was reported to be minor compared with that between teleost and mammalian species (Fontaine *et al.*, 1972). The present study was not intended to compare the effectiveness of gonadotropin preparations from the different sources, but to determine any oocyte response to these hormones throughout the 24-hr cycle.

Experimental results suggested that the stimulatory action of PGE_2 on cyclic AMP synthesis was more potent than that of PGF_{2a} on mature oocytes of the mullet. In general, PGE_2 has been found to be more potent than PGF_{2a} in promoting progesterone synthesis *in vitro* (Speroff and Ramwell, 1970), but less potent in the luteolysis of rat corpora lutea (Labhsetwar, 1975). Okamura *et al.* (1972), Jalabert and Szöllösi (1975), and Jalabert (1976) indicated that PGF_{2a} may play an important role in follicle rupture (ovulation) through the stimulation of smooth muscle fibers present in the ovarian stroma as well as in the follicle walls. Kuehl (1974) further suggested that PGF_{2a} was more selective in stimulating cyclic GMP, and that its role in the production of cyclic AMP was minor.

In the 24-hr cycle, marked increases in cyclic AMP accumulation in the oocytes were recorded at certain times after exposure to the light phase, regardless of the time of day. The circadian rhythm of oocyte response to gonadotropins and PGE_2 appeared to be « timed » by the onset of the light phase. The photosensivity rhythm of organisms determines whether light will stimulate the hypothalamo-hypophyseal gonadal axis (Stetson *et al.*, 1975) or not. The circadian rhythm of the endogenous cyclic AMP level might be reflecting a temporal relationship between the oocyte response and the pituitary gonadotropin level or gonadotropin-surge in the plasma. De Vlaming and Vodicnik (1977) reported that the gonadal response to gonadotropin treatment was maximal when the pituitary gonadotropin level was minimal for *N. crysoleucas* maintained in a 15 1/2 L/8 1/2 D photoperiod regime at 15 °C, Gonadal responses might therefore be related to the gonadotropin-surge in the plasma, and the hormones act as triggers iniating biochemical reactions that persist after the hormones are no longer detectable. In the absence of data on diurnal rhythms in pituitary gonadotropin level or gonadotropin-surge in the grey mullet, this relationship has not yet been determined. The increase of cyclic AMP accumulation in the oocytes responding to exogenous LH or SG-G100 *in vitro*, was observed in most cases, if not all, when the endogenous cyclic AMP levels were relatively high. The results further indicate the additive effect of these gonadotropins in activating adenyl cyclase system.

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Résumé. Ce travail décrit le rythme circadien de la réponse des ovocytes d'un poisson téléostéen aux gonadotropines exogènes et aux prostaglandines.

Des femelles matures, *Mugil cephalus*, sont divisées en deux groupes et acclimatées pendant 45 jours à une photopériode constante 12 L-12 N à 21 $\pm$ 1 °C. Le début de la période claire est à 6 h pour un groupe et 18 h pour l'autre. La réponse des ovocytes à NIH-LH, NIH-FSH, SG-G 100, et aux prostaglandines E_2 et $F_{2\alpha}$ est examinée toutes les 3 h. La sensibilité des ovocytes aux hormones est testée par le dosage de l'AMP cyclique.

Le rythme circadien de la réponse des ovocytes aux gonadotropines et à la prostaglandine E est déterminé par le début de la période claire. Une augmentation notable de l'accumulation d'AMPc se produit généralement à 3 et 12 h après l'allumage. Pendant la période sombre on n'observe pas d'augmentations synchronisées. Le rythme circadien du niveau d'AMPc endogène apparaît comme un reflet de la relation temporelle entre la réponse de l'ovocyte et la décharge de gonadotropine dans le plasma. Il semble exister un effet additif de LH et SG-G 100 pour activer le système adényl cyclase.

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The genetic control of sexual maturation in the teleost, *Xiphophorus maculatus* (Poeciliidae) ; a review

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Summary. A sex-linked gene which controls the age at which the gonadotropic zone of the pituitary gland develops and becomes physiologically active has been identified in the platyfish. The alleles for early (P^e) and late (P^l) differentiation are linked to pigment genes that serve as genetic markers. The average age of sexual maturation was 12.5 weeks (range, 10-15) for $P^e\ P^e$ males, 20 weeks (range, 16-29) for $P^e\ P^l$ males and 26.5 weeks (range, 18-40) for $P^l\ P^l$ males. $P^e\ P^e$ females matured between 10 to 14 weeks of age and $P^e\ P^l$ females between 16 to 20 weeks. No overlap in maturation time is found when fish of different genotypes are raised under identical conditions. The presence or absence of the gonadotropic zone is well correlated with the differentiation of the gonad. However, even in the absence of a gonadotropic zone oocytes may proceed up to the yolk droplet stage and testis will form spermatogonia. In males, androgen from the developing testis, in turn, controls the metamorphosis of the anal fin into a gonopodium. The growth rate of immature males and females is the same, however it declines sharply in males (but not females) at the time of sexual maturity. Thus, early-maturing males are significantly smaller than late-maturing ones. Female genotypes do not exhibit size differences and eventually become larger than all males.

This polymorphism effecting gonadotrop differentiation is a natural component of wild populations and of laboratory stocks derived from them.

Intraspecific variation in a variety of endocrine parameters including those dealing with the hypothalamo-hypophysial-gonadal (HPG) axis are known to be under genetic control. Most of our information comes from studies of laboratory animals and genetically controlled aberrant endocrinological conditions in man. Many of the differences between laboratory stocks are merely of a quantitative nature and thus make the understanding of the basic mechanisms involved difficult. Recently, we have discovered in the platyfish, *X. maculatus*, a gene that controls an all-or-nothing response of the pituitary-gonadal axis.

A sex-linked gene, *P*, affects the onset of sexual maturity in *X. maculatus* by controlling directly or indirectly the age or size at which the gonadotrops differentiate and become physiologically active. Five *P* alleles have been identified in natural populations and laboratory stocks (Kallman and Borkoski, 1978). The various *P* factors are closely linked to a number of color genes that serve as genetic markers. The same *P* allele may be associated with a variety of color genes. The existence of platyfish with

« early » and « late » genotypes that differ only by the sex chromosomes they carry but are genetically identical in all other respects (platyfish have 23 pairs of autosomes), has provided a model system to study how the genotype controls the development of the pituitary-gonadal axis, how the pituitary gland regulates gonadal structure and function, and how age and size separately affect gonad maturation.

Originally, it was found that in the Belize stock males homozygous for a certain pigment gene, red iris (*Ir*), matured at 13 weeks and at a size of 24 mm, and males homozygous for a second pigment gene, red body (*Br*) matured at 26 weeks and at 31 mm (table 1). Heterozygous males were intermediate for both traits (Kallman and Schreibman, 1973 ; Schreibman and Kallman, 1977 ; Kallman, Schreibman and Borkoski, 1973). The *P* allele linked to *Ir* was subsequently described as P^3 and the one linked to *Br* as P^4. Males and females homozygous for P^1 mature at approximately 8 weeks and at 21 mm whereas 50 p. 100 of the females homozygous for P^5 still had undeveloped ovaries at 60 weeks, although they exceeded 40 mm in length (Kallman and Borkoski, 1978).

TABLE 1

Age of sexual maturity and adult size of 3 genotypes of male platyfish, Xiphophorus maculatus *(Belize stock)*

n	Maturity weeks s. e.	Size mm s. e.	n	Maturity weeks s. e.	Size mm s. e.
	Ir P^3 *Ir* P^3 males *			*Ir* P^3 *Br* P^4 males *	
11	13.3 ± 0.57	24.4 ± 0.83	24	18.4 ± 0.74	27.9 ± 0.67
17	12.6 ± 0.33	23.3 ± 0.23	19	19.7 ± 0.52	27.7 ± 0.43
	Ir P^3 *Br* P^4 males **			*Br* P^4 *Br* P^4 males **	
40	20.2 ± 0.53	27.0 ± 0.42	46	26.5 ± 0.60	31.3 ± 1.43

* From W - + Y-*Ir* female × Y-*Ir* Y-*Br* male (Kallman and Schreibman, 1973).
** From W - + Y-*Br* female × Y-*Ir* Y-*Br* male (Schreibman and Kallman, 1977).

Neonatal platyfish have pituitary glands that, with the exception of the gonadotrops, contain all the cell types present in mature fish (Schreibman, 1964). In immature animals, regardless of age, the gonadotropic zone is represented by only a few chromophobes in the peripheral caudal pars distalis. The activity of the *P* gene is correlated with the age and size at which the chromophobes proliferate and differentiate to form a well-developed zone of active gonadotrops. Depending upon genotype this event may occur as early as 5 weeks or not until the fish are well past one year of age. Ultimately the region of gonadotrops in early and late developers are indistinguishable. Whether this gene operates directly on the pituitary gland, the hypothalamus or some other level of the endocrine or sensory systems is still unknown and under investigation. We are especially interested in the events that occur in the hypothalamus concomitantly with the maturation of the hypophysis.

The development of the gonadotropic zone precedes, and is essential for, complete gonadal maturation. In the absence of a functional zone oocytes develop through the oil

droplet stage and are surrounded by active follicle cells and a prominent zona pellucida. Yolk deposition, however, does not occur. Spermatogenesis proceeds up to the spermatocyte stage and efferent ducts display little activity.

The anal fin of male platyfish undergoes a complex transformation into an intromittent organ. Each of the 6 clearly-defined successive stages of this process are dependent upon increasing levels of androgens. Thus the stage of development of the anal fin serves as an indicator of sexual maturation and of sex steroid levels. It permits us to postulate the course of steroidogenesis as indicated in figure 1. All males, but not females, enter into stage one at 5 weeks of age. The transition of the unmodified anal fin into stage 1 is not contingent upon the presence of a Y chromosome, since the same transformation occurs in males with the exceptional genotypes, XX (Kallman, 1968). All males remain in this stage until the pituitary-gonadal axis becomes active which, depending upon genotype, may occur as early as 5 weeks in $P^1\ P^1$ males or as late as 25 weeks in $P^2\ P^5$ males. The rate of anal fin metamorphosis (stage 2-stage 6) is directly related to the age at onset of maturity ranging from 3.2 weeks for $P^1\ P^1$ to 7 weeks for $P^2\ P^5$ males (Kallman and Borkoski, 1978).

Androgens are also responsible for retarding the rate of growth of poeciliid fish (Pickford and Atz, 1957). Immature males and females grow at the same rate, however this rate declines sharply in males (but not females) at the time of sexual maturity. This leads to significant differences in the final adult size that are a permanent phenotypic expression of the *P* locus and of activity in the HPG axis.

Figure 1 illustrates our findings that several processes associated with sexual maturation are retarded in the later-developing genotypes. Gonopodial differentiation, a sensitive indicator of androgen (Turner, 1942) and indirectly of gonadotropin production, takes almost twice as long to go from stage 2 to stage 6 when early and late genotypes are compared (Schreibman and Kallman, 1977). Similarly, histological observations indicate that the development of the gonadotropic zone and of the gonads proceeds more slowly in the late genotypes. Ultimately all fish, regardless of their genetic makeup, have histologically indistinguishable pituitaries and gonads.

The relationship between age and size in determining the onset of sexual maturity was studied in three genotypes of females. $P^1\ P^1$ (early) females matured at 21 mm regardless of age. Some $P^5\ P^5$ (late) females initiated maturity after week 34 regardless of size, but even at 60 weeks, half of them were still immature although they exceeded 40 mm in length. An inverse relationship between age and size existed for $P^1\ P^5$ females. They had ripe ovaries as early as 11 weeks provided they had attained a size of 30 mm, but they became mature as small as 23 mm at 25 weeks of age. Keeping age and size constant, females homozygous for P^5 always had a smaller number of eggs than females of the other two genotypes. Up to a size of 30 mm, $P^1\ P^1$ females had significantly more ova than $P^1\ P^5$ females. This suggests that fecundity and the gonadosomatic index are genotype specific (Kallman and Borkoski, 1978).

Our analysis of the HPG axis raises a number of obvious questions that go beyond the study of the platyfish. Investigation of these problems may provide some insight into general endocrinological mechanisms and phenomena.

1. What triggers the *P* gene ? How is it related to size and age ? How do internal and external stimuli affect activity of the various *P* alleles ?

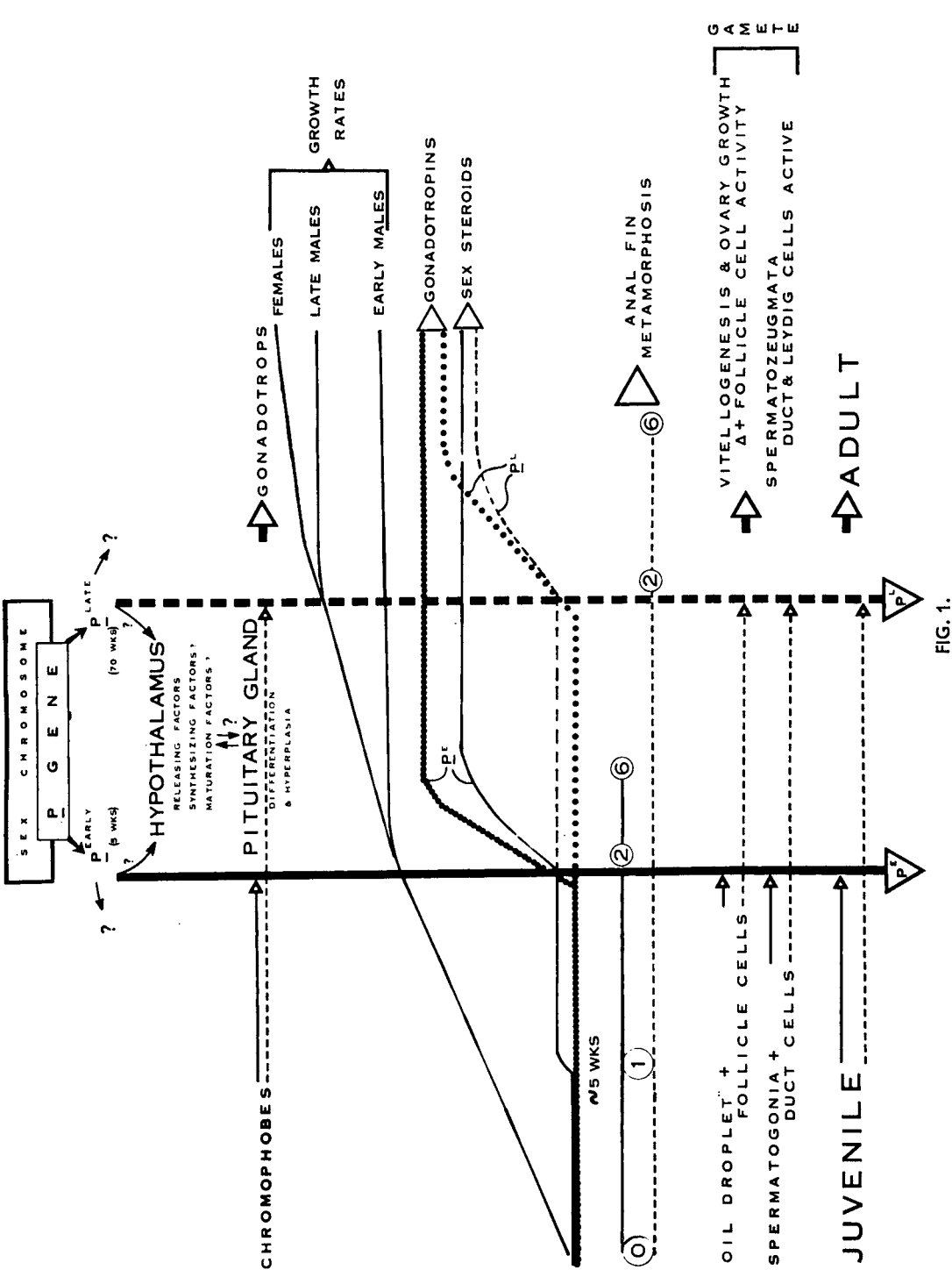
SEX CHROMOSOME
P GENE
P EARLY (5 WKS)
P LATE (10 WKS)
?
HYPOTHALAMUS
RELEASING FACTORS
SYNTHESIZING FACTORS?
MATURATION FACTORS?
PITUITARY GLAND
DIFFERENTIATION & HYPERPLASIA
CHROMOPHOBES
GONADOTROPS
FEMALES
LATE MALES
EARLY MALES
GROWTH RATES
GONADOTROPINS
SEX STEROIDS
PE
PL
ANAL FIN METAMORPHOSIS
VITELLOGENESIS & OVARY GROWTH
Δ+ FOLLICLE CELL ACTIVITY
SPERMATOZEUGMATA
DUCT & LEYDIG CELLS ACTIVE
GAMETE
ADULT
JUVENILE
OIL DROPLET + FOLLICLE CELLS
SPERMATOGONIA + DUCT CELLS
≈5 WKS
0
1
2
6
2
6

FIG. 1.

2. What is the site and mechanism of action of the *P* gene ? It is important to know if the gene turns on a single event which then serves to trigger successive processes in a « chain-reaction » type of phenomenon or if it affects several organs or processes simultaneously. Does it dictate when other genes become activated ?

3. What is the role of the hypothalamus in determining the onset of sexual maturation ? Although it is clear that the hypothalamus produces releasing and inhibiting factors, its role in regulating the rate of synthesis of pituitary hormones is somewhat less lucid (Vale *et al.*, 1977). Does the hypothalamus develop at approximately the same time as the pituitary or does it contain releasing factors long before the gonadotrops differentiate. Perhaps a hypophysiotropic substance is produced that *induces* maturation of the gonadotropic zone (a gonadotrop-maturing factor ?). This problem may be analyzed by transplanting pituitary glands and hypothalami from genetically early maturing fish to genetically late fish and vice versa. The availability of isogenic stocks (Kallman, 1975) makes this feasible.

4. What is the mechanism by which the size of the gonadotropic zone increases ? Recent examination of a homozygous late-maturing (P^5) female suggests that proliferation of the chromophobes occurs prior to their activation. This requires additional study.

5. The structural changes that take place in the anal fin at approximately five weeks of age in all males signals the beginning of gonopodial stage one. Since this occurs in the absence of a gonadotropic zone it suggests that low levels of androgens may already be present months before the gonadotrops develop. Since we could not demonstrate active Leydig cells with our methods the question arises as to the origin of the steroids. It also suggests that androgens do not initiate the maturation of the HPG axis.

6. What is the explanation for the protracted development of various segments of the HPG axis and of the maturation process in general ? Is it simply a case of older tissues having lost some of their responsiveness (loss of hormone receptors ?) or do the various *P* genes produce their effects at different rates ?

This polymorphism which affects maturation of the HPG axis is a natural component of wild populations and laboratory stocks derived from them. This system is developing as an important model for investigating the genetic control of endocrine gland structure and function and for the study of basic endocrine mechanisms.

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Résumé. Un gène lié au sexe qui contrôle l'âge auquel la zone gonadotrope de l'hypophyse se développe et devient physiologiquement active a été identifié chez le Xiphophore. Les allèles pour la différenciation précoce (P^e) et tardive (P^l) sont liés à un gène de pigmentation qui sert de marqueur génétique. L'âge moyen à la maturation sexuelle est 12,5 semaines (extrêmes 10-15) pour les mâles $P^e\,P^e$, 20 semaines (16-29) pour les mâles $P^e\,P^l$ et 26,5 semaines (18-40) pour les mâles $P^l\,P^l$. Les femelles $P^e\,P^e$ maturent entre 10 et 14 semaines d'âge et celles $P^e\,P^l$ entre 16 et 20 semaines. Aucun chevauchement dans l'époque de maturation n'a été trouvé lorsque les poissons des différents génotypes ont été

élevés dans des conditions identiques. La présence ou l'absence de zone gonadotrope est bien corrélée avec la différenciation des gonades. Cependant même en l'absence de zone gonadotrope, les ovocytes peuvent se développer jusqu'au stade de globule lipidique et les testicules jusqu'au stade spermatogonie. Chez les mâles, les androgènes du testicule en développement à leur tour contrôlent la métamorphose de la nageoire anale en gonopode. Le taux de croissance des mâles et femelles immatures est le même, mais diminue fortement chez les mâles (mais pas chez les femelles), lors de la maturité sexuelle. Donc les mâles précoces sont significativement plus petits que ceux à maturité tardive. Le génotype femelle ne montre pas de différence de taille et finalement devient plus gros que tous les mâles.

Ce polymorphisme affectant la différenciation gonadotrope est un composant naturel des populations sauvages et des populations de laboratoire qui en dérivent.

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Synthetic releasing hormones LH/FSH-RH and LH-RH : effect of intracerebral and intramuscular injections on female carp (*Cyprinus carpio* L.) maturation

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Summary. The influence of intracerebral and intramuscular injections of synthetic LH/FSH-RH on the maturation of female carp was investigated. The results indicate that intrahypophyseal injections of synthetic LH/FSH-RH at 1 μg/kg body weight stimulated ovarian maturation in female carp. Maturation was measured in terms of the percentage of oocytes with the nucleus shifted under the micropyle and of those after GVBD. Neither intramuscular injection of synthetic LH/FSH-RH or LH-RH at 1 μg/kg of body weight nor intraventricular injections of synthetic LH-RH had any effect on ovarian maturation in female carp.

Introduction.

The injection of fish hypothalamic extract as well as that of synthetic releasing hormones stimulates the release of gonadotropin from fish hypophysis (Breton *et al.*, 1972 ; Breton and Weil, 1973 ; Crim and Cluett, 1974 ; Hirose and Ishida, 1974 ; Weil *et al.*, 1975 ; Lam *et al.*, 1975 ; Crim *et al.*, 1976). Since experiments on mammals showed that the effect of synthetic LH-RH was more potent when given by intracerebral infusion than by intravenous or intramuscular route (Ben-Jonathan *et al.*, 1974), we used intraventricular and intrahypophyseal infusions in this study as well as intramuscular injections of synthetic releasing hormones LH/FSH-RH for comparison. In this paper, we report their effect on oocyte maturation in female carp in the final stage of maturity.

Material and methods.

Fifty-five 4-year old female carp weighing on an average 2.5 kg were used. They were given gonadotropin releasing hormone and 2 experiments (group I and group II) were carried out using 2 releasing hormone from different origin.

Group I, comprising 9 females given intramuscular injections and 16 given intrahypophyseal injections of synthetic LH/FSH-RH (Hoescht). The latter group was

divided into two subgroups, D_1 and D_2, according to the maturity stage of the oocytes before the first injection (2 Feb). Subgroup D_1 : 42 p. 100 oocytes with the nucleus shifted peripherally ; subgroup D_2 : 54 p. 100 (fig. 1). The control comprised 4 females receiving intramuscular and 4 given intrahypophyseal injections of NaCl physiological solution.

Group II, comprising 6 females given intramuscular and 8 given intraventricular injections of synthetic LH-RH (Lot 760003, Calbiochem, San Diego, Calif). The fishes were designated with a symbol, D. The control comprised 4 females receiving intramuscular and 4 intraventricular injections of the NaCl physiological solution.

Control females in both groups were designated with a symbol, K. Intracerebral injections were performed with a needle located in the hypophysis or in the third brain ventricle, according to the stereotaxic technique of Peter (1970). Hormones were given once a day for 9 consecutive days at a dose of 1 μg/kg of body weight. Water temperature during the time of experimentation ranged between 18 and 20 °C.

To investigate the effect of injections on ovarian maturation, the *in vivo* method of ovarian sampling was used (Bieniarz and Epler, 1976), and the maturity stage of an ovary was estimated on the basis of oocyte nucleus shift. A distinction was made among oocytes with the nucleus situated in the center or slightly shifted towards the periphery (stage 1 + 2), oocytes with the nucleus shifted under the micropyle (stage 3 + 4) and oocytes after germinal vesicle breakdown (GVBD). The degree of oocyte maturity was estimated before starting an experiment and prior to each successive injection.

Results.

Group I.

Intrahypophyseal injection (fig. 1). — As a result of 9 intrahypophyseal injections of synthetic LH/FSH-RH, the number of oocytes with the nucleus shifted peripherally (stage 3 + 4) increased in group D_1 from 42 p. 100 (2 Feb.-day of first injection) to 78 p. 100 + 4 p. 100 oocytes after GVBD ; in group D_2, from 54 p. 100 to 77 p. 100 + 22 p. 100 oocytes after GVBD ; in group K, from 57 p. 100 to 63 p. 100 at the end of the experiment.

Intramuscular injections (fig. 2). — The number of oocytes (stage 3 + 4) before the first injection (12 April) was 59 p. 100 in females of group K and 63 p. 100 in females

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FIG. 1. — *Intrahypophyseal injections of* 1 μg *synthetic LH/FSH-RH per kg.*

FIG. 2. — *Intramuscular injections of* 1 μg *synthetic LH/FSH-RH per kg.*

FIG. 3. — *Intraventricular injections of* 1 μg *synthetic LH-RH per kg.*

FIG. 4. — *Intramuscular injections of* 1 μg *synthetic LH-RH per kg.*

FIG.1

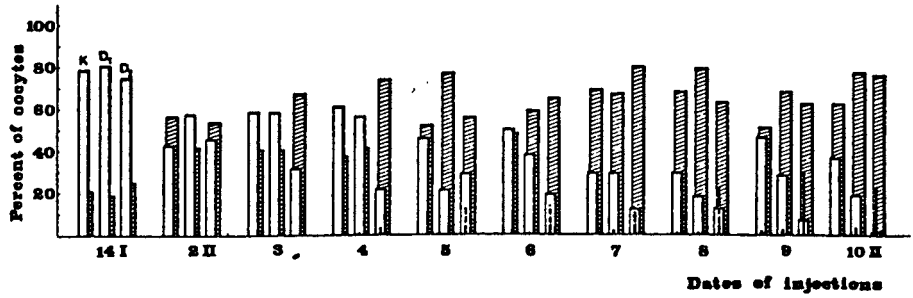


FIG.2

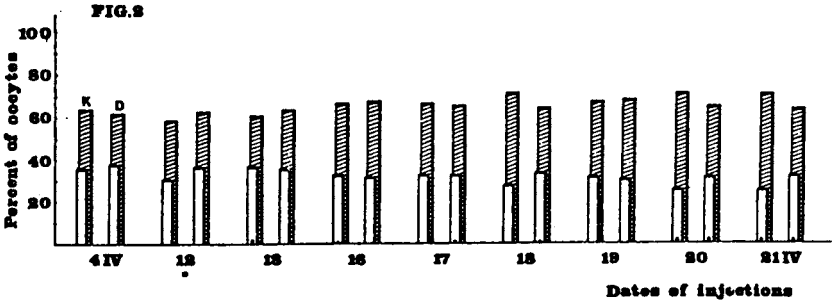


FIG.3

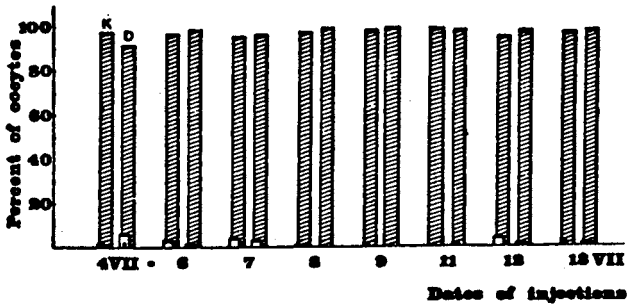
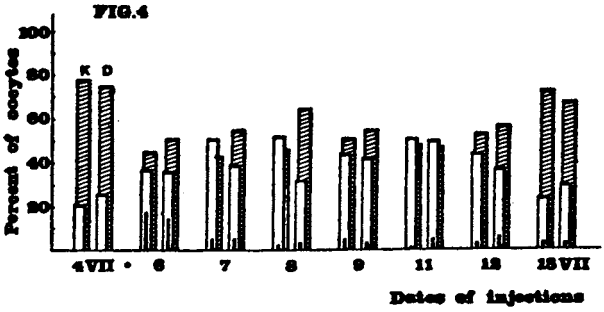


FIG.4



□ Stage 1-2
▨ Stage 3-4
| Oocytes after GVBD
• Day of first injection

of group D, while it was 72 p. 100 in group K and 65 p. 100 in group D after the last injection.

Group II.

Intraventricular infusions and intramuscular injections of synthetic LH-RH did not stimulate sexual maturity in female carp when measured in terms of the percentage of oocytes with the nucleus shifted under the micropyle and of those after GVBD (fig. 3 and 4).

Discussion.

Intrahypophyseal infusion of synthetic LH/FSH-RH at a dose of 1 μg/kg of body weight proved effective in accelerating oocyte maturity in female carp. A more potent effect, expressed in terms of a higher percentage of the oocytes with the nucleus shifted peripherally and of those after GVBD, was observed in a group of fishes which from the very beginning of the experiment had a greater number of mature oocytes (stage 3 + 4).

Hypophyseal sensitivity to an exogenic factor releasing gonadotropin in carp depends not only on external factors, but also on the condition of the hypophysis and gonad maturity (Weil *et al.*, 1975). Synthetic LH/FSH-RH given intramuscularly had no effect on stimulation of oocyte maturation, which suggests that when using a dose of 1 μg/kg of body weight, the site of hormone administration is an important factor.

Intraventricular and intramuscular injections of synthetic LH-RH had no effect on ovarian maturation of female carp. A dose of 1 μg/kg synthetic LH-RH seemed too low to cause an increase in the blood gonadotropic level high enough to have an effect on maturation which would be expressed by an elevated percentage of oocytes with the nucleus shifted towards the periphery. Perhaps infusion directly to the hypophysis would give a more pronounced effect, as in the case of hormone LH/FSH-RH, although hormone diffusion to other parts of the brain, as well as to the hypophysis cannot be excluded in the case of intraventricular injections.

Conclusions.

Intrahypophyseal injections of synthetic LH/FSH-RH at 1 μg/kg of body weight stimulated ovarian maturation in female carp. This was measured in terms of the percentage of oocytes with the nucleus shifted under the micropyle and of those after GVBD.

Intramuscular injections of synthetic LH/FSH-RH or LH/RH at 1 μg/kg of body weight as well as intraventricular injections of synthetic LH-RH had no effect on ovarian maturation in female carp.

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Résumé. Nous avons étudié l'influence de l'injection intracérébrale et intramusculaire de LH/FSH-RH synthétique sur la maturation de la carpe femelle. Les injections intrahypophysaires de 1 µg/kg de LH/FSH-RH stimulent la maturation ovarienne estimée par le pourcentage d'ovocyte dont le noyau a migré sous le micropyle et de ceux qui ont subi la GVBD. Les injections intramusculaires ou intraventriculaires des mêmes doses de LH/FSH-RH sont sans effet sur la maturation ovarienne.

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Precocious induction of maturation and ovulation in northern pike (*Esox lucius*)

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Summary. The efficiency of partially purified salmon gonadotropin (PPSG) and human chorionic gonadotropin (HCG), administered alone or in association with a progestagen (17α-hydroxy, 20β-dihydroprogesterone : 17α-20β P, or 17α-hydroxyprogesterone : 17α P) was investigated in submature females using the germinal vesicle in non-peripheral position as the criterium for initial oocyte stage. A dose of 0.1 mg PPSG/kg gave satisfactory ovulation (89 p. 100) and fertilization (83 p. 100) rates ; a minimum dose of 0.03 mg/kg was required to induce maturation without ovulation. The same dose of PPSG (0.03 mg/kg) combined with 17α-20β P (3 mg/kg) or 17α P (3 mg/kg) 1 day later led to full maturations and partial ovulations. 17α-20β P (3 mg/kg) administered alone caused maturation in all animals and partial ovulation or no ovulation. In all the above cases using the combined treatment or 17α-20β P alone, fertilization rates were low (20 to 41 p. 100). HCG alone or associated with 17α-20β P (3 mg/kg) was unable to induce ovulation. It is emphasized that treatment should be started as soon as possible after capture. Three-day captivity prior to treatment led to ovarian atresia and a significant decrease in ovulation rates.

Introduction.

Hormonal control of ovulation is of particular interest in northern pike (*Esox lucius*) since the normal sequence of oocyte maturation and ovulation does not occur under confinement, even in submature females captured after the completion of vitellogenesis. Furthermore, most females quickly undergo ovarian atresia and may die after a few weeks (Portal, 1947 ; Chimits, 1947, 1956 ; De Montalembert *et al.*, unpublished data). Performed in one or several injections, hypophysation with carp pituitaries has been somewhat successful (Sorenson *et al.*, 1966), but precise data on the state of maturity of recipient females, as well as the actual efficiency of the treatment (ovulation and fertilization/hatching rates), are lacking. Moreover, the females were held for several days in tanks before the treatment was started. Recent experiments in carp (Jalabert *et al.*, 1977) showed that the combined use of fish pituitary extracts and a steroid, 17α-hydroxy-20β-dihydroprogesterone (17α-20β P) can be more efficient in marginal temperature conditions than the classical hypophysation method which requires ten times more pituitary material.

Consequently, the purpose of the present study was double. Firstly, we wished to assess the efficiency of various hormonal treatments (gonadotropic preparations and/or steroids) on *in vivo* maturation and ovulation in pike. Since another salmon gonadotropic preparation (SG-G100) was found active on *in vitro* maturation of northern pike oocytes (Jalabert and Breton, 1973), we used a partially purified salmon gonadotropin in the absence of homologous pituitary extracts. Human chorionic gonadotropin (HCG) was also tested ; this substance is unable to induce *in vitro* oocyte maturation in northern pike (Jalabert and Breton, 1973) or in trout and goldfish (Jalabert *et al.*, 1972, 1973), but may trigger *in vivo* ovulation in a number of fish species (see review by Shehadeh, 1975). The two steroids employed were (1) 17α-20β P which is the most efficient steroid found so far on *in vitro* maturation of northern pike oocytes (Jalabert and Breton, 1973), and (2) an inexpensive substitute, 17α-hydroxyprogesterone (17α P).

We also studied the possible effect of captivity prior to treatment on the efficiency of the latter.

Material and methods.

The experiments were performed in March and April 1977. Natural ponds (« Etangs des Dombes ») were emptied at various periods. Two to 3-year old northern pike breeding females weighing 0.8 to 2 kg were collected and kept in darkness inside floating cages in the Rhone river at a temperature of 9 to 10 °C. The hormonal treatment was started in the above conditions on these fish at different time intervals after capture. The spawners were then transported to the laboratory and again kept in darkness at a temperature of about 12 °C. The fishes were anesthetized in a solution of 2-phenoxyethanol (Merck) (0.5 ml/l) before every ovarian sampling or injection.

Injected material. — The products were injected intraperitoneally and dosed in order to introduce the same volume of physiological saline (1 ml/kg) into each fish.

Partially purified salmon gonadotropin (PPSG) was prepared from salmon (*Oncorhynchus tschawytscha*) pituitary glands according to the method described by Idler *et al.* (1975) and modified by Breton *et al.* (1978). This preparation was slightly more active than SG-G 100 (Donaldson *et al.*, 1972) on *in vitro* maturation of trout oocytes and 5.7 times less active than purified S-GTH (Jalabert and Breton, unpublished data). The preparation of 17α-20β P (crude preparation, 4 pregnen-17α, 20β-diol-3 one) has been described by Jalabert *et al.* (1977). 17α P (4 pregnen-17α-ol-3 one) was purchased from Roussel ; HCG (Human Chorionic Gonadotropin, « Chorillon ») was provided by Intervet.

Prior to injection, steroids were first dissolved in pure ethanol as a stock solution, then precipitated as a suspension in physiological saline just before injection (20 p. 100 ethanol in the injected products).

Determination of oocyte stage. — Some oocytes from the females were sampled before treatment and during each experiment by gentle manual pressure. They were then put into Stockard's solution for a short time to visualize the germinal vesicle (GV) and determine the oocyte stage according to the following classification (Jalabert *et al.*, 1976) :

i : GV in non-peripheral position (« immature » stage),
1 : GV in peripheral position,
2 : Maturing oocyte (GV still visible),
3 : Matured oocyte (after GV breakdown),
4 : Matured and ovulated oocyte.

Only females with oocytes at stage i were used in the experiments. In order to avoid excessive handling and after preliminary observation, females were checked for ovulation on the third day after treatment and on the next 2 days whenever necessary.

Determination of ovulation rate. — All females were killed to check the stage of the ovarian remnants either after ovulation and egg sampling or after 8 days, if ovulation had not occurred. The sampled ova and the ovarian remnants were weighed ; ovulation rate was determined as the ratio of ovulated oocyte weight to initial ovary weight (ovulated oocytes plus remnants).

Determination of fertilization rate. — Three hundred ova per female were inseminated in 10 ml DIA_{TG} diluent (Billard, 1977) with 10 µl sperm pooled from 10 males (dilution rate : 1/1 000) and incubated in a fine mesh trough at about 12 °C. The fertilization rate was determined as the percentage of embryos at 40 degree-days.

Statistical analysis. — The mean values of the ovulation and fertilization rates and their confidence limits at the 95 p. 100 level of probability were computed using the angular (arc-sine) transformation of the percentages. The levels of significance were determined by the F-test after angular transformation of the percentages.

Results.

Ovulatory response (table 1).

Four types of responses were observed depending on the nature of the treatment and the animal :

Total ovulations were clear-cut (ovulation rate : 100 p. 100). The highest dose of PPSG (0.1 mg/kg) was the only treatment able to induce such a response (6 fishes out of 14).

Partial ovulations occurred in some females after treatment with the highest dose of PPSG or after sequential treatment. With PPSG (0.1 mg/kg) the remaining oocytes were degenerating. With sequential treatment (PPSG, 0.02 to 0.03 mg/kg + 17α-20β P or 17α P, 3 mg/kg) and with 17α-20β P alone, the remaining oocytes either matured (stage 3) or remained at stage i and started to degenerate.

Maturation of some oocytes without ovulation : low doses of PPSG (0.02-0.03 mg/kg) and 17α-20β P alone or associated with a priming dose of PPSG (0.02 mg/kg) induced the maturation of a number of oocytes in some fishes. Other oocytes were in stage i and degeneration.

No response : with a low priming dose of PPSG (0.02 mg/kg) alone or in conjunction with 17α-20β P, some of the females did not show any response ; the oocytes were in stage i and undergoing degeneration.

TABLE 1

Effects of various hormonal treatments on maturation and ovulation in pike

N° of fish	Treatment	Day of treatment	Response to treatment after 4 days				Mean ovulation rate in p. 100	Mean fertilization rate in p. 100
			Total ovulation	Partial ovulation	Maturation without ovulation	No response		
14	PPSG 0.1 mg/kg	D_0	6	8	0	0	89 (75-98)	83 (79-87)
8	PPSG 0.03 mg/kg	D_0	0	0	8	0		
16	PPSG 0.02 mg/kg	D_0	0	0	9	7		
7	HCG 2 000 IU/kg	D_0	0	0	0	7		
9	17 α-20 β P 3 mg/kg	D_0	0	3	6	0	21	20
8	PPSG 0.03 mg/kg + 17 α-20 β P 3 mg/kg	D_0 D_1	0	8	0	0	48 (31-65)	41 (30-52)
8	PPSG 0.02 mg/kg + 17 α-20 β P 3 mg/kg	D_0 D_1	0	2	3	3	38	24
7	PPSG 0.03 mg/kg + 17 α P 3 mg/kg...	D_0 D_1	0	6	1	0	40 (10-74)	23 (18-28)
7	HCG 400 IU/kg + 17 α-20 β P 3 mg/kg	D_0 D_1	0	0	←7→			
15	Control : physiological saline.....	D_0	0	0	0	15		

— Interval between capture and treatment did not exceed 24 hrs in any of these data.
— The mean ovulation and fertilization rates, and their confidence limits at the 95 p. 100 level of probability have been calculated after angular transformation of the percentages.
— PPSG : partially purified salmon gonadotropin ; HCG : Human chorionic gonadotropin.
— 17 α-20 β P : 17 α-hydroxy-20 β dihydroprogesterone ; 17 α P : 17 α hydroxyprogesterone.

In female treated with HCG as well as in controls there was no change but degeneration.

Fertilization rate (table 1).

The best results (mean fertilization rate : 83 p. 100) were obtained with the highest dose of PPSG (0.1 mg/kg). With the sequential treatment (PPSG, 0.03 mg/kg + 17α-20β P, 3 mg/kg) the fertilizability of the ovulated oocytes (41 p. 100) was significantly lower ($P < 0.001$). Although 17α P and 17α-20β P associated with PPSG exhibited a similar efficiency on ovulation rates (40 and 48 p. 100) the PPSG-17α-20β P combination led to significantly higher fertilization rates (41 p. 100, versus 23 p. 100 with the PPSG-17α P association).

Effects of various time intervals between capture and treatment on ovulation and fertilization rates (fig. 1).

Mean ovulation rate was 96 p. 100 when treatment was initiated on the very day of capture. It dropped to 40 p. 100 when the females were confined during three days before treatment (the difference is significant at the 1 p. 1 000 level of probability). However, the fertility of ovulated oocytes was not affected.

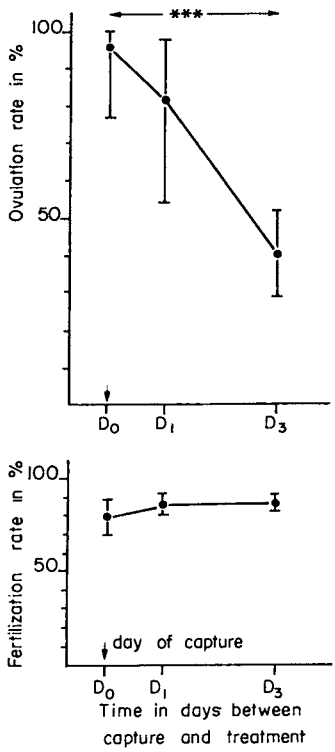

FIG. 1. — *Effects of various time intervals between capture and treatment* (0.1 mg PPSG/kg) *on ovulation and fertilization rates.* Vertical bars show confidence limits at the 95 p. 100 level of probability. *** : highly significant difference ($P < 0.001$).

Discussion.

Our results unequivocally indicate the efficiency of an adequate hormonal treatment (a single injection of partially purified salmon gonadotropin administered at the end of vitellogenesis and before completion of the germinal vesicle migration) on *in vivo* maturation and ovulation in northern pike female. The ova were of good quality, as shown by the high fertilization rates estimated at 40 degree-days (mean : 83 p. 100). Hatching rates were not checked during this investigation, but complementary observations using similarly treated females have shown that embryonic loss does not exceed 15 p. 100 between 40 degree-days and the end of hatching (Bry, unpublished data).

The most striking fact demonstrated here is the considerable loss of responsiveness to hormonal treatment, occurring in all the females after a few days of captivity. A concomitant increase in the proportion of degenerating oocytes was observed from day to day in the control females. Degeneration was characterized by the disappearance of the nucleus and a loss of the homogeneous aspect of the yolk. Handling stress and confinement are certainly responsible for such drastic and irreversible changes. Similar reactions have been noticed in females captured at the stage of oocyte maturation and then transferred under confinement ; ovulation does not occur and degeneration takes place (Bry, unpublished observations). In the natural environment, a sudden drop in water temperature may stop normal ovarian evolution, usually before completion of the germinal vesicle migration, and cause widespread atresia (Bry, unpublished observations ; Preudhomme, personal communication). The reproductive process may also be interrupted after ovulation (no spawning) by stressing factors such as a cold spell or a drop in water level (June, 1970). Thus, northern pike females appear to be particularly sensitive to various kinds of disturbances in their environment. Regarding the mechanisms involved in the appearance of ovarian atresia in submature captive females, we can only speculate at this moment and evoke a possible lack of gonadotropin or an hormonal unbalance such as hypothetic high levels of some corticosteroids. Our data clearly show that a minimum amount of gonadotropin is absolutely necessary to obtain both complete ovulations and high fertilization rates.

The situation of hormonal receptivity in pike is similar to that in rainbow trout, where 17α-20β P can induce *in vivo* oocyte maturation and ovulation only in females treated at the peripheral or subperipheral nucleus stage (Jalabert *et al.*, 1976). If treated at more precocious stages, most females undergo maturation without ovulation. The connection between the time-related physiological events of maturation and ovulation seems to be lacking at this stage but can be restored by a pituitary priming before the steroid injection ; this suggests a pituitary controlled acquisition of a specific mechanism for ovulation (Jalabert *et al.*, 1978).

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Résumé. L'efficacité d'une préparation d'hormone gonadotrope de saumon partiellement purifiée (PPSG) et de la gonadotropine chorionique (HCG), associées ou non à un progestagène (17α-hydroxy, 20β-dihydroprogestérone : 17α-20β P ou 17α-hydroxyprogestérone : 17α P) a été testée chez des femelles de Brochet submatures (stade initial des ovocytes : vésicule germinative non périphérique).

a) Une dose de 0,1 mg/kg de PPSG a conduit à des taux satisfaisants d'ovulation (89 p. 100) et de fécondation (83 p. 100) ; une dose minimum de 0,03 mg PPSG/kg a permis d'induire la maturation sans l'ovulation.

b) La même dose de PPSG (0.03 mg/kg), suivie à 24 h d'intervalle par de la 17α-20β P (3 mg/kg)ou de la 17α P (3 mg/kg) a provoqué des maturations complètes et des ovulations partielles.

La 17α-20β P (3 mg/kg), injectée seule, a induit la maturation chez tous les animaux traités et des ovulations partielles ou pas d'ovulation. Dans tous les cas précités (§ b), la fécondabilité a été médiocre (20 à 41 p. 100).

c) HCG, seule ou en association avec la 17α-20β P (3 mg/kg) n'a en aucun cas provoqué d'ovulation. Il est essentiel de débuter le traitement hormonal dès que possible après la capture. Trois jours de captivité avant le traitement ont provoqué une atrésie ovarienne et une diminution significative des taux d'ovulation.

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Precocious induction of oocyte maturation and ovulation in rainbow trout (*Salmo gairdneri*) : problems when using 17 α-hydroxy-20 β-dihydroprogesterone

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Summary. The efficiency of 17 α-hydroxy-20 β-dihydroprogesterone (17 α-20 β P) administered alone or after pituitary priming, was investigated *in vivo* before peripheral migration of the germinal vesicle 4 to 8 weeks prior to natural ovulation. Treatmert with 17 α-20 β P alone (2 injections of 3 mg/kg at a 2-day interval) induced oocyte maturation in 94 p. 100 of the fish, but only 25 p. 100 ovulated. Treatment with 17 α-20 β P (3 mg/kg once) 2 days after pituitary priming (1 ml/kg of trout pituitary extract, TPE, containing 3.25×10^{-3} mg/ml of trout gonadotropin, t-GTH) induced maturation in all fish, 59 p. 100 of which ovulated. In both cases, fish in which ovulation did not follow oocyte maturation were killed 15 days after the first injection ; ovarian follicles were either dissected by hand to remove mature oocytes, or incubated *in vitro* with prostaglandin $F_{2\alpha}$ which induced successful ovulation. In all cases, oocytes obtained from *in vivo* ovulation, *in vitro* ovulation by $PGF_{2\alpha}$, or manual dissection were fertilized to some extent.

These observations demonstrate that :

1. Fertilizable mature oocytes can be produced 4 to 6 weeks in advance of natural spawning by injection of 17 α-20 β P *in vivo* ;
2. Although ovulation can occur *in vivo*, or *in vitro* with $PGF_{2\alpha}$, the specific stimulus for ovulation is lacking in most of the fish injected with 17 α-20 β P only, and appears to some extent when a pituitary priming is given prior to 17 α-20 β P.

The possible involvement of gonadotropin in the synthesis and storage of some mediator (or its precursor) specific for the induction of ovulation is discussed in relation to the plasma gonadotropin level in the different groups of females.

Introduction.

Previous work on rainbow trout (Jalabert *et al.*, 1976) has already shown that 17 α-hydroxy-20 β-dihydroprogesterone (4 pregnen-17 α, 20 β diol-3 one ; abbreviation : 17 α-20 β P) is able to induce normal oocyte maturation, i. e. resumption of meiosis characterized by clearing of the yolk and germinal vesicle breakdown (GVBD), and ovulation when injected to females presenting oocytes with the germinal vesicle

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(GV) in subperipheral position. But recent work in Coho salmon (*Oncorhynchus kisutch*) (Jalabert *et al.*, 1978) and in northern pike (*Exos lucius*) (de Montalembert, Jalabert and Bry, 1978) has shown that injection of 17 α-20 β P alone before GV peripheral migration results in oocyte maturation without ovulation. On the other hand, 17 α-20 β P is completely ineffective in carp (*Cyprinus carpio*) when administered alone, but induces successful maturation and ovulation in marginal temperature conditions when given after pituitary priming (Jalabert *et al.*, 1977).

The present experiment was undertaken in trout before peripheral migration of GV, 3 to 8 weeks in advance of expected natural ovulation. We tried to answer the following questions : — Does 17 α-20 β P induce oocyte maturation without ovulation when injected at these precocious stages, as it does in Coho salmon and in pike ? — What is the nature of the blockade between maturation and ovulation, if any ? — Using a low dose of pituitary extract, what is the effect of preliminary pituitary priming on further 17 α-20 β P action ?

Material and methods.

The experiment was carried out in December 1976 using 2-year old rainbow trout (*Salmo gairdneri*) weighing 400 to 600 g and kept in water of about 12 °C. Sixty-six females were chosen according to the state of maturity of a few oocytes ; the criterium used was that the oocytes be 3.5 to 4.5 mm in diameter and without apparent GV at the periphery. They were squeezed out by abdominal stripping after anesthesia in a 0.5 p. 100 aqueous solution of 2-(phenoxy) ethanol (Merck). As seen in control fish without handling, the experimental fish would have ovulated naturally 3 to 8 weeks later. They were separated into 4 groups and submitted to different treatments (table 1) on day 0 (beginning of the experiment) and on day 2 (2 days later). The dose was injected intraperitoneally and calculated in order to always introduce the same volume of vehicle (physiological saline, 1 ml/kg).

TABLE 1

Experimental treatments

Lot	No. of fish	Treatments	
		on day 0	on day 2
1	15	Physiological saline	Physiological saline
2	17	TPE (0.5 mg/kg)	Physiological saline
3	16	17 α-20 β P(3 mg/kg)	17 α-20 β P(3 mg/kg)
4	17	TPE (0.5 mg/kg)	17 α-20 β P(3 mg/kg)

17 α-20 β P : 17 α-hydroxy-20 β dihydroprogesterone (4 pregnen-17 α, 20 β diol-3 one).

TPE : Trout pituitary extract from dry acetonic powder. 0.5 mg/kg is equivalent to 3.25×10^{-3} mg/kg of pure t-GTH measured by *in vitro* trout maturation assay.

Trout pituitary extract (TPE) is a crude preparation made by homogenizing, in a glass-teflon homogenizer, acetone-dried trout pituitary powder suspended in physiological saline and taken from females at peripheral GV stage. The dose injected (1 ml/kg) contains 3.25×10^{-3} mg/ml of pure t-GTH (Breton, Jalabert and Reinaud, 1976), as measured by *in vitro* trout maturation assay (Jalabert, Breton, and Billard, 1974) ; it represents 1/10th of the dose known to induce maturation and ovulation in submature fish. Pure 17 α-20 β P was prepared according to Fostier *et al.* (1973).

Before each injection and at varied intervals until day 15 after the first injection, the fish were anesthetized and submitted to ovarian and blood sampling. Blood samples of 0.3 ml were taken from a caudal vessel in the tail by puncture using a 1 ml syringe previously rinsed with an heparine solution (700 IU/ml). The plasma obtained after centrifugation was kept frozen until subsequent determination of trout gonadotropin (t-GTH) by radioimmunoassay according to Breton and Billard (1977). When treatment resulted in oocyte maturation (characterized by GVBD) without ovulation, the fish were killed after 15 days and the ovaries removed. Mature oocytes were then either dissected out of the follicle using watchmaker's forceps to tear off the follicular envelope, or incubated *in vitro* within the follicle during 48 hrs. at 10 °C in trout balanced salt solution (TBSS) (Jalabert, 1978) with or without prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$ 10^{-5} M, Upjohn Co., Kalamazoo, Michigan) to induce ovulation.

Matured oocytes obtained after normal ovulation *in vivo*, manual dissection, or after ovulation *in vitro* by $PGF_{2\alpha}$ were inseminated with diluted sperm (1/100) according to Billard *et al.* (1974) ; fertilizability was estimated from the proportion of eggs with apparently normal embryos after 10 days of development.

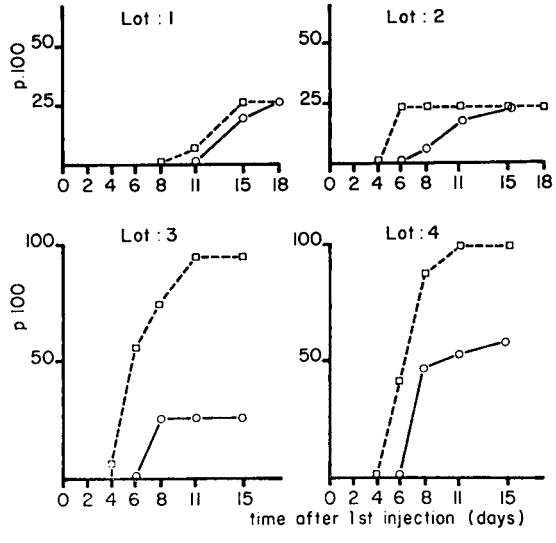

FIG. 1. — *p. 100 fish exhibiting complete oocyte maturation* (GVBD : ——□——) *or complete ovulation* (—— o ——) *in the 4 experimental groups.*

Lot 1, day 0, day 2 : physiological saline.
Lot 2, day 0 : TPE 0.5 mg/kg ; day 2 : saline.
Lot 3, day 0, day 2 : 17 α-20 β P 3 mg/kg.
Lot 4, day 0 : TPE 0.5 mg/kg ; day 2 : 17 α-20 β P 3 mg/kg.

Results.

Maturation and ovulation responses of fish in the different lots are shown on figure 1. 27 p. 100 of control fish (lot 1) mature and ovulate after 15 to 18 days ; in lot 2 (TPE priming only), roughly the same proportion of animals mature and ovulate, but are slightly precocious. In both cases, ovulation always follows maturation. In lot 3 (2 injections of 17 α-20 β P), apparently normal maturation occurs in all fishes within 6 to 11 days, but ovulation follows in only 25 p. 100. When priming treatment with TPE is given before 17 α-20 β P (lot 4), maturation still occurs in all fishes, but the proportion of those ovulating reaches 59 p. 100.

Plasma gonadotropin levels are presented in figure 2. In controls, basal levels are around 3.5 ng/ml at the beginning of the experiment and reach about 17 ng/ml in ovulating fish. In both lots 2 and 4, TPE priming injection induces a surge in plasma t-GTH up to 100 ng/ml, followed by a slow decrease. In lot 3 (2 injections of 17 α-20 β P), there is a small rise in gonadotropin beginning on day 6 and reaching 12 ng/ml on day 15.

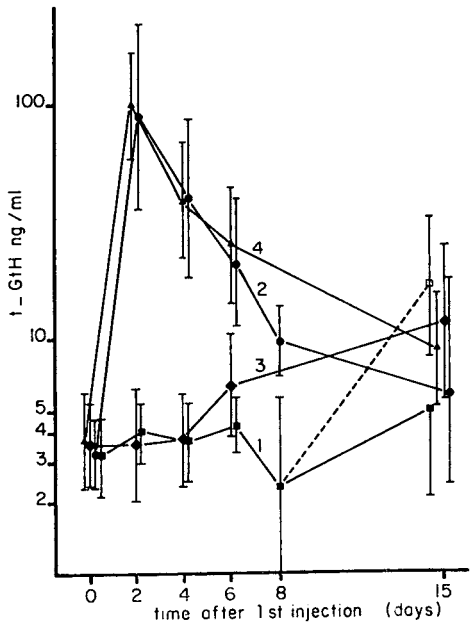

FIG. 2. — *Mean plasma gonadotropin level* (t-GTH) *in the 4 experimental groups.* Vertical bars show the standard deviation.

Symbols for the different groups : 1 : — ■ — (non-maturing controls) or □ (2 maturing and ovulating controls) ; 2 : — ● — ; 3 : — ◆ — ; 4 : — ▲ —.

Table 2 shows the results of incubation *in vitro* of follicles from fish exhibiting oocyte maturation without ovulation and killed on day 15 (lots 3 and 4). Even though some spontaneous ovulation occurs, $PGF_{2\alpha}$ very significantly enhances the proportion of ovulation *in vitro*.

TABLE 2

In vitro ovulation of follicles from fish exhibiting oocyte maturation without ovulation (100 follicles per fish incubated for 48 hrs at 10 °C in TBSS with or without prostaglandin $PGF_{2\alpha}$, 10^{-5} M)

Lot	Treatment *in vivo*	No. of fish	Incubation of follicles *in vitro*		
			Treatment	Mean p. 100 ovulation	
3	17 α-20 β P/17 α-20 β P	7	control	8.5	**
			$PGF_{2\alpha}$	77.4	
4	TPE/17 α-20 β P	4	control	6.3	**
			$PGF_{2\alpha}$	74.1	

TABLE 3

Mean percent of embryonic development in eggs from in vivo *ovulation* (200 eggs per fish)

Lot	Treatment	Number of fish	Mean p. 100 of successful embryonic development
1	Phys. saline/Phys. saline	4	80.4
2	TPE/Phys. saline	4	93.2
3	17 α-20 β P/17 α-20 β P	4	90.9
4	Phys. saline/17 α-20 β P	10	87.5

TABLE 4

p. 100 *embryonic development in eggs inseminated either after manual dissection, or after* in vitro *ovulation by* $PGF_{2\alpha}$

No.	Lot	Fish No.	Manual dissection		Ovulation *in vitro* by $PGF_{2\alpha}$	
	in vivo treatment		No. of eggs	p. 100 embryonic development	No. of eggs	p. 100 embryonic development
3	17 α-20 β P/17 α-20 β P	16	54	0	57	8.8
		21	53	75.5	20	33.3
		31	65	66.2	77	2.6
		36	23	0	48	4.4
		41	49	69.4	82	43.9
		66	55	43.6	79	5.1
		71	47	25.5	—	—
		74	50	0	78	1.3
4	TPE/17 α-20 β P	03	49	10.2	82	20.7
		23	45	4.4	49	2.0
		63	52	21.2	85	0
		81	53	9.4	16	0

Regarding fertilization data, eggs from *in vivo* ovulation (table 3) exhibit a normal amount of embryonic development without any significant difference between experimental and control groups. In eggs from matured but non-ovulated fish (table 4), inseminated after manual dissection or *in vitro* ovulation, the success of embryonic development is more irregular. Although a high percentage of development can be observed in the eggs of many fishes, particularly after manual dissection, eggs from some females give poor results and sometimes none. But comparison of data after manual dissection or after *in vitro* ovulation by $PGF_{2\alpha}$ shows that fertilization and development can occur in the eggs from any non-ovulated fish in groups 3 and 4. This fact is shown in the particular case of fish No. 71 (table 4) in which 50 p. 100 of the oocytes were found to remain in an immature state, while the others were fully matured. The latter, dissected out of the follicle, exhibited 25.5 p. 100 development after insemination.

Discussion.

Induction of oocyte maturation without ovulation after injection of steroid hormones has already been observed in the amphibian *Discoglossus pictus* by Alonso-Bedate *et al.* (1971) and in various fishes : *Misgurnus fossilis* (Kirshenblat, 1952) ; northern pike (de Montalembert, Jalabert and Bry, 1978).

The present experiment using 17 α-20 β P, which appears as the most likely mediator of oocyte maturation in trout (Jalabert, 1976), demonstrates that this steroid is also able to induce oocyte maturation well in advance of the natural process (3 to 8 weeks) and that these oocytes can be fertilized and develop normally until at least 10 days. However, this maturation is not necessarily followed by ovulation, in which case the mature oocytes to be inseminated must be removed from the follicular envelope by artificial means.

It must be underlined that the fishes chosen for the experiment were certainly heterogeneous as to expected time of natural spawning because of the absence of reliable criteria ; the follicle size only gives an approximation due to individual variations, and the non-peripheral position of the GV only indicates that natural maturation would normally occur more than 2 weeks later. This may explain why 25 p. 100 of the fish mature and also ovulate after treatment by 17 α-20 β P only, since this is the kind of response already found in females with oocytes at subperipheral GV stage (Jalabert *et al.*, 1976). Moreover, roughly the same proportion of fish ovulate at TPE priming only (lot 2), or spontaneously in controls after 15 to 18 days (lot 1). In fact, probably due to severe handling stress, these are in advance as compared with controls without such regular handling (anesthesia, blood and oocyte sampling). Thus, it can be assumed that a same proportion of fish in every group was advanced enough to mature and ovulate normally in response to 17 α-20 β P, TPE priming or handling stress.

The rate of embryonic development is normally high in eggs from normally ovulated fish, whatever the treatment ; this confirms that these females are probably closer to natural maturation, as discussed above. More surprising is the fact that embryonic development is always found in eggs of fish with mature but non-ovulated oocytes, some of them being probably very far from natural maturation. The discrepancy between data either after manual dissection or ovulation *in vitro* by $PGF_{2\alpha}$

emphasizes that the conditions were not optimum in either case for taking mature oocytes out of their follicle. In addition, mature oocytes were kept *in vivo* in the follicle much longer than they normally would be after natural maturation in order to make sure that ovulation was really blocked before killing the fish ; thus, some aging could have occurred within the follicle. Despite these unfavorable conditions, it remains that some embryonic development was always found in the eggs from all experimental females after 17 α-20 β P-induced maturation (lots 3 and 4). However, the success of embryonic development was evaluated by fixation in Stockard's solution as soon as 10 days after insemination, and it is not known if these embryos would have developed normally to hatching or further.

Another interesting point is that follicles containing mature oocytes, which do not ovulate spontaneously *in vivo* after *in vivo* 17 α-20 β P treatment, are able to contract and ovulate *in vitro* in response to $PGF_{2\alpha}$ as in naturally mature fish (Jalabert and Szöllösi, 1975). This implies that the lack of *in vivo* ovulation cannot be attributed to insufficient differentiation of the smooth muscle cells of the theca, but more probably to the absence of a specific mediator initiating follicle contraction, or of a precursor which should be synthesized and stored before 17 α-20 β P action. As preliminary priming with a low dose of TPE enhances the proportion of fish which ovulate in response to 17 α-20 β P treatment, it can be hypothesized that gonadotropin t-GTH is the pituitary factor which, at low doses, favors the synthesis and storage of such an ovulation mediator. This hypothesis coincides with the fact that 17 α-20 β P was able to induce maturation followed by successful ovulation when administered to fish at a later stage (subperipheral GV), characterized by plasma t-GTH levels of about 6 to 7 ng/ml (Jalabert *et al.*, 1976) as compared to 3 to 5 ng/ml at the beginning of the present experiment.

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Résumé. L'efficacité de la 17 α-hydoxy-20 β dihydroprogestérone (17 α-20 β P) seule ou après sensibilisation par un extrait hypophysaire a été testée chez des animaux dont le stade ovocytaire était antérieur à la migration périphérique de la vésicule germinative (V. G.) (4 à 8 semaines avant ovulation naturelle). Le traitement par la 17 α-20 β P (2 injections de 3 mg/kg à 2 jours d'intervalle) a induit la maturation ovocytaire (reprise de la méïose caractérisée par l'éclaircissement du vitellus et l'éclatement de la V. G.) chez 94 p. 100 des poissons, dont 25 p. 100 seulement ovulèrent normalement. Le traitement par la 17 α-20 β P (3 mg/kg) après sensibilisation hypophysaire (Extrait hypophysaire de Truite, 1 ml/kg, contenant l'équivalent de $3{,}25 \times 10^{-3}$ mg/ml de gonadotropine de Truite, t-GtH) a induit la maturation ovocytaire chez tous les poissons, parmi lesquels 59 p. 100 ovulèrent. Dans les deux cas, les poissons chez lesquels l'ovulation ne se produisit pas normalement après maturation furent sacrifiés 15 jours après la 1re injection ; les follicules ovariens furent soit disséqués pour extraction des ovules mûrs, soit incubés *in vitro* en présence de prostaglandine $PGF_{2\alpha}$ (10^{-5} M) qui induisit l'ovulation avec succès. Les ovules mûrs récoltés après ovulation *in vivo* présentèrent une fécondabilité (estimée d'après le p. 100 de développements embryonnaires 10 jours après insémination) normalement élevée. Les ovules des animaux maturés mais non ovulés, récoltés après dissection manuelle ou ovulation *in vitro*, présentèrent une fécondabilité plus irrégulière, en fonction de la technique d'obtention, mais tous les animaux eurent des œufs fécondés.

Ces observations démontrent que :

1. Des ovules mûrs fécondables peuvent être produits 4 à 8 semaines en avance sur la fraie naturelle par l'injection de 17 α-20 β P.

2. Bien que l'ovulation puisse se produire (spontanément *in vivo* chez certains animaux, ou sous l'action de $PGF_{2\alpha}$ *in vitro* chez les autres), le stimulus spécifique de l'ovulation paraît faire défaut chez les poissons recevant la 17 α-20 β P seule, et réapparaît dans une certaine mesure lorsqu'une injection de sensibilisation par un extrait hypophysaire est administré avant la 17 α-20 β P.

L'implication possible de l'hormone gonadotrope t-GtH dans la synthèse et le stockage d'un médiateur (ou d'un précurseur) spécifique de l'induction de l'ovulation est discutée, en liaison avec les niveaux de gonadotropine plasmatique dans les différents groupes de femelles.

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Induced spawning of *Sparus aurata* (L.) by means of hormonal treatments

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Summary. This paper reports experiments done in induced spawning of *Sparus aurata* during two breeding seasons : 1975/76 and 1976/77. Reared in captivity in the Elat Mariculture Laboratory, this species did not spawn spontaneously. In the 1975/76 season, HCG was injected IM using doses up to 2 700 IU/kg of fish. Each treatment was run in a separate tank which held a few females and males. Many of the fish shed either unripe or aged eggs. The experimental method did not allow detailed analysis of treatments.

During the 1976/77 season, each female used in the experiments was held in a separate tank. HCG calibration experiments, based on the vitellogenetic stage of the oocytes, were carried out. Doses found to be sufficient to cause ovulation and spawning of viable eggs ranged from 100 to 1 200 IU/kg fish. *S. aurata*'s natural spawning season is believed to last six weeks. Due to the HCG treatments, the spawning season was extended to five and a half months.

Introduction.

One of the basic requirements for fish culture programs is that the entire life cycle of the species should be completed in captivity. Many of the fish which serve in such programs do not breed spontaneously under such conditions. Hence, an induced spawning technique must be applied.

The gilthead seabream *Sparus aurata* is thought to have a great potential for mariculture. In its natural environment in the eastern Mediterranean, *S. aurata* breeds once a year during a six-week period from the middle of December through the end of January. In captivity this species does not spawn spontaneously (Arias, 1976 ; Villani, 1976). Our detailed histological studies (Zohar, 1976 ; Zohar *et al.*, in preparation) showed that in *S. aurata* ovarian development is not completed ; oocytes develop to the last stages of vitellogenesis and then undergo atresia. On the other hand, testicular development is completed.

The most potent ovulation and spawning-inducers in hypophysectomized fish and in fish in which spawning does not occur spontaneously were found to be fish pituitary extracts, fish gonadotropins and mammalian gonadotropins (mainly LH, HCG and PMS) (see reviews by Shehadeh, 1970 ; de Vlaming, 1974).

Ovulation and spawning in *S. aurata* have been induced by means of HCG treatments (Barnabé and René, 1973 ; Lumare and Villani, 1973 ; Alessio and Bronzi, 1974 ; Alessio *et al.*, 1975, 1976 ; Arias, 1976 ; Barnabé, 1976 ; San Feliu *et al.*, 1976 ; Villani, 1976). Effective doses ranged from 3 500 to 15 000 IU/fish. A previous study done in our laboratory showed that HCG doses of 800 to 2 000 IU/kg fish were effective in inducing spawning, whereas carp pituitary extract was ineffective.

This paper reports on experiments carried out during the years 1976 and 1977 to study (1) the efficiency of HCG as an ovulation and spawning-inducing agent in captivity-reared *Sparus aurata* in Elat and (2) the relations between oocyte vitellogenetic stage and the HCG dose required to induce completion of oocyte development, ovulation and spawning.

Materials and methods.

The fish used in the present study were collected as fry from their natural habitat in the Mediterranean and were stocked in outdoor experimental tanks. Two and three-year old fish were treated. Experiments were run during two breeding seasons. In 1976, experiments started on January 15th and lasted two months. During this period, HCG was tested as an ovulation and spawning-inducing agent. During 1976/1977, experiments started on November 23rd and the spawning period ended in the middle of May.

All treated fish were anesthetized in 1 : 20 000 dilution of MS 222 (Sandoz). An ovarian biopsy was done prior to each treatment by sucking a small tissue sample into an hematocrite capillary tube inserted into the ovary through the ovipore. The fresh biopsy was used to determine oocyte developmental stage. Only females with vitellogenetic oocytes were used in the study. Following the biopsy, females were injected with the hormone either intraperitonially or intramuscularly. Control fish were injected with 0.9 p. 100 NaCl solution. The total dose of the hormone was administered by 1 or 2 injections. A second injection was given 48 hrs after the first one, following a second ovarian biopsy. Every treatment was followed by a chloramphenicol injection at a dose of 50 mg/kg fish. During the 1976 experiments, a few females exposed to treatment were stocked in the same outdoor tank together with a few mature untreated males. During the 1976/77 experiments, each treated female was stocked in a separate tank together with two males. An open circulation system supplied the experimental tanks with Gulf of Elat seawater. Fish were maintained on artificial feed.

Spawned eggs were fertilized naturally by untreated males present in the experimental tanks. The eggs were collected and counted daily and fertilization percentage was determined.

Results.

The results of the work carried out during the 1976 and 1977 spawning periods of *S. aurata* are presented in table 1 and table 2, respectively. During the 1976 spawning experiments, more than one female was held in each tank, hence the results are related to treatments and not to individual fishes. In the following year, each treated female was held in its own tank ; therefore results are related to individual fishes. During both years, most treated females completed oocyte maturation and ovulation but not all of them spawned. Unspawed eggs underwent rapid reabsorption.

In general terms, the experiments of winter 1976 demonstrated that HCG doses ranging from 700-2 700 IU/kg fish were effective in inducing completion of oocyte development, ovulation and spawning (table 1). In winter 1976/1977, it was shown

TABLE 1

The effect of human chorionic gonadotropin (HCG) (1) *on ovulation and spawning of females of* Sparus aurata *during winter* 1976

No. of fish	No. of injections	Dose per injection/kg fish (2)	Total dose UI/kg fish	Ovulation +	Ovulation —	Spawning	Eggs quality
3	1	700	700	3 (3)	0	+	poor
2	2	500-700 (4)	1 200	2	0	+	poor
2	2	500-1 200	1 700	1	1	—	
15	2	700-1 000	1 700	11	4	+	partly good
4	2	700-1 200	1 900	2	2	+	partly good
3	3	500-1 000-500	2 000	3	0	+	poor
2	2	1 200-1 000	2 200	1	1	—	
4	3	700-1 000-1 000	2 700	4	0	+	poor

(1) Sigma Chemical Company CG-2.
(2) All females were injected intramuscularly.
(3) Figures indicate number of fish.
(4) First, second and third figures correspond to first, second and third injections, respectively.

that HCG doses as low as 100-200 IU/kg fish were very effective in inducing the same process, if administered to females in which oocytes were in a more advanced vitellogenetic stage. It was also found that the quantity of HCG needed to induce completion of oocyte development, ovulation and spawning was in inverse relation to the developmental stage of the vitellogenetic oocytes (table 2). As a result of the HCG treatment during winter 1976/1977, the spawning season of *Sparus aurata* lasted from November 30th to May 21st. A large number of viable eggs were spawned by each female (up to 1 million) and the percentage of fertilization was found to be high (table 2). Survival rates of larvae hatched from eggs which had been spawned by hormone-treated females did not differ from those of larvae hatched from eggs which had been spawned naturally in 2 unusual cases (unpublished data).

TABLE 2

The effect of human chorionic gonadotropin (HCG) on ovulation and spawning of females of Sparus aurata *during winter 1976/77*

Initial egg ø	No. of fish	No. of injections	Dose per injection/kg fish	Total dose UI/kg fish	Ovulation +	Ovulation —	Spawning +	Spawning —	No. of eggs (1)	p. 100 fertilization
	2	3	100 (2)	300	0	2 (3)	0	2	—	—
200-300	1	3	200	600	1	0	0	1	—	—
	3	3	400	1 200	3	0	2	1	not counted	low
	2	3	600	1 800	2	0	1	1	not counted	low
	2	3	800	2 400	2	0	0	2	—	—
	1	2	150 (IP)	300	1	0	1	0	641 210	95-100
301-400	1	2	400-200 (4)	600	1	0	0	1	—	—
	2	2	400	800	2	0	0	2	—	—
	1	2	200	400	1	0	1	0	33 000	50-90
401-450	1	1	400	400	1	0	1	0	37 400	92
	2	2	400-200	600	2	0	1	1	90 000	50
	2	2	400	800	2	0	2	0		
	1	1	200	200	1	0	1	0	35 000	95-1p0
451-500	3	2	200	400	2	1	2	1	150 000	85
	1	2	400-200	600	0	1	0	1	—	—
	1	1	150	150	1	0	0	1	—	—
501-525	3	1	200	200	3	0	2	1	40 000	95
	1	2	150 (IP)	300	1	0	1	0	33 000	80
	1	2	200-100	300	1	0	1	0	100 000	0
	2	2	200-150	450	2	0	2	0	95 000	100
	1	1	100	100	1	0	0	1	—	—
526-550	1	1	150 (IP)	150	1	0	1	0	297 450	95-100
	1	1	150	150	1	0	0	1	—	—
	3	1	200	200	3	0	3	0	1 067 500	50-100
	1	2	150-150	300	1	0	1	0	27 000	100
	1	1	100	100	1	0	1	0	42 250	100
551-575	5	1	150	150	5	0	4	1	417 900	0-100
	3	1	200	200	3	0	1	2	4 000	100
	2	1	100	100	2	0	2	0	995 600	100
576-600	5	1	150	150	5	0	5	0	2 711 440	90-100
601-625	2	1	150	150	2	0	2	0	1 796 880	30-100
Total	58				54	4	38	20		
200-626	6	2	0.9 p. 100 saline (5)		0	6	0	6		

(1) Only viable eggs were counted.
(2) Females were injected intramuscularly except where IP (intraperitonially) indicated.
(3) Figures indicate number of fish.
(4) First and second figures correspond to first and second injections, respectively.
(5) Control group, two 0.9 p. 100 saline injections given.

Discussion.

Induced spawning of *Sparus aurata* by means of HCG treatments was achieved previously (see references in the Introduction). Spawning was induced by several

injections of HCG totaling 3 500 to 5 000 IU/kg fish, administered to females at the time of their natural spawning season. None of the previous studies with fish correlated the dose of the hormone used to oocyte developmental stage. In the present study, the HCG dose needed to induce oocyte development, ovulation and spawning at various stages of vitellogenesis was determined. Efficient HCG doses were found to be one to two orders of magnitude lower than doses used up to now. These doses were shown to relate inversely to the vitellogenetic stage of the oocytes.

The biopsy technique was found to have many potentialities : (1) It enabled us to determine the developmental stage of the oocytes, and hence to select only the appropriate females for treatment. (2) It allowed us to administer the selected hormone to the treated female in an effective way. Using this technique, the spawning season of *Sparus aurata* was extended to five and a half months. This result is a great advantage for mariculture since it permits a constant supply of larvae over a long period of the year.

The vast majority of the HCG treatments induced oocyte development and ovulation, whereas spawning response was induced in only part of the treated females. It is quite certain that each of the processes — maturation, ovulation and spawning of oocytes — involves different hormonal agents including gonadotropins, ovarian steroids and neurohypophysial hormones (Jalabert, 1976). The entire chain of events leading up to spawning of mature ovulated oocytes involves an elaborate complex of interactions. It is very probable that the gonadotropin treatments triggered this chain of events in most of the cases, but it was not completed in all of them, resulting in spawning responses.

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Résumé. Des expériences d'induction de la ponte de Dorades (*Sparus aurata*) pratiquées au cours de 2 saisons de reproduction 1975-76 et 1976-77 sont rapportées dans cet article. Les dorades élevées en captivité au laboratoire de mariculture d'Elat ne frayent pas spontanément. Au cours de la saison 1975-76 des injections intra-musculaires de HCG à des doses allant jusqu'à 2 700 UI/kg de poids vif corporel ont été pratiquées. Chaque traitement a été pratiqué en bassins séparés contenant quelques individus mâles et femelles. La plupart des femelles ont émis des ovules non matures ou surmatures. Cette méthode d'expérience n'a pas permis une analyse détaillée des traitements.

Au cours de la saison 1976-77, chaque femelle expérimentale a été placée en bassin séparé. Les doses d'HCG administrées ont été établies d'après l'état des ovocytes (stade de vitellogenèse). Les doses suffisantes pour induire l'ovulation d'ovules viables et la fraie vont de 100 à 1 200 UI/kg de poids vif. La saison de reproduction de la dorade en condition naturelle est supposée durer 6 semaines. Du fait des traitements avec HCG, la durée de la période de reproduction a été étendue à 5 mois et demi.

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In vitro ovulation of European eel (*Anguilla anguilla* L.) oocytes following *in vivo* stimulation of sexual maturation

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Summary. In 27 European female eels, a considerable advance in sexual maturation was induced by HCG and carp hypophyseal homogenate injections although no ovulation followed. In view of this, ovarian samples were taken from 4 females for culture *in vitro* supplemented with hormones. During 24-hr. incubation, a supplement of HCG + hypophyseal homogenate to the culture medium resulted in 50 p. 100 oocyte ovulation, while addition of prostaglandin or adrenaline induced ovulation in 80 to 90 p. 100 of the oocytes. An attempt to fertilize the ovulated eel oocytes *in vitro* with fully vital male eel spermatozoa failed to give positive results.

Introduction.

Studies on artificial reproduction in European eels have been undertaken since the 1930s, and many attempts were initially made to accelerate sexual maturation in males (Fontaine, 1936), resulting in the obtention of fully mature male eels (Boetius and Boetius, 1967 ; Meske and Cellarius, 1973 ; Bieniarz and Epler, 1977).

A much more difficult problem was to accelerate sexual maturity in female eels. The first to obtain fully ripe eggs of European eels were Fontaine *et al.* (1964), Villani and Lumare (1975), Boetius and Boetius (1976). Attempts to fertilize the eggs, however, proved unsuccessful.

In studies on the acceleration of sexual maturity in European eels undertaken in Poland in 1973, we obtained fully ripe male eels (Bieniarz and Epler, 1977) and a significant increase in the growth of female oocytes (Bieniarz and Epler, 1974).

The aim of this study was to induce ovulation in mature female eels and to obtain offspring of the European eel in artificial conditions. For this purpose, we carried out *in vivo* studies similar to those of Fontaine *et al.* (1964), Villani and Lumare (1975) or Boetius and Boetius (1976), as well as investigations *in vitro* like those done on other species of fish by Yamuchi and Yamamoto (1974) and Jalabert (1976).

Material and methods.

We observed 27 females weighing 0.6 to 0.7 kg, placed in aquaria filled with artificial sea water of 30 p. 100 salinity with 7 to 8 mg O_2/l at 21 to 22 °C. After the fishes had been laboratory-acclimated for 4 days, they were given hormonal injections twice a week containing HCG and carp hypophyseal homogenate (table 1). Hypophyses were collected from 3-year old carp in autumn, fixed in acetone and dried. For *in vitro* studies, ovarian samples from 4 females were used. Fifteen ovarian samples were taken from each fish killed. Every fragment containing 120 to 150 oocytes 1.0 to 1.1 mm in diameter was placed in culture medium supplemented with different hormones (table 2).

TABLE 1

Injection of HCG and pituitary carp homogenate in female eels

Days of investigation	HCG (kg body weight)	Carp hypophyseal homogenate (mg/kg body weight)
1	250	—
4-22	500	—
23-26	500	1
27-30	500	2
31-33	500	3
34-37	500	4
38	500	5

TABLE 2

Percentage of ovulated oocytes of European eels after 24-hour incubation in vitro

Hormone	female 1	female 2	female 3	female 4
Estriol	+	—	+	+
Estron	+	—	+	+
Estradiol	+	—	+	+
Testosterone	+	—	+	+
Progesterone	+	—	+	+
Corticosterone	+	—	+	+
DOC	+	—	+	+
DOCA	+	—	+	+
Cortisone	+	—	+	+
HCG	+	—	+	+
Carp hypophysis	+	merging of fatty balls	+	+
Carp hypophysis + HCG	++	merging of fatty balls	++	++
Prostaglandin	+++	+	+++	+++
Adrenaline	+++	+	+++	+++
Control	+	—	+	+

\+ Single ovulating oocytes.
++ About 50 p. 100 ovulating oocytes.
+++ About 80-90 p. 100 ovulating oocytes.

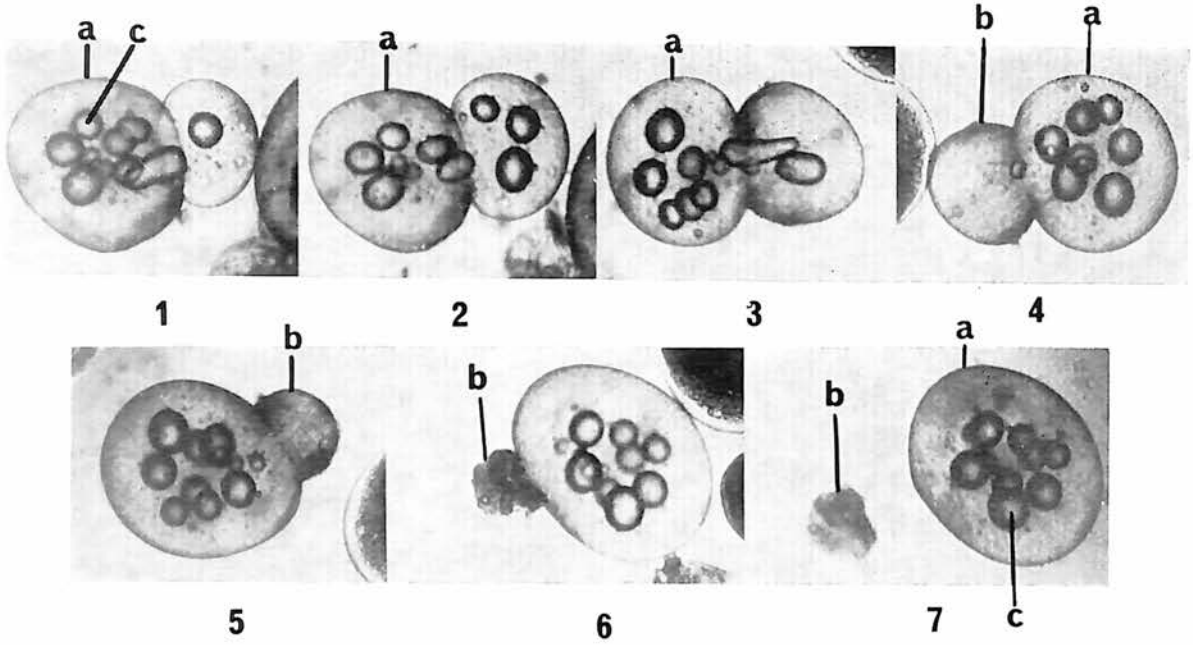

PLATE 1

Stages of oocyte ovulation in vitro *in European eel* (× 30). *a*) oocyte ; *b*) follicular envelope ; *c*) fatty ball.

The *in vitro* oocytes were incubated in BSS Cortland supplemented with 0.3 p. 100 NaCl (Wolf and Quimby, 1969) at 18 to 20 °C for 24 hrs. Except for the control, this medium was supplemented separately with the hormones (table 2) in the following concentration : steroids 1 μg/ml, HCG 100 IU/ml, adrenaline 5 μg/ml ; prostaglandin $F_{2\alpha}$ 3 μg/ml, carp hypophyseal homogenate 100 μg + HCG 100 IU/ml of medium.

Results and discussion.

Since the injections failed to induce ovulation in females, 4 of them were killed ; oocytes of various sizes were found in their ovaries. The largest of these (1.0 to 1.1 mm in diameter) were transparent and 60 p. 100 were lacking the germinal vesicle, while the germinal vesicle in 40 p. 100 had shifted towards the periphery. These oocytes also presented a fatty ball consisting of a single big ball or a few smaller ones grouped together.

Following 24-hr. incubation *in vitro* individual ovulating oocytes were found in the control fish as well as in the presence of steroids, HCG or carp hypophyseal homogenate ; 50 p. 100 of ovulating oocytes were found to occur in the presence of carp hypophyseal homogenate + HCG, while 80 to 90 p. 100 of ovulating oocytes were ascertained in the presence of adrenaline or prostaglandin $F_{2\alpha}$ in the medium (table 2). This result was typical of the oocytes in females N^{os}. 1, 3 and 4, whereas the oocytes obtained from female N° 2 ovulated individually only after treatment with prostaglandin $F_{2\alpha}$ or adrenaline. The oocytes placed in media supplemented with the other hormones, or in the control, failed to ovulate. However, in the oocytes placed in the media supplemented with carp hypophyseal homogenate + HCG, fatty ball fusion was observed.

In the ovulation process of eel oocytes *in vitro* (plate I), the oocyte is expulsed through the follicular envelope surrounded by the zona radiata alone. During this time, the follicular envelope rolls up, contracts and falls off after the ovulation process has been completed. An attempt to fertilize the ovulated eel oocytes *in vitro* with fully vital spermatozoa of male eel, failed to give positive results. No embryonic development was observed.

Our studies seem to indicate that prostaglandin or adrenaline plays a significant role in inducing ovulation in eel oocytes as some other authors found for trout oocytes (Jalabert, 1976). The results also suggest that gonadotropin is directly or indirectly involved in the ovulation process, although a synergistic mechanism of the activity of carp hypophyseal homogenate + HCG is not elucidated.

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Résumé. Chez des anguilles européennes traitées par un mélange d'HCG et d'extrait hypophysaire total de carpe, la maturation des ovaires est stimulée ; l'ovulation ne se produit pas spontanément. Celle-ci peut être provoquée *in vitro* par culture de fragments ovariens en présence d'hormones. Après 24 h d'incubation, l'addition d'HCG et de poudre hypophysaire au milieu de culture entraîne l'ovulation de 50 p. 100 des ovocytes ; l'addition de prostaglandine ou d'adrénaline induit l'ovulation de 80 à 90 p. 100 des ovocytes. Les œufs ovulés obtenus après culture ne sont pas fécondables *in vitro*.

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The effect of storage conditions on the biological activity of salmon gonadotropin

par E. M. DONALDSON, Helen M. DYE, B. F. WRIGHT

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Summary. The purpose of this study was to determine the effect of environmental conditions on the biological activity of salmon gonadotropin (SG-G100) during storage. At low relative humidity (RH) there was no significant loss or gain in gonadotropic activity at 35 °C or 50 °C over 21 days. At high RH there was a significant loss of potency at 35 °C after 10 and 21 days while at 50 °C after 21 days, there was almost complete loss of activity. When SG-G100 was held in saline for 7 or 21 days, there was a significant loss of activity at 20-25 °C after 7 days and at 9 °C after 21 days. In the second phase SG-G100 was held for 2, 4, 6 and 16 months at 35 °C and low RH, prior to bioassay. Activity was maintained for 6 months but by 16 months had declined relative to gonadotropin held at — 40 °C.

Introduction.

Partially purified salmon gonadotropin SG-G100 (Donaldson *et al.*, 1972) has been used in reproductive studies on several species of fish (Donaldson, 1973) however, to date, no investigation has been made of its biophysical properties with regard to shipping and storage. This study was undertaken to facilitate a project in Malaysia on the spawning of chinese carps for the purpose of fry production. The experiments were designed to determine the effect of transportation and storage of the hormone at high ambient temperatures and low and high humidity. In addition an experiment was carried out to establish whether the gonadotropin could be kept in saline solution for several days without deterioration.

Material and methods.

Treatment of gonadotropin samples. — The gonadotropin used in this study was partially purified chum salmon (*Oncorhynchus keta*) gonadotropin, SG-G100, prepared in this laboratory for the International Development Research Center. The study was conducted in two phases. In the first phase the effect of storing the SG-G100 at low and high humidity for 3-21 days and in solution for 7-21 days was investigated. In the second phase the effect of storing the SG-G100 at low humidity at 35 °C for 2 to 16 months was investigated.

In phase 1 the conditions of humidity and temperature were obtained by placing the samples in a covered water bath (Grant Instruments, Cambridge Type SSB4). The flasks containing the high humidity samples were open to the atmosphere of the incubator via an inverted « U » tube while the low humidity samples were sealed in flasks containing silica gel desiccant. Samples stored in solution were dissolved in 0.85 p. 100 NaCl, 0.1 p. 100 bovine serum albumin at concentrations of 125 and 250 μg/ml and stored either in a refrigerator at 9 °C or at room temperature (20-25 °C). In the second phase the closed vials of SG-G100 were placed in a beaker containing silica gel which was covered with « Parafilm » and placed in Blue M Model 100A incubator oven at 35 °C.

Bioassay. — Relative potencies of the treated SG-G100 samples were determined using the augmentation of radiophosphate (^{33}P) uptake by the chick testis (Florsheim *et al.*, 1959 ; Breneman *et al.*, 1962 ; Follett and Farner, 1966 ; Donaldson *et al.*, 1972). Control (untreated) SG-G100 was stored at — 40 °C prior to assay. Dosages of treated and untreated SG-G100 ranged from 6.25 to 50 μg/chick. Computer analysis was of a completely randomized four-point design. Analysis of variance tested parallelism, regression and preparation. The computer program also tested for the significance of the difference between potency ratios.

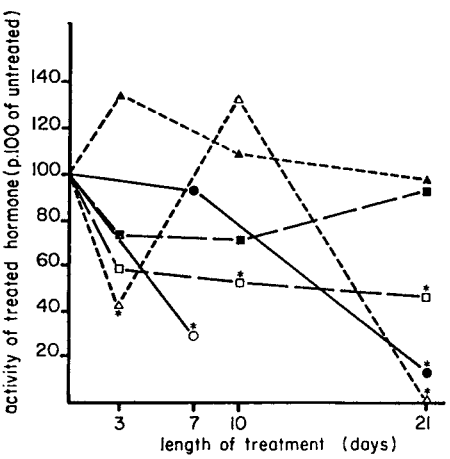

FIG. 1.

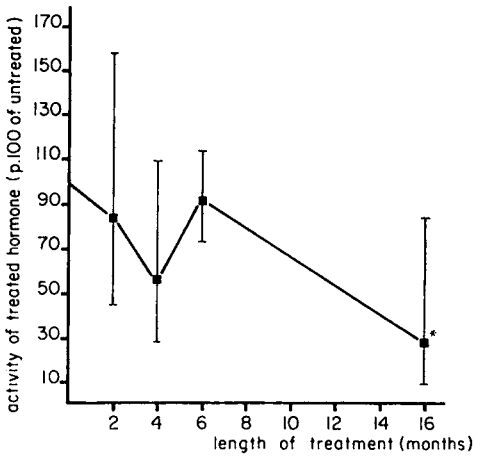

FIG. 2.

FIG. 1. — *Effect of storage for 3-21 days at 35 °C and 50 °C at low and high humidity and in solution at 9 °C and 20-25 °C on the gonadotropic potency of salmon gonadotropin SG-G100.* Potencies are expressed as a percentage of the potency of control SG-G 100 held at — 40 °C. Asterisks indicate potencies that were significantly different from the control ($P < 0.05$).

●——● 9°, ○——○ 20-25° : in solution. ■——■ 35° low, □——□ 35° high, ▲- - -▲ 50° low, △- - -△ 50° high : relative humidity.

FIG. 2. — *Effect of storage for 2-16 months at 35 °C and low humidity on the gonadotropic potency of salmon gonadotropin SG-G100.* Potencies expressed as a percentage of the potency of control SG-G100 held at — 40 :C. Asterisk indicates a potency that was significantly different from the control ($P < 0.05$).

Indices of precision for the various bioassays ranged from 0.19 to 0.39 in the first phase and from 0.15 to 0.34 in the second phase. The potencies of the salmon gonadotropin stored under various conditions have been expressed as a percentage of the potency of salmon gonadotropin stored at — 40 °C.

Results.

The results for the first phase of the experiment which involved storage for 3-21 days are shown in figure 1. The results for the second phase of the experiment which involved storage for 2 to 16 months are shown in figure 2. No significant changes in potency occurred when SG-G100 was stored at 35 °C or 50 °C for 21 days at low humidity. Storage at high humidity on the other hand resulted in a significant loss of potency after 10 and 21 days at 35 °C and after 3 and 21 days at 50 °C. In fact there was virtually no gonadotropic activity in the latter sample (fig. 1). Storage in solution at 9 °C for 7 days resulted in no significant loss of potency while storage in solution at 9 °C for 21 days or 20-25 °C for 7 days resulted in significant loss of potency. Untreated SG-G100 minus albumen had the same potency as untreated SG-G100 with albumen, suggesting that albumen is unimportant in the injection solution. In the second phase storage for 2, 4 and 6 months at 35 °C in a low humidity atmosphere resulted in no significant loss in activity while storage for 16 months resulted in a significant loss to approximately one quarter of the original potency (fig. 2).

Discussion.

This investigation is probably the first systematic storage study which has been conducted on a lower vertebrate gonadotropin preparation. While the general trend of the findings confirmed our expectations, it would not have been possible to determine safe storage times in the dry state and in solution without conducting the study.

Storage of salmon gonadotropin in a warm humid atmosphere for even a short period of time resulted in rapid loss of gonadotropic potency. Storage in a warm dry atmosphere on the other hand did not lead to a significant deterioration in potency during short and medium term storage. This finding permits the shipping of salmon gonadotropin at ambient temperature to tropical regions of the world providing that suitable precautions are taken to ensure an environment of low humidity. In this laboratory this is accomplished by placing the SG-G100 in plastic stoppered glass vials and placing the vials in a vapour proof polyester film pouch, containing silica gel desiccant, which is then heat sealed.

Most investigators prefer to prepare injection solutions on the day they are to be used, however, our data indicate that when necessary SG-G100 can be stored in saline solution without significant loss of activity for 7 days providing that it is held at 9 °C or below.

While SG-G100 can be shipped and stored at 35 °C for up to 6 months without significant loss of biological activity, losses did occur by 16 months. Thus long term storage is best accomplished by placing the hormone in suitable containers in the freezer. While we prefer — 40 °C for extended storage — 20 °C is probably adequate for

most purposes. When a vial containing SG-G100 is removed from the freezer it is allowed to stand until it reaches room temperature before being opened to prevent atmospheric water condensing upon the contents.

Conclusions.

Salmon gonadotropin can be shipped at ambient temperature providing it is in a low humidity atmosphere. Long term storage is preferably conducted in a low temperature freezer. When necessary SG-G100 may be stored in saline solution for up to one week in a refrigerator.

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Résumé. Nous avons étudié l'effet des conditions de stockage sur l'activité biologique de la gonadotropine de saumon (SG-G100). Quand l'humidité relative (RH) est faible, l'activité gonadotrope reste stable à 35 °C ou 50 °C pendant plus de 21 jours. Quand l'humidité relative est élevée, l'activité baisse significativement à 35 °C après 10 et 21 jours, tandis qu'à 50 °C la perte d'activité est à peu près totale après 21 jours. Quand la gonadotropine est conservée dans une solution saline pendant 7 ou 21 jours, la perte d'activité est significative à 20-25 °C après 7 jours et à 9 °C après 21 jours.

Durant le stockage sur une période prolongée de 2, 4, 6 ou 16 mois, l'activité biologique est maintenue jusqu'à 6 mois à 35 °C en humidité relative faible, et pendant 16 mois à une température de conservation de — 40 °C.

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Plasma gonadotropin, estradiol, and vitellogenin and gonad phosvitin levels in relation to the seasonal reproductive cycles of female brown trout

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Summary. Profiles for plasma gonadotropin, estradiol, and vitellogenin were obtained in female brown trout during the seasonal reproductive cycle. The accumulation of yolk lipophosphoprotein (phosvitin) in the gonad was also followed. Plasma estradiol and vitellogenin concentrations rose progressively and parallel increases in ovary yolk phosvitin were observed. Gonad development continued while plasma gonadotropin levels remained low and unchanged until oocyte maturation was underway. LH-RH treatment failed to stimulate vitellogenesis in fish in the early stages of the reproductive cycle ; however, gonad stimulation and increases in plasma hormone values were observed after females were treated with an extract of whole pituitaries taken from vitellogenic Pacific salmon.

Introduction.

Seasonal gonad recrudescence requires the integrated activities of the pituitary gland, the liver, and the gonads. In common with other lower vertebrates, a vitellogenic protein is produced in the liver of the female teleost under estrogen stimulation. This material is transported via the blood to the gonad, under pituitary mediation (Campbell and Idler, 1976).

The triggers for and regulation of the vitellogenic processes are poorly understood. The present study was designed to collect data for profiles of gonadotropin (GtH), estradiol (E_2), and vitellogenin (V_g) levels related to the reproductive cycles of the female brown trout, *Salmo trutta*. The effect of LH-RH and pituitary extract on these plasma indicators of sexual development was examined.

Materials and methods.

Experimental animals. — Wild adult brown trout, *Salmo trutta*, were collected from a local pond using fyke nets. The fish were transported to the laboratory and held without feeding in aquaria provided with a single pass freshwater supply and simulated natural photoperiod.

Experimental design. — All fish were bled 1 day following capture, and again 7 and 14 days after the beginning of hormone treatment. Blood samples from the caudal vasculature were drawn into heparinized syringes and the resulting plasmas were divided into aliquots and stored frozen until hormone assays were performed.

Hormone preparations. — Synthetic LH-RH (amide form) was dissolved in 0.05 M tris, pH 7.7 containing 3.75 p. 100 w/v gelatin. Fish treated with LH-RH received 100 μg i.p. in 0.3 ml/454 g body weight. Pituitaries from vitellogenic sockeye salmon were homogenized in tris buffer. The salmon gonadotropin hormone potency of this extract (84.8 μg/ml-salmon GtH radio-immunoassay) was 74.1 SG * units/ml. Fish treated with crude pituitary extract received 2 pit. equivalents i.p. in 0.3 ml/454 g body wt. Control fish received 0.3 ml vehicle solution only. All groups of fish received treatment twice each week, a total of 4 injections.

Hormone assays. — Radioimmunoassay techniques (RIA) were used to measure plasma concentrations of gonadotropin (GtH), estradiol (E_2), and vitellogenin (V_g). The method for measuring salmonid GtH was previously reported (Crim, Watts and Evans, 1975). A similar method has been developed for V_g RIA (Idler, Hwang and Crim, unpublished). Briefly, a rabbit antibody was produced against twice precipitated Atlantic salmon yolk lipophosphoprotein. A highly purified yolk lipophosphoprotein preparation was iodinated by the Chloramine T method (Greenwood, Hunter and Glover, 1963). The antibody to the purified yolk lipophosphoprotein was shown to cross react with the major plasma lipophosphoprotein. The E_2 RIA was developed according to the instructions obtained with the E_2 antiserum from Dr. G. Abraham. The protocol was modified for E_2 purification from plasma samples by using Sephadex LH-20 (DeJong, Hey and Van Der Molen, 1973).

Gonad analysis. — Sections of ovaries were examined histologically to evaluate oocyte development by the classification of Ishida, Takagi and Arita (1961). Small pieces of ovary were removed for the analysis of yolk lipophosphoprotein (Pv) by V_g RIA. Tissues were homogenized in 0.5 M NaCl, 5 mM EDTA and held at 4 °C for 30 min. After centrifugation for 60 min. at 24 000 g the supernatant fluid was removed for RIA.

Statistical analysis. — The student « t » test was used to compare groups.

Results.

Seasonal reproductive development of the female brown trout under natural conditions begins in the summer in preparation for the fall spawning period. Female trout were collected at the beginning, during, and at the completion of gonadal growth and development. Significant changes in gonadosomatic index (GSI) were not apparent until August (table 1) ; GSI rapidly increased thereafter and peaked in October. Histological evidence suggested that vitellogenesis was underway in June since all oocytes had progressed to the primary yolk stage. Increased yolk Pv accumulation

* 1 SG unit = 1 μg NIH-LH-S18 in the chick bioassay.

in June compared to May ($P < 0.005$) supported this conclusion. Ovary Pv concentration increased in August but declined at the October sampling ; with ovary weights growing larger, total ovary Pv continued to increase as full maturity approached in October.

Table 1

Changes in gonadosomatic index (GSI), *ovary phosvitin* (Pv) *concentrations and total* Pv *accumulation, and oocyte stage of development of female brown trout associated with the seasonal reproductive cycle*

Date of sacrifice	N	GSI (p. 100)	PV concentration (µg/mg)	PV total (µg/g body wt.)	Stage of oocyte (b) development			
					OG	PY	SY	OV
May 10	7	0.57 ± 0.09 (a)	0.29 ± 0.17 (a)	2.26 ± 1.56 (a)	3	4	—	—
June 10 ..	12	0.67 ± 0.05	1.31 ± 0.21	8.85 ± 1.42	—	12	—	—
August 26 .	5	4.63 ± 0.85	4.92 ± 0.45	236.01 + 61.86	—	4	1	—
October 21	5	18.82 ± 4.58	1.78 ± 0.19	334.29 ± 52.03	—	—	3	2

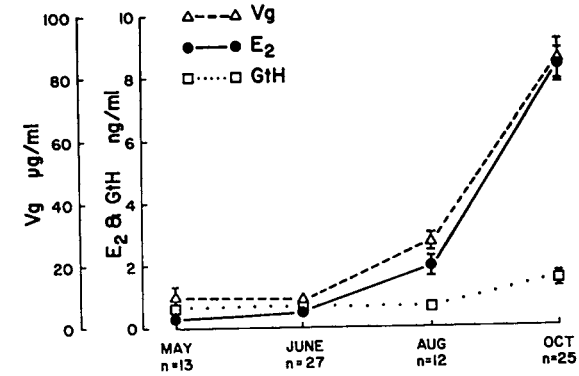
(a) = Mean ± SE.
(b) = stages of vitellogenesis (Ishida, Takagi and Arita, 1961) ; OG = oil globule, PY = primary yolk, SY = secondary yolk, OV = ovulated but not spawned.

Plasma profiles for GtH, E_2, and V_g levels associated with the female trout reproductive cycle are presented in figure 1*a*. Plasma GtH was very low throughout the cycle. Although these fish normally spawn in November, significant GtH increases were observed in fish nearing oocyte maturation in October ; E_2 and V_g both significantly increased in August with further increases observed in the October sample.

Manipulation of the female trout reproductive cycle was attempted with vitellogenic fish in June using either LH-RH or an extract of whole sockeye salmon pituitary glands. Intraperitoneal treatment with LH-RH over a 2 week period failed to produce changes in plasma GtH, E_2 or V_g values (fig. 1*b*). Likewise, GSI and ovary Pv values were not significantly altered (data not shown). Treatment of female brown trout with a crude extract of maturing salmon pituitary glands, however, significantly increased ($P < 0.02$) both GSI (0.95 ± 0.27 from 0.67 ± 0.16) and total ovary Pv (20.76 ± 5.38 from. 8.85 ± 1.42) and significant elevations in plasma E_2 ($P < 0.01$) and GtH ($p < 0.005$) were sustained for the 2 week treatment period. Plasma V_g levels were not significantly altered.

Discussion.

The levels of E_2 and V_g in the plasma progressively rise over the course of the female seasonal reproductive cycle and parallel increases occur in yolk Pv levels in the gonad as development continues. Gonadotropin levels are low and steady during much of the gonad development period. Very modest GtH changes were previously reported (Crim, Watts and Evans, 1975) for trout and salmon during the early vitellogenic period and also for the time of accelerated ovarian development. Late in the reproductive cycle dramatic increases in GtH are usually found in spawning fish.



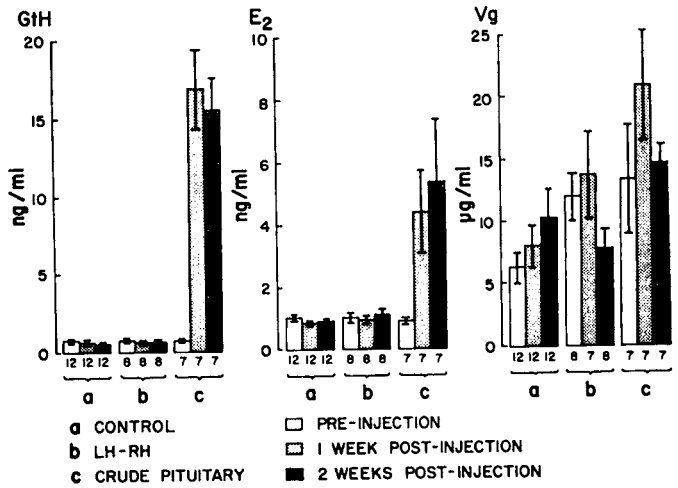


FIG. 1*a*. — *Profiles of plasma GtH, E_2, and V_g associated with the seasonal reproductive cycle of female brown trout.* Symbols represent means and vertical lines ± SEM. When SEM is not shown it is too small for the scale.

FIG. 1*b*. — *Plasma GtH, E_2, and V_g values in female brown trout before and after treatment with LH-RH or a crude extract of salmon pituitary gland.* Bars and lines represent means ± SEM. The n values appear beneath the bars.

Rainbow trout show increasing plasma GtH and E_2 levels during the late stages of vitellogenesis, but while GtH continues to increase during germinal vesicle breakdown, E_2 values are reported to decline (Breton *et al.*, 1975).

Although synthetic LH-RH stimulates GtH release in the sexually mature carp and trout (Weil, Breton, and Reinaud, 1975 ; Crim and Cluett, 1974), LH-RH in the present study failed to increase plasma GtH, E_2, and V_g levels of the trout during the early phases of vitellogenesis. Therefore, the mechanism for neural regulation of the vitellogenic process remains to be determined. Species differences may exist with respect to the LH-RH sensitivity of fish at various stages of the reproductive cycle

because Chan (1977) showed that LH-RH promotes ovary growth and maturation in the regressed Japanese Medaka.

The results of the present experiment indicate that the salmon pituitary gland contains a vitellogenic factor(s), possibly gonadotropin, capable of increasing plasma estradiol levels and enhancing gonad accumulation of phosvitin. Other pituitary substances in addition to classical glycoprotein gonadotropin, have been shown to stimulate vitellogenesis (Campbell and Idler, 1976) and the nature of the vitellogenic material in the salmon pituitary remains to be elucidated.

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Résumé. L'évolution de la gonadotropine, de l'estradiol et de la vitellogénine dans le plasma de truites Fario femelles a été suivie pendant le cycle saisonnier de reproduction. L'accumulation de lipophosphoprotéines (phosvitine) dans le vitellus a été également suivie. L'estradiol et la vitellogénine plasmatique augmentent progressivement, en même temps que la phosvitine dans le vitellus. Pendant le développement de l'ovaire, le niveau plasmatique de la gonadotropine reste bas jusqu'à la phase de maturation finale des ovocytes. Un traitement au LH-RH ne stimule pas la vitellogenèse durant les premiers stades du cycle de reproduction, cependant on observe une stimulation de l'ovaire et une élévation du niveau de la gonadotropine dans le plasma quand les femelles sont traitées par un extrait hypophysaire préparé à partir d'hypophyses de saumon prélevées en période de vitellogenèse.

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Estrogens and estrogenic effects in *Tilapia aurea* (Cichlidae, Teleostei)

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Summary. Estradiol (E_2) level in plasma of non-breeding female *Tilapia aurea*, measured by RIA, was 0.33 $\pm$ 0.02 ng/ml (mean $\pm$ SEM, n = 6). This level is about 10 p. 100 of the level found in breeding females, about 4 times higher than in ovariectomized females and similar to the level found in non-breeding males.

Twenty-four hours following a single i. p. injection of 0.5 mg E_2, the estradiol level in plasma of ovariectomized *Tilapia* was within the physiological range (0,2-10 ng/ml). This level was maintained for at least 4 days. Comparison of the vitellogenic potency of 3 estrogens in ovariectomized *T. aurea* shows that estriol was more potent than estradiol, which, in turn, was more potent than estrone. Since E_2 increased in the plasma of estrone-injected fish, it is assumed that estrone was converted to estradiol in the recipient fish.

Introduction.

Raising water temperature above 22° induces ovarian growth and spawning in *Tilapia aurea* (Fishelson, 1966). By employing the isotopic derivative technique it was shown that in female *T. aurea* exposed to 30° the plasma level of testosterone, 11-ketotestosterone, 11β-hydroxytestosterone and deoxycorticosterone (DOC) is considerably higher than in females kept at 18° (Katz and Eckstein, 1974). The increase of DOC level was explained by its possible role in the maturation of oocytes and ovulation, in line with the findings of Goswami and Sundararaj (1971) in *Heteropneustes fossilis*. However, the increased level of the three androgens remained unexplained. Also estrone (E_1) and estradiol-17β (E_2) were isolated and identified in ovarian extracts of this fish, but they could not be detected in the peripheral plasma, using the isotopic derivative method (Katz *et al.*, 1971 ; Katz and Eckstein, 1974).

Studies on the functional significance of ovarian steroids in bony fishes have been hampered for a long time by the peculiar morphological organization of the ovary and oviduct. « Spaying a female teleost means in most cases that not only the source of the hormones is removed, but also an important part of the potential target organ. » (Reinboth, 1972.) However, E_2 is known to induce the synthesis and secretion of vitelloproteins by the liver in teleosts, as in other non-mammalian vertebrates (Chester-Jones *et al.*, 1972 ; Campbell and Idler, 1976 ; Emmersen and Emmersen, 1976).

In a recent report from our laboratory (Yaron *et al.*, 1977), we have shown that the plasma of *Tilapia aurea* contains immuno-reactive estradiol-17β (3.1 ± 0.7 ng/ml ; mean ± SEM ; n = 26). The specificity of the antibody employed in our radioimmunoassay (RIA) was examined by cross-reaction with all the steroids previously reported in this species. The highest cross-reaction with E_2 was of E_1 (1.4 p. 100). However, the similarity of E_2 determinations in whole versus chromatographically-separated plasma extract indicated that neither E_1 nor estriol (E_3), if present in the plasma of this fish, could contribute to the E_2 determinations. A positive correlation was found between plasma E_2 level and ovarian weight. The plasma E_2 level dropped to 0.08 ± 0.04 ng/ml nine days after ovariectomy. Concentrations of plasma calcium and proteins, indirect parameters of plasma vitelloprotein level, decreased following ovariectomy but could be restored to the initial level by injection of E_2.

This paper presents data on E_2 level in plasma of non-breeding females and males, compares the potency of E_1 and E_3 with that of E_2 on the above-mentioned vitellogenic parameters, and describes the disappearance of injected estradiol from the circulation of ovariectomized *Tilapia*.

Materials and methods.

Fish source, fish maintenance, surgery, blood sampling, protein, calcium and E_2 determinations were as described earlier (Yaron *et al.*, 1977). Plasma E_2 was measured by RIA using the rabbit anti-17β-estradiol-6-BSA-serum. For details on the technique, specificity and validity of the assay, the reader is referred to the latter article. Results are expressed as group means ± SEM.

Results and discussion.

E_2 level in non-breeding T. aurea. Plasma E_2 level was measured in 6 fish of each sex collected after the breeding season (October) and kept for 10 days at 17° and photoperiod of 12 L/12 D. The E_2 and protein levels were similar in both sexes ($p > 0.05$), but calcium level in the plasma of the females was higher than that of the males ($p = 0.044$, table 1).

TABLE 1

Estradiol, calcium and protein in plasma of non-breeding Tilapia aurea [a]

Sex	E_2		Ca		Protein	
	n	ng/ml	n	mg p. 100	n	gr p. 100
females	6	0.330 ± 0.02	6	13.64 ± 0.80	6	4.05 ± 0.28
males	6	0.516 ± 0.086	7	11.88 ± 0.21 [b]	7	4.33 ± 0.24

[a]) Fish were collected after the breeding season (October) and kept at 17 °C for 10 days.
[b]) Significantly different from the female value ($p = 0.044$). Student's t-test.

The E_2 level in non-breeding female *T. aurea* is about one-tenth of the level recorded in females kept at 25° during the breeding season (3.1 $\pm$ 0.75 ng/ml), but 4 times higher than that found in ovariectomized fish (0.08 $\pm$ 0.04 ng/ml). The calcium concentration in the non-breeding females was higher than in males (table 1) and in ovariectomized fish (11.4 $\pm$ 0.2 mg p. 100 ; Yaron *et al.*, 1977). It is assumed, therefore, that E_2 is secreted by the ovary of non-breeding *T. aurea* and that their higher Ca level indicates the presence of vitelloprotein in the plasma even beyond the breeding season.

Disappearance of injected E_2 from circulation. — In many instances a relatively high dose of estrogen was required to elicit a pronounced vitellogenic effect. Since the physiological level of estrogen is rather low, such high doses as used in experimental procedures were justly criticized (Reinboth, 1972). The following experiment was performed in order to clarify the circulatory level of injected estradiol in *T. aurea*. For this study we used ovariectomized *Tilapia* of about 150 g body weight in which E_2 level was lower than 1 ng/ml. Plasma levels of E_2 were measured 5, 24, 120 and 315 h following a single i. p. injection of 0.5 mg E_2 dissolved in sesame oil.

Five hours after the injection, plasma E_2 was extremely high (fig. 1). Between 24 h and 120 h the level of the exogenous steroid was within the physiological range, comparable to the levels encountered in female *Tilapia* with a gonadosomatic index of 1.8 to 4.0 (Yaron *et al.*, 1977). The long intervals between samplings did not permit the calculation of the half-life for this steroid. It is obvious, however, that following an i. p. injection of 0.5 mg to *Tilapia* the « pharmacological » level is sustained less than 24 h, while in the 4 subsequent days the E_2 is maintained at a physiological level.

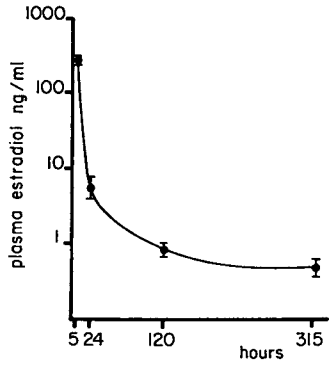

FIG. 1. — *Disappearance of injected estradiol-17β from the circulation of* Tilapia aurea. Estradiol-17β (0.5 mg, 1.2×10^{-5} mole/kg bw) dissolved in sesame oil (0.2 ml) was injected i. p. to 8 ovariectomized fish one month post operation.

Plasma E_2, Ca and protein following the injection of E_1, E_2 or E_3. — This experiment was designed to compare the estrogenic effects in *T. aurea* of three C_{18} steroids known as estrogens in various vertebrates (Chester-Jones *et al.*, 1972). E_1, E_2, E_3 or the vehicle (1.3 ml/kg) were injected i. p. (1.3×10^{-5} mole/kg bw) to female *T. aurea* one month after ovariectomy. Injections were repeated on days 3, 6, 10 and 13. Blood was sampled before the first injection, on day 10 before injection, and on day 18. Plasma calcium and protein were determined as indirect parameters of the vitellogenic response to the injected estrogens (Yaron *et al.*, 1977). The results of day 10 and day 18 were averaged for each fish. For each pair of treatments, the means of these averaged

levels were compared using Student's t-test (unequal variances). The highest vitellogenic response was found in fish treated with E_3. Treatment with E_1 was followed by an increase of the plasma calcium but not of protein. The effect of E_2 on these parameters was significant and similar to that reported earlier in this fish (Yaron *et al.*, 1977) (fig. 2*a*, *b*).

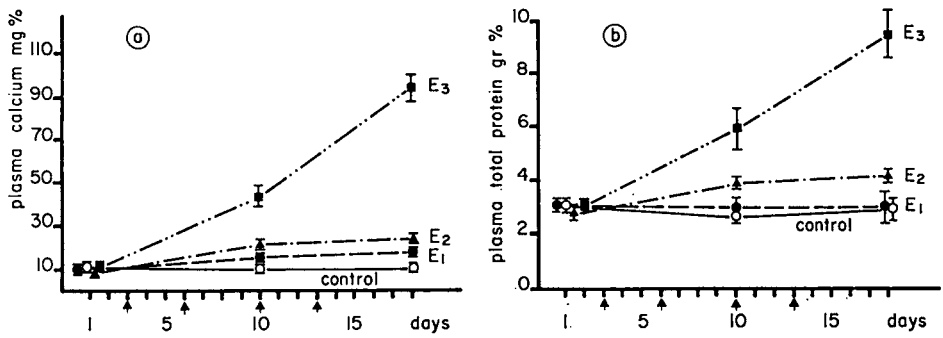

FIG. 2. — *Vitellogenic parameters in* Tilapia aurea *injected with estrone, estradiol or estriol.* The steroids (1.3×10^{-5} mole/kg bw) dissolved in sesame oil (1.3 ml/kg) were injected i.p. to ovariectomized females (*ca.* 150 gr bw) on days marked with arrows. Eight fish in each group. On day 10 blood was sampled before the injection. *a*) Plasma calcium ; *b*) Plasma protein.

In order to ascertain whether the vitellogenic effects of the steroids investigated resulted from the injected substances *per se*, the level of the immuno-reactive E_2 was measured in the plasma of fish from all groups (fig. 3). E_2 level in the plasma of oil-injected, ovariectomized fish remained low throughout the experiment. Similar levels were found in E_3-treated fish. It was not surprising to find higher E_2 levels in plasma of E_2 injected fish, but unexpectedly, the plasma E_2 level of E_1 injected fish increased significantly at day 10 and was similar to the level found in E_2-injected fish. This may indicate a conversion of the injected E_1 to E_2. The inter-conversion between these two steroids is a common phenomenon in vertebrate tissues (Ozon, 1972). If, indeed, the injected E_1 was promptly transformed into E_2 it would be difficult to explain

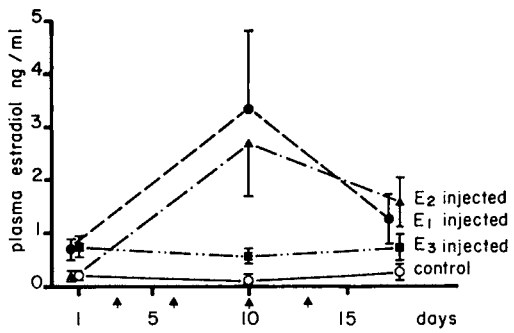

FIG. 3. — *Plasma level of immuno-reactive estradiol in fish injected with* E_1, E_2, E_3 *or oil.* Details as in figure 2.

the low vitellogenic response to the injected steroid. We assume, therefore, that the increase of plasma E_2 in E_1-treated fish resulted from either : (i) a low rate of clearance and a consequent accumulation of E_1 in the plasma (a level of *ca.* 200 ng/ml E_1 may be measured as *ca.* 3 ng/ml E_2 in the RIA used, due to a 1.4 p. 100 cross reaction) ; (ii) a conversion of estrone to an unknown steroid with an affinity to the antiserum similar to that of E_2 but with a low vitellogenic potency ; (iii) the main conversion of E_1 to E_2 occuring close to day 10 after injection, and thus not yet allowing the manifestation of the vitellogenic response ; or (iv) a combination of these possibilities. Nevertheless, the high vitellogenic potency of E_3 may be attributed to this steroid *per se*, since no increase in plasma E_2 was detected in this group. It should be emphasized, however, that estriol has never been reported in *Tilapia aurea*.

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Résumé. Le niveau d'estradiol (E_2) dans le plasma de *Tilapia aurea* femelles en dehors de la période de reproduction, mesuré par dosage radioimmunologique, atteint $0{,}33 \pm 0{,}02$ ng/ml (moyenne $\pm$ SEM, $n = 6$). Il représente à peu près 10 p. 100 du niveau trouvé chez les femelles au cours de cette dernière période ; il est environ 4 fois plus élevé que chez les femelles ovariectomisées et égal au niveau trouvé chez les mâles en dehors de la saison de reproduction.

Vingt-quatre heures après une seule injection intrapéritonéale de E_2 (0,5 mg), la concentration plasmatique d'estradiol de *tilapia* ovariectomisées est de l'ordre de celle trouvée chez les femelles normales (0,2-10 ng/ml). Ce niveau se maintient au moins 4 jours. La comparaison de l'effet vitellogénique des trois estrogènes chez les *Tilapia aurea* ovariectomisées montre que l'estriol est plus actif que l'estradiol, et ce dernier plus actif que l'estrone. Puisque E_2 a augmenté dans le plasma de poissons ayant subi une injection d'estrone, ce composé a probablement été converti en estradiol par les poissons injectés.

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In vitro stimulation of vitellogenin incorporation into trout oocytes by salmon pituitary extracts

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Summary. Follicles, isolated from ovaries of rainbow trout before the onset of active vitellogenesis, incorporate ^{14}C-labelled vitellogenin when incubated *in vitro* for 18 hrs. An aqueous extract of lyophilised salmon pituitaries stimulated the incorporation of vitellogenin, but not other serum proteins.

A pituitary fraction which is not bound to Con A-Sepharose (during two chromatographies) stimulates incorporation in a similar fashion to the total extract.

Less than 20 p. 100 of the total radioactivity incorporated into the follicles was associated with follicular cells and removal of these tissues after incubation showed that hormonal treatment resulted in a real stimulation of vitellogenin incorporation into the oocytes.

The results of this study suggest that the incorporation of vitellogenin by trout oocytes may be under direct control by a pituitary gonadotropin.

Introduction.

In teleosts the process of vitellogenesis apparently involves synthesis of a yolk precursor protein by the liver (Plack and Frazer, 1971 ; Emmersen and Emmersen, 1976 ; Campbell and Idler, 1976) which has been found in the blood by many workers (Ho and Vanstone, 1961 ; Thurston, 1967 ; Utter and Ridgeway, 1967 ; Aida *et al.*, 1973 ; Heesen and Engels, 1973 ; Emmersen and Petersen, 1976). Campbell and Idler (1976) found that a pituitary factor could reinitiate incorporation of this protein into the ovary of hypophysectomized flounder. The use of hypophysectomy precluded the possibility of action of injected preparations via the pituitary but gave no indication whether action was direct or involved a relay by other endocrine tissues.

Wallace *et al.* (1970) began a series of experiments showing that oocytes taken from HCG-treated *Xenopus laevis* continued to incorporate vitellogenin when maintained *in vitro*. This system (Wallace and Jared, 1976) was adopted to examine the direct action of extracts of salmon pituitaries on protein incorporation by isolated trout follicles which had not yet begun active vitellogenesis.

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Materials and methods.

Hatchery reared rainbow trout were maintained in a closed-circuit freshwater raceway system between 10 and 20 °C. Between February and July oocytes in the primary vitellogenic state were isolated from ovaries by treatment with collagenase. Protein incorporation was evaluated by use of proteins labelled with ^{14}C by reductive alkylation (Wallace and Jared, 1976 ; Rice and Means, 1970). The proteins were isolated using Triethyl aminoethyl cellulose (Jared and Wallace, 1968 ; but using a linear gradient) and/or by chromatography on Ultrogel AcA 22 (LKB). Serum from estrogenised trout was extensively purified by distilled water precipitation followed by TEAE and/or Ultrogel chromatography. This preparation contained vitellogenin with two minor contaminants detected by polyacrylamide gel electrophoresis. Serum from non-vitellogenic female trout was chromatographed on TEAE cellulose. The eluate fraction (C6a) which would have contained vitellogenin was selected for use as a control. In electrophoresis C6a contained five principal proteins and a barely detectable amount of a protein with a similar mobility to vitellogenin.

Lyophilised pituitary glands from spawning *Oncorhynchus tsawytscha* were extracted following Idler *et al.* (1975*a*) to yield a soluble total extract (TE) of which a portion was subjected to Con A-Sepharose chromatography. The unbound (Con AI) fraction was chromatographed twice to reduce the contamination by proteins normally bound to the gel (Con AII) (Campbell and Idler, 1977). All extracts were tested at concentrations equivalent to 0.07 pituitary glands per incubation, i. e., 200 μg/ml for TE ; 170 μg/ml for Con AI and 30 μg/ml for Con AII. When assayed in the immature trout ovarian cyclic AMP assay (Idler *et al.*, 1975*b*) the gonadotropin contents of TE, Con AI and Con AII were 80, < 4 and 725 SG units/mg of protein respectively.

Individual oocytes were incubated in 20 μl of trout balanced salt solution (Jalabert, 1975) containing penicillin-streptomycin, labelled protein and pituitary preparation or as control an equal concentration of bovine serum albumin (BSA). Protein concentrations were made up to 200 μg/ml in Con AI and Con AII media by addition of BSA. Incubations were performed in microtest plates (Cooke Microtiter) for 18 hours under a water saturated O_2/N_2 atmosphere at a constant temperature appropriate to the current seasonal temperature of the fish (10, 15 and 20 °C ; no attempt was made to evaluate effects of temperature since for each experiment all test treatments were evaluated under the same conditions).

After incubation oocytes were examined using a disecting microscope and those which had maintained their original appearance were rinsed three times with an isotonic balanced salts solution. Post-incubation denuding of oocytes was assured by further collagenase treatment to remove thecal layers followed by mechanical treatment to remove all granulosa cells which could be stained by toluidine blue. Oocytes were dissolved by either Protosol (NEN) or trypsin in preparation for scintillation counting.

Separate experiments using groups of 27 oocytes from each of two or three fish were performed. Data were converted to $\mu g/mm^2$ and combined for comparison by analysis of variance.

Results.

In a preliminary group of experiments treatment of entire follicles with TE increased incorporation of ^{14}C-labelled vitellogenin by 21 p. 100 over BSA controls ($P \leqslant 0.01$, table 1*a*).

Incubation of oocytes of 16 fish with the Con AII subfraction resulted in levels of incorporation similar to control oocytes (table 1*b*). Both Con AI and total extract significantly stimulated ($P \leqslant 0.01$) vitellogenin incorporation. The Con AI fraction was significantly less active than the total extract ($P \leqslant 0.01$). Equal amounts of the Con AI fraction obtained from two separated extractions of salmon pituitary both gave a 17 p. 100 stimulation.

TABLE 1

Total incorporation of ^{14}C-labelled vitellogenin by individual trout follicles incubated with 2 mg/ml vitellogenin and equivalent amounts of total pituitary extract (TE), *Con AI or Con AII fractions with bovine serum albumin* (Control C) *used to adjust to equal protein concentrations for each group* (ng vitellogenin/mm² oocyte surface/18 hrs ; mean ± SE : number of oocytes in parentheses)

	Treatment			
	C	TE	Con AI	Con AII
1*a*	109 ± 3 (108)	132 ± 4 (124)	—	—
1*b*	148 ± 4 (380)	172 ± 4.0 (395)	164 ± 4 (376)	153 ± 4 (368)

In further experiments with five fish the result of treatment with total extract or Con AI was an apparent 13 and 4 p. 100 stimulation ($P \leqslant 0.01$) of incorporation (table 2*a*). Thecal layers removed after incubation contained 14 p. 100 of the total

TABLE 2

ng of vitellogenin incorporated/mm² oocyte surface/18 hrs of incubation when oocytes were incubated in the presence of 2 mg/ml vitellogenin and treated with TE, Con AI, Con AII or BSA. 2*a* : incorporated vitellogenin found in entire follicles and follicles from which the thecal cells were removed after incubation ; 2*b* : incorporated vitellogenin found in oocytes from which all follicle cells were removed after incubation (mean ± SE, number of oocytes in parentheses)

	Treatment			
	C	TE	Con AI	Con AII
2*a* : entire follicle.....	188 ± 4 (127)	213 ± 5 (124)	197 ± 4 (121)	187 ± 4 (127)
oocyte + granulosa.....	152 ± 4 (101)	183 ± 5 (113)	178 ± 5 (110)	163 ± 5 (111)
2*b* : naked oocyte.....	218 ± 4 (64)	246 ± 6 (65)	242 ± 6 (69)	223 ± 6 (66)

protein incorporated partially masking the real stimulation of 21 and 17 p. 100 respectively.

Oocytes of three fish were totally denuded after incubation. An approximately equal 12 p. 100 stimulation ($P \leqslant 0.01$) by total extract or Con Al fraction represents a stimulation of incorporation into the oocyte rather than into the follicle cells (table 2*b*).

In three experiments eight oocytes were damaged during denuding and the chorion alone was taken for counting. Three percent of the radioactivity in a naked oocyte was found associated with the chorion.

Total extract stimulated incorporation of vitellogenin ($P \leqslant 0.01$) but not non-vitellogenic serum protein (C6a) (table 3). The quantity of vitellogenin incorporated was 17-18 times greater than that of C6a and the differences in quantities found in washed follicles and denuded oocytes, representing the protein association with follicular tissues, was only 10 p. 100 for vitellogenin but 60 p. 100 for C6a.

TABLE 3

Incorporated vitellogenin (V25) *and non-vitellogenic serum protein* (C6a) *found in control and total-extract-stimulated oocytes after complete denuding expressed as ng/mm²/18 hrs and also as a percentage of the total incorporation into oocyte and follicular tissues* (1 mg/ml of test protein + 200 µg/ml BSA or TE ; mean of 2 fish ± SE, number of oocytes io parentheses)

Protein	Treatment			
	C		TE	
	ng	p. 100	ng	p. 100
V25	139 ± 5 (36)	86	150 ± 5 (47)	93
C6a	8 ± 0.3 (39)	41	8 ± 0.3 (47)	43

Discussion.

The oocytes liberated from the ovarian tissues after experimental incubation were still surrounded by the granulosa and thecal layers and the data show that approximately 14 p. 100 of the total vitellogenin « associated » with the intact follicle is in the thecal tissue (table 2). The difference between vitellogenin incorporated into oocytes which were denuded and the total follicular « association » presented in table 3 approximates to this figure suggesting that little vitellogenin is « associated » with granulosa cells. The association with follicular tissues was greatest for C6a (table 3). For vitellogenin at least, this association may be a non-specific artifact in the system used. Table 2*a* shows that stimulations of incorporation by 21 and 17 p. 100 were masked and detected as only 13 and 4 p. 100 respectively when total follicles were processed.

The denuding of oocytes after incubation has shown that most vitellogenin « associated » with entire follicles, during *in vitro* incubation, was in fact associated with the oocyte. Examination of the chorion of several oocytes has indicated that the vitellogenin was incorporated into the oocyte and very little remained at the cell membrane. Wallace *et al.* (1970) found that vitellogenin incorporation by unstimulated oocytes of

X. laevis was minimal but augmented by five times if the donor had been treated with human chorionic gonadotropin (HCG) 24 hrs in advance. The vitellogenin incorporation by control oocytes suggests that this process might contribute to primary vitellogenic growth. However, this type of oocyte is normally exposed to concentration of < 20 µg vitellogenin/ml of serum at this stage (Crim and Idler, 1978) rather than the 1 or 2 mg/ml used experimentally so that vitellogenin incorporation must be a minor factor. Despite the high basal incorporation by control oocytes, vitellogenin incorporation was increased by treatment of oocytes with pituitary extracts for 18 hrs. The small augmentation seen may be due to some limiting factor in incubation conditions since Wallace *et al.* (1973*a*, *b*) showed that the incorporation of vitellogenin by oocytes from HCG-stimulated *X. laevis* was extremely sensitive to the ionic composition of the incubation media. In addition it is possible that 18 hour incubations are too short to demonstrate a large stimulation of incorporation.

The basal incorporation activity is relatively specific for vitellogenin and the stimulation of protein incorporation by pituitary extract is specific for vitellogenin (table 3). Wallace and Jared (1976), Wallace *et al.* (1970) showed that in HCG stimulated *X. laevis* oocytes vitellogenin was incorporated more actively than several other test proteins.

Treatment of trout oocytes with Con AI fraction or total extract of salmon pituitary glands stimulated vitellogenin incorporation. No such effect was produced by Con AII treatment. These results support a hypothesis of direct action by a Con AI gonadotropin on vitellogenic follicles in the trout. A Con AI preparation from *Hippoglossoides platessoides* pituitaries has already been found to promote yolk incorporation into the gonads of *Pseudopleuronectes americanus in vivo* (Campbell and Idler, 1976). The salmon gonadotropin capable of inducing oocyte maturation and ovulation (Jalabert *et al.*, 1974), having activity in the trout ovary cyclic AMP assay and stimulating ^{33}P-incorporation into chick testes (Idler *et al.*, 1975*a*) was isolated from the Con AII fraction. The results of Campbell and Idler (1977) suggest that the gonadotropin which will induce maturation and ovulation of *P. americanus* oocytes is also found in the Con AII fraction.

For these two species vitellogenin incorporation is stimulated by a gonadotropin which is distinguishable from previously isolated teleost gonadotropins by its lack of affinity to concanavalin A.

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Résumé. Les follicules ovariens isolés, prélevés sur des truites avant le début de la vitellogenèse, accumulent de la vitellogénine marquée au ^{14}C quand ils sont incubés *in vitro* pendant 18 h. L'incorporation de la vitellogénine est augmentée par l'action d'un extrait total d'hypophyse de saumon.

La fraction non liée à la Con A Sépharose (après deux passages) et l'extrait total stimulent l'incorporation de la vitellogénine de la même manière. Les cellules folliculaires

incorporent moins de 20 p. 100 de la vitellogénine ; il y a donc une vraie incorporation dans l'ovocyte. Ces résultats préliminaires montrent que l'incorporation de vitellogénine peut être directement contrôlée par l'hypophyse.

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Ultrastructural studies on experimentally induced vitellogenesis in juvenile rainbow trout (*Salmo gairdneri* R.)

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Summary. Juvenile rainbow trout (weighing 10 or 20 g) were treated thrice weekly for 10 weeks with salmon-gonadotropin (S-GTH), salmon pituitary extract (S-PE), S-GTH plus estradiol-17β, and estradiol-17β alone. The effects of these treatments on the oocytes were studied at ultrastructural level.

Saline-injected control fish contained oocytes at previtellogenic stage of development. S-GTH (0.1 or 0.5 μg/g) induced a synthesis of endogenous yolk in the oocyte cytoplasm but failed to initiate incorporation of exogenous yolk or vitellogenin into the oocytes. S-GTH (0.1 μg/g) in combination with estradiol-17β (1 μg/g) produced similar results to those with S-GTH alone. Estradiol-17β alone failed to induce vitellogenesis. In contrast, S-PE (10 μg/g) induced the incorporation of exogenous yolk into the oocyte indicated by an extensive pinocytotic activity at the oocyte membrane.

It is suggested that gonadotropin is necessary for the induction of endogenous yolk synthesis and that some other pituitary hormone(s) is involved in the process of incorporation of exogenous yolk or vitellogenin into the oocyte.

Introduction.

Vitellogenesis has been regarded as a process of yolk accumulation in the oocyte. During this process a part of the yolk is synthesized in the oocyte cytoplasm by various organelles (Raven, 1961 ; Norrevang, 1968) ; the rest of the yolk material is synthesized at an extra-ovarian site and then transported via circulation to the ovary and finally deposited into the oocyte by the process of pinocytosis (Norrevang, 1968 ; Wallace and Bergink, 1974).

The influence of pituitary on vitellogenesis has been demonstrated in several teleost species ; hypophysectomy results in the degeneration of yolky oocytes and replacement therapy using pituitary extracts reinitiates vitellogenesis (de Vlaming, 1974). However, the number of pituitary hormones involved in the regulation of vitellogenesis in teleosts is still controversial.

This report presents the effects of purified salmon-gonadotropin, salmon pituitary extract and estradiol-17β on the oocyte development in juvenile rainbow trout (*Salmo gairdneri*) with a view to finding out whether vitellogenesis can be induced experimentally in juvenile fish and the number of pituitary hormones involved in this process.

Materials and methods.

Juvenile rainbow trout (*Salmo gairdneri* R) weighing aproximately 10 or 20 g were injected thrice weekly for 10 weeks with salmon-gonadotropin (S-GTH) male and female (0.1 μg/g and 0.5 μg/g body weight), S-GTH (0.1 μg/g) plus estradiol-17β (1 μg/g), salmon pituitary extract male and female (10 μg/g), estradiol-17β (1 μg/g), and fish saline (0.9 p. 100).

Salmon-gonadotropin was purified from male and female *Onchorynchus tshawytscha* pituitaries. The purification procedure for S-GTH has been described elsewhere (Breton, Prunet and Reinaud, 1978). Salmon pituitary extract (S-PE) was prepared by homogenizing male and female *O. tshawytscha* pituitaries in a glass-teflon homogenizer. Estradiol-17β was obtained from Steraloids (USA).

At the end of treatment, the fish were killed by decapitation. Small pieces of ovary were immediately put in a fixative containing paraformaldehyde (1 or 2 p. 100), glutaraldehyde (2 or 3 p. 100) and picric acid (0.1 p. 100) in 0.15 M cacodylate buffer pH 7.3, for 2 hrs at room temperature followed by post-fixation in 1 p. 100 osmium tetraoxide in the same buffer for 1 hour at room temperature. After dehydration tissues were infiltrated and embedded in an epon-araldite mixture. Ultrathin sections stained with uranyl acetate and lead citrate were observed under a Siemens Elminskop.

Results.

Saline-injected control fish contained oocytes measuring 43 to 130μ in diameter. All the oocytes were at previtellogenic stage of development as no evidence for yolk accumulation was observed in their cytoplasm.

S-GTH purified from male and female pituitaries had similar effects on oocyte development. The oocytes in S-GTH-(0.1 or 0.5 μg/g) treated fish measured up to 495 μ in diameter. A correlation was observed between the oocyte diameter and stage of development. Apart from previtellogenic oocytes, the more advanced oocytes could be classified into three categories on the basis of their diameter : small (175 to 233 μ), medium (285 to 350 μ), and large (400 to 495 μ). The small oocytes were characterized by the presence of multi-vesicular bodies (MVB) at the perinuclear cytoplasm (fig. 1). The medium-sized oocytes exhibited lipid bodies in addition to the MVB. These lipid bodies were found scattered amongst the MVB (fig. 2). The large oocytes

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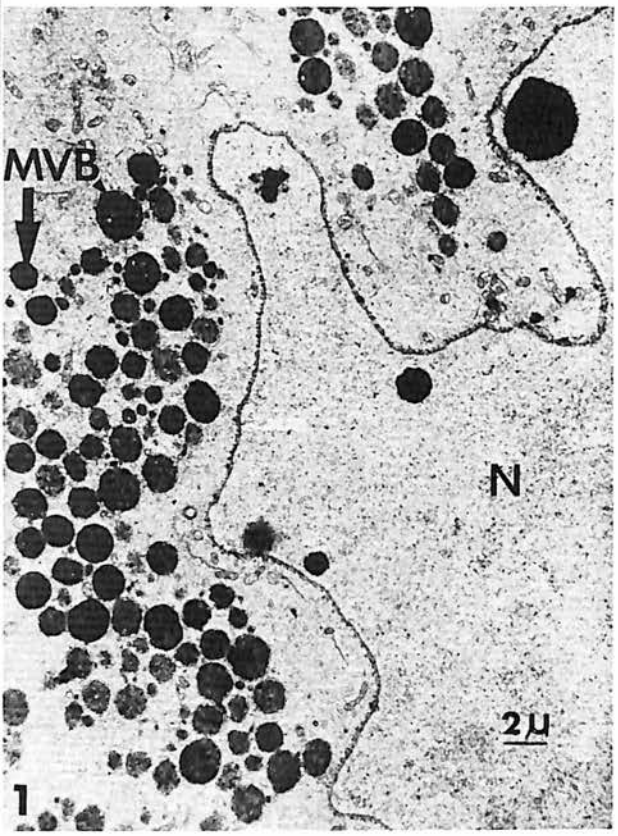
Electron micrographs of portions of oocyte from the ovary of juvenile rainbow trout treated with salmon-gonadotropin.

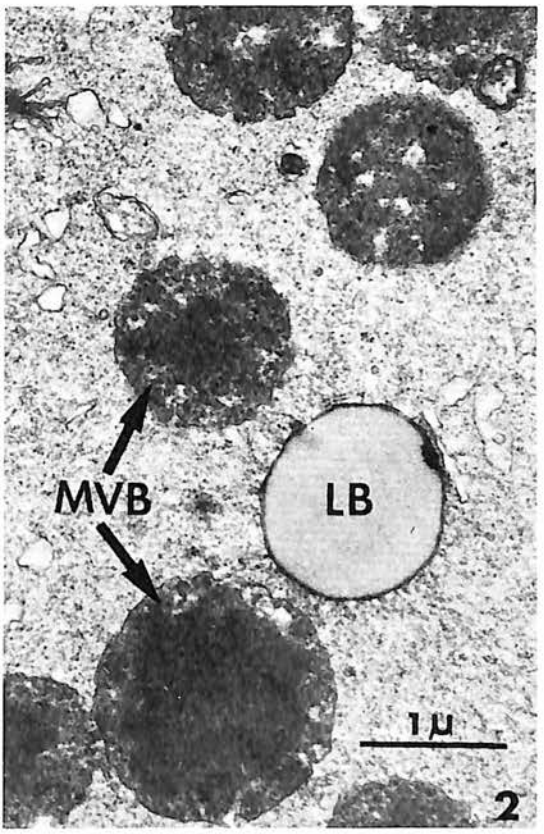
FIG. 1. — *A large number of multi-vesicular bodies* (MVB) *are seen around the oocyte nucleus* (N).

FIG. 2. — *Lipid bodies* (LB) *are found scattered amongst the multivesicular bodies* (MVB).

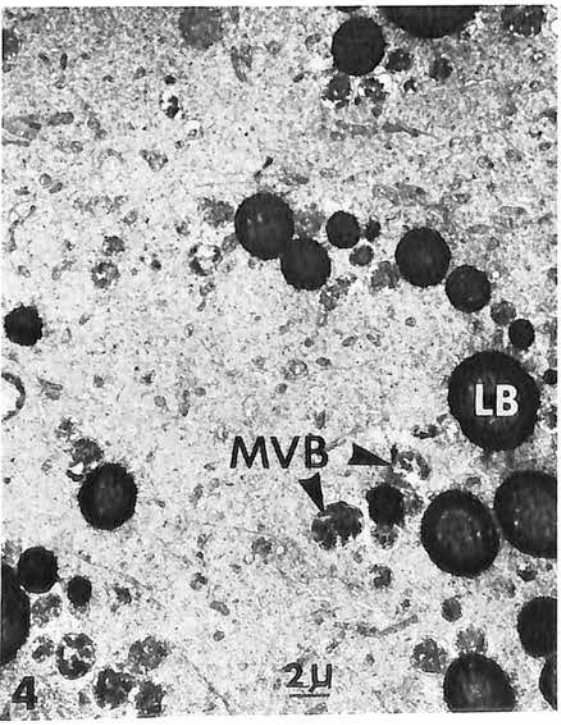
FIG. 3 and 4. — *Multi-vesicular bodies* (MVB) *and lipid bodies* (LB) *are dispersed in the cytoplasm.* Note large lipid bodies exhibiting concentric zonation. N : oocyte nucleus.

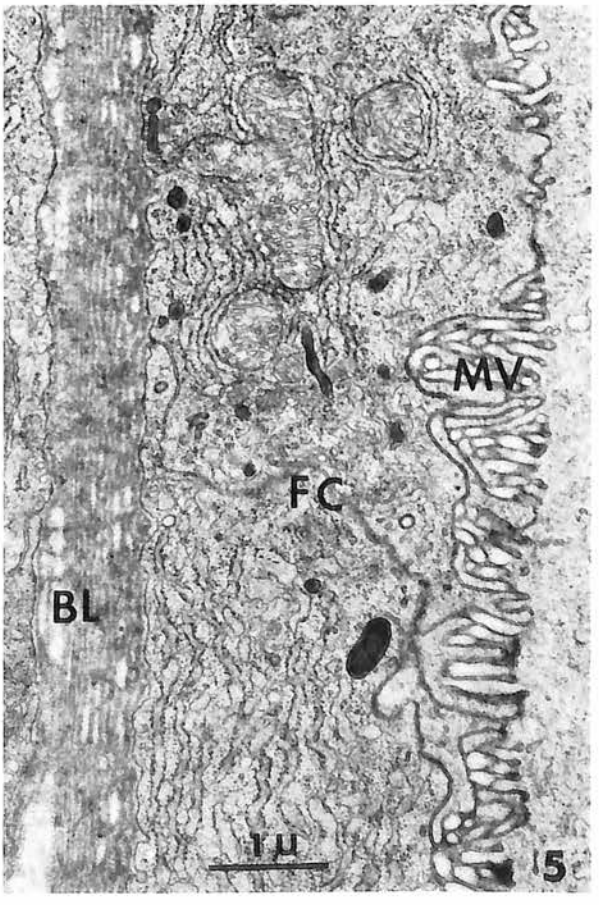
FIG. 5. — *Oocyte bound with a basal lamina* (BL) *and a single layer of follicle cells* (FC). Note microvilli (MV) projecting from the oocyte surface.


MVB
N
2μ
1


MVB
LB
1μ
2


MVB
N
LB
2μ
3


LB
MVB
2μ
4


MV
FC
BL
1μ
5

had MVB and lipid bodies dispersed throughout the cytoplasm (fig. 3 and 4). The most obvious difference observed in the oocytes of this size range was that the lipid bodies exhibited concentric zonation (fig. 3 and 4). In all the three categories of oocytes no zona radiata was formed and no evidence for the deposition of exogenous yolk by the process of pinocytosis was observed (fig. 5). Therefore, MVB and lipid bodies can be considered as constituent of endogenous yolk formed in the oocyte cytoplasm in response to S-GTH treatment.

Treatment with S-GTH (0.1 μg/g) in combination with estradiol-17β (1 μg/g) produced similar results as described for the treatment with S-GTH alone. However, treatment with estradiol-17β alone did not initiate synthesis of endogenous yolk in the oocyte cytoplasm.

The oocytes of fish treated with S-PE (10 μg/g) were considerably advanced as compared to those seen after treatment with S-GTH alone or in combination with estradiol-17β. The oocytes measuring 580 to 787 μ in diameter were characterized by the formation of a distinct and continuous zona radiata (fig. 6), cytoplasmic vesicles (probably yolk vesicles) of variable sizes distributed throughout the cytoplasm (fig. 7), and an extensive pinocytotic activity at the oocyte membrane (fig. 8). The induction of pinocytotic activity following S-PE treatment indicates the incorporation of exogenous yolk or vitellogenin into the oocyte. S-PE prepared from male and female pituitaries had similar effects.

Discussion.

This study demonstrates that S-GTH, whether purified from male or female pituitaries, does not differ in its qualitative properties. S-GTH (0.1 or 0.5 μg/g) induces an endogenous synthesis of yolk in the oocyte cytoplasm but fails to initiate the incorporation of exogenous yolk into the oocyte which would be necessary for the completion of vitellogenesis. S-GTH in combination with estradiol-17β has a similar effect on the oocyte development. Estradiol-17β alone does not induce vitellogenesis. In contrast, S-PE treatment induces an extensive pinocytotic activity at the oocyte membrane in addition to an endogenous synthesis of yolk in the oocyte cytoplasm. The pinocytotic activity has been considered as an indicative of the incorporation of exogenous yolk or vitellogenin into the oocyte (Wallace and Bergink, 1974).

A comparison of these results suggests that there may be other pituitary hormone(s) involved in the process of the incorporation of exogenous yolk or vitellogenin

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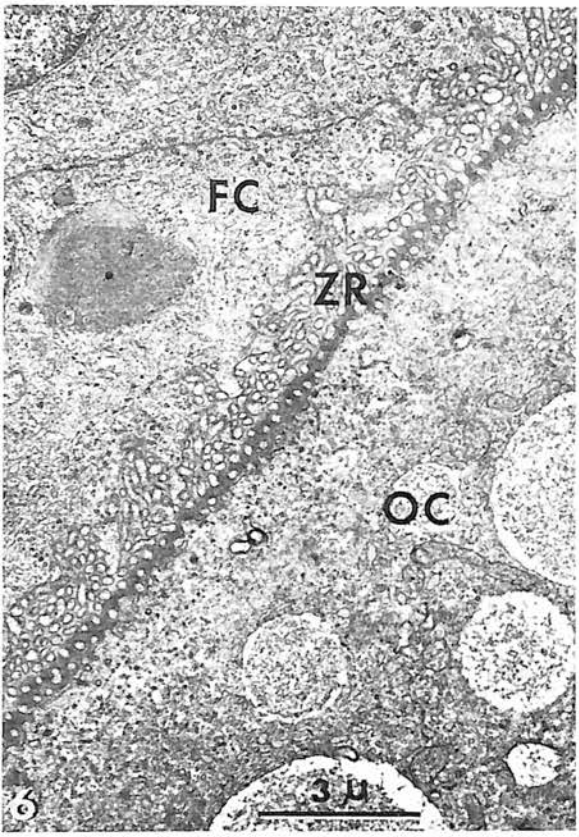
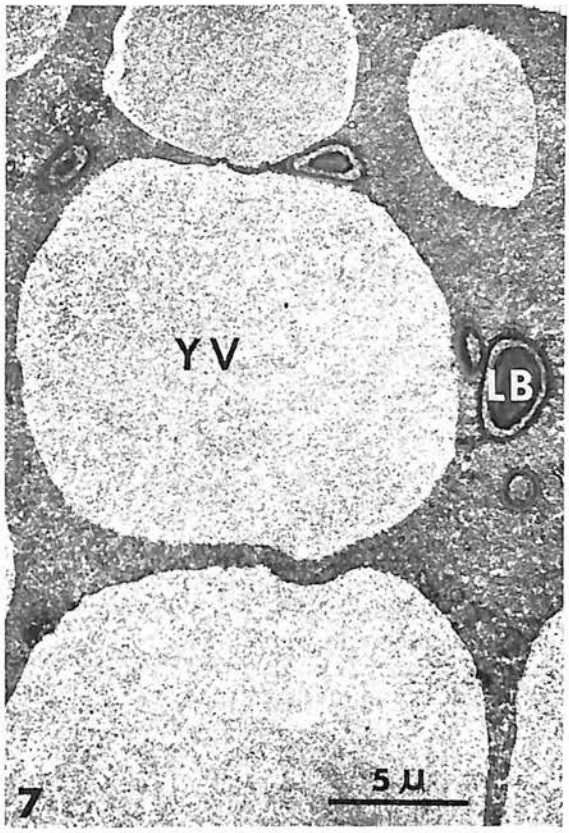
Electron micrographs of portions of oocyte from the ovary of juvenile rainbow trout treated with salmon-pituitary extract.

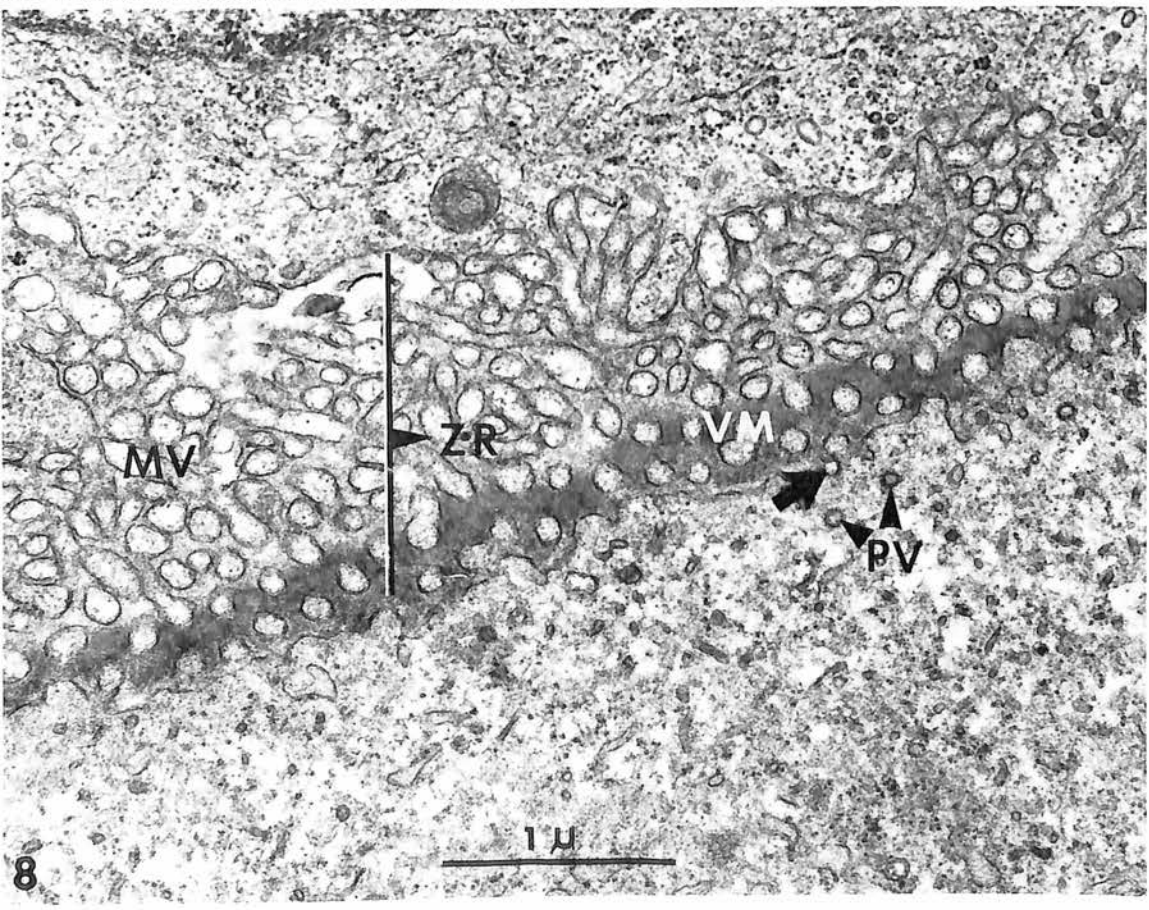
FIG. 6. — *A continuous zona radiata* (ZR) *is observed between follicle cells* (FC) *and the oocyte* (OC).

FIG. 7. — *Oocyte cytoplasm showing yolk vesicles* (YV).

FIG. 8. — *Zona radiata* (ZR) *is composed of an inner zone of vitelline membrane* (VM), *an outer zone of microvilli* (MV). Note extensive pinocytotic activity at oocyte periphery. Pinocytotic vesicles (PV) arise at the base of microvilli (arrow).


FC
ZR
OC
3 μ
6


YV
LB
5 μ
7


MV
ZR
VM
PV
1 μ
8

into the oocyte, which may act alone or in synergism with gonadotropin. Campbell and Idler (1976) reported that a glycoprotein fraction from the pituitary of *Hippoglossoides platessoides* containing gonadotropic activity failed to stimulate incorporation of yolk in the hypophysectomized *Pseudopleuronectus americanus*.; in contrast a non-glycoprotein fraction of these pituitaries stimulated yolk incorporation into the ovary.

The present results are in contrast to the conclusions of Donaldson (1973) and Burzawa-Gérard (1974), who believe that a single pituitary gonadotropin is capable of stimulating all the processes related to oocyte development in teleosts.

S-GTH has been found to stimulate the steroidogenic features of ovarian interstitial cells and protein synthetic activity in the liver parenchymal cells of female juvenile rainbow trout (Upadhyay, 1977) ; the latter action is most probably mediated by ovarian estrogens.

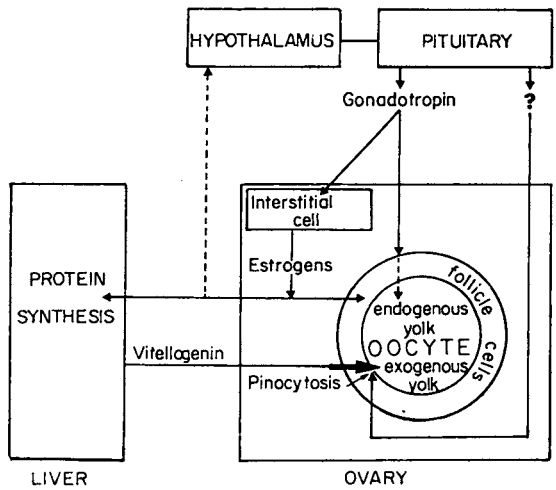


FIG. 9. — *Generalized scheme showing major vitellogenic relationship and response of* Salmo gairdneri.

In conclusion, the major vitellogenic responses and relationships in rainbow trout are illustrated schematically in figure 9.

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Résumé. Des truites Arc-en-ciel juvéniles pesant entre 10 et 20 g ont reçu trois fois par semaine pendant 10 semaines des injections intrapéritonéales de gonadotropine de saumon (S-GTH), des extraits hypophysaires (S-PE), de S-GTH + estradiol 17β (E_2) et E_2 seul. Les effets de ces traitements sur les ovocytes ont été observés en microscopie électronique.

Les animaux témoins recevant une solution saline ont des ovocytes au stade prévitellogenèse. S-GTH (0,1 or 0,5 μg/g) induit une synthèse de vitellus endogène dans le cytoplasme de l'ovocyte, mais ne peut induire l'incorporation de vitellus exogène ou vitello-

génine dans les ovocytes. S-GTH (0,1 µg/g) associé à E_2 produit des effets similaires à S-GTH seule. Au contraire S-PE (10 µg/g) induit l'incorporation de vitellus exogène dans l'ovocyte comme l'indique une intense activité de pinocytose au niveau de la membrane de l'ovocyte.

Il est suggéré que GTH est nécessaire pour l'induction de la synthèse endogène de vitellus et qu'une ou plusieurs autres hormones hypophysaires sont impliquées dans l'incorporation du vitellus exogène dans l'ovocyte.

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Studies on the rainbow trout (*Salmo gairdneri* Rich.). I. Correlation between gonadal development and serum protein pattern

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Summary. Maturity was studied on 273 rainbow trout of both sexes. The female gonads were classed into 3 maturation stages and the male gonads into 4. Characteristic pherograms of both serum protein electrophoresis and total serum protein corresponded with maturation stage. Electrophoretic globulin and total protein fractions increased considerably with spermatogenetically active testes and mature ovaries ; the A/G ration decreased accordingly. These parameters can be used to determine maturity.

Introduction.

Several reports have been published on the serological parameters of fish. The general serology of rainbow trout has also been studied (Perrier *et al.*, 1973, 1974, 1976; Saifi, 1969, Delcroix, 1972). The serum protein pattern has been shown to be affected by diseases (Riedmüller, 1971), toxic substances (Thurston, 1967) and seasonal variation such as water temperature (Schlotfeldt, 1975). Haider (1970) has reported pherogram differences according to age. Considerable work has been carried out on female fish indicating that specific female proteins occur in the blood during gonad maturations (Drilhon and Fine, 1963 ; Thurston, 1967). The present study was done to determine if there are also changes in serum protein during testis growth. The gonads were classed according to maturation stage and the serum protein pattern changes occuring at different stages of testis and ovary maturation were noted.

Material and methods.

Rainbow trout of one and the same group were used. From October 1975 to May 1977, blood samples were taken from 168 spawning fish (medium length : 35.1 cm; medium weight : 477.4 g ; age : 31 to 50 months) and 95 fingerlings (medium length: 23.4 cm ; medium weight : 98.1 g ; age : 8 to 20 months). Ten rainbow trout of a different group from another pond were used as controls. Blood samples were usually taken once a week from all the 273 fish by cardiac puncture (Lehmann and Stürenberg, 1974). This technique required the use of a holder. The unanesthetized fish

was held down and a sterile needle (Supra Rekord Typ M, 40 mm long, 1.40 mm ⌀) injected carefully and nearly vertically through the skin exactly between the pectoral fins into the heart ventricle. Immediately after the needle penetrated the cardiac wall, the blood was withdrawn drop by drop with the heartbeats. After the first 2 drops were discarded, the blood was collected in a small glass tube. Each sample was stored for 10 min. at 4 °C to allow clotting ; it was then centrifuged at 5 000 g for 10 min. Only the serum of sound fish was fractionated ; some was used at once for analysis, and the rest was deep-frozen at — 80 °C for further utilization. Several organs were excised for further study.

Electrophoresis. — Micro-electrophoresis on cellulose acetate strips (Boskamp) was used to separate the serum proteins. In preliminary experiments the usual method of electrophoresis on cellulose acetate was modified. The optimal separation was reached at a running time of 24 min., voltage 250, Michaelis buffer at pH 8.5 ; strip staining was done with amido black staining liquid. Pherogram quantity was evaluated with an automatic pherostat (Super Cellomatic, CELTEC).

Determination of total protein and albumin. — The total serum protein of each trout was analysed. We used a modified Biuret method (Weichselbaum, 1946) with 20 µl serum and 2 ml Biuret reagent. With copper salts the proteins formed a stained complex ; the staining intensity of this complex was proportionate to protein concentration and was determined photometrically. For hyperlipemic or hemolytic serum, we subtracted blank-value absorbance from that of the Biuret complex.

Albumin was analyzed photometrically using the Bromocresol blue technique (Schirardin and Ney, 1972). This works very well with human serum, but with fish serum the values may differ from those obtained with electrophoresis, especially when hyperlipemic serum is involved. Nevertheless, with this technique a protein fraction can be classed as an albumin or a globulin. Further mention of « albumin » « globulin » or the « albumin-globulin (A/G) ratio » in this report must be regarded as limited since we did not exactly identify the single protein fractions during this study.

Quality control. — A definite human control serum and a self-made serum pool consisting of about 200 rainbow trout serums were used for control of integrity and precision. This quality control was carried out for the electrophoretic and photometric determinations. Statistical variations were in the range of $\pm$ 2.5.

Histological preparation. — The gonads were fixed in Bouin solution and embedded in paraffin. The 6 to 8 µ-thick sections were stained using the Aldehyde-Fuchsin-Goldner method.

Results.

Gonad maturation stages.

The ovaries were divided into 3 stages :

Stage I : ovary with small, immature eggs ; zona radiata and yolk mass not present ;

Stage II : nearly mature eggs with yolk, shortly before ovulation ; follicular epithelium and zona radiata already entirely developed ;

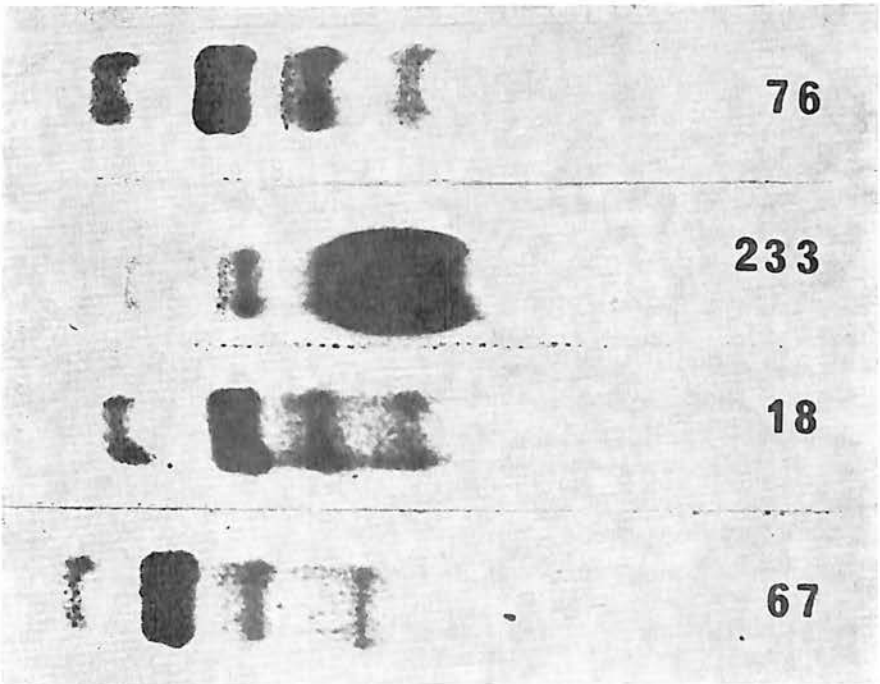


FIG. 1. — *Electrophoretic pherograms of male trout.* 76 : maturation stage I ; 233 : maturation stage II ; 18 : maturation stage III ; 67 : maturation stage IV.

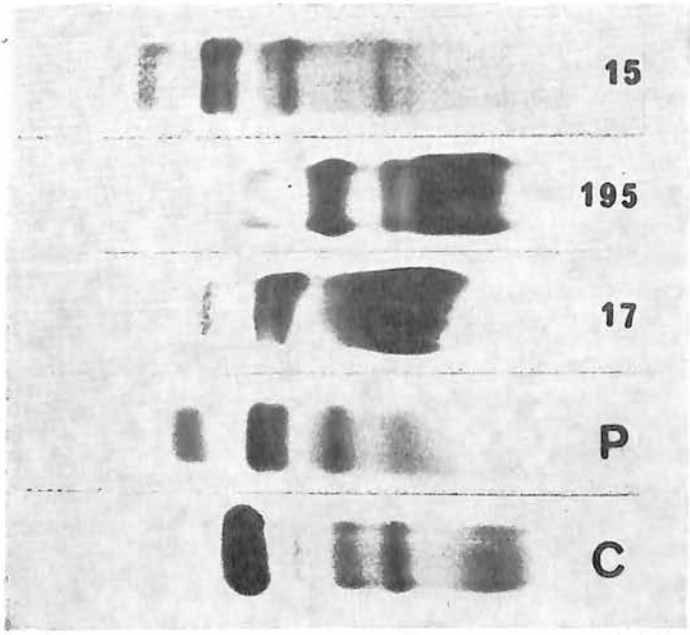


FIG. 2. — *Electrophoretic pherograms of female trout.* 15 : maturation stage I ; 195 : maturation stage II ; 17 : maturation stage III ; C : human control serum ; P : serum pool of 200 rainbow trout.

Stage III : mature eggs with big yolk mass lying free in the coelom ; follicular cells already discarded.

The testes were classified into 4 maturation stages :

Stage I : juvenile testis with small tubules not yet clearly separated ; interstitium only weakly developed ;

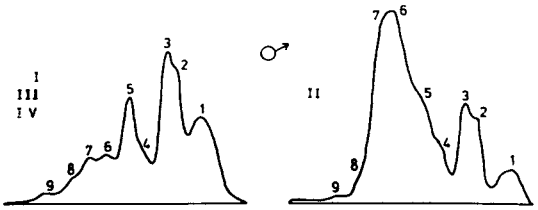
Stage II : testis at onset of spermatogenesis ; tubule diameter and interstitium highly enlarged ;

Stage III : nearly mature testis with highly enlarged interstitium and tubules with spermatogonia, spermatocytes and spermatozoa in the lumen ;

Stage IV : mature testis with no new formation of spermatozoa ; tubule lumina filled with mature sperms.

Electrophoresis.

Differences corresponding to maturation stages appear in the pherograms of male and female fish. Figures 1 and 2 show the pherograms of some subjects at different stages of maturity. The strong increase of the protein fractions in fishes 233 (fig. 1), 195 and 17 (fig. 2) is especially evident.



Fraktion	I		II		III		IV	
	g/100 ml	S	g/100 ml	S	g/100 ml	S	g/100 ml	S
1	0,50	0,23	0,28	0,23	0,82	0,22	0,65	0,30
2	0,65	0,18	0,43	0,38	0,88	0,16	0,54	0,23
3	0,58	0,19	0,35	0,28	0,59	0,29	0,67	0,17
4	0,21	0,08	0,14	0,10	0,26	0,09	0,21	0,07
5	0,36	0,12	1,90	1,02	0,56	0,21	0,60	0,13
6	0,24	0,08	2,95	2,79	0,32	0,12	0,24	0,07
7	0,35	0,12	1,41	1,32	0,36	0,11	0,25	0,11
8	0,08	0,04	0,18	0,13	0,10	0,05	0,11	0,06
9	0,01	0,01	0,01	0,01	0,03	0,07	0,03	0,05

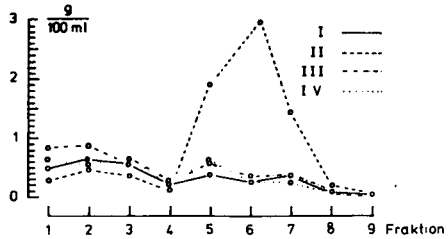
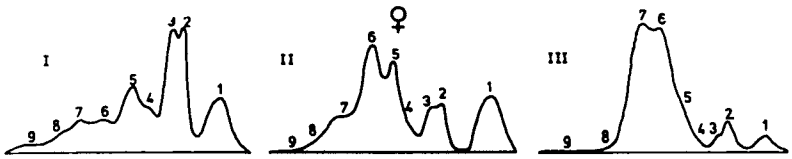


FIG. 3. — *Integral graphs showing pherograms, average means, standard deviations ; graphs of single protein fractions of male rainbow trout at maturation stages I, II, III and IV.*

As single protein fractions have not yet been characterized biochemically, they are indicated by numbers 1 to 9, corresponding to decreasing mobility. The integral graphs and the table of figure 3 show that there are no differences in maturation stages I, III and IV in the males. In maturation stage II the fractions 5, 6, and 7 quantitatively increase sharply. The same may be said of the female animals (fig. 4) in which protein fractions 5, 6 and 7 increase strongly with advancing maturity.



Fraktion	I		II		III	
	g/100 ml	S	g/100 ml	S	g/100 ml	S
1	0,52	0,20	0,49	0,22	0,32	0,16
2	0,59	0,26	0,60	0,33	0,29	0,25
3	0,60	0,22	0,50	0,23	0,30	0,19
4	0,21	0,09	0,20	0,06	0,15	0,10
5	0,47	0,12	0,67	0,18	0,66	0,42
6	0,29	0,11	1,03	1,06	1,92	1,25
7	0,35	0,16	0,59	0,33	0,63	0,82
8	0,10	0,07	0,37	0,10	0,11	0,06
9	0,01	0,01	0,01	0,04	0,01	0,05

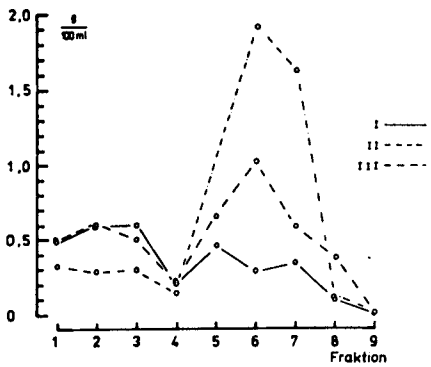


FIG. 4. — *Integral graphs showing pherograms, average means, standard deviations ; graphs of single protein fractions of female rainbow trout at maturation stages I, II and III.*

Total protein, A/G ratio and gonad weight.

The total protein rise with progressive maturity from a mean of 3 g p. 100 to 5 g p. 100 in the females (fig. 5). In the males there is a clear maximum of 7.5 g p. 100 at maturation stage II, while the values of the other maturation stages lie between 3 and 4 g p. 100.

A comparison of the photometric albumin analysis with the electrophoretic values shows that the protein infractions 1, 2 and 3 must be albumins. The A/G ratio was ascertained accordingly. This ration is graphically the reverse of total protein (fig. 5). Gonad weight also increases with maturation.

Discussion.

Electrophoretic and total protein fractions in the serum of male and female rainbow trout change with the stage of gonadal maturation. During this process the total protein increases in stage II males ; this increase mainly results from an increase of serum protein electrophoretic fractions 5, 6 and 7 which are globulins. The A/G ratio decreases accordingly at maturation stage II. This maturation stage is characterized

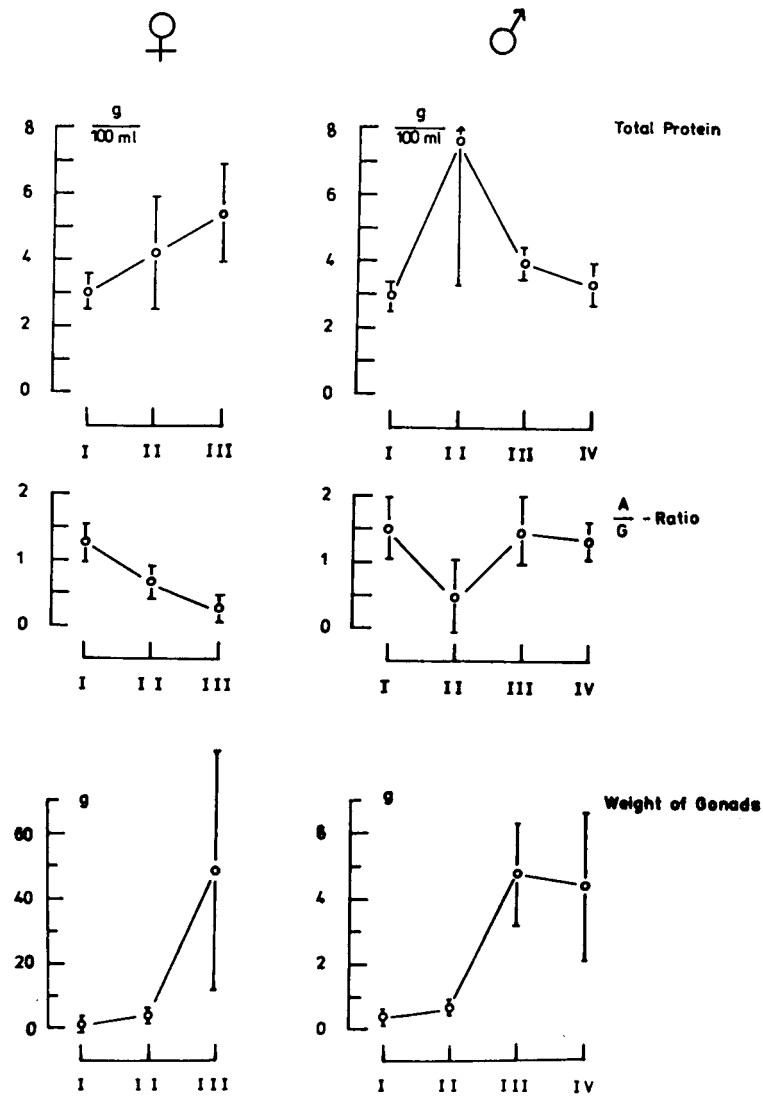


FIG. 5. — *Graphs showing total protein, A/G ratio and weight of male* (right) *and female* (left) *gonads at maturation stages I, II, III and IV and I, II and III, respectively.*

by strong spermatogenic activity and increased tubule diameter. Large quantities of building material are probably necessary for testicular synthesis and the development of spermatocytes and spermatozoa ; this material is transported in the blood to the testis as a protein of globulin fractions 5, 6 and 7. However, only little or no building protein is needed if spermatogenic activity is weak (maturation stage III), or if the testes have mature sperms (maturation stage IV). These differences found in the serum protein of male trout during spermatogenesis have not been previously reported by other workers.

Conditions are slightly different in the female fish. The gonadal products are not formed over a short period and stored in the gonads until spawning time. On the contrary, the maturation of ovary and eggs, and especially yolk accumulation, continue over a longer interval. Accordingly, during maturation stages II and III, the total protein and globulin fractions 5, 6 and 7 are high as they are probably precursors of yolk protein. These results in female trout serum are comparable to those found by Thurston (1967) and Drilhon and Fine (1963) who reported a special protein fraction in the serum of mature females.

It is also evident that the pherograms of immature adult fish do not differ from those of fingerlings. Therefore, there are no age differences in any parameters studied, as described by other authors (Haider, 1970) ; the differences only reflect maturation stages.

As this is a preliminary study, the results will be confirmed with other parameters, and the separated electrophoretic fractions will have to be defined by physicochemical characters.

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Résumé. La maturité sexuelle a été étudiée sur 273 truites Arc-en-ciel des deux sexes. Les gonades femelles ont été classées en 3 stades de maturation et les gonades mâles en 4 stades. Les caractéristiques de l'électrophorègramme des protéines sériques identifiées par électrophorèse et les protéines sériques totales correspondent aux stades de maturation. Les globulines électrophorétiques et des fractions de protéines totales augmentent considérablement avec l'activité spermatogénétique des testicules actifs et la maturité des ovaires ; le rapport A/G décroît parallèlement. Ces paramètres peuvent être utilisés pour déterminer la maturité.

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The effects of alterations in photoperiod on ovarian development and spawning time in the rainbow trout (*Salmo gairdneri*)

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Summary. The present study investigates the changes in serum œstradiol 17β, total calcium and phosphoprotein (as a measure of vitellogenesis) under a normal seasonal 12 month photoperiod cycle, normal yearly cycles compressed into 9 and 6 months, and a constant 12 hour light/12 hour dark regime and the effects of these experimental photoperiod regimes on the times of spawning of both male and female rainbow trout.

Spawning was advanced by 6 weeks under the 9 months photoperiod regime, and by 12 weeks under the 6 month regime in comparison with the normal cycle. The levels of œstradiol 17β, calcium and phosphoprotein demonstrated under the simulated normal yearly cycle were markedly altered in female fish under the other photoperiod regimes and in each case coincided with the alterations in spawning time, thus indicating the involvement of these materials in the control of the processes of vitellogenesis and spawning.

These results indicate that photoperiod is the major environmental factor in the control of the sequence of endocrine and other physiological changes which ultimately lead to spawning in salmonids and suggest that photoperiod may act by modifying an inherent reproductive rhythm.

Introduction.

In seasonally-breeding fish many factors from both the external and internal environments are thought to act as cues for the initiation of the complex series of neuroendocrine changes which exert overall control of the processes of gonadal maturation and spawning. Amongst the large number of factors reported in the literature, photoperiod and temperature are the most frequently mentioned (see de Vlaming, 1972) although the relative importance of each factor varies with different species of fish. Previous work suggests that photoperiod exerts the major controlling influence in salmonids (Alison, 1951 ; Hazard and Eddy, 1951 ; Nomura, 1962 ; Henderson, 1963 ; Carlson and Hale, 1973 ; Kunesh *et al.*, 1974) although in some of these studies other environmental factors besides photoperiod varied during the course of the experiments.

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Additionally, apart from Sundararaj and Sehgal (1970) with catfish and Breton and Billard (1977) and Whitehead *et al.* (1977*a*, 1977*b*) with rainbow trout, few workers have studied the various physiological and endocrine changes which occur in response to these environmental triggers, and which initiate and control the onset and course of maturation and spawning.

In view of these difficulties the present work, whilst maintaining close control of all other environmental variables, investigates firstly the effect of various photoperiod regimes on spawning in the rainbow trout and, secondly, the dynamic changes in serum levels of oestradiol 17β, calcium and phosphoprotein (as a measure of vitellogenesis) which accompany this process.

Materials and methods.

Four groups of 20 immature, 2-year old, male and female rainbow trout (*Salmo gairdneri*), each weighing approximately 1.3 kg, were maintained in light-proof aquaria and exposed to four different photoperiod regimes provided by fluorescent light and controlled by electric time clocks adjusted once per week. The 4 photoperiod regimes under investigation (shown in fig. 1) were as follows :

1. Normal 1 year seasonal cycle (control) in which July 4th was the longest day and December 20th the shortest.
2. Normal seasonal cycle compressed into 9 months in which June 4th was the longest day and October 20th the shortest.
3. Normal seasonal cycle compressed into 6 months in which May 4th was the longest day and August 8th the shortest.
4. Constant 12 hr light/12 hr dark regime.

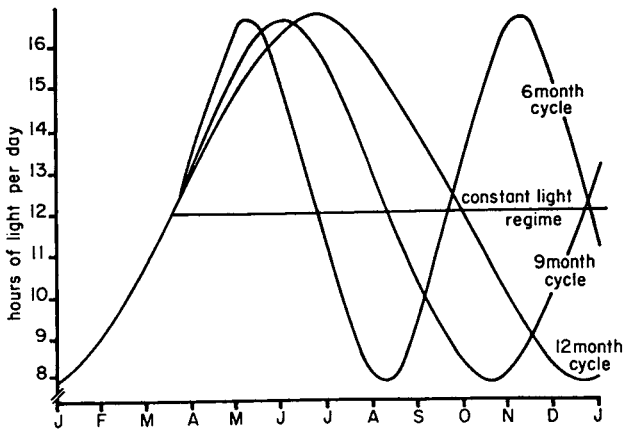

FIG. 1. — *Photoperiod regimes under investigation.*

In all groups, experimental conditions were closely controlled ; water from a borehole supply was maintained at 9 °C throughout the experiment, with a constant flow rate, a dissolved oxygen content of 100 p. 100 in the effluent, and a pH of 6.6.

The fish were fed on a proprietary diet at 0.5 p. 100 of the body weight per day. Blood samples were withdrawn from 10 fish in each group, via the Cuverian vein, in March at the start of the experiment and each month thereafter. The number of female fish in each group varied as indicated in tables 1-4. The serum thus obtained was assayed for oestradiol 17β, total calcium and phosphoprotein by the following methods :

1. *Oestradiol* 17β was assayed by radioimmunoassay using a modification of the method of Leonard and Craig (1974) in which a serum sample of only 200 μl was used and in the extraction phase the sample was brought within the correct standard range by taking a 500 μl aliquot of the original 5 ml extractant. The standard curve on all occasions exhibited full parallelism with dilutions of rainbow trout sera. The cross-reactivity of the antisera was 10 p. 100 with oestriol, 3 p. 100 oestrone and 1 p. 100 with testosterone relative to oestradiol.

2. *Total serum calcium* was assayed by a fluorimetric method using a Corning calcium analyser model 940.

3. *Serum phosphoprotein* was assayed by measurement of its phosphorus content (Boehringer Corporation Test Handbook, 1969) with the following modifications : A 100 μl serum sample was taken and the proteins precipitated with 5 ml trichloroacetic acid. The precipitate was isolated, washed successively with a series of organic solvents to remove lipid (Wallace and Jared, 1968) and the resultant protein pellet dried and assayed for phosphoprotein phosphorus. These levels may be converted to vitellogenin by multiplying 71.4, assuming that fish, like other vertebrates investigated have 1.4 p. 100 of their vitellogenin as phosphoprotein phosphorus.

Results.

The various photoperiod regimes produced no adverse physiological or behavioural changes in the experimental fish and, apart from variations in spawning time, they were indistinguishable from fish of the same age maintained in outside ponds under natural environmental conditions. The eggs obtained from each group were fertilized by hand-stripping and showed normal development. Egg size was uniform, irrespective of spawning time (4.7 $\pm$ 0.2 mm), and there were no differences in fecundity (2 000 eggs/kg body weight). In all groups the egg survival up to hatching was 75-80 p. 100. Food conversion was stable between 1.3 and 1.6 until the fish were in spawning condition, at which time a slowing down of feeding occurred and preliminary data also suggests an associated loss of conversion efficiency.

As maturation proceeded, gross changes were seen in the serum levels of oestradiol 17β, phosphoprotein and calcium in all female fish, although the onset and duration of these changes was affected in different ways by each of the four photoperiod regimes. All fish, irrespective of sex or treatment, had similar resting or basal levels of total calcium (12 $\pm$ 2 mg p. 100, i. e. mean $\pm$ standard error of the mean) and phosphoprotein phosphorous (25 $\pm$ 7 μg/ml) during the months April to July and oestradiol 17β (130 $\pm$ 15 pg/ml) in April and May.

TABLE 1

Levels of oestradiol 17β, phosphoprotein phosphorus and total calcium in serum of female rainbow trout under a normal 12 month photoperiod regime (n = 7)

	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.
Oestradiol 17β (pg/ml)	122	128	234	371	886	3 471	4 808	4 107	2 729	310	130
± standard error	11	11	29	50	126	549	1 105	666	667	109	12
Phosphoprotein phosphorus (μg/ml)	15.3	20.0	39.3	34.7	54.3	93.3	176.3	206.6	255.9	400.9	132.9
± standard error	5.4	2.1	8.0	6.6	7.5	14.9	25.7	38.8	55.3	66.9	23.1
Total calcium (mg p. 100)	10.5	11.9	14.2	14.3	15.6	21.9	33.0	36.1	43.6	57.5	30.3
± standard error	0.8	0.2	0.2	0.5	0.5	1.0	3.5	5.0	7.2	5.4	2.8

TABLE 2

Levels of oestradiol 17β, phosphoprotein phosphorus and total calcium in serum of female rainbow trout under a normal seasonal photoperiod compressed into 9 months (n = 5)

	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.
Oestradiol 17β (pg/ml)	22	157	184	292	707	2 690	3 310	2 380	639	107	153
± standard error	8	7	21	48	133	257	429	297	339	17	11
Phosphoprotein phosphorus (μg/ml)	15.9	17.1	26.0	19.1	64.8	273.5	308.0	344.2	601.6	324.0	82.0
± standard error	9.6	5.0	4.7	3.0	14.9	78.9	25.9	47.8	79.4	90.3	33.0
Total calcium (mg p. 100)	12.5	12.4	12.8	12.7	16.2	41.7	57.1	63.3	81.0	54.4	20.9
± standard error	0.2	0.3	0.6	0.7	1.5	9.4	12.3	13.8	10.9	9.9	7.0

Although the time of ripening and spawning of the male fish under each of the photoperiod regimes occurred at the same time as the female fish in each of the four experimental groups, the serum oestradiol 17β, phosphoprotein phosphorous and total calcium remained at basal level throughout the experiment, irrespective of the photoperiod regime. The changes in the female fish induced by the various light regimes are described in more detail below.

1. *Normal 1 year seasonal cycle* (see table 1). — The fish in this group spawned in mid-January at exactly the same time as broodstock maintained outside in open ponds. Between June and July, serum oestradiol 17β levels increased in the female fish from basal levels to a peak of 4 800 ± 1 000 pg/ml in October and then returned to resting levels before the onset of spawning in mid-January (fig. 2). By October the

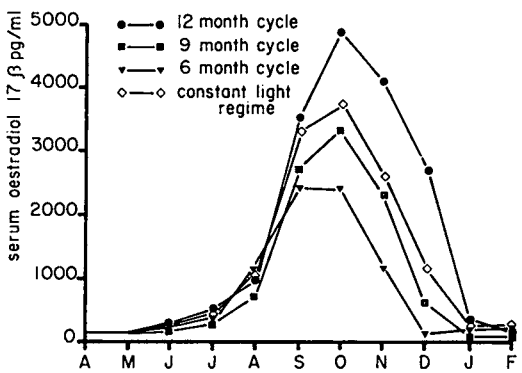

FIG. 2. — *Sequential changes in serum oestradiol 17β under a normal 12 month photoperiod, normal cycles compressed into 9 and 6 months, and a constant 12-hr light/12 hr dark regime.*

serum levels of phosphoprotein phosphorous and calcium of female fish had both begun to rise, reaching peaks of 400 ± 60 pg/ml and 58 ± 10 mg p. 100 respectively in January (figs. 3 and 4).

2. *Normal seasonal cycle compressed into 9 months* (see table 2). — Spawning in this group occurred in early December, 6 weeks earlier than fish from either the control photoperiod or the outside ponds. Between June and July serum levels of oestradiol 17β in the female fish started to increase, reaching a peak of 3 300 ± 400 pg/ml in October, before returning to resting levels just prior to spawning in early December (fig. 2). Serum levels of phosphoprotein phosphorous and calcium in the female fish were both increased in August, reaching peaks of 600 ± 80 μg/ml and 80 ± 20 mg p. 100 respectively, just prior to spawning (figs. 3 and 4).

3. *Normal seasonal cycle compressed into 6 months* (see table 3). — In this group, spawning occurred in mid-October, approximately 3 months earlier than fish from either the control photoperiod or the outside ponds. Serum levels of oestradiol 17β in the females started to increase in July, reaching a peak of 2 500 ± 500 pg/ml in late September, but returning to basal levels in December (fig. 2). By August, levels

of serum phosphoprotein phosphorous and total calcium had significantly increased, reaching peaks of 680 ± 70 μg/ml and 88 ± 10 mg p. 100 respectively in October just before spawning (figs. 3 and 4).

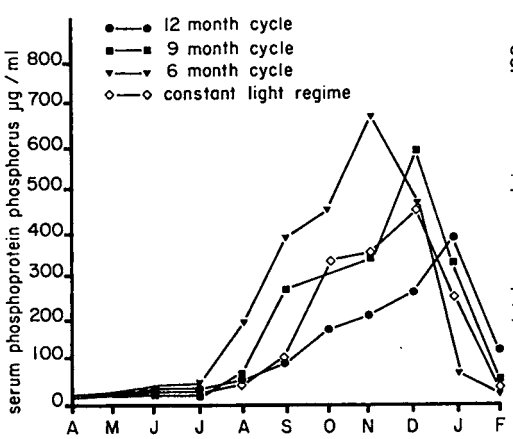

FIG. 3.

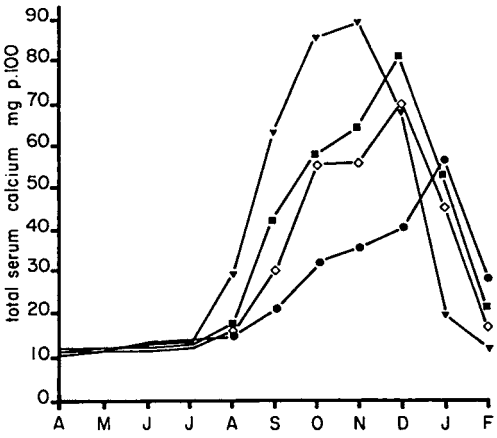

FIG. 4.

FIG. 3. — *Sequential changes in serum phosphoprotein phosphorus under a normal* 12 *month photoperiod, normal cycles compressed into* 9 *and* 6 *months, and a constant* 12 hr *light*/12 hr *dark regime.*

FIG. 4. — *Sequential changes in total serum calcium under a normal* 12 *month photoperiod, normal cycles compressed into* 9 *and* 6 *months, and a constant* 12 hr *light*/12 hr *dark regime.*

4. *Constant* 12 *hr light*/12 *hr dark regime* (see table 4). — Spawning in this group occurred in January at approximately the same time as the control group. Serum levels of oestradiol 17β in the females started to increase in July, reaching a peak of 3 800 ± 820 pg/ml in October before returning to basal levels in January, at which time spawning occurred (fig. 2). Both serum phosphoprotein phosphorous and total calcium, began to increase during August up to peaks of 480 ± 60 μg/ml and 70 ± 14 mg p. 100 respectively in December, just before the onset of spawning (figs. 3 and 4).

Discussion.

The present study demonstrates clearly that, in the rainbow trout, photoperiod plays a major role in the initiation of the series of changes in reproductive function which ultimately leads to spawning.

Under the influence of shortened photoperiod, spawning was advanced by 6 and 12 weeks in regimes where the normal yearly cycle was compressed into 9 and 6 months respectively. In both groups early spawning was accompanied by modifications of the normal sequential cycle of serum oestradiol 17β, calcium and phosphoprotein. Under each of the experimental regimes these changes appeared to be initiated by the decreasing phase of the light regimes which occurred after the longest day. The normal sequential changes in serum oestradiol 17β, calcium and vitelloge-

TABLE 3

Levels of oestradiol 17β, phosphoprotein phosphorus and total calcium in serum of female rainbow trout under a normal seasonal photoperiod compressed into 6 months (n = 4)

	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.
Oestradiol 17β (pg/ml)	129	158	229	320	1 191	2 425	2 413	1 170	108	172	178
± standard error	17	19	23	30	219	589	380	679	33	60	14
Phosphoprotein phosphorus (μg/ml)	19.4	23.3	35.8	41.3	195.5	397.3	456.8	671.5	459.5	77.5	26.0
± standard error	10.5	4.6	7.9	5.5	47.7	47.8	20.5	76.8	113.0	29.3	6.6
Total calcium (mg p. 100)	13.1	12.2	13.4	14.0	29.1	64.6	85.6	90.1	69.9	20.3	13.1
± stantard error	0.4	0.6	0.7	1.3	4.1	7.5	6.7	6.2	15.1	5.5	0.7

TABLE 4

Levels of oestradiol 17β, phosphoprotein phosphorus and total calcium in serum of female rainbow trout under a constant 12 hr light/12 hr dark photoperiod (n = 4)

	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.
Oestradiol 17β (pg/ml)	130	140	220	425	1 106	3 300	3 725	2 550	1 162	82	147
± standard error	20	22	43	58	346	1 153	827	496	181	9	22
Phosphoprotein phosphorus (μg/ml)	15.6	14.4	32.2	24.0	52.5	107.0	337.0	345.3	481.3	241.5	60.3
± standard error	8.6	1.5	7.4	1.7	14.0	27.0	75.2	91.8	61.1	39.9	12.1
Total calcium (mg p. 100)	12.0	13.0	13.3	13.2	15.8	30.6	57.0	55.6	69.2	46.1	18.1
± standard error	0.3	0.3	0.3	0.2	0.9	6.1	13.1	13.3	9.7	5.9	1.7

nin seen in fish under natural conditions (Whitehead *et al.*, 1977*a*) were altered significantly by shortened light regimes and under both regimes the serum levels of oestradiol 17β, total calcium and phosphoprotein reached peaks before the onset of spawning with the oestradiol returning to basal levels before ovulation. Since fish, like amphibians and birds, deposit large quantities of yolk in their ova, it is probable that the changes in oestrogen, the hypercalcaemia and increased phosphoprotein production are functionally inter-related. The sequence of events suggests that the control of vitellogenesis in female teleosts is similar to that already demonstrated in amphibians (Wallace and Bergink, 1974 ; Wallace and Jared, 1968). Similar changes have also been observed in the cod (Plack *et al.*, 1971). Furthermore, the rates of increase of serum calcium and phosphoprotein and the levels achieved before spawning were far greater in the groups under the 6 and 9 months photoperiod regimes than in the controls, probably reflecting an increased rate of vitellogenesis in preparation for early spawning in these groups.

Under the constant light and dark regime the changes in serum components, and spawning, occurred just before those under the normal yearly cycle apparently in the absence of a cue from decreasing photoperiod. This suggests that rainbow trout like many other seasonally-breeding vertebrates may have an endogenous rhythm of reproductive function, which is modified under the influence of photoperiod. This aspect could be further investigated in experiments using constant light regimes of different photoperiod length.

Although the measurements of gonadotropins are not reported here results of a collaborative research programme with Breton and Billard (unpublished) indicate that there are progressive increases in levels of serum gonadotropin during oocyte maturation and vitellogenesis which fall to basal values before the occurrence of a much higher spike of activity just before spawning. This preliminary data on gonadotropins together with the results of the present study clearly supports the hypothesis of a photoperiod control of reproduction by the pituitary probably via the hypothalamus with a subsequent induction by gonadotropic hormone of release of steroids from the gonad.

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Acknowledgements. — The authors are grateful to the British Oxygen Company for facilities at BOC Shearwater, to Dr. Clive Craik for his suggestions on the modification of the phosphoprotein assay and to the Interdisciplinary Higher Degrees Scheme at Aston for an IHD award to one of us (C. Whitehead).

Résumé. Cette étude porte sur les variations des taux sériques d'estradiol 17β, du calcium total et des phosphoprotéines (comme mesure de la vitellogenèse) durant un cycle photopériodique normal de 12 mois, des cycles annuels normaux compressés en 9 et 6 mois et un régime constant de 12 heures de lumière/12 heures d'obscurité. Les effets de ces régimes photopériodiques expérimentaux sont appréciés par les époques de reproduction des truites Arc-en-ciel mâles et femelles.

L'époque de reproduction est avancée de 6 semaines sous le régime photopériodique de 9 mois et de 12 semaines sous celui de 6 mois par rapport au cycle normal. Les niveaux d'estradiol 17β, calcium et phosphoprotéines observés sous le cycle annuel normal simulé

sont fortement modifiés chez les femelles soumises aux autres régimes photopériodiques et coïncident dans chaque cas avec les modifications de l'époque de reproduction, ce qui montre que ces composants sont impliqués dans le contrôle des processus de vitellogenèse et de fraie.

Ces résultats indiquent que la photopériode est le principal facteur de l'environnement impliqué dans le contrôle de la séquence des variations endocriniennes et physiologiques qui conduisent finalement à la reproduction des salmonidés et suggèrent que la photopériode peut agir en modifiant un rythme de reproduction inhérent.

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Seasonal effects of exposure to temperature and photoperiod regimes on gonad growth and plasma gonadotropin in goldfish (*Carassius auratus*)

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Summary. High temperatures inhibited goldfish (*Carassius auratus*) oogenesis at three different season(fall, winther, spring) in spite of an increase in plasma gonadotropin level. In winther, long photoperiod stimulated gonad growth. When fish were kept at a constant high temperature for 9 consecutive months from September to January, the gonads remained regressive during the first 4 months and then developed normally.

Introduction.

In the northern hemisphere, cyprinid fish spawn in spring when the water temperature reaches its maximum. In several species of cyprinid fish (*Couesius plumbeus* : Ahsan, 1966 ; *Carassius auratus* : Gillet *et al.*, 1977), a sudden rise of temperature in winter can apparently block gametogenesis in laboratory condition. In other species as *Lepomis cyanellus*, an increase of temperature associated with long photoperiod stimulates gonadal development in winter (Kaya and Hasler, 1972). These experiments suggest that temperature and photoperiod interact in the control of gametogenesis. In the present work, we have tried to analyze this phenomenon in the goldfish Endocrinological implications have also been investigated.

Material and methods.

In this experiment, we compared the effects of temperature either during a short 3-month period or over a long period extending from fall to summer. Adult goldfish from natural ponds were maintained :

— in fall from 19 September to 15 January at 20 °C ;
— in winter from 5 January to 6 March at 20 °C ;
— in spring from 15 March to 15 June at 20 °C or 30 °C.

At each period the control group was kept at the corresponding natural seasonal pond temperature (12 °C in fall and spring ; 10 °C in winter). In the long-term experi-

ment, the rearing temperatures were 30 °C. In all cases, two photoperiodic regimes (16L-8D and 8L-16D) were studied.

Thirty fishes, weighing about 50 g each were used in each experimental group. They were kept in 150-liter tanks with the temperature regulated to the nearest 1 °C. During acclimation water temperature was increased by 1.5 °C per day. Photoperiod was controlled by electric clocks. Fish were fed *ad libitum* twice daily with pellets « Aqualim » for carp.

Blood plasma gonadotropin (c-GTH) levels were measured at the end of each experiment and during the acclimation period in spring on 30 fisches. In all cases, blood samples were taken at the onset on the light phase. GTH levels were measured by radioimmunoassay, according to the technique described by Breton *et al.* (1971).

The gonado-somatic index (GSI) was also determined at the end of the experiments on 10 fishes. Results were analysed using one or two-way variance analysis.

Results.

I. *Effects of seasonal adaptation to high temperature.*

a) *GSI* (fig. 1A, B, C).

During fall, high temperature inhibed oogenesis recrudescence. On the contrary in the control group, ovaries which were very small at the beginning of the experiment were significantly ($P < 0.05$) enlarged at the end. Interaction ($P < 0.05$) between photoperiod and temperature were also observed ; growth of ovaries was stimulated at 12 °C under short photoperiod (8L-16D) and at 20 °C under long photoperiod.

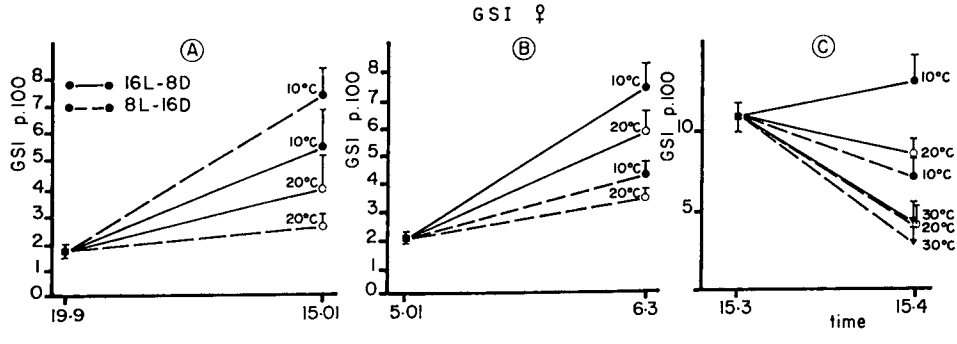

FIG. 1. — *Seasonal effects of adaptation of goldfish to high temperature on gonadosomatic index* (GSI). *a*) in fall ; *b*) in winter ; *c*) in spring.

In winter, the effects of high temperature were similar to those obtained in fall ($P < 0.005$) but there was no interaction between photoperiod and temperature. Long photoperiods strongly stimulated oogenesis whatever the temperature ($P < 0.001$).

In March, maintaining the animals at a warm temperature induced regression of the ovaries ($P < 0.05$) which were well developed at the beginning of the experiment. This regression occurred within one month and was accentuated with short photoperiod ($P < 0.05$).

b) *Gonadotropin secretion* (figs. 2A, B, C).

Seasonal effects of light and temperature. No differences were found between gonadotrophin levels in any of the experiments in reference to the photoperiod. Temperature stimulates plasma c-GTH levels in fall and spring, but there were no effects of this environmental factor in winter.

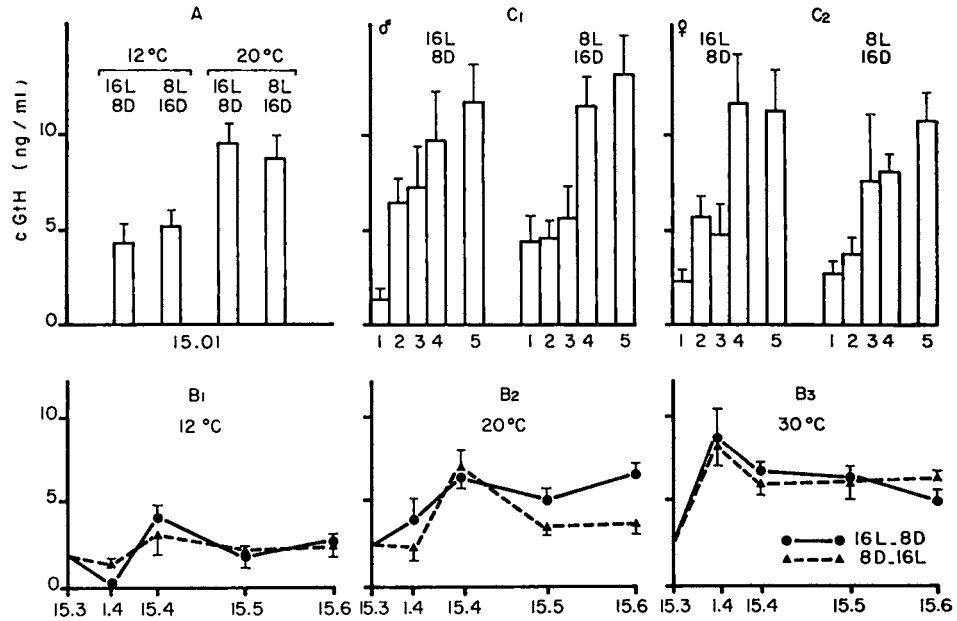

FIG. 2. — *Seasonal effects of adaptation of goldfish to high temperature on plasma gonadotropin levels. a)* in fall ; *b)* in spring ; *c)* effects of acclimation on plasma c-GTH in spring : 1 15 °C March 18, 2 20 °C March 21, 3 25 °C March 24, 4 30 °C March 27, 5 30 °C April 1.

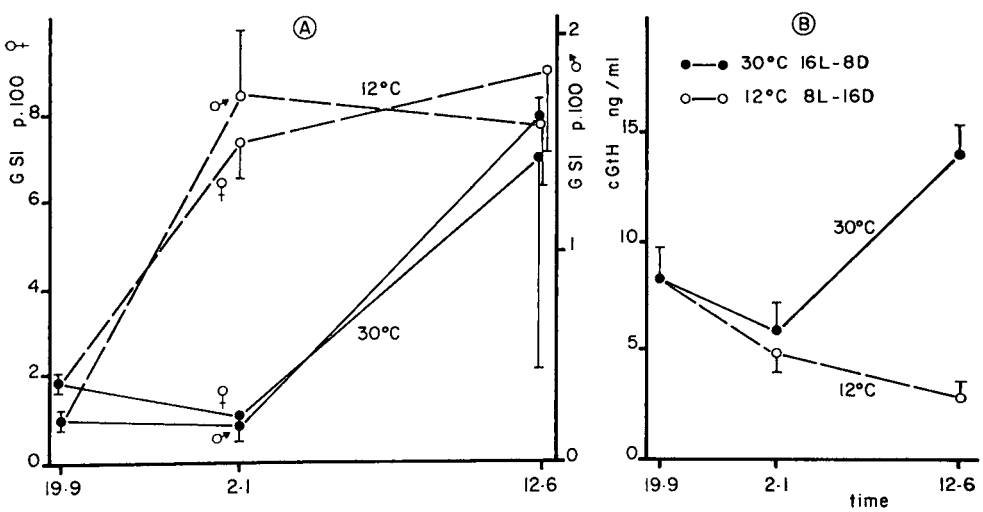

FIG. 3. — *Effects of prolonged exposures to high temperature. a) on GSI ; b) on plasma c-GTH.*

Acclimation effect on GTH secretion. In spring, c-GTH levels increased regularly with the temperature, and independently of photoperiod and sex of the animals.

II. *Effects of prolonged exposure to high temperature* (fig. 3-A, B, C).

In animals maintained at 30 °C, gonads (ovaries and testes) regressed until January and they developed normally from January to June. At this stage, there was no statistical difference between the GSI of these animals and that of the controls maintained at 12 °C during the whole period.

In the control group, the plasma gonadotropin levels remained very low from September to June. At 30 °C, GTH level increased between January and June ($P < 0.01$).

Discussion.

Gonadotropins and temperature. — These results demonstrate that in the goldfish long exposure to high temperature stimulated gonadotropin secretion. Similar results have been obtained in the rainbow trout (*Salmo gairdneri* : Breton and Billard, 1977) and in the tench (*Tinca tinca* : Breton *et al.*, 1975). On the other hand, in the carp (*Cyprinus carpio*), pituitary responsiveness to exogenous releasing-hormone stimulation appears in spring when water temperature increases and is enhanced from April to July (Weil *et al.*, 1975). Thus, the effects of temperature on gonadotropin secretion could be modulated by activation of the hypothalamic-hypophyseal complex for GTH secretion.

Recent work (Peter and Crim, 1978) has demonstrated the existence of nycthemeral GTH secretion cycles in the goldfish. These cycles differ with temperature and photoperiod. In our experiments, blood was sampled every morning at the beginning of the light period. Thus, the absence of photoperiod effect in our experiments indicates that the interaction between light and temperature did not affect GTH secretion at this time of day. Different hours of sampling during the day must be compared in further studies to define this problem.

Gametogenesis and temperature. — Increased GTH secretion was not always correlated with stimulation of gonadal development. In fall and winter, high temperatures inhibited ovary recrudescence. In spring, the same high temperatures induced gonadal regression. Temperature may have a direct action on the gonads by modifying ovary responsiveness to GTH. Nevertheless, the inhibitory action of high temperature on gametogenesis was not irreversible. After a 4-month inhibitory period, animals developed normal gametogenesis. A similar result was reported by Clemens and Reed, (1967). The results can also be compared with those obtained on carp (*Cyprinus carpio* (Gupta, 1975). When animals are acclimated at more than 20 °C as soon as hatching, they develop a total reproductive cycle in hot water after a precocious puberty and rapid growth.

Increasing the photoperiod never balances the inhibitory effects of the high temperature. As described earlier by Fenwick (1970) studying the same species in winter, a long photoperiod strongly stimulates oogenesis. The exact role of photoperiod remains to be defined in relation to temperature conditions. As mentioned by de

Vlaming (1972), both photoperiod and temperature may be important environmental factors in the determinism of the cyprinid reproductive cycle.

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Résumé. Nous avons testé les effets de la température et de la photopériode sur la gamétogenèse du Poisson rouge (*Carassius auratus*) à trois saisons différentes (l'automne, l'hiver et le printemps). Dans tous les cas, les températures élevées inhibent l'ovogenèse malgré une augmentation du taux de gonadotropine plasmatique. Les photopériodes longues stimulent la croissance des gonades en hiver seulement. Lorsque les poissons sont élevés à 30 °C pendant une période prolongée (de septembre à juin), leurs gonades demeurent régressées jusqu'au mois de janvier. Après cette période, les gonades se développent normalement.

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Photoperiod induced off-season spawning of coho salmon (*Oncorhynchus kisutch*)

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Summary. Three groups of coho salmon were reared under artificially controlled photoperiod regimes. One group was exposed to a normal first, second and third year photoperiod cycle. The other two groups were maintained on a normal first but modified second year cycle designed to advance or delay spawning by 120 days. The third cycle was of normal duration but shifted out of phase to maintain the time differences that had been established during the second altered photoperiod cycle.

In December 1975 (the end of the normal second cycle), numbers of precocious males (jacks) and immature fish were determined. Gonadosomatic indices of a subsample of precocious males, immature males and immature females are reported.

Sexually mature adult coho salmon with viable gametes were produced 78-90 days prior to the normal spawning period and 148 days after the normal period. Mortality to swim-up in the fry stage was 8.5 p. 100 in the normal group, 16 p. 100 for the 120 day advanced group and 52 p. 100 for the 120 day delayed group.

Nineteen per cent of the 120 day advanced fish, 17 p. 100 of 120 day delayed fish and 6 p. 100 of the normal fish did not respond to light treatment. They retained their marine colouration and were histologically identified as immature females.

Introduction.

This study was initiated to determine whether controlled adjustments to the normal rate of change of daylength in the annual photoperiod cycle would induce off-season functional sexual maturity in coho salmon (*Oncorhynchus kisutch*) similar to that previously demonstrated in pink salmon (*O. gorbuscha*) (MacQuarrie, Markert and Vanstone, 1978).

Coho salmon usually mature 3 years after egg fertilization. However, sexually precocious 2 yr-old males are observed frequently and 4 yr-old adults occasionally in returns to the spawning grounds. We proposed to develop broodstocks of coho salmon that would spawn 120 days prior to and 120 days later than a normal group of

3 yr-old adult fish by compressing or expanding the normal rate of change of daylength in their second annual photoperiod cycle.

The rationale for conducting this study was based on the desirability of having groups of swim-up coho fry available regularly throughout the year as test animals for our diet development and salmon aquaculture programs.

Material and methods.

The period extending from the time of egg fertilization (December 1, 1973) until October 31, 1974, constituted the first phase of this experiment. Up to this time the fish had been reared in well water (10 °C) on a normal photoperiod. If the normal photoperiod cycles continued all fish were expected to reach sexual maturity and spawn in early December, 1976. However, on November 1, 1974 these yearling coho salmon were randomly divided into three groups, each containing 125 fish. These groups of fish were placed into three light proof 3 m diameter fibreglas tanks containing flowing aerated well water (10 °C) as previously described by MacQuarrie *et al.* (1978). The interior of each tank was illuminated by four 40 watt Vita Lite fluorescent tubes controlled by a manually adjustable time clock.

During phase II, the fish in each group were subjected to the photoperiod regime they would normally receive to December 20, 1975, however, the duration of this phase of the cycle was either compressed by 120 days (group 1) normal (group 2) or expanded by 120 days (group 3). Manually controlled time clocks were adjusted every 10 days during this phase of the experiment. Daylengths (i. e. the time of sunrise and sunset) remained constant for all groups of fish for the initial 10 day interval. However, for the following 10 day intervals the time of sunrise and sunset had been advanced 14 days for group 1, 10 days for group 2 and 8 days for group 3. Thus, a normal 13.7 month cycle was compressed into 9.7 months for group 1 or expanded into a 17.7 month cycle for group 3 (fig. 1).

The experiment was continued into phase III using a normal, but still artificially controlled photoperiod shifted out of phase by time clocks (which automatically adjust themselves for seasonal changes in daylength) to maintain the time differences established during phase II. Spawning was expected to occur in early December 1976 for group 2 fish and in what was apparently early December 1976 for groups 1 and 3.

During the experiment the fish were fed Oregon moist pellets (OMP II) ad lib. twice daily.

Smolts in group 2 were acclimated to flowing aerated salt water at ambient temperature in May 1975 as occurs normally for wild fish. Fish in groups 1 and 3 were acclimated to salt water in what was apparently May 1975 (March 1975-group 1 ; July 1975-group 3).

The fish were sampled in December 1975. i. e. the end of the normal second cycle for group 2. Weights, gonadosomatic indices and numbers of precocious males and immature fish were recorded (table 1). The number of fish/tank was also reduced at this time so that group 1 had 53, group 2, 55 and group 3, 62 fish.

FIG. 1. — *Off season spawning in coho salmon.* Experimental protocol showing specific periods during which coho salmon in each of the three groups were on either normal photoperiod or altered photoperiod from initiation in 1973 to termination in 1977.

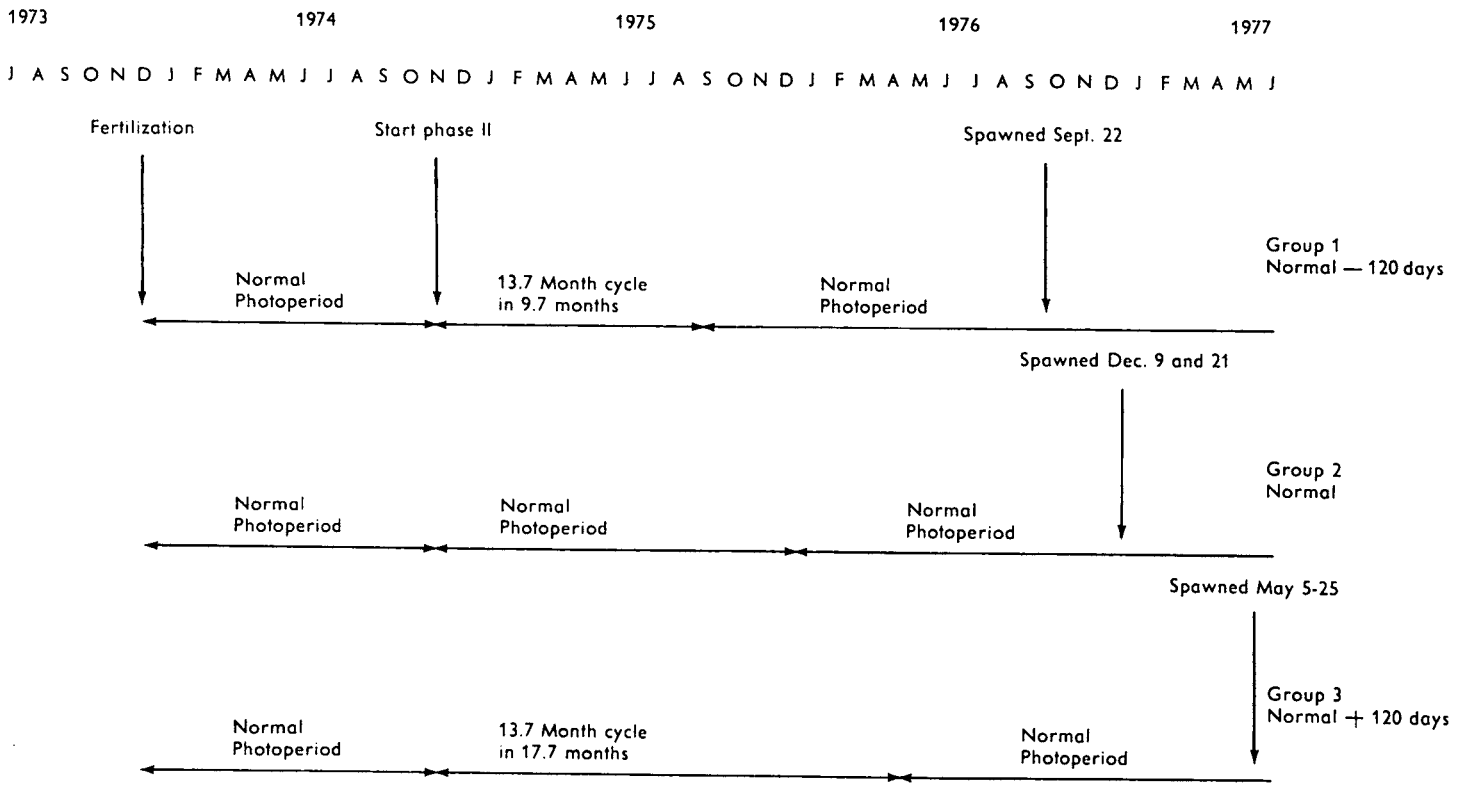

TABLE 1

Mean weight and GSI $\bar{x} \pm$ SD of coho salmon exposed to normal or altered photoperiod regimes

Group	1	2	3
No. of fish (end of Phase II for normal group)	91	108	117
No. of precocious adult males	9	25	37
Body weight	166.6	189.0	282.7
GSI *	7.0 $\pm$ 1.0	6.9 $\pm$ 2.0	3.9 $\pm$ 0.9
No. of immature fish	82	83	80
Average weight (♂ + ♀)	99.3	202.8	90.9
Male GSI *	0.05 $\pm$ 0.01	0.07 $\pm$ 0.02	0.07 $\pm$ 0.01
Female GSI *	0.35 $\pm$ 0.09	0.40 $\pm$ 0.12	0.40 $\pm$ 0.07

* Estimated following a sampling of 4 to 7 fish/group.

All groups of fish were reacclimated to 10 °C well water when they started to take on their breeding colouration. They were lightly anaesthetized with 2-phenoxyethanol (0.07 ml/l) and examined for maturity prior to, during and after the expected spawning period. When the fish were ready to spawn, they were stunned, weighed and measured and then the eggs were stripped and fertilized. Weights and numbers of eggs were determined within 15 min of fertilization. The eggs were allowed to water harden for 2 hr and were then placed in Heath trays and incubated in flowing (12 l/min) aerated 10 °C well water. Dead eggs were removed after the eyed stage, cleared using Stockard's solution and numbers of fertilized and non-fertilized ova were recorded (table 4).

Results.

On December 30, 1975 (the end of the normal second cycle) an inventory of each group revealed (table 1) that 9.9 p. 100 (group 1) 23.1 p. 100 (group 2) and 31.6 p. 100 (group 3) of the fish were precocious males (jacks). The gonadosomatic indices of these fish were significantly different : 120 day advanced $>$ 120 day delayed ; normal $>$ 120 day delayed. There was no significant difference between the normal group and the 120 day advanced group (one way ANOVA and Scheffe's test $P = 0.05$). A subsample (4-7 fish/group) from the remaining immature fish showed that there was no significant difference between the gonadosomatic indices of immature males or between immature females in the three groups (one way ANOVA).

Data in table 2 indicate that the first mature male coho salmon was observed just prior to (groups 2 and 3), or just after (group 1) the expected spawning date. However, the time interval between the expected spawning date (time 0), the observation of the first partially ovulated female (a) and the actual time the majority of the females were spawned (b) range from : (a) + 37 days (b) + 53 days for group 1 ; (a) — 8 days

(b) + 9 days for group 2 ; to (a) + 19 days, (b) + 35 days for group 3. Only the normal group (group 2) spawned close to the expected date.

TABLE 2

Expected, actual and apparent spawning dates for 3 groups of coho salmon exposed to normal or altered photoperiod regimes

Group	1	2	3
Spawning date			
Expected	August 1, 1976	December 1, 1976	April 1, 1977
Actual	September 22, 1976	December 9 and 21, 1976	May 2, 5, 17 and 25, 1977
Apparent	January 20, 1977	December 9 and 21, 1976	January 18 and 21, 1977 February 2 and 10, 1977
First partially ovulated female	September 7, 1976	November 22, 1976	April 19, 1977
First mature male	August 2, 1976	October 27, 1976	March 25, 1977

Numbers, lengths and weights of breeding males and females are recorded in table 3.

The ovulatory response of breeding coho salmon females is also reported in table 3. All of the females in group 1 had completely ovulated. Eighty-six per cent of the females in group 2 and 64 p. 100 of the group 3 females had also reached this stage.

TABLE 3

Lengths (mean ± SD), weights (mean ± SD), gonadosmotic index (mean ± SD) at spawning for coho salmon exposed to normal or altered photoperiod regimes

Group	1	2	3
Mature females			
Total number	11	35	11
Length (mm)	361.8 ± 23.2	402.9 ± 22.7	405.5 ± 31.1
Weight (g)	608.2 ± 116.7	798.9 ± 139.3	776.5 ± 163.2
No. completely ovulated	11	30	7
No. partially ovulated	0	3	3
No. non-ovulated	0	2	1
Mature males			
Total number	14	13	9
Length (mm)	350.7 ± 42.5	380.0 ± 25.0	395.8 ± 42.3
Weight (g)	506.1 ± 173.8	630.0 ± 124.3	562.2 ± 255.8
Silver fish			
Total number (♂ + ♀)	6	3	4
Length (mm)	300.5 ± 38.9	280.0 ± 60.0	340.0 ± 33.7
Weight (g)	312.8 ± 117.8	271.1 ± 152.8	448.5 ± 125.0
GSI females	0.95 ± 0.32	0.81 ± 0.04	4.56 ± 8.1 *
male		1.12	

* One had GSI of 16.7.

Information in table 3 also indicates that group 1 had 19.4 p. 100 and group 3, 16.7 p. 100 immature silver fish present. Only 5.9 p. 100 of these immature fish were observed in group 2 during the spawning period. With only one exception, all of the immature fish were histologically identified as females. One immature male was present in group 2 (had one blind eye).

Female fecundity (table 4) was also greatly reduced in the three experimental groups when compared to wild females and ranged from 645 to 803 eggs/female. It was apparent that longer growing periods resulted in larger mean weights for the ova (group 3 > group 2 > group 1). It was also evident that egg and alevin mortality (table 4) increased slightly for group 1 (16.15 p. 100) and markedly for group 3 (52.0 p. 100) when compared with the normal group (8.51 p. 100). The high egg

TABLE 4

Female fecundity ; mean egg weight and viability of ova and alevins obtained from 3 groups of coho salmon exposed to normal or altered photoperiod regimes

Group	1	2	3
Number of eggs stripped.....	7 096	24 934	5 733
Number of eggs/female (av.).....	645	792	803
Number of eggs/kg body weight ..	1 060.3	990.8	942.8
Average egg weight (g)	0.160	0.174	0.187
Percentage of eyed eggs	84.9	93.6	50.4
Percentage of fry at swim-up	83.9	91.5	48.0

losses experienced by group 3 can be attributed to the very large percentage of non-fertilized eggs which were present (47.4 p. 100 non-fertilized group 3, 15.1 p. 100 non-fertilized group 1 and 6.4 p. 100 non-fertilized in group 2).

Discussion.

Photoperiod manipulation induced off-season functional sexual maturity in coho salmon. However, if the main spawning date for each group is taken into consideration (group 1-September 22, 1976 ; group 2 — December 9, 1976 ; group 3 — May 5, 1977) then group 1 spawned 78 days prior to and group 3 spawned 148 days later than the normal group rather than at the expected 120 day interval. A similar 4-6 week spawning delay was also reported for pink salmon reared on manipulated photoperiods (MacQuarrie *et al.*, 1978). This spawning delay might be attributed to the effects of captivity such as high rearing density, artificial lighting, an artificial diet, nutritional status, temperature cycles out of phase with photoperiod, handling, etc. Wild adult coho salmon spawn from October to January.

Scott and Crossman (1973) state that average lengths and weights of adult coho salmon range from 457 to 610 mm and from 3 628 to 5 443 g, respectively. It is readily apparent that fish in all experimental groups reached only a fraction of the size they

could have attained in the wild environment. Foerster and Pritchard (1941) suggest that small adult females produce fewer eggs. This observation might explain the marked reduction in fecundity of the experimental fish when compared to the 2 100 to 2 789 egg average reported for British Columbia's adult coho (Scott and Crossman, 1973). The eggs generated by fish in groups 1 and 2 were of good quality as demonstrated by their relatively low rates of mortality to swim-up.

Prespawning mortalities occured in group 3. Four mature males and 2 maturing females (GSI 15.4 and 15.8) died within one month of spawning. Forty-seven per cent of the ova produced by this group were not fertilized. As these eggs were treated in the same manner as ova in groups 1 and 2 with respect to methods of collection, insemination and incubation it implies that either the eggs or sperm were of poor quality. However, as ripe males were observed in group 1 seven weeks and in group 3 six to eight weeks prior to spawning, one might conclude that the sperm produced by both groups of fish would have the same potential to fertilize the eggs. Immature, silver fish which did not respond to the photoperiod treatment (Hazard and Eddy, 1951 ; MacQuarrie *et al.*, 1978) were also observed in this experiment (these fish might mature as 4 yr olds). It was interesting to note that all but one of these fish were females. MacQuarrie *et al.* (1978) indicated that all immature pink salmon (*O. gorbuscha*) present at spawning were females. It appears evident that it is much more difficult to induce off-season functional sexual maturity in female Pacific salmon. Even if successful in inducing these fish to mature, not all of the females will produce viable ova as the more complicated, rigorously scheduled progression of events that occurs during vitellogenesis might be disrupted by manipulative changes in the photoperiod cycle.

The present work on off-season induction of sexual maturity in coho salmon has demonstrated that it is feasible to produce viable alevins at spaced intervals throughout he year to satisfy the requirements of various experimental programs or to initiate new studies at times when swim-up fry are not available.

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Résumé. Trois groupes de saumons coho sont élevés sous des photopériodes artificielles contrôlées. Un groupe est exposé à trois cycles photopériodiques annuels normaux. Pour les deux autres groupes, la première année le cycle est normal, mais la seconde année il est modifié pour avancer ou retarder la fraie de 120 jours. Le troisième cycle est de durée normale, mais déplacé pour maintenir les différences temporelles établies au cours du second cycle photopériodique modifié.

En décembre 1975 (fin du second cycle normal), le nombre de mâles précoces (Jacks) et de poissons immatures est déterminé. Les rapports gonadosomatiques d'un échantillon de mâles précoces, de mâles immatures et de femelles immatures sont rapportés. Les mâles adultes sexuellement matures, sont produits 78-90 jours avant la période normale de fraie et de 148 jours après la période normale. La mortalité jusqu'au stade de nage libre des

alevins est de 8,5 p. 100 dans le groupe normal, 16 p. 100 pour le groupe avancé de 120 jours et 52 p. 100 pour le groupe retardé de 120 jours.

19 p. 100 de poissons avancés de 120 jours, 17 p. 100 de poissons retardés de 120 jours et 6 p. 100 de poissons normaux ne répondent pas au traitement lumineux. Ils conservent leur coloration marine et sont identifiés en histologie comme des femelles immatures.

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Décalage de la période de reproduction par raccourcissement des cycles photopériodique et thermique chez des poissons marins *

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Summary. *Shift in the reproduction period of salt-water fish using shortened photoperiod and temperature cycles.*

Sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) and gilthead sea bream (*Sparus aurata*) spawners were kept in 2 identical 40 m^3 tanks, one outdoors (control) and one indoors (test) during a 5-year experiment. The control tank was under natural yearly photoperiod and the temperature cycles of western Brittany. The test tank was under similar variation for 3 years but with the cycles compressed into 10 months. The 3 species spawned naturally in these artificial conditions at various stages of the experiment. After 3 years, the fish in the test tank spawned an average 5 months ahead of the others.

Introduction.

L'existence d'un cycle de reproduction annuel est un élément gênant pour des programmes d'élevage larvaire, les possibilités d'expérimentation se trouvant concentrées sur une période qui peut être limitée à quelques semaines par an. En dehors des régions équatoriales, les cycles photopériodique et thermique annuels constituent conjointement l'élément régulateur des cycles reproducteurs de nombreux organismes dont la plupart des poissons. Les effets respectifs de la photopériode et de la température ne sont pas toujours clairs. Il semble cependant que la photopériode soit l'élément régulateur essentiel de la maturation sexuelle, la température jouant surtout un rôle sur le déclenchement de la ponte (Kuo *et al.*, 1974 ; de Vlaming, 1975).

Les deux facteurs ont été utilisés de différentes manières pour provoquer des pontes hors saison de poissons marins. Leong (1971) et Lasker (1974) ont démontré qu'il est possible d'obtenir des pontes spontanées à longueur d'année à partir d'un stock d'un millier d'Anchois (*Engraulis mordax*) maintenus en conditions constantes (15 °C et 4 h de jour). Laurence (comm. pers.) a obtenu des pontes de tautogue noir (*Tautoga onitis*) et de spare doré (*Stenotomus chrysops*) 4 à 6 mois avant la période normale, en intervenant simultanément sur la température et la photopériode.

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Le schéma expérimental testé ici visait à obtenir progressivement des pontes naturelles décalées de 6 mois chez des reproducteurs captifs, en prenant un minimum de risques expérimentaux. Température et photopériode n'ont donc pas été dissociées, mais les cycles annuels naturels de la région ont été comprimés sur 10 mois pendant 3 ans. A ce terme, le décalage voulu étant acquis, les cycles ont été ramenés à leur valeur normale de 12 mois pour le maintenir à niveau constant.

Ce schéma a été appliqué simultanément à trois espèces, le bar (*Dicentrarchus labrax*), le turbot (*Scophthalmus maximus*), et la daurade dorée (*Sparus aurata*). Lorsque l'expérience a été lancée, la ponte naturelle en captivité dans des bassins de la dimension employée n'avait encore été obtenue que chez le bar (Boulineau, 1974). Chez cette même espèce, une expérience de maturation décalée, par raccourcissement de moitié des conditions thermiques normales des 4 mois précédant la ponte, avait permis d'obtenir des œufs 1 mois à l'avance, avec des injections hormonales (Barnabe, 1974). Mais moins de 3 p. 100 d'entre eux étaient entrés en développement.

Matériel et méthodes.

Deux bacs parallélipipédiques, en polyester armé (4 × 6 × 2 m de haut), sont équipés de doubles-fonds de sable percolé, avec des exhausteurs (« air-lifts ») aux quatre coins. Pour des volumes utiles de 40 m³ environ, les débits de renouvellement sont fixés entre 3 et 4 m³/h. L'eau de mer, non filtrée, est prise sur les circuits d'alimentation normaux du laboratoire, sans modification de température pour le bac témoin, après ajustage à la valeur voulue pour le bac expérimental.

Le bac témoin, placé à l'extérieur, est soumis aux cycles de température et de photopériode naturels de la région. Le cycle imposé au bac expérimental reproduit les mêmes variations (à partir d'une moyenne des 3 années précédentes pour la température), mais comprimées sur 10 mois (fig. 1). Cette reproduction est très simplifiée. Le cycle évolue par paliers, à partir d'un réglage hebdomadaire de température et de photopériode. En période diurne, comme en période nocturne, l'intensité de l'éclairage est constante. En période diurne, 17 tubes fluorescents (« grolux », type lumière du jour), totalisant 680 watts, fournissent un éclairement moyen de surface voisin de 1 500 lux en surface, et 30 lux au fond. L'allumage des tubes se fait en deux temps, par moitiés, à 5 mn d'intervalle. Leur extinction se fait en un seul temps, et provoque l'allumage d'une veilleuse bleue de 25 watts destinée à faciliter les visites.

Les deux bacs sont chargés de poissons entre novembre 1973 et février 1974, au fur et à mesure des disponibilités. Ils reçoivent chacun 8 turbots de 4 à 12 kg, 35 à 40 bars de 100 à 150 g, et 20 à 25 daurades de 50 à 100 g. Les turbots ont été pêchés un an auparavant, dans la région. Les bars et les daurades ont été péchés aux environs du gramme, et employés d'abord à des expériences de nutrition (Alliot *et al.*, 1974 ; Sabaut et Luquet, 1973). Les bars sont d'origine essentiellement méditerranéenne, les daurades toutes d'origine atlantique.

Du fait de leur provenance, les bars du bassin expérimental continuent à recevoir quotidiennement le granulé dont ils se nourrissaient auparavant (P50 L12 de Alliot *et al.*, 1974), et refusent tout autre aliment, jusqu'au mois de juin 1976. Ils acceptent ensuite, après 1 à 2 mois de jeûne suivant les individus, les morceaux de maquereau

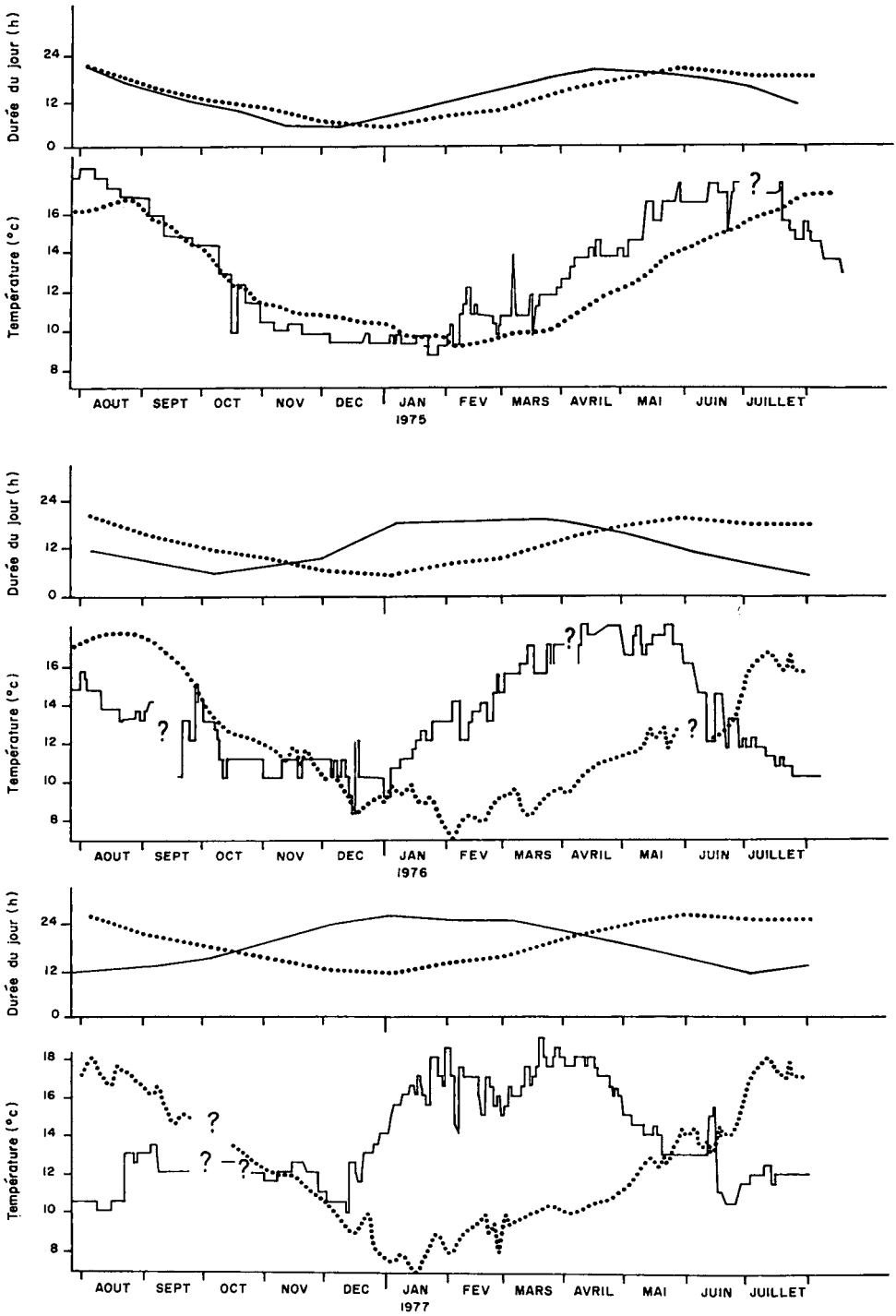

FIG. 1. — *Cycles photopériodique et thermique subis par les deux bassins de l'expérience*
——— Bassin expérimental ; ..... Bassin témoin.

(*Scomber scombrus*), de tacaud (*Trisopterus luscus*), ou de chinchard (*Trachurus trachurus*), qui sont distribués, dans les deux bacs, 3 fois par semaine en été, 2 fois par semaine en hiver, en quantités ajustées à la demande. Des langoustines (*Nephrops norvegicus*), des crabes verts (*Carcinus moenas*) et de la chair de bucarde de Norvège (*Laevicardium crassum*) ou de cythérée fauve (*Cytherea chione*), sont ajoutés en complément 2 à 3 fois par mois et sont consommés en quasi-totalité par les daurades. La distribution de la nourriture est toujours faite en milieu de journée.

Les 7 premiers mois de 1974 sont consacrés au rodage des installations de contrôle de la température et de la photopériode, et le cycle comprimé débute le 1er août.

Les œufs, collectés automatiquement au niveau des trop-pleins, sont mis à incuber dans des paniers (Girin, 1976) ou dans des barquettes carrées (44 cm de côté pour 17 cm de haut) à fond en toile calibrée de 250 μ, en circuit ouvert d'eau de mer filtrée. Tous les œufs collectés sont dénombrés. Les diamètres moyens sont mesurés sur des échantillons de 20 œufs par ponte. A partir de 1976, les taux de viabilité des œufs au moment de la récolte (proportion de ceux dont le développement débute normalement), et les taux d'éclosion, sont enregistrés systématiquement.

Dans le texte qui suit, le terme ponte sera employé pour désigner l'ensemble des œufs collectés un jour donné et dont le stade de développement indique qu'ils ont été fécondés dans un intervalle de l'ordre de l'heure. Il est évident qu'une ponte, au sens employé ici, peut impliquer des œufs de plusieurs femelles.

Résultats.

Depuis le début de l'expérience, le système de contrôle de la photopériode a donné toute satisfaction. Cela n'a pas été le cas du système de thermorégulation, dont la puissance de refroidissement et la fiabilité ont laissé à désirer (fig. 1).

Aucune mortalité n'a été enregistrée chez les daurades. 5 bars du lot témoin sont morts à quelques mois d'intervalle, en 1975 et 1976, avec des symptômes similaires à ceux que Barahona-Fernandes (1977) a décrit chez le juvénile. Ils n'ont pas été remplacés. 7 turbots du bac expérimental ont été perdus en juillet 1975, à la suite d'un incident technique, et immédiatement remplacés par des poissons de même origine. 2 turbots en 1974, 3 en 1975 et 5 en 1976, sont morts dans le bassin témoin, après la saison de ponte, pour des raisons indéterminées. Les animaux morts en 1976 n'ont pu être remplacés qu'après la saison de ponte de 1977.

En juillet 1977, les poids des animaux pouvaient être estimés entre 800 g et 1,5 kg pour les daurades, 600 g et 3 kg pour les bars, 4 et 10 kg pour les turbots, soit une charge globale de 2,5 à 3 kg/m^3.

Les trois espèces ont fourni des pontes naturelles, dans des proportions variables. Les principales caractéristisques des pontes de bars et de turbots sont rassemblées dans le tableau 1. Les bars, encore petits, et manipulés peu avant la saison de ponte, n'ont fourni qu'un millier d'œufs en 1974. Ils ont ensuite pondu tous les ans, dans les deux bacs. Les turbots ont pondu normalement dès 1974, fournissant 23 pontes sur l'ensemble des deux bacs, entre le 1er mai et le 4 juillet. Les pontes se sont ensuite poursuivies, sauf dans le bac témoin en 1977, faute de femelle survivante. Les daurades hermaphrodites protandriques, dont le changement de sexe s'effectue aux environs

TABLEAU 1

Caractéristiques des pontes de Turbot Scophthalmus maximus *et de Bar* Dicentrarchus labrax

Espèce	Bassin	Année	Nombre de pontes	Période de ponte			Total des œufs récoltés	Nombre moyen d'œufs par ponte	Diamètre moyen des œufs (mm)	p. 100 d'œufs viables	p. 100 d'éclosion
				Première ponte	Moyenne	Dernière ponte					
Turbot	Expérimental	1975	22	25/04	**23/05**	17/06	1 174 000	53 000	1,04	?	?
		1976	30	5/03	**28/03**	26/04	5 825 000	194 000	1,04	43	34
		1977	43	23/12/76	**30/01**	28/02	3 037 000	71 000	1,12	47	16
	Témoin	1975	30	1/05	**30/05**	7/07	3 500 000	117 000	1,04	?	?
		1976	16	23/04	**13/05**	5/06	4 210 000	263 000	1,08	67	61
		1977	0	—	—	—	—	—	—	—	—
Bar	Expérimental	1974	1	—	**1/04**	—	1 000	1 000	?	?	?
		1975	20	26/02	**19/03**	17/04	207 000	10 000	1,16	?	?
		1976	20	13/01	**1/02**	23/02	2 328 000	116 000	1,14	68	51
		1977	18	14/11/76	**4/12**	30/12/76	905 000	50 000	1,19	83	52
	Témoin	1974	0	—	—	—	—	—	—	—	—
		1975	10	14/04	**21/04**	29/04	347 000	350 000	1,23	?	?
		1976	26	29/02	**12/04**	19/05	5 138 000	198 000	1,24	81	49
		1977	39	2/03	**25/04**	4/06	8 993 000	230 000	1,23	100	63

du kilo dans la région, n'ont fourni leurs premières pontes qu'en 1977. 20 pontes, totalisant 34 000 œufs, en grande majorité fécondés, ont été récoltées du 22 juin au 22 août dans le bassin expérimental.

Discussion.

Indépendamment du décalage des saisons de ponte, l'obtention de pontes naturelles fécondées chez le turbot et la daurade représente déjà un élément intéressant. Chez le turbot, les seules relations antérieures de pontes naturelles fécondées en captivité (Malard, in Anthony, 1910) impliquaient un bassin de plusieurs centaines de m^3. Dans des bacs de 20 m^3 ou moins, comme Jones (1972), nous n'avions obtenu que des pontes non fécondées. Chez la daurade, il ne semble pas y avoir eu de relation écrite de pontes naturelles fécondées chez des animaux maintenus en captivité plusieurs mois avant la saison de reproduction, encore que des résultats de ce genre aient été obtenus au printemps 1977, en Israël (Gordin, comm. pers.). Les pontes fournies par les bars confirment les observations de Boulineau (1974) en bacs de 20 m^3.

Dans le bassin témoin, la période de ponte et la taille moyenne des œufs sont assez constantes d'une année à l'autre, particulièrement chez les bars. Il n'apparaît ni l'avancée progressive de la période de ponte, ni l'augmentation de taille des œufs que plusieurs auteurs ont observées chez d'autres espèces à mesure que les femelles grandissent (Gall, 1974).

Dans le bassin expérimental, il semble y avoir, surtout chez le turbot, une certaine résistance des poissons à accepter une compression de leur cycle de reproduction. Les pontes du lot expérimental se décalent progressivement, mais toujours avec quelques semaines de retard sur la date à laquelle elles devraient théoriquement intervenir. Chez le bar, la compression du cycle semble s'assortir d'une stagnation du nombre des pontes, et de leur dimension, avec des œufs plus petits que dans le lot témoin. Ce dernier point est important, la taille de l'œuf influant sur les chances de survie et la croissance de la larve, dans une espèce déterminée (Gall, 1974 ; Hempel et Blaxter, 1967 ; Reagan et Conley, 1977).

Mais les changements de régime alimentaire peuvent être mis en cause dans les résultats obtenus. Chez le turbot, les remplacements de reproducteurs et l'absence de pontes dans le lot témoin en 1977 ne permettent pas d'analyser les variations observées dans le nombre, la dimension des pontes ou celle des œufs. Chez la daurade, il faudra attendre les premières pontes dans le bassin témoin pour disposer d'un terme de comparaison. Elles devraient intervenir vers janvier 1978, si l'on s'en rapporte aux variations du rapport gonado-somatique chez les animaux sauvages dans la région, ce qui fixerait le décalage obtenu entre 5 et 6 mois.

Les données disponibles sur les taux de viabilité et d'éclosion sont encore trop fragmentaires pour permettre des conclusions précises. Le niveau de viabilité des œufs augmente dans chaque lot avec le temps, et il est en règle générale plus faible dans le bassin expérimental, aussi bien chez le bar que le turbot. Il en est de même des taux d'éclosion mais qui sont à considérer avec précaution, les conditions d'incubation n'étant pas standardisées. Les données concernant les élevages larvaires, en cours de dépouillement, feront l'objet d'une note séparée.

Le nombre de pontes obtenues chez les turbots, au moins 8 par femelle en 1977 dans le bassin témoin, met bien en évidence l'aspect séquentiel de la ponte chez cette espèce. Ce n'est pas le cas chez le bar.

Les conditions très artificielles du bac expérimental sont donc compatibles avec la ponte naturelle des trois espèces étudiées et son décalage sans manipulation des animaux. Mais les premiers résultats présentés ici laissent encore nombre de questions en suspens. Certaines d'entre elles, comme l'effet du régime alimentaire des poissons, les effets respectifs d'un simple décalage ou d'une compression du cycle annuel, la qualité des pontes obtenues dans ces conditions, le rendement de l'ovulation, devraient trouver une réponse dans les deux ans à venir, par la poursuite des expériences. D'autres, comme la décomposition des rôles respectifs de la photopériode et de la température dans la maturation sexuelle et le déclenchement de la ponte des animaux, supposeraient une expérimentation complémentaire, qu'il n'a pas encore été possible de mettre en place.

Symposium sur la Reproduction des Poissons
Paimpont, France, 19-21 *septembre* 1977.

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Ovarian rematuration of ablated sugpo prawn *Penaeus monodon* Fabricius *

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Summary. Spent *Penaeus monodon* females were tagged around one eyestalk with numbered brass tags, stocked in a concrete tank with males, and periodically examined for ovarian rematuration over a six-month period (February to August, 1977). The females were either artificially-induced (ablated spawners from concrete tanks and marine pens) or wild spawners. Most of the wild spawners were ablated after spawning ; some remained unablated to serve as controls. Out of a total of 349 experimental females, 35 or 10.1 p. 100 had a second spawning and 5 or 1.4 p. 100 a third spawning ; none of the 74 controls spawned subsequently. The average number of days for maturation and rematuration were : ablation to first spawning, 22.6 days ; first spawning to second spawning (for females ablated before the first spawning), 11 days ; first spawning to second spawning (for females ablated after the first spawning), 15 days ; and second spawning to third spawning, 10.4 days. Fecundity of rematured spawners is comparable to that of first spawners ; there is insufficient data on hatching rate and fry survival.

Introduction.

Completion of the life cycle in captivity of the giant tiger prawn or sugpo *Penaeus monodon* using the eyestalk ablation method was first achieved in the Igang Sea Farming Station of the SEAFDEC Aquaculture Department (Santiago, 1977). Destruction or removal of the eyestalk in prawns and other decapod crustaceans induces ovarian maturation by eliminating the production and storage sites of the ovary-inhibiting hormone (Adiyodi and Adiyodi, 1970).

Up to 1976, most of the females spawned in the Department came from the wild ; for January to June 1976, wild spawners constituted 98 p. 100 of total supply. The successful mass production of spawners by unilateral ablation in marine prawn pens and land-based tanks has increased the proportion of ablated spawners to 84 p. 100 for the March to August, 1977 period.

* Contribution No. 17, SEAFDEC.

To what extent spent females can remature and spawn successive broods is an important question in terms of recycling spawners in a commercially viable operation. Corollary to the rate of rematuration is the quantity and quality of fry from rematuring females in comparison to those from first spawnings.

Materials and methods.

Spent *P. monodon* females from wild (unablated) and broodstock (ablated) sources spawned in the hatchery and wet laboratory of the SEAFDEC Aquaculture Department in Tigbauan, are routinely tagged around one eyestalk with numbered brass tags (Rodriguez, 1977) for rematuration studies. The ablated females come from concrete tanks in the Tigbauan station or marine pens in the Batan station. Wild spawners are ablated after tagging ; some remain unablated to serve as controls. Spawning is verified the following day by checking the outline of the ovaries while holding the female against a bright light in a darkroom ; both partially and completely spawned females are used. Partial spawners may spawn or resorb remaining eggs in the next few days ; it is standard procedure to keep them in the spawning tank only for the first night.

This study covers the 6-month period of February to August, 1977. All females were stocked in a 4.85 × 4.85 × 2.80 m concrete tank with approximately an equal number of males. There was no bottom substrate in the tank and water depth was at least one m. Feeding was once to twice daily salted or fresh brown mussel (*Modiolus metcalfei*) meat at 10 p. 100 estimated prawn body weight. Sampling for rematuration of females was done every five days during which the tank was cleaned and the water completely changed.

Rematuring females were taken to the wet laboratory for spawning and rearing of the larvae. One to three spawners were placed in one-ton fiberglass tanks with seawater of 26-29 °C temperature and 30-34 °/₀₀ salinity. The tank was covered with a dark cloth or corrugated plastic and provided with two airstones. The numbers of eggs and nauplii were estimated by taking five one-l beaker samples, counting individual eggs and nauplii and computing the average number per sample multiplied by the total water volume in the tank.

TABLE 1

Number of spawnings and survival in ablated and unablated P. monodon

	No. of spawnings			No. of days survival			
	1st	2nd	3rd	Ave.	S. D.	Min.	Max.
Ablated before first spawning (pond stock)	316	33	5	9.2	6.8	0	40
Ablated after first spawning (wild)	33	2	0	8.4	6.5	0	27
Unablated	74	0	0	7.9	6.8	1	44
Total	423	35	5				

Results.

Of 349 experimental females, only 10.1 p. 100 had a second spawning and 1.4 p. 100 a third spawning (table 1). It takes an average of 23 days and a minimum of 12 days after ablation for a prawn with immature ovaries to mature and spawn (table 2). For

TABLE 2

Number of days between ablation and spawning and between successive spawnings of P. monodon

	No. of spawners	No. of days			
		Ave.	S. D.	Min.	Max.
Ablation to first spawning	70 *	22.6	6.3	12	44
First spawning to second spawning					
a) ablated before first spawning	33	11.0	6.1	5	23
b) ablated after first spawning	2	15.0	—	8	22
Second spawning to third spawning	5	10.4	—	5	18

* Exact date of ablation is known only for 70 of a total 316 females ablated before first spawning ; the remaining females were ablated in other SEAFDEC stations.

TABLE 3

Data on second spawning of ablated P. monodon

Date	Tag nos.		Total no. spawners	No. of eggs	No. of nauplii (N_5N_6) *	Hatching rate (p. 100)	Remarks
	Partial	Complete					
4-6-77	059	043	2	158 000	0	0.0	Eggs did not hatch.
4-13-77		181, 057	2	129 000	46 000	35.7	Discarded-larvae weak.
4-14-77		045	1	197 000	46 000	23.4	Discarded-larvae weak.
4-18-77		185	1	107 000	0	0.0	Eggs did not hatch.
4-20-77		108	1	104 200	75 000	72.0	Discarded-larvae weak.
4-25-77		100	1	58 000	0	0.0	Eggs did not hatch.
4-28-77		151	1	77 160	30 180	39.1	Discarded-larvae weak.
5-7-77		195	1	307 800	262 000	85.1	Harvested 10 000 P_1 (3.8 p. 100 survival from N_5N_6).
5-28-77	478		1	126 000	0	0.0	Eggs did not hatch.
6-2-77	461		1	106 000	76 800	72.4	Harvested 5 000 P_2 (6.5 p. 100 survival from N_5N_6).
6-3-77		536	1	138 000	0	0.0	Eggs did not hatch.
6-8-77		543, 421, 503	3	773 600	278 000	35.9	Harvested 13 000 P_1 (4.7 p. 100 survival from N_5N_6).
				300 000	No data	No data	
7-12-77		592	1	107 000	45 000	42.0	Discarded-larvae weak.
7-22-77		393	1	217 000	105 600	48.7	Discarded-larvae weak.
7-22-77		517	1	523 500	247 500	47.3	Discarded-larvae weak.
7-22-77	378		1	208 800	159 000	76.2	Discarded-larvae weak.
8-6-77	380			204 800	196 200	95.0	Harvested 11 300 P_3 (5.8 p. 100 survival from N_5N_6).
Average				182 993		36.7 **	

* Count was made during the fifth and sixth substages of the nauplius.
** Weighted average.

previously ablated females, there is an average of 11 days between first and second spawnings whereas it takes 15 days for wild females ablated after the first spawning. The average between second and third spawnings is 10.4 days.

The kind of first spawning did not affect the length of time it takes for rematuration and subsequent spawning ; complete spawners (n = 24) took on the average 10.7 days for a second spawning as compared to 12.6 days for partial spawners (n = 11) (A subsequent spawning does not include completion of earlier partial spawning.)

Second spawnings average 180 000 eggs (table 3) while third spawnings average 140 000 eggs (table 4), both with a hatching rate of 35 p. 100 or greater in contrast with 110 000 to 120 000 eggs with a hatching rate of 64 p. 100 for first spawnings from wet laboratory data. Although rematuring females show a slight increase in fecundity and a decrease in hatching rate compared to first spawnings, the small number of samples makes generalization difficult.

TABLE 4

Data on third spawning of ablated P. monodon

Date	Tag nos.		Total no. spawners	No of eggs	No. of nauplii (N_5N_6) *	Hatching rate (p. 100)	Remarks
	Partial	Complete					
5-2-77	100		1	—	—	—	No count.
5-8-77		108	1	86 000	0.0	0.0	Eggs did not hatch.
5-30-77		504	1	130 000	110 000	84.6	Harvested 27 000 P_i (24.5 p. 100 survival from N_5N_6).
6-25-77		503	1	124 000	0	0.0	Eggs did not hatch.
7-27-77		378	1	220 000	85 000	38.6	Discarded-larvae weak.
Average				140 000	48 750	34.8	

* Fifth and sixth substages of the nauplius.

Discussion.

To a large degree, the low rate of rematuration is due to high spawner mortality ; the females survived an average of only 8 to 9 days after spawning (table 1). High mortality may be traced to poor tank conditions, mainly the build-up of excess feeds on the bottom before every water change.

Moreover, a few rematurations and spawnings may have occurred in the culture tank itself during the 4-day interim between samplings. More frequent sampling could not be undertaken because of stress on the prawns and the large size of the tank. Although no unablated wild females (controls) spawned a second time, only histological studies can definitely establish non-rematuration (this work is now in progress).

The minimum of 12 days and average of 3 weeks between ablation and spawning confirm earlier observations (Aquacop, 1977 ; Primavera *et al.*, 1978). The average number of days between successive spawnings from one ablated female is 11 with a

minimum of 5 days (table 2). In comparison, 6 unilaterally ablated *P. monodon* females in Aquacop (1977) had 18 spawnings over three months for an average of one spawning per female per month with a minimum of eight days between spawnings. A unilaterally ablated *P. orientalis* female gave four spawnings with intervals of six days between spawnings (Arnstein and Beard, 1975).

In contrast, maturation data of unablated penaeids in captivity show a longer period between spawnings. Based on data up to the F_2 generation, Beard *et al.* (1977) state that a *P. merguiensis* female might be expected to spawn once every 2.6 months on the average. Laubier-Bonichon and Laubier (1976) obtained 87 depositions of spawn from 60 *P. japonicus* females over four months or an average of one deposition of spawn every 2.8 months under controlled photo- and thermoperiod conditions.

In the wild, *P. monodon* probably spawn more than once in their lifetime. This is suggested by a bimodal pattern of carapace length measurements of 56 and 61 mm on 104 gravid females caught from the wild (Motoh, pers. comm., 1977). Total length measurements of wild females of some Arabian Gulf penaeids suggest a total of five spawnings during lifetime for *P. semisulcatus* and five spawnings before attaining maximum size for *Trachypenaeus granulosus* (Badawi, 1975). For both species as well as *Metapenaeus stebbingi*, there appears to be a gap between spawnings of nearly two months, probably representing the length of time for ova to fully mature. Employing the gonad index method, Pillay and Nair (1971) conclude that the breeding season of the prawn *M. affinis* extends over several months of the year with distinct peaks of gonadal activity and indicate the possibility of production of successive broods of eggs by one female during a single breeding season.

Conclusion.

If the rate of rematuration is increased and the hatching rate and fry quality from subsequent spawnings improved by good nutrition, throughflow water and other better rearing conditions, rematuration of spent *P. monodon* spawners may further decrease broodstock costs.

Symposium sur la Reproduction des Poissons Paimpont, France, 19-21 septembre 1977.

Résumé. Des femelles *Penaeus monodon* matures marquées et stockées en bassins bétonnés ont été périodiquement examinées pendant une période de 6 mois (février à août 1977) afin d'identifier les rematurations ovariennes. Les femelles étaient soit des géniteurs à reproduction artificiellement induite par ablation des pédoncules oculaires et provenant de bassins ou de cages en mer, soit des géniteurs sauvages. La plupart des géniteurs sauvages ont subi une ablation après la fraie et d'autres sans ablation ont servi de contrôle. Sur un total de 349 femelles expérimentales, 35 (soit 10 p. 100) ont présenté une seconde fraie et 5 (soit 1,4 p. 100) une troisième. Aucun des 74 témoins n'a frayé. Le nombre moyen de jours nécessaires pour la maturation ou la rematuration était de 22,6 entre ablation et première fraie, 11 entre première et deuxième fraie (cas d'ablation après la première) et 10,4 entre la deuxième et la troisième fraie. La fécondité des géniteurs à leur première maturité est comparable à celle des maturités suivantes. Les données concernant les taux d'éclosion et la survie larvaire sont insuffisantes.

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Cryopreservation of the sperm of some freshwater teleosts

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Summary. The sperm of 7 freshwater fishes was cryopreserved using the pellet technique of Nagase (1964). The fertilization rate in the rainbow trout was about 80 p. 100 when the sperm was equilibrated for 15 mins before deep freezing. Under the same conditions, the fertilization rate in the brown trout was about 70 p. 100. All the other species included in the experiments also showed high activity of frozen and thawed spermatozoa, but the fertilization rate was low or zero. It was more difficult to cryopreserve the sperm of cyprinid fishes than the sperm of salmonid fishes, although the activity of the thawed spermatozoa was the same. There are few papers describing experiments and fertilization rates with cryopreserved cyprinid sperm, and further experimentation is thus necessary.

Introduction.

Some of the genetic progress in the breeding of domestic animals can only be obtained by applying the technique of sperm-cryopreservation. This technique would also open the way to new opportunities in the propagation of cultivated freshwater fishes if their sperm could be preserved.

The present investigations were aimed at developing a technique for deep freezing the sperm of freshwater fishes living under the climatic conditions of West Germany.

Materials and methods.

The subjects under investigation during the breeding season of 1976/77 were the rainbow trout (*Salmo gairdneri* Richardson), the brown trout (*Salmo trutta forma* fario L.), the brook trout (*Salvelinus fontinalis* Mitchill), the danube salmon (*Hucho hucho* L.), the grayling (*Thymallus thymallus* L.), the pike (*Esox lucius* L.) and the carp (*Cyprinus carpio* L.).

The sperm was taken by hand stripping from the males and collected in syringes. In the period between collection and cryopreservation the syringes were stored in pond water in order to maintain a constant temperature.

One drop of the sample of each male was examined microscopically before and after dilution with water and dilution medium. Only samples without activity before

dilution and high activity after dilution with water were used for cryopreservation experiments.

The sperm was cryopreserved according to the technique described by Nagase (1964). After dilution the sperm was dropped on carbon ice and stored in liquid nitrogen. The extender always contained 10 p. 100 DMSO. The dilution ratio was 1 : 3 and the size of the pellets 0.2 ml. For thawing, 3 of the pellets were put in 10 ml of a 1 p. 100 $NaHCO_3$ solution. The thawing process was accelerated by rapid shaking. Immediately after thawing the pellets were poured over the eggs or examined microscopically. The sperm was thawed after a storage period of 7 days. According to the results of earlier experiments (Stein, 1976 ; Stein and Lamina, 1976) we tested two different extenders without equilibration time and with an equilibration time of 15 and 20 min. because we found that the equilibration time differs depending on the extender and the species.

Extender 1 : 750 mg NaCl, 200 mg $NaHCO_3$, 53 mg Na_2HPO_4, 23 mg $MgSO_4.7\ H_2O$, 38 mg KCl, 46 mg $CaCl_2.2\ H_2O$, 100 mg glucose, 500 mg glycine, 100 ml H_2O, 20 ml egg yolk (= V2).

Extender 2 : 750 mg NaCl, 200 mg $NaHCO_3$, 38 mg KCl, 100 mg glucose, 100 ml H_2O, 20 ml egg yolk (= V2e).

TABLE 1

Results of fertilization experiments of the breeding season 1976/77

Species	Dilution medium	Equil. time	n	Fertil. rate ± SD*	Number of eggs/sample	Spermatozoa/egg	
						frozen	fresh
Rainbow trout	V2	0	15	78,1 ± 6,9	500	$4\text{-}6.10^6$	
	V2e	15	26	81,9 ± 3,8	—	—	
	control		30	87,9 ± 17,3	—		$6\text{-}10.10^6$
Brown trout	V2	0	15	41,2 ± 17,6	—	—	
	V2e	15	5	77,0 ± 6,5	—	—	
	control		20	90,2 ± 9,3	—	—	
Grayling	V2	0	6	46,8 ± 19,0	1 500	$1\text{-}2.10^6$	
	V2	20	9	55,0 ± 15,4	—	—	
	control		6	95,3 ± 2,4	—		$2\text{-}3.10^6$
Brook trout	V2	0	4	27,4 ± 7,3	500	$4\text{-}6.10^6$	
Danube salmon	V2	0	2	22,5 ± 1,6	500	$4\text{-}6.10^6$	
Pike	V2 (650)	0	2	25,0 ± 2,4	5 000	$0{,}8.10^6$	
Carp	V2	0	2	—	20 000	$0{,}4.10^6$	
	control		1	81,0	—		1.10^6

* SD : standard deviation.

In the pike the NaCl content of Extender 1 was reduced to 650 mg/100 ml because there was a better motility.

The F-test was used to test the significance between different treatments.

Results.

The most interesting results of the fertilization experiments of the breeding season 1976/77 are summarized in table 1.

There was alway high motility in the dilution medium before and after freezing. More than 70 p. 100 of the spermatozoa were motile. The duration of the motility of the thawed spermatozoa was about 30 s. In the rainbow trout the difference in the fertilization rate between V2 and V2e with equilibration is statistically significant ; in the brown trout the difference is highly significant. The difference between equilibrated and non-equilibrated sperm in the grayling is not significant.

There was high motility in the thawed sperm of the carp too, but no fertilizing capacity.

In the brook trout, danube salmon and pike we could not obtain fresh eggs and fresh sperm at the same time, so control tests were impossible.

Discussion.

In the rainbow trout and brown trout the fertilization rate was improved by applying a new extender and an equilibration time of 15 minutes. As compared to our earlier experiments it is now possible to cryopreserve the sperm of the rainbow trout with a fertilization rate of 80 p. 100 and with small variance. In the brown trout the new technique succeeded with a significantly higher fertilization rate but there is still a difference between the two trout species. This difference is very marked in V2 without equilibration time. The number of experiments with the sperm of the brook trout, the danube salmon and the pike was low because we had no more males and females. But the experiments corroborated our earlier results showing that it is possible to cryopreserve the sperm of these species with a relatively low fertilization rate. Further improvement is certainly possible but it is difficult to procure the material.

We could not do more experiments on the grayling either to demonstrate a possible significant increase of the fertilization rate with equilibrated sperm. In the thawed semen of the carp there was high motility, but no fertilizing capacity. The eggs were possibly affected by the thawing solution or the extender. It seems to be very difficult to cryopreserve the sperm of cyprinid fishes. Thus, there are only two papers describing successful fertilization tests with cryopreserved sperm in cyprinid fishes (Moczarski, 1976, 1977).

Conclusion.

The experiments point out that the sperm of salmonid and esocid fishes can be cryopreserved using the technique of Nagase (1964). In the rainbow trout a practical application of this technique would bring a relatively small loss of fertilizing capacity.

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Résumé. La congélation du sperme de 7 téléostéens d'eau douce a été expérimentée en utilisant la technique de Nagase (1964). Avec 15 mn d'équilibration, les taux moyens de fécondation obtenus après décongélation ont été de 80 p. 100 chez *Salmo gairdneri* et environ 70 p. 100 chez *Salmo trutta forma* fario. Le sperme des autres espèces s'est révélé motile, mais peu fécondant, après décongélation. La conservation du pouvoir fécondant du sperme après congélation semble plus difficile à obtenir chez les Cyprinidés que chez les Salmonidés

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Short-term and cryopreservation of rainbow trout (Salmo gairdneri Richardson) sperm

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Summary. This paper reports a series of experiments conducted to obtain data on short-term preservation of trout semen. Using both motility of activated spermatozoa and fertilizing ability as criteria, it was found that rainbow trout semen, if not frozen, could be best stored if kept undiluted at — 2 °C in an oxygen atmosphere. Stirring proved to be unfavorable. Antibiotics inhibited bacterial growth but had a detrimental effect on spermatozoa when added at levels as high as 9 000 i. u. penicillin and 9 000 μg streptomycin/ml. Mixing milt from different males did not seem to effect storing ability. When taking these factors into account, rainbow trout semen could be stored for more than 3 weeks without loss of fertilizing ability.

In freezing semen from rainbow trout the best results were obtained when diluting one part semen with 1 or 3 parts of the following diluent mixture : 592 mg NaCl, 172 mg KCl, 67.9 mg $CaCl_2$, 15.1 mg $MgSO_4$ and 2 420 mg Tris made up to 100 ml with water ; citric acid was added to bring the pH to 7.25 ; to this was added 400 mg bovine serum albumin, 7 50 mg Promine D-soybean protein and 12 ml DMSO. Samples were frozen by pelleting on dry ice. The pellets were stored in liquid nitrogen and thawed in a 1 p. 100 $NaHCO_3$-solution. Results were variable, covering a range of 2.6-80.3 p. 100 hatching (corrected for controls).

Introduction.

Attempts to store salmonid sperm either in a liquid or frozen state have been reported or reviewed mainly by Scheuring (1924), Barrett (1951), Henderson and Dewar (1959), Truscott *et al.* (1968) and Horton and Ott (1976), but up to now no fully satisfactory techniques have been produced.

Material and methods.

Milt from 2-3 year old male rainbow trout was collected into pre-cooled test tubes which were kept on water-ice for about 3 h before further processing.

Preservation of liquid semen.

We investigated the effect of dilution rate, temperature, gas atmosphere, addition of antibiotics, mixing of milt from several males and occasional stirring on the motility and fertility of rainbow trout semen stored in a liquid state.

Experiment 1. — Samples of 1.5 ml of either undiluted semen or semen diluted at a ratio of 1 : 1 or 1 : 16 with a buffered isotonic diluent resembling the inorganic composition of seminal plasma (Holtz *et al.*, 1976) were placed in open glass vials with a surface area of 3.14 cm^2. The vials were placed in 1 l flasks (6 vials/flask) which were kept in total darkness either at 4 °C or 20 °C. Twice daily the flasks were opened and gassed by one of a number of gasses (table 1). At the same time the samples were swirled gently to counteract sedimentation of spermatozoa. A small drop of semen from each vial was checked daily for motility (Holtz *et al.*, 1977).

Experiment 2. — Applying the same technique used in experiment 1, milt was kept at 4 °C under O_2, at — 2 °C under air or at — 2 °C under O_2 for a period of 23 days. Motility was judged as good, fair or poor depending on the minimal rates of motility which were 40, 10 or 1 p. 100 respectively. There were 13 replicates per treatment.

Experiment 3. — Applying the same three treatments a fertility trial was conducted. Over a period of 23 days, semen was collected every other day and stored. Half of the strippings were stored individually ; the other half were made into mixtures from 3 different males. On day 23, each sample was added to 200 freshly collected eggs. Two weeks later all eggs were cleared in an 8 p. 100 acetic acid solution and examined microscopically for embryonic development. There were 2 replicates per treatment.

Experiment 4. — To inhibit bacterial growth antibiotics were added to semen kept under storage conditions of — 2 °C under O_2, checking for motility and fertility of spermatozoa. Penicillin was added at increasing levels up to 25 000 i. u. per ml semen. Subsequently penicillin and streptomycin were added at a ratio of 1 i. u. : 1 µg at levels of 0-40 000 (4 replicates per treatment). Eventually a fertility trial was conducted comparing levels of 0, 250, 1 000 and 2 000 i. u./µg. There were 8 replicates per treatment, 4 of which were left untouched while the other 4 were stirred gently twice daily. Half the samples were added to eggs after 10 days of storage and the other half after 16 days.

Cryopreservation.

For deep freezing rainbow trout semen a diluent was developed based on the composition of seminal plasma. The constituents of the diluent were : 592 mg NaCl, 172 mg KCl, 68 mg $CaCl_2$, 15 mg $MgSO_4$, 2 420 mg Tris made up to 100 ml with distilled water. Citric acid was added to adjust the pH to 7.25 before addition of 400 mg bovine serum albumin, 500-1 500 mg Promine D (a soybean product of « Central Soya ») and 12 ml dimethylsulfoxide (DMSO). Immediately after mixing semen and diluent at a ratio of 1 : 1 or 1 : 3, pellets (size 0.04 ml) were prepared on dry ice and stored in liquid nitrogen for 3-4 months. About 100 pellets were placed in 20 ml of a 1 p. 100 $NaHCO_3$-solution (Stein, 1975) at 1 °C. The resulting sperm suspension was immediately added to about 600 eggs which were then transferred to an incubator until the time of hatching.

Results and discussion.

Preservation of liquid semen.

Experiment 1. — A temperature of 20 °C was totally unsuited to the storage of trout semen. Thus the results presented in table 1 refer only to samples stored at 4 °C. They suggest that at that temperature dilution had an unfavourable effect on the survival rate and so did anaerobic conditions. Pure O_2 proved to be superior to air or a 1 : 1 mixture of N_2 and O_2. This result is in good agreement with earlier findings by Scheuring (1924).

TABLE 1

Duration in days ($\bar{x} \pm s$) *of spermatozoan motility in trout milt stored at* 4 °C *under different gas atmospheres at different dilution rates.* The number of replicates is given in parenthesis

Dilution rate[a]	Gas atmosphere					
	Air	O_2	$O_2 \pm N_2$ (1 : 1)	N_2	$N_2 + H_2 + CO_2$ (8 : 1 : 1)	CO_2
Undiluted	8.3 ± 2.7 (16)	12.4 ± 1.8 (14)	9.0 ± 0 (2)	3.6 ± 1.3 (10)	0 (4)	0 (6)
1 : 1	1.0 ± 0 (6)	—[b]	—	1.0 ± 0 (6)	—	0 (6)
1 : 16	0 (6)	—	—	0 (6)	—	0 (6)

[a] Semen : diluent ratio.
[b] Not tested.

Experiment 2. — The results presented in figure 1 suggest that a gas atmosphere of O_2 and storage temperature of — 2 °C rendered the best results ; several samples retained a degree of motility up to 23 days.

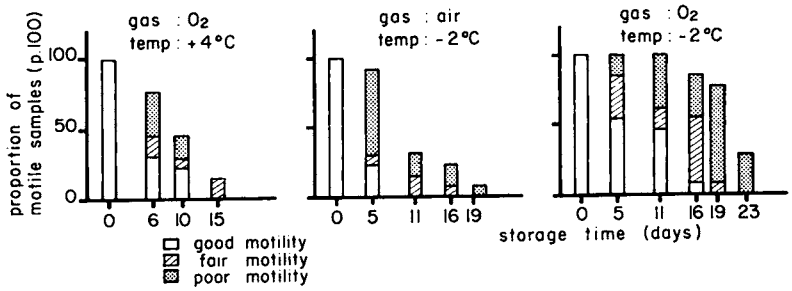

FIG. 1. — *The proportion* (p. 100) *of samples with good* (> 40 p. 100), *fair* (10-40 p. 100) *or poor* (1-10 p. 100) *motility after different storage times under either air or oxygen at temperatures of either* + 4 °C or — 2 °C.

Experiment 3. — The results of the fertility trial are presented in figure 2. The fertilizing capacity of semen stored under O_2 at 4 °C was retained for a period of 21 days. At — 2 °C under air it was 17 days, and at — 2 °C under O_2 it had not subsided by the end of the experiment (23 days). While motility dropped to a low level during the

more advanced stages of storage with the exception of a few individual samples fertilizing ability remained high right to the end. There appeared to be no effect of pooling semen samples from several males. A few samples had to be eliminated on account of decay, mainly due to progressively increasing numbers of bacteria.

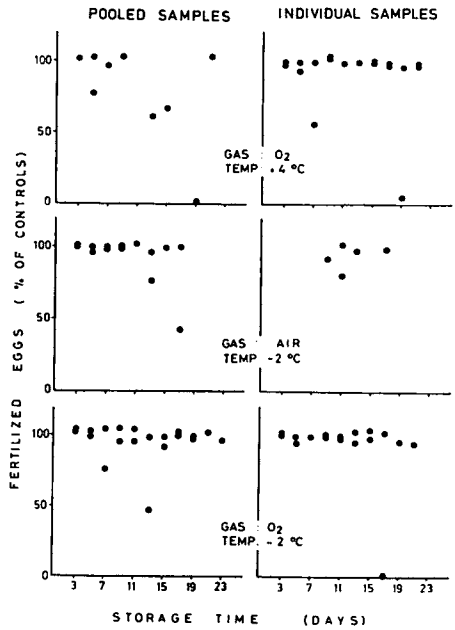

FIG. 2. — *The proportion of eggs fertilized after different storage times of semen kept under air or oxygen at temperatures of either* + 4 °C or — 2 °C. Each point represents one sample. Some samples had to be eliminated due to poor quality of eggs (second graph on right) or decay of semen due to bacterial growth.

Experiment 4. — During a 10-day storage period penicillin alone up to a level of 25 000 i. u./ml semen had no effect on spermatozoan motility. When combining penicillin and streptomycin at a 1 i. u. : 1 μg ratio, motility was decreased as soon as quantities of 9 000 i. u. respectively, μg were added. No difference was detected between

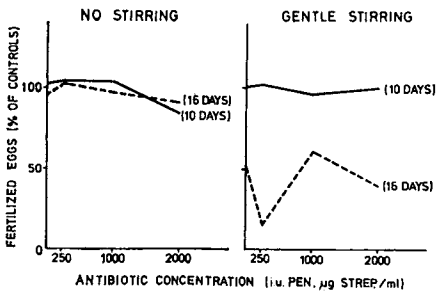

FIG. 3. — *Proportion of fertilized eggs after* 10 *or* 16 *days of storage under oxygen at* — 2 °C *with the addition of different levels of antibiotics.* Part of the samples were stirred gently twice daily.

samples to which penicillin and streptomycin were added at levels of 500-6 000 i. u., respectively, μg per ml semen. Beyond 9 000 i. u., respectively, μg, however, there was an immediate drop in spermatozoan motility. The lowest level tested was sufficient to prevent bacterial growth.

As illustrated in figure 3, the antibiotic levels tested did not effect the fertilizing ability of semen. Bacterial growth was inhibited in every case and no samples had to be eliminated. Repeated slight agitation of semen proved to be harmful. Fertility of samples stirred gently twice a day, while still good after 10 days, was significantly reduced after 16 days ($P < 0.01$).

Cryopreservation.

Since dilution rate did not seem to make a difference, samples in table 2 diluted at ratios of 1 : 1 and 1 : 3, were lumped together. This table presents the hatching results. The large variation of individual results was striking and leads to the conclusion that more research is needed. Our results compare favourably with those of other researchers (Bratanov and Dikov, 1960 ; Graybill and Horton, 1969 ; Ott and Horton, 1971*a*, *b*). Only Stein (1975) reports better success.

TABLE 2

The proportion of hatched eggs after fertilization with semen frozen after addition of a diluent with different levels of Promine-D and with fresh semen ($\bar{x} \pm s$)

Concentration of Promine-D (mg/100 ml)	No. of replicates	Fertilization rate (p. 100 eggs hatched)	
		Frozen semen	Fresh semen
0	2	12.7 ± 9.5	69.0 ± 38.3
500	3	35.1 ± 18.6	94.7 ± 5.0
750	4	45.0 ± 22.4	89.8 ± 16.1
1 000	3	21.4 ± 29.4	58.1 ± 16.2
1 500	1	2.5	96.0

Some of the results appear encouraging, although the cause of their variation must be determined before the procedure can be considered suitable for practical application.

Conclusions.

It has been shown that rainbow trout milt in a liquid state can be stored up to periods of more than 3 weeks with practically no loss of fertilizing capacity, provided that it is kept at a temperature of — 2 °C under O_2 and is not unduly agitated. The results obtained with cryopreserved milt are encouraging, although their considerable variation indicates the need for further study.

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Résumé. Une série d'expériences a été conduite sur la conservation à court terme du sperme de truite, utilisant la motilité et le pouvoir fécondant. Plusieurs paramètres ont été testés : température (— 2, 4 et 20 °C), agitation, antibiotiques, oxygénation. L'agitation s'est révélée défavorable ; d'addition d'antibiotiques au sperme (9 000 UI de pénicilline et 9 000 µg de streptomycine/ml) inhibe la croissance bactérienne, mais est défavorable à la conservation du pouvoir fécondant. Le cas le plus favorable à la conservation est le maintien du sperme à — 2 °C sous oxygène ; dans ces conditions, le pouvoir fécondant a pu être conservé pendant 3 semaines. Le mélange de sperme de plusieurs mâles ne nuit pas à la conservation.

En ce qui concerne la congélation de la semence de truite Arc-en-ciel les meilleurs résultats ont été obtenus avec un mélange qui contenait une part de semence et 1 ou 3 parts de dilueur de la composition suivante : 592 mg de NaCl, 172 mg de KCl, 67,9 mg de $CaCl_2$, 15,1 mg de $MgSO_4$ et 2 420 mg de Tris. Ce mélange est ajusté à 100 ml avec de l'eau et amené à pH 7,25 avec de l'acide citrique. On ajoute ensuite 400 mg de sérum albumine bovine, 750 mg de Promine D (protéine de soja) et 12 ml de DMSO. Ensuite quelques gouttes du mélange sont déposées sur de la neige carbonique et les « boulettes » ainsi obtenues sont stockées dans l'azote liquide et dégelées dans une solution de $NaHCO_3$ à 1 p. 100 avant insémination. Les résultats obtenus sont trés variables et se situent entre 2,6 et 80,3 p. 100 de taux d'éclosion.

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Conservation à court terme des gamètes de Salmonidés à des températures voisines de 0 °C

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Summary. *Short term preservation of gametes in Salmonids.*

Preservation of gametes was attempted in two species of salmonids brown trout (*Salmo trutta*) and rainbow trout (*Salmo gairdneri*). Tests were carried out with gametes stored at 0 °C during 7 days. The following results were obtained : after one day a drop of fertilizability (13 p. 100) was observed for ova in brown trout but not in rainbow trout. However, after 5 days the fertilizability of the female gametes is the same for both species (about 57 p. 100). Ova of brown trout vary more widely in response to aging than those of rainbow trout. The loss of ova fertilizability may be partly compensated by using larger doses of sperm (12.10^6 spermatozoa/egg). Sperm of rainbow trout lose 25 p. 100 of their fertilizing ability after one day and 10 p. 100/day during the following days. There is a significant interaction between aging of sperm and ova which is not of a simple type additif or multiplicatif.

Introduction.

La conservation des gamètes a de nombreux avantages notamment leur transport lorsque les lieux de prélèvements et d'insémination sont différents, la facilité du travail en pisciculture ou des améliorations génétiques (croisements, hybridation). Pour le sperme, les possibilités de conservation sont de deux ordres :

— congélation à — 190 °C qui permet une conservation à long terme et qui est possible pour des poissons marins (Billard et Dupont, 1975), mais très aléatoire pour les salmonidés (Stoss *et al.*, 1978, Billard, 1978) ;

— conservation à court terme à des températures voisines de 0 °C dans des milieux divers : colloïdes (Bratanov et Dikov, 1961), DMSO et éthylène glycol (Truscott *et al.*, 1968 ; Sanchez-Rodriguez et Billard, 1977).

Pour les ovules, l'aptitude à être fécondés peut se prolonger chez les salmonidés pendant au moins 8 jours (Escaffre *et al.*, 1977 ; Bry, communication personnelle), mais après prélèvement, la durée de la fécondabilité est variable et dépend de facteurs variés comme la température, la lumière, le milieu de conservation (Takano *et al.*, 1973).

Dans le présent travail, les possibilités de conservation des ovules et du sperme à des températures voisines de 0 °C seront testées en prenant pour critère de survie l'aptitude à la fécondation pour les ovules et le pouvoir fécondant et la motilité pour le sperme. En outre, l'évolution de la composition minérale (Na^+, K^+) du liquide séminal, déjà étudiée chez le Saumon (Hwang et Idler, 1969) sera examinée pendant la période de conservation.

Matériel et méthodes.

Matériel animal. — L'étude porte sur la Truite Arc-en-ciel (*Salmo gairdneri*) et la Truite fario (*Salmo trutta*). Les géniteurs d'un poids de 300 à 400 g proviennent de pisciculture privées. Les femelles fario avaient ovulé spontanément à une date indéterminée avant leur arrivée au laboratoire qui s'est effectuée une semaine avant le début de l'expérience ; l'ovulation des femelles Arc-en-ciel a été induite par traitement hormonal (t-GTH, 0,1 γ/g poids vif) et les ovules destinés à l'expérimentation ont été prélevés 3 j après l'ovulation. Les mâles utilisés sont pris dans un groupe d'individus isolés au début de l'expérience et en spermiation depuis environ un mois.

Effets du vieillissement des gamètes sur le pourcentage de fécondation. — Au début de l'expérience, les ovules sont prélevés sur plusieurs femelles (10 truites Arc-en-ciel et 3 fario) et placés séparément en boîtes plastiques de 400 ml avec le liquide coelomique homologue (10 ml de fraction de 200 ovules). Pour chaque mâle, 3 ml de sperme sont placés en tubes à hémolyse de 5 ml maintenus bouchés. Les gamètes ainsi conditionnés sont amenés de la température ambiante (10-12 °C) à la température de conservation (0 °C) en 20 mn, puis stockés à l'obscurité en étude réfrigérée. La fertilité des ovules et le pouvoir fécondant du sperme sont appréciés à des temps croissants : 0, 1, 3, 5 et 7 jours après prélèvement ; les ovules sont inséminés par lots de 200 environ additionnés de 10 ml de dilueur avec un mélange de sperme frais provenant de plusieurs mâles ; d'autre part, le pouvoir fécondant du sperme vieilli est testé sur des lots également de 200 ovules provenant du mélange de la production de 3 à 5 femelles fraîchement ovulées. En vue de tester les effets cumulés du vieillissement des gamètes mâles et femelles sur le pourcentage de fécondation, on a mis en présence à J_1, J_3, J_5 et J_7 des ovules et du sperme conservés dans les conditions définies ci-dessus. Dans chaque cas, l'insémination est pratiquée à + 4 °C à deux taux de dilution différents (4.10^{-3} et 2.10^{-4}), selon la méthode décrite par Billard *et al.* (1974) en mettant en œuvre le dilueur 532 (Billard, 1977). Après insémination, les œufs sont mis en incubation en eau recyclée et thermorégulée à 10 ± 0,5 °C et le pourcentage d'œufs embryonnés (stade œillé) qui représente une estimation par défaut du taux de fécondation est établi à 200 degré-jours. En outre, le pourcentage d'individus survivants est dénombré en fin de résorption vitelline.

Effets du vieillissement du sperme sur la durée de motilité et le rapport Na^+/K^+. — La conservation du sperme est d'autre part testée d'après l'évolution de la concentration en Na^+ et K^+ mesurée par spectrophotométrie de flamme dans le liquide séminal et d'après la durée de motilité. Cette dernière est définie comme étant la durée pendant laquelle la motilité observée sous microscope se maintient à son intensité initiale (après dilution du sperme dans un iso-volume de dilueur 532). Dans cette expérience, le

sperme de 10 mâles truite Arc-en-ciel est prélevé séparément et conservé pendant 6 jours dans les conditions définies ci-dessus. Les mesures de motilité, pouvoir fécondant et Na^+-K^+ sont pratiquées à J_0 et J_6.

Analyse statistique. — Les résultats sont testés par analyse de variance avec répétition en modèle factoriel à effets fixes. Dans les cas de pourcentages, la transformation angulaire a été utilisée pour stabiliser la variance résiduelle (Snedecor, 1956).

Résultats.

Conservation des ovules.

Chez la Truite Fario (fig. 1) l'aptitude des ovules à être fécondés (dilution de 4.10^{-3}) diminue significativement ($P < 0{,}01$) de 13 p. 100 après 24 h (passage de 73 à 60 p. 100), puis se maintient à ce même niveau jusqu'à la fin de l'expérience (7 jours) ; les mêmes résultats sont observés à la dilution de 2.10^{-4}. La mortalité embryonnaire mesurée en fin de résorption vitelline et exprimée en pourcentage par rapport au nombre d'œufs œillés est significativement plus élevée ($P < 0.025$) dans les lots d'œufs conservés 7 jours (J_7) que dans les lots de départ (J_0).

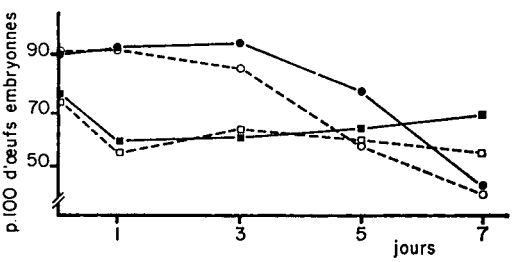

FIG. 1.

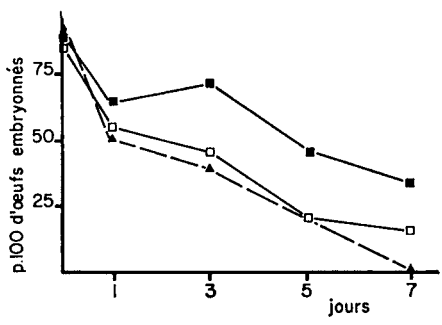

FIG. 2.

FIG. 1. — *Effet de la conservation à 0 °C dans le liquide coelomique et à l'obscurité d'ovules de truite fario* (o) *et arc-en-ciel* (□), *sur leur aptitude à être fécondé*, estimée d'après le pourcentage d'œufs embryonnés à 200 degré-jours. Taux de dilution du sperme lors de l'insémination : 4.10^{-3} ——— ; 2.10^{-4} - - - - -.

FIG. 2. — *Evolution du pouvoir fécondant des gamètes de truites arc-en-ciel conservés pendant 7 jours à 0 °C et à l'obscurité.* — Sperme vieilli et ovules frais lors de l'insémination : ■ 4.10^{-3} (soit 1,2 million de spermatozoïdes/ovules ; □ 2.10^{-4} (soit 20.000 spermatozoïdes par ovules). — Insémination d'ovules vieillis avec du sperme vieilli ▲ taux de dilution 4.10^{-3}.

Pour la Truite Arc-en-ciel, il n'y a pas de diminution de l'aptitude à la fécondation pendant les trois premiers jours de conservation (fig. 1). Entre J_3 et J_7, il y a par contre une diminution significative ($P < 0{,}01$) du pourcentage d'œufs embryonnés. A J_3 et J_5, on observe un effet favorable des faibles dilutions en spermatozoïdes qui compense les effets du vieillissement. Entre J_0 et J_7 il n'y a pas de différences dans les taux de survie post-embryonnaire (entre éclosion et résorption).

Conservation du sperme de Truite Arc-en-ciel.

— *Evolution du pouvoir fécondant* (fig. 2). — Le pouvoir fécondant chute brutalement de 25 p. 100 après 24 h de conservation. Au cours des jours suivants, la chute journalière est moins importante (de l'ordre de 10 p. 100). En outre, le pouvoir fécondant varie selon la dilution : au taux le plus élevé ($2{,}10^{-4}$) la chute du pouvoir fécondant est significativement ($P < 0{,}01$) plus forte de 10 à 25 p. 100 par rapport aux faibles dilutions ($4{,}10^{-3}$).

— *Vieillissement cumulé des gamètes.* — Lorsque les ovules et les spermatozoïdes ont vieilli pendant 7 jours, le taux de fécondation (fig. 2), toujours mesuré à 200 degré-jours, diminue plus fortement que lorsque le vieillissement porte seulement sur les ovules ou le sperme séparément (fig. 1 et fig. 2 pour le sperme). Il existe une interaction significative entre le vieillissement du sperme et celui des ovules, mais d'après le test χ^2 il ne s'agit pas d'un mode simple de type additif ou multiplicatif.

— *Relation entre pouvoir fécondant, durée de motilité et rapport* Na^+/K^+ (tabl. 1). — Après 6 jours de conservation, le pouvoir fécondant du sperme et la durée de motilité sont significativement diminués ($P < 0{,}005$). Le rapport Na^+/K^+ du liquide séminal ne présente pas de variations notables pendant cette période. Il n'y a pas cependant de relations significatives entre durée de motilité et pouvoir fécondant à l'intérieur de chacun des groupes (J_0 ou J_6). Il apparaît d'autre part que pour des spermes dont les durées de motilité sont de 35 à 50 sec., le pouvoir fécondant est d'environ 70 p. 100 à J_0 et 15 p. 100 seulement à J_6, de sorte que la durée de motilité ne rend pas compte de tous les phénomènes de vieillissement qui affectent le sperme.

TABLEAU 1

Appréciation par différents critères de la qualité du sperme lors du prélèvement (J_0) *et après conservation pendant 6 j* (J_6) *à 0 °C*
Entre parenthèses : nombre d'individus. Comparaison statistique entre J_0 et J_6 par analyse de variance

Critères d'appréciation de la qualité du sperme	J_0	J_6	Comparaison statistique
Durée de la motilité (en sec.).....	$53 \pm 6{,}04$ (10)	$26 \pm 6{,}92$ (9)	$P < 0{,}005$
Pourcentage de fécondation	$72 \pm 2{,}15$ (10)	$11 \pm 2{,}40$ (9)	$P < 0{,}005$
Rapport Na/K+	1,22 (10)	0,87 (8)	

Discussion.

Des ovules de truite extraits de la cavité générale ont été conservés à des températures voisines de 0 °C pendant 3 jours au moins pour les truites Arc-en-ciel chez lesquelles les œufs étaient fraîchement ovulés. La chute de l'aptitude à la fécondation des ovules de truite Fario est plus rapide (diminution significative après 24 h de conservation), mais dans ce cas, les ovules avaient séjournés dans la cavité générale depuis plus de 8 jours. Il faut noter néanmoins qu'après 7 jours de conservation *in vitro* la fécondabilité des ovules de truite Fario est plus élevée que celle des ovules de truite

Arc-en-ciel, bien que le temps écoulé depuis l'ovulation soit plus court. Le vieillissement se traduit en outre, chez la truite Fario, par une mortalité plus élevée pendant la période de résorption, aucun effet significatif n'ayant été décelé pour la truite Arc-en-ciel. Cette différence peut à nouveau être mise en relation avec la durée totale du vieillissement (*in vivo* + *in vitro*) après l'ovulation qui est plus longue dans le cas de la truite Fario. Cela montre d'autre part que le pourcentage de fécondation ou le pourcentage d'œufs embryonnés ne rend pas totalement compte de la qualité des ovules puisque des mortalités se produisent pendant la résorption vitelline pour les lots vieillis.

D'une façon plus générale, les durées de conservation des ovules de Salmonidés rapportées dans la littérature sont très variables et de nombreux facteurs peuvent expliquer les différences entre auteurs. Des durées de conservation des ovules de 24 h pour *S. gairdneri* (Hamor, 1966) et de 2 à 4 jours pour *O. nerka* (Barrett, 1951 ; Foerster, 1965) ont été rapportées. La durée de conservation est de quelques jours dans le liquide coelomique (Ginsburg, 1968; Takano *et al.*, 1973), et n'est que de quelques heures à sec (Billard, 1976). D'autres facteurs comme la température sont aussi importants (Withler et Morley, 1968). Il est possible de compenser au moins dans le cas de la truite Arc-en-ciel les effets du vieillissement sur la fécondabilité des ovules en augmentant le nombre de spermatozoïdes lors de l'insémination.

Dans les conditions de la présente expérience, la durée de conservation du sperme reste relativement brève (diminution significative du pouvoir fécondant après 24 h). Pour obtenir des durées plus longues, il est nécessaire d'avoir recours à des procédés plus complexes : soit addition de cryoprotecteurs (Sanchez-Rodriguez et Billard, 1977), soit mise sous atmosphère d'oxygène (Stoss *et al.*, 1978). Dans ces deux cas, la température de stockage était inférieure à 0 °C (respectivement — 4 et — 1 °C). Lors du vieillissement, le pouvoir fécondant du sperme chute beaucoup plus rapidement que la durée de motilité (tabl. 1) et, à l'intérieur de chaque groupe J_0 et J_6, il n'y a pas de corrélation entre ces deux paramètres. La durée de motilité n'est donc pas en mesure de constituer un bon critère d'appréciation du pouvoir fécondant, excepté dans les cas où le sperme est non motile car alors, il n'est jamais fécondant. Cependant des motilités faibles et brèves peuvent conduire à quelques fécondations, mais à condition que le taux de dilution ne soit pas trop élevé ; on observe d'autre part que le pouvoir fécondant du sperme est amélioré lorsque le nombre de spermatozoïdes (fig. 2) utilisé lors de l'insémination est augmenté ; la quantité moyenne de spermatozoïdes par ovule est ainsi plus élevée et la probabilité que le micropyle reçoive un spermatozoïde est plus grande, compensant de la sorte, au moins partiellement, la perte de motilité en durée et en intensité. Ce n'est donc que dans des cas limites et seulement dans un sens négatif que le critère durée de motilité peut être utilisé en pratique salmonicole : il n'est pas possible de sélectionner des spermes à haut pouvoir fécondant, mais il est possible d'éliminer ceux dont le pouvoir fécondant est faible.

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Cryopreservation of gametes and embryos of salmonid fishes

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Summary. After incubation in a Tris-citrate diluent (pH 8.9), two-day old rainbow trout milt increased its fertility from 7 and 13 p. 100 to 78 and 79 p. 100 (equal to fresh milt). Atlantic salmon milt, frozen to —8 °C, retained fertility of 84 and 94 p. 100. After storage at — 196 °C, spermatozoa were motile but not fertile, probably due to osmotic pressure in one diluent and the extender in another. Vacuum-dried spermatozoa have been fertile, but the drying method needs refinement. Salmonid eggs and embryos survived freezing to — 55 °C when frozen immediately after fertilization in salt solution or after eying. Frozen eggs (— 20 °C) were fertilized after thawing.

Practical techniques for long or short-term storage of fish gametes and embryos would contribute to increased productivity in aquaculture. Several techniques have been published on cryopreservation of salmonid spermatozoa (Hodgins and Ridgway, 1964 ; Hoyle and Idler, 1968 ; Ott and Horton, 1971), but they have not generally been successful when repeated (Zell, 1974 ; Graham and Holtz, 1977, personal communication). In some cases an inadequate criterion such as motility, which was used initially to evaluate sperm fertility, could explain the lack of reproductibility. In some cases variations in sperm quality might account for the observed discrepancies. In other cases there are no explanations.

Some diluents used to protect sperm from mechanical damage during freezing, will reduce sperm fertility. For example, solutions with K^+ concentrations close to zero activate salmonid sperm, which then irreversibly lose their fertility (Billard and Jalabert, 1974 ; Zell, unpublished). On the other hand, some solutions improve sperm fertility. In one experiment presented in this paper, the fertility of rainbow trout milt after two days storage at 0 °C was 7 and 12 p.100. Incubation for 20 min. at 8 °C increased the fertility to 78 and 79 p. 100 equal to that of fresh milt.

In contrast to the numerous studies on fish sperm cryopreservation, only one unsuccessful attempt (Blaxter, 1953) has been published on the cryopreservation of fish eggs or embryos. However several studies demonstrating the effect diluents can have on salmonid eggs (Billard *et al.*, 1974 ; Ginsburg, 1963 and Szöllösi and Billard, 1974) have provided data necessary to attempt freezing experiments. In addition, the survival of mammalian embryos after storage in liquid nitrogen (Leibo, 1977 ; Whittingham *et al.*, 1972) has illustrated the possibility of preserving vertebrate embryos.

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In this paper experiments are summarized that (1) test various protocols for preserving salmonid spermatozoa (2) identify experimental variables that most critically affect spermatozoan survival and fertility during cryopreservation and (3) develop techniques for cryopreserving salmonid eggs and embryos.

Material and methods.

Gametes from brook trout (*Salvelinus fontinalis*), rainbow trout (*Salmo gairdneri*) and both sea-run and land-locked Atlantic salmon (*Salmo salar*) were used. Gametes were collected either at the peak or end of a spawning season, depending on when they could be provided by the hatchery. Milt was obtained by stripping several unanesthetized, ripe males. It was mixed before use, and eggs from at least two females were combined. Eggs were fertilized by the dry method or, for experiments in which unfertilized eggs or zygotes were to be frozen, they were fertilized in a Hanks' (1X) salt solution (HSS) (see table 1). About 0.5 ml of milt was added to each sample of approximately 250-1 850 eggs. Fertility was measured as the percentage of eggs that either hatched or developed to the eyed stages. After eying, eggs from control samples were used in freezing experiments.

Media. — The media used are shown in table 1. All Hanks' media were prepared from a commercial solution of Hanks' (10X) balanced salt solution (GIBCO, Grand Island, N. Y.), to which sodium bicarbonate was added. Glycine was added only to the media used for sperm preservation. Ion concentrations and osmolalities shown in table 1 are average values. In the Hanks' media sodium or chloride concentration varied as much as 15 p. 100 depending on the quantity of HCl or NaOH used to adjust the pH. Potassium concentrations were constant within 1 or 2 mmol/l.

For freezing sperm, two different diluents were used, a modified Poulik's tris-citrate buffer with 10 p. 100 polyvinylpyrrolidone (PVP) and a modified Hanks' 2.6X, phosphate-buffered salt solution, with from 8-16 p. 100 dimethyl sulfoxide (DMSO) and from 0 to 16 p. 100 fetal calf serum (FCS). For incubating sperm, the Poulik's diluent proved most effective. For freezing eggs, the Hanks' double (2X) salt solution or the Hanks' 2.6X diluent with 10 p. 100 PVP plus 8 p. 100 DMSO (pH 7.9) gave best results. Eggs were fertilized in the Hanks' (1X) salt solution (HSS).

Preparation of milt. — Between the time of collection and use for incubation or fertilization, milt was held in 20-30 ml centrifuge tubes, covered with parafilm and stored on ice. Tubes were never filled more than 1/2 to 3/4 full.

Incubation of milt. — Successful sperm incubation depends on a combination of temperature, time, diluent composition and dilution. A 2-6 ml solution of milt plus diluents was gently mixed in a parafilm-covered, 20 ml test tube and placed in a stationary water bath. Temperatures of 0, 2-3, 7-8 and 10 °C and incubation times of 1/4 to 2 1/2 hrs were tested. Although milt incubated for 2 1/2 hrs at 10 °C remained fertile, one 20-min. incubation or a combination of a 15 min. pre-thaw and a 15 min. post-thaw incubation at 7-8 °C was selected as optimal combinations for experimental efficiency and spermatozoan fertility. Milt was incubated in either Hanks' or Poulik's diluent before it was frozen. Some samples were used directly for insemination after freezing and thawing. Other samples were rediluted with Poulik's diluent

TABLE 1

Composition of dilution media

Name [a]	Use	Extender (p. 100)	pH	Osmolality mOsm	glycine	HCO_3^-	Na^+	K^+	Cl^-	Mg^{++}	Ca^{++}
					(in mmol./l)						
Sperm preservation											
Poulik's	Incubation										
	Freezing	10 PVP	8.6-9.0	425	85	56	58	35-50	35-50	0	0
Hanks' 2.6 X	Freezing	8-16 DMSO	7.6-8.6	2 100-3 700 [b]	85	6	355	15	350	2.2	2.4
Egg preservation											
Hanks' 1 X	Freezing										
	Fertilization	None	7.9	10	—	9	153	6	141	1 [c]	1 [c]
Hanks' 2 X	Freezing	None	7.9	600	—	18	303	12	272	2 [c]	2 [c]
Hanks' 2.6 X	Freezing	10 PVP 8 DMSO	7.9	1 620	—	9	366	14	332	2.2	2.4

[a] The Hanks' media without extender are referred to as Hanks' (1 X) salt solution (HSS) or Hanks' (2 X) double salt solution (HDS). Hanks' 2.6 X with extender is called Hanks' diluent. [b] Osmolality was measured by freezing point depression, after a 1 : 1 dilution of diluent with water. [c] Concentrations are estimates based on atomic absorption analyses of Mg^{++} and Ca^{++} in the Hanks' 2.6 X diluent.

and re-incubated before insemination. After incubation in each diluent or in a combination of the two, fertility of unfrozen sperm was tested. In addition the effect of Poulik's diluent on the fertility of aging milt was tested with rainbow trout milt that had been stored undisturbed in sealed tubes at 0 °C for 2 days.

Freezing milt. — Diluted milt was frozen in plastic straws to various temperatures (i. e., — 6, — 20 or — 80 °C) at about — 5 C/min. in a Linde biological freezer (BF-4-1). After reaching — 80 °C in the Linde unit, samples to be frozen to — 196 °C were placed in liquid N_2. Since it was difficult to hold the samples at a constant temperature in the Linde chamber, samples were kept frozen only so long as their minimum temperature was constant (about 2 or 3 min.) ; then they were thawed rapidly in 50-60 °C water. Some samples in liquid N_2 were thawed shortly after freezing, while other samples were thawed after three or more days of storage. After they were thawed, sperm were either incubated in Poulik's diluent or used immediately for fertilization.

Vacuum drying milt. — Whole milt or milt diluted 1/2 in Poulik's diluent (1.5 to 2 ml total volume) cooled to 0 °C was vacuum dried for about 45 min. in 5 ml vials fitted on the outside of a freeze-drying unit (Virtis, Model 10-010 with a maximum pump pressure of 1.10^{-4} torr Hg). So long as drying continued, the vials stayed cold, but actual temperature of milt during the drying process was not measured. Vials were placed on ice while drying, so the samples did not warm above 0 °C. Maximum vacuum, measured with a McCleod gauge was 0.2 mm Hg. In one experiment two freshly dried samples of whole rainbow trout milt were reconstituted with 1.5 to 2 ml distilled water and immediately used to fertilize eggs. Other vials were stored at + 4 °C or — 11 °C for one year before being tested for fertility, at which time fresh samples were also dried.

Freezing eggs and embryos. — Unfertilized eggs, zygotes (eggs fertilized in HSS) and eyed eggs were frozen. They were frozen at various rates in several sizes of polyethylene vials in the Linde biological freezing unit to various temperatures between — 5 and — 80 °C. In initial experiments survival after freezing to — 55 °C was high but inconsistent, and no eggs survived after freezing to colder temperatures. Subsequently, in an attempt to identify and standardize the successful conditions, eggs were frozen only to temperatures between — 5 and — 55 °C. Requirements for pre-freeze incubation of eggs has not been determined, but eggs were generally equilibrated for at least 15 min. before freezing. Eggs were held at their minimum temperature in the Linde chamber for as long as their temperature could be kept constant, usually less than 5 min. Then the frozen eggs were thawed slowly as the Linde chamber warmed. Thawing was completed at room temperature. Alternatively, eggs were thawed rapidly in hot (40-60 °C) water. None of the egg samples were stored after freezing.

Results and discussion.

Sperm preservation.

Incubation of fresh sperm. — Table 2 shows the results of incubating fresh, rainbow trout sperm in Hanks' and Poulik's diluents. Fertility after incubation corres-

ponded to the osmolality and ionic strength of each solution. As the final osmolality of the incubation medium decreased, sperm fertility increased.

The higher DMSO concentration in the 1/4 dilution of milt in Hanks' (12 p. 100 DMSO) compared to that in the 1/2 dilution (8 p. 100 DMSO) cannot account for the lower fertility of sperm in the 1/4 dilution. In one experiment rainbow trout sperm were incubated after a 1/2 or a 1/4 dilution in Hanks', and the final DMSO concentration in each solution was 6 p. 100. The relative fertility of sperm in the 2 dilutions was comparable to that in table 1.

Likewise a reduced concentration of undetermined, but critical components in the seminal plasma cannot explain the lower fertility of sperm in the 1/4 dilution in Hanks'. (1) Sperm in the 1/8 double dilution (first 1/2 in Hanks' and then again 1/4 in Pouliks) had a higher fertility than sperm in the 1/2 dilution in Hanks' alone. (2) Sperm diluted 1/2 or 1/4 in Poulik's had a fertility equal to that of sperm in control, undiluted milt (table 2).

TABLE 2

Fertility of fresh rainbow trout sperm after incubation for 30 min. at 0 °C

Diluent	Dilution	N° eggs	p. 100 eyed
Hanks'	1/2	722	37
		965	58
	1/4	1 309	24
		1 342	25
Poulik's	1/2	1 183	98
		1 278	97
	1/4	1 354	97
		1 196	98
Double	1/8	943	64
		988	68
Control	—	1 296	98
	—	1 551	98

Hanks' : 16 p. 100 DMSO, 0 FCS, pH 7.6. Poulik's : 10 p. 100 PVP, pH 8.6. Double : Milt diluted 1/2 in Hanks' for 15 min. then rediluted 1/4 in Poulik's for 15 min. Dilution refers to the milt (i.e., a 1/2 dilution of milt results from a 1 : 1 mixture of milt and diluent).

The higher the pH of either Hanks' or Poulik's diluent, up to the highest pH used (pH 8.9), the greater was the fertility of sperm after incubation. When incubated in a 1/2 dilution of Hanks' diluent with pH of either 7.0 or 8.6, sperm had a higher fertility in the pH 8.6 solution (45 p. 100 compared to 39 p. 100 in the pH 7.9 solution). However pH cannot be the primary factor responsible for the different fertility in the 2 diluents. When milt was incubated in a 1/2 dilution of either Hanks' or Poulik's, each adjusted to pH 8.6, fertility in Hanks' was only 15 p. 100 compared to almost 70 p. 100 in Poulik's.

Incubation of aging sperm. — Table 3 shows the increased fertility of 2-day-old rainbow trout milt after incubation in Poulik's diluent. The diluent apparently has

a restorative effect on sperm fertility and may be useful for short-term preservation of sperm without freezing.

TABLE 3

Fertility of fresh and 2-day-old rainbow trout sperm before and after incubation in Poulik's diluent for 20 min. at 8 °C

	Fresh milt		2-day-old milt	
	N° eggs	p. 100 eyed	N° eggs	p. 100 eyed
Unincubated	847	84	798	13
	658	78	621	7
Incubated	534	87	741	78
			830	79

Milt was diluted 1/2 in Poulik's (10 p. 100 PVP ; pH 8.9).

Freezing sperm. — Freezing sperm to various temperatures reveals the warmest freezing temperature at which fertility is lost. Table 4 shows the results of freezing brook trout sperm to — 6 °C and Atlantic salmon sperm to — 8 °C. After being frozen to — 8 °C in the Poulik's solution Atlantic salmon sperm had a fertility of 84 and 93 p. 100 compared to 96 p. 100 for control sperm.

TABLE 4

Fertility of sperm after « Freezing »

	°C	Diluent	Initial dilution	Post-thaw incubation	Final dilution	N° eggs	p. 100 eggs hatched
Brook trout							
	0	Control	—	—	—	934	91
			—	—	—	1 031	94
	— 6	Poulik's	1/2	+	1/4	253	51
		Hanks'	1/2	+	1/4	405	35
			1/4	+	1/8	351	2
			1/4	—	1/4	471	15
Atlantic salmon							
	0	Control	—	—	—	675	96
	— 8	Poulik's	1/2	+	1/4	484	84
			1/2	+	1/4	506	93

After initial dilution, incubate 15 min. at 7.5 °C. Post-thaw incubation 15 min., 7.5 °C in Poulik's. Poulik's : 10 p. 100 PVP, pH 8.6 ; Hanks' for 1/2 dilution of milt : 12 p. 100 DMSO, 12 p. 100 FCS, pH 7.6 ; Hanks' for 1/4 dilution of milt ; 8 p. 100 DMSO, 8 p. 100 FCS, pH 7.6.

It is not known whether the sperm had completely frozen, but after a 1/4 dilution of milt in Hanks' (3 000 mOsm), the medium was approximately 2 300 mOsm, which would freeze at — 1.24 °C. Note that fertility after freezing in Hanks' was better without a post-thaw dilution in Poulik's, even though the double dilution of incubated, unfro-

zen sperm (table 2) increased sperm fertility. This suggests that the osmotic response of sperm that have been frozen to — 6 °C in Hanks' differs from that of fresh, incubated sperm.

Fertility of sperm in Hanks' after freezing to — 20 °C or lower has been unpredictable and low. In Poulik's, it has been completely lost. Perhaps the PVP in Poulik's is an unsatisfactory extender for salmonid spermatozoa at temperatures as low as — 20 °C, even though it was effective at — 8 °C. At the ultrastructural level sperm seemed intact after thawing from — 196 °C in Hanks' (Summers and Gregory, 1974). In another experiment, rainbow trout sperm appeared motile after 7 months storage at — 196 °C in Hanks'. They had not been fertile when thawed immediately after freezing. In one early study with extremely poor rainbow trout gametes (control fertility was 0 and 0.2 p. 100) fertility after freezing to — 196 °C in Hanks' without post-thaw incubation in Poulik's was 0 p. 100, but with the post-thaw dilution it was 6 and 4 p. 100. In this study, thawed sperm were diluted in Poulik's only after a delay of about 40 min at 0 °C. In an earlier study, without post-thaw incubation, fertility after freezing to — 196 °C in Hanks', was 7 p. 100 compared to about 40 p. 100 for control, fresh milt.

TABLE 5

Fertility of rainbow trout sperm after vacuum « drying » and storage

Treatment	Storage (0 °C)	N° eggs	p. 100 hatch
January, 1974			
Control	—	1 489	70
« Dried »			
Undiluted	1/2 hour	1 082	64
Undiluted	1 hour	1 000 *	0
	Storage (— 11 °C)	**N° eggs**	**N° eyed**
April, 1975			
Control	—	1 192	262
Control	—	1 133	181
« Dried »			
Undiluted	10 hours	1 204	20
Undiluted	1 week	910	36
Undiluted	1 week	917	17
Diluted	1 week	1 039	31
Diluted	1 week	1 333	41
Undiluted	11 days	1 718	52
Undiluted	2 weeks	1 481	7
Undiluted	2 weeks	1 807	3
Diluted	2 weeks	1 676	22
Diluted	2 weeks	1 364	0
Undiluted	1 year	1 854	11

Milt was « dried » either undiluted or after a 1 : 1 dilution with Poulik's diluent (pH 8.7). Extent of dehydration was not measured. In the April 1975 experiment 12 samples of « dried » diluted or undiluted milt, stored for from 10 hours to 2 weeks and 1 sample stored for 1 year had no fertility. A total of 8 eyed eggs resulted from three additional samples. * Number is an estimate.

Clearly, for successful freezing below — 8 °C, several changes in the protocol are necessary. The following seem advisable : (1) use DMSO as the cryoprotectant in the modified Poulik's diluent, (2) gradually dilute the Hanks' diluent after thawing, thus reducing osmotic damage of (3) use a more physiological concentration of Hanks' for freezing protection.

Vacuum drying sperm. — Vacuum drying as an alternative to freezing would eliminate problems with storage at very low temperatures. There are reports of successful inseminations with freeze-dried sperm of rabbits (Yushchenko, 1960) and bulls (Meryman and Kafig, 1959, and Vandemark, 1961) and other reports of motility after « drying » sperm of bulls (Singh and Roy, 1966) and poultry (Polge *et al.*, 1949). However the method has not been reproducible (Saacke and Almquist, 1961 and Sherman, 1963).

The results of vacuum drying rainbow trout sperm have also been unreproducible (table 5). In the first experiment, 64 p. 100 in one sample of eggs fertilized with reconstituted, « dried » sperm and 0 p. 100 in the other sample hatched. Milt in both samples looked dry and flaky, but no measurement or control of percentage dehydration and no control over the temperature reached by samples during drying was possible.

When the experiment was repeated in the spring of 1975 some samples were diluted with Poulik's diluent before drying. « Dried » undiluted milt formed clumps when reconstituted with water, but diluted milt dispersed readily after rehydration. The results shown in table 5 reveal little relationship between fertility and pre-drying dilution. The small number of eggs that eyed after insemination with « dried » sperm may reflect the poor quality of eggs. Only a small percentage of control eggs eyed and a smaller percentage hatched. Because of the egg quality and the inconsistent fertility of « dried » sperm, the results are presented as the number rather than the percentage eggs that eyed.

A few samples, including some with resultant fertility, contained visible moisture after storage with a dessicant in sealed containers at — 11 °C. Samples stored at + 4 °C for one year had decomposed. Although the amount of water removed was not measured and was not the same in separate samples, it is certain that the samples were not completely dry. However enough water had been removed to allow some survival of « dried » sperm after 6 hrs in liquid N_2, since in one experiment two eggs fertilized with such sperm had eyed.

Sample temperatures that were measured during drying varied. Minimum values ranged from — 7 to — 35 °C. The freeze-drying equipment was modified in an attempt to control sample temperatures and thus control the rate of dehydration, but the changes were unsuccessful. In subsequent experiments sperm were structurally disrupted and the tails were often tangled. For lack of available equipment needed to control the rate and extent of sample dehydration, the experiments have been discontinued.

Although results show that vacuum-dried milt can be fertile, it is clear that the method needs significant modification and refinements before it can be a useful procedure. Perhaps freezing milt with a diluent to as low a temperature as practical before drying it, would eliminate the mechanical damage observed in the present

experiments in which a vacuum was applied to unfrozen samples of milt. Reconstituting « dried » milt with more water than was removed during drying could activate sperm. Either an alternative to using water or a careful measurement of the quantity of water needed might improve retention of fertility after rehydration.

Egg and embryo preservation. — The data in table 6 represent the results of freezing unfertilized eggs, zygotes and eyed eggs (embryos) from one set of experiments with rainbow trout and book trout gametes. Unfertilized eggs, frozen to — 20 °C were fertile after being thawed, and high percentages of zygotes or eyed eggs survived after being frozen to temperatures as low as — 50 °C. Additional experiments with Atlantic salmon and rainbow trout eggs have yielded similar results. So far the minimum temperature at which zygotes and eyed eggs have survived has been — 55 °C with gametes from Atlantic salmon.

TABLE 6

Hatching of eggs after freezing : no storage

Species	Stage frozen	Fertilization medium	Freezing medium	Minimum temp. °C	N° eggs	p. 100 hatch
Rainbow	Unfert.	HSS	HSS	— 20	360	66
	Zygote	HSS	HSS	— 20	370	68
	Eyed	Water	HSS	— 5	75	96
	Eyed	Water	HSS	— 20	75	39
	Control	HSS	—	+ 3 to + 5	*	(75) *
	Control	Water	—	+ 3 to + 5	643	74
	Control	Water	—	+ 3 to + 5	700	80
Brook	Zygote	HSS	HSS	— 50	750	20
	Zygote	HSS	Hanks'	— 50	230	70
	Control	HSS	—	+ 3 to + 5	505	80
	Control	HSS	—	+ 3 to + 5	693	71
	Control	Water	—	+ 3 to + 5	862	87

Control eggs ; minimum temperature is approximate. * Sample had about 1 000 eggs ; An incubator accident shortly after eying destroyed majority of eggs. Most had eyed and about 75 p. 100 of few remaining eggs hatched. HSS : Hanks' (1 X) salt soln., Hanks' : diluent with 10 p. 100 PVP, 8 p. 100 DMSO.

After fertilization and before eying, the egg is especially delicate. Fortunately by fertilizing salmonid eggs in a salt solution that allows the sperm to enter the micropyle but prevents the elevation of the fertilization membrane, one can have a zygote that is physiologically like an unfertilized egg (Ginsburg, 1963). In several experiments, about 75-100 p. 100 of the control eggs that were fertilized in the Hanks' (1X) salt solution (pH 7.9) hatched. A similar percentage of these eggs (zygotes) hatched after they were frozen to — 5 or — 12 °C.

Hatching of all eggs that were supercooled or frozen to — 5 or — 12 °C has been uniformly high. Survival after freezing to colder temperatures has been less consistent. The biggest technical difficulty has been controlling the optimal rate of cooling. A sudden release of the latent heat of fusion in a sample raises its temperature by as much as 15 °C or more and causes a high mortality. It is probable that

eggs surviving at — 40 and — 55 °C would have survived colder temperature as well, but the successful method of cooling has not been standardized adequately for testing survival at lower temperatures.

So far an initial freeze rate of about 3-5 °C/min., followed by a somewhat faster rate below — 8 °C has been most successful. Optimal thaw rate seems to depend on the freeze rate and perhaps also on the stage of development. Unfertilized eggs and zygotes have survived best with a slow thaw ; eyed eggs have done best with a rapid thaw. The techniques are still in a preliminary stage of development and storage of frozen eggs has not been tested. Methods are now being developed for controlling the release of latent heat during freezing and for storing the frozen samples at different temperatures.

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Résumé. Après conservation pendant deux jours à l'état non dilué, du sperme de truite Arc-en-ciel *Salmo gairdneri* est mis en incubation dans un diluent Tris-citrate (pH 8,9) ; le pouvoir fécondant passe alors de 7 et 13 p. 100 à 78 et 79 p. 100, atteignant le niveau initial du sperme frais. Du sperme de saumon atlantique *Salmo salar* congelé à — 8 °C conserve une fécondance de 84 à 94 p. 100 ; après conservation à — 196 °C ; les spermatozoïdes retrouvrent leur motilité, mais non leur fécondance; ceci est vraisemblablement dû à la pression osmotique et au cryoprotecteur des dilueurs. Des spermatozoïdes lyophylisés conservent leur pouvoir fécondant mais la méthode doit être améliorée. Des œufs pris immédiatement après insémination et des embryons au stade œillé survivent après passage à — 55 °C. Des ovules congelés à — 20 °C ont pu être fécondés après décongélation.

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The development of the gonadal and immune systems in the Atlantic salmon (*Salmo salar* L.) and a consideration of the possibility of inducing autoimmune destruction of the testis

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Summary. Gonad development was studied histologically and morphologically in eggs and fry of *Salmo salar* L. First signs of gonad tissue were found 6 days prior to hatching. Sex differentiation of the gonads appeared to be complete by about 60 days post-hatch. The development of the lymphoid system was also studied. The thymus is the first organ to become lymphoid (22 days post-hatch). Membrane bound immunoglobulin M (mIgM) and Mixed Lymphocyte Reactivity (MLR) appeared simultaneously about 45 days post-hatching, coincident with the start of feeding.

Experiments using testicular extracts injected into salmon parr have indicated that it is possible to induce autoimmune gonad destruction.

Introduction.

The efficiency of salmonid farming would be greatly increased if gonad maturation could be stopped. Food would be converted to somatic tissue more efficiently and problems associated with precocious maturation, such as disease, eliminated. In several species of mammals experimental autoimmune rejection of testis germinal epithelium and autoimmune orchitis has been achieved using isogenic or allogenic tissue as antigen in Complete Freund's Adjuvant (CFA) (reviewed by Rumke and Hekman, 1975). The antigens producing the lesions are associated with mature and maturing germ cells and are of a polysaccharide-polypeptide complex of low molecular weight (Bishop et Carlson, 1971) or the enzyme sorbitol dehydrogenase (Mancini and Andrada 1971).

This paper presents the results of preliminary investigations into the ability of injected testis homogenate to produce auto-immune destruction of gonad tissue in salmon and to determine the earliest stage of development when stimulation of the

immune system could bring about such a lesion. The differentiation of the gonads, and the immune system with respect to both humoral and cell-mediated immunity is described.

Materials and methods.

Salmon eggs and fry were kept in running water at 10 °C, hatching occurred 45 days post-fertilization : these were used for the study of gonad development. A separate batch of eggs was kept in running water at 4 °C rising 7 °C, hatching 111 days post-fertilization for the study of the development of the immune system. For the experimental work year old salmon parr and smolts, mean length 10 cms, were kept in aquarium tanks. Some of these fish were likely to mature in the next two months. Testes were extracted from a freshly killed maturing male parr. The whole testes were ground up and washed in phosphate buffered saline (PBS). 3 ml of this suspension were mixed with CFA using an ultrasonic probe. This was injected intraperitoneally into 5 parr in October 1976. A follow-up injection of sperm in PBS (0.2 ml/fish) was given 4 weeks later. A second set of 5 parr received no treatment and were held in similar conditions. Two fish from each group died, the remainder were killed in May 1977. For all the histology carried out, tissues were fixed in 10 p. 100 buffered formol-saline and paraffin embedded sections cut at 5 μ. Routine staining was in haematoxylin and eosin, and Mallory's trichrome, in addition Unna Pappenheim and fluorescent

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PLATE 1

Differentiating gonad ventral to kidney, 6 days prior to hatching. k, kidney tubule ; i, intestine ; g, gonad. × 350.

PLATE 2

Gonad containing large cells at time of hatching. g, gonad. × 220.

PLATE 3

Female gonad containing oogonia and primary oocytes 60 days post-hatching. × 110.

PLATE 4

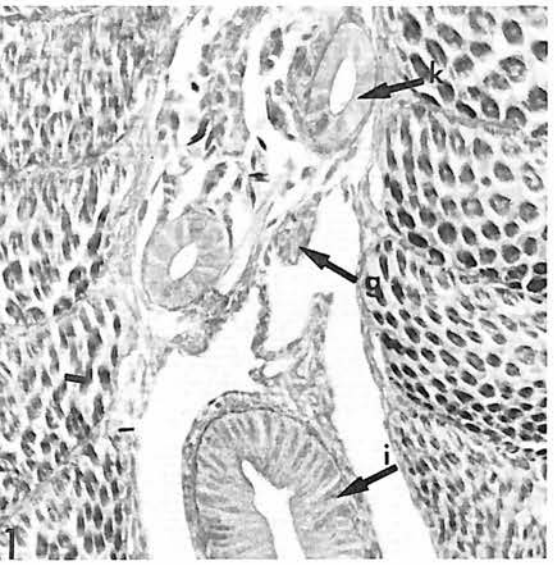
Thymus containing small thymocytes, 12 days prior to hatching. t, thymus ; g, gill bar. × 110.

PLATE 5

Autoimmune lesion in testis showing absence of gametes and extensive lymphocyte infiltration within the gonad tissue. Haematoxylin and eosin. × 160.

PLATE 6

Autoimmune lesion in testis showing infiltration by plasma cells and small lymphocytes. p, plasma cells ; l, lymphocytes. Stained with Unna-Pappenheim. × 250.



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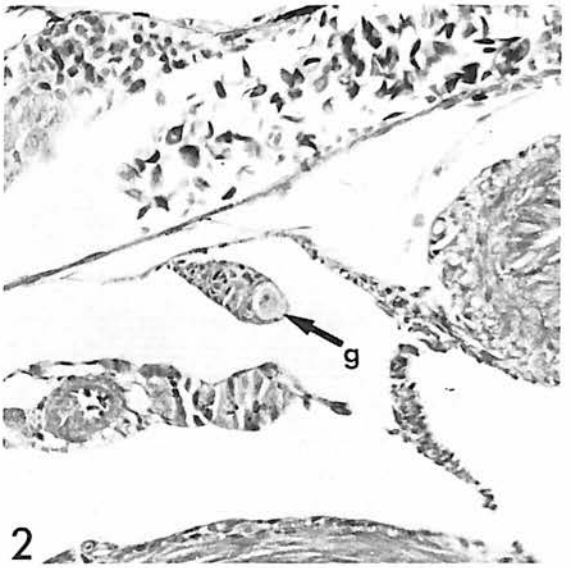

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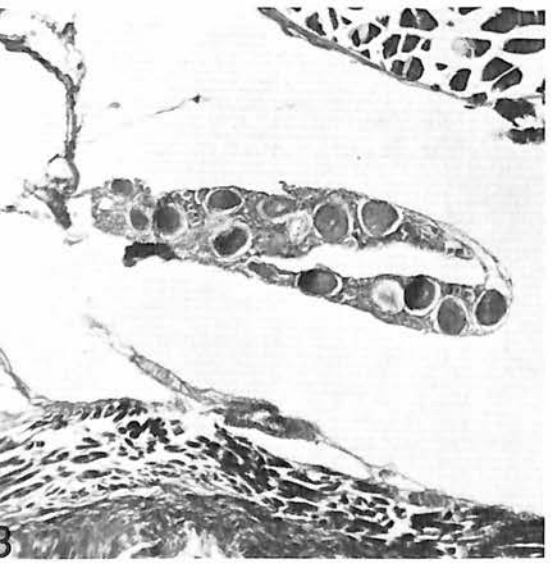

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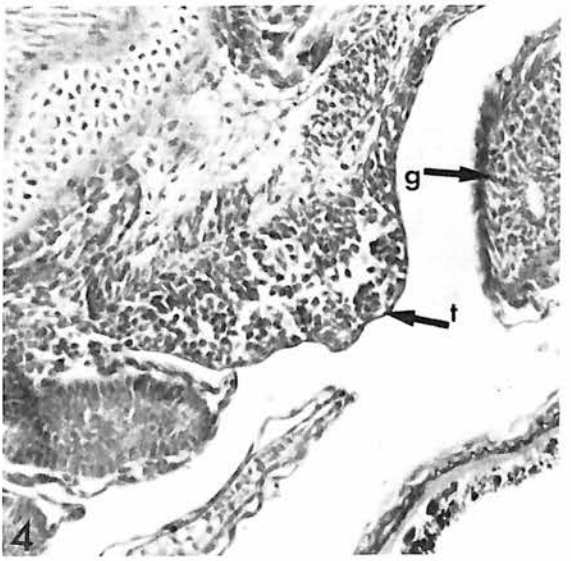

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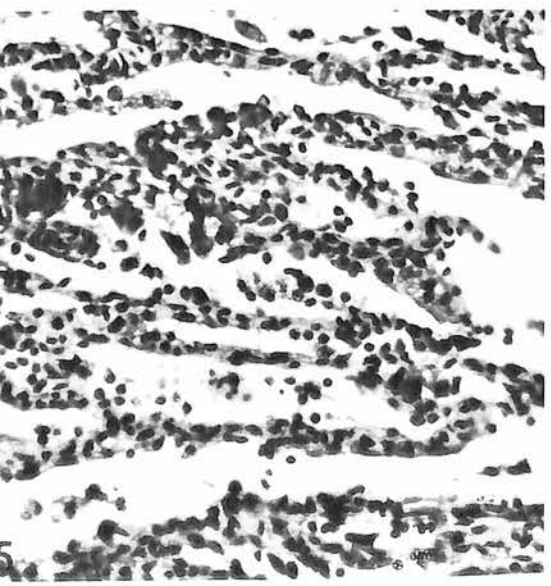

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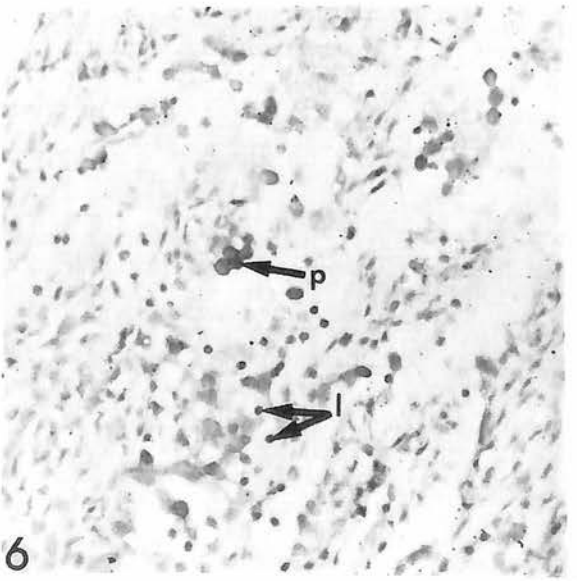

6

antibody techniques were performed. Methods for the immunofluorescent assay for mIgM and MLR have been reported in detail elsewhere (Waith and Hirschhorn, 1973 ; Ellis, 1977).

For sections of fish embryos, the shell was removed and the yolksac cut off before sectioning.

Results.

A. *Development of the gonad system.* — Gonads first appeared 6 days prior to hatching and were visible as ridges in the peritoneum at the base of the kidney (plate 1). The gonad ridge extended from a point above the anterior end of the stomach to a point above the anus. Germ cells appeared to be formed in the endoderm surrounding the gut as suggested for amphibia, birds and mammals (Berrill and Karp 1976), and to migrate through the dorsal mesentry to the gonad primordia. The gonads increased in size and became separated from the base of the kidney, although still attached to the dorsal mesentry. At the time of hatching, larger cells (14-16 μ diameter) were visible in the gonad (plate 2). The sexes were distinguishable by 60 days after hatching. The female gonad had increased in size, oogonia and primary oocytes are developing (plate 3). The male gonad increased in size more slowly, spermatogonia appearing after the oogonia in the female.

B. *Histological and functional development of the lymphoid system.* — The thymus was the first organ in which lymphoid differentiation took place. Development occurred within three separate pockets located over the first three gill arches, within the pharyngeal epithelium (plate 4). The anlage differentiated 22 days post-hatch and subsequently coalesced (table 1). The kidney contained haemopoietic stem cells prior to 23 days pre-hatch, but lymphoid cells were not apparent prior to 14 days pre-hatch. The spleen differentiated late in ontogeny, at 42 days post-hatch. This organ is never prominently lymphoid. Circulating lymphocytes were first observed in sections of blood vasculature 14 days pre-hatch.

TABLE 1

Histological and functional development of the lymphoid system in Salmo salar *kept at 4 °C rising to 7 °C at hatch*

Events	Days pre-hatch
Spawning	111
Lymphoid cells present in embryo homogenates	22
Thymocytes differentiated	22
Circulating lymphocytes	14
Kidney lymphocytes	14
	Days post-hatch
Feeding behaviour	41
Spleen differentiated	42
mIgM present on lymphocytes	42
MLR positive	45

Prior to feeding (41 days post-hatch) the lymphoid cells were negative for mIgM. In succeeding days the proportion of lymphocytes bearing mIgM increased and by day 48 post-hatch 80 p. 100 of lymphocytes were positive. Results for the mixed lymphocyte reaction are shown in table 2.

TABLE 2

MLR using homogenates of whole larvae. Disintegrations per minute (DPM) are averages of assays done in triplicate for 3 individual larvae

Days post-hatch	38	45	60	85	90
Control DPM (av)	3 400	2 700	8 000	3 000	12 000
MLR DPM (av).....	3 300	3 800	18 000	11 000	35 000
Ratio MLR/Control	1	1.4	2.2	3.6	3

C. *Auto-immune destruction of testicular tissue.* — The three surviving control fish developed gonads in the same way as the wild smolts. Three treated fish also survived and smolted.

Fish 1. This was a male fish and both superficial examination and subsequent histology indicated that it had certainly been maturing at the time of injection. The testes showed signs of degeneration and adhered to the body wall in some places. Histological examination showed that extensive degeneration of the testes had taken place, together with oedoema and proliferation of fibrous tissue (plate 5). Neighbouring muscle tissue was not damaged. Staining with Unna Pappenheim showed pyroninophilic cells which are plasma cells associated with antibody production in immune reactions (plate 6). Fluorescent antibody tests (Sainte-Marie, 1962) also showed antibody-producing cells. No germ cells could be seen in section.

Fish 2. Gross examination suggested that this male fish had not matured, the gonads were two long, thin strands. Histological examination showed that the testes had responded to the injection. Compared with a normal testis from a smolt which had not previously matured there was an increase in fibrous tissue, antibody producing cells were present and germ cells were absent. Histological examination of kidney, liver and spleen from this fish showed no abnormalities, suggesting that the reaction produced in the testes was not merely the result of the adjuvant. This is supported by results from other experiments where Freund's Complete Adjuvant has been used.

Fish 3. Gross and histological examination showed this fish to be a normally developing female except for a small area of fibrous tissue in the region of the injection. Growth and external appearance of the treated fish seemed to be normal.

Discussion and conclusions.

These preliminary experiments indicate that it is possible to alter gonad development by stimulating auto-immune rejection. It appears that this rejection is both sex- and organ-specific. More extensive studies are being carried out to investigate the

nature of the reaction and its long term effects. In order for the method to be used commercially, Complete Freund's Adjuvant would need to be replaced by a less toxic adjuvant and it would also be preferable to isolate soluble antigens, possibly administering them osmotically (Amend and Antipa, 1976) at the earliest possible stage of the fishes development. It appears from these results that the immune system reaches a competent stage of development to be stimulated at the time of first feeding.

Symposium sur la Reproduction des Poissons
Paimpont, France, 19-21 *septembre* 1977.

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Résumé. Le développement des gonades a été étudié histologiquement et morphologiquement sur des embryons et alevins de *Salmo salar* L. Les premiers signes de formation des gonades apparaissent 6 jours avant l'éclosion. La différentiation sexuelle des gonades apparaît achevée environ 60 jours après l'éclosion. Le développement du système lymphoïde a aussi été étudié. Le thymus est le premier organe qui devient lymphoïde (22 jours après l'éclosion). Des immunoglobulines M liées aux membranes (mIgM) et une immunoréactivité mixte des lymphocytes (MLR) apparaissent simultanément environ 45 jours après l'éclosion et coïncident avec la première prise de nourriture.

Des expériences utilisant des extraits testiculaires injectés à des parrs indiquent qu'il est possible d'induire une destruction autoimmune des gonades.

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TABLE DES MATIÈRES

INTERNATIONAL SYMPOSIUM ON REPRODUCTIVE PHYSIOLOGY OF FISH

Paimpont (France), 19-21 septembre 1977

I. — Gonadotropine hypophysaire.

II. — Axe hypothalamo-hypophysaire.

TABLE DES MATIÈRES

III. — Histo-physiologie de la gonade.

IV. — Endocrinologie du cycle sexuel : variations hormonales, contrôle de la maturation et de l'ovulation.

V. — Vitellogenèse.

VI. — Effet de la photopériode et de la température.

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