PHYSIOLOGICAL CONTROL OF REPRODUCTION IN FEMALE WINTER FLOUNDER

(pseudopleuronectes americanus WALBAUM)

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PHYSIOLOGICAL CONTROL OF REPRODUCTION IN FEMALE WINTER FLOUNDER

(*PSEUDOPLEURONECTES AMERICANUS* WALBAUM)

A Thesis

Presented to

The Department of Biology

Memorial University of Newfoundland

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

 Christopher Moger Campbell, B.Sc. (Wales)

February 1975

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ABSTRACT

Vitellogenesis in *Pseudopleuronectes americanus* Walbaum involves a transfer of yolk material synthesised by the liver into the oocytes in the gonad. The hormones controlling these processes were studied. Hormones which can induce maturation and/or ovulation of oocytes were identified.

The gonadal lipid accumulation during winter starvation is not accounted for by the decrease in the concentration of liver lipid. Vitellogenesis must consist of three phases; mobilization of lipid and other energy reserves into the circulation, synthesis of yolk proteins by the liver, and uptake of yolk into oocytes.

Hypophysectomy suppressed both liver synthesis and oocyte uptake of yolk protein. Injection of estradiol-benzoate restores the liver synthesis of yolk, resulting in accumulation of yolk proteins in serum because none is incorporated by the gonad. A glycoprotein fraction from *Hippoglossoides platessoides* Fabricius pituitaries, which is capable of stimulating ovulation, did not promote uptake into the gonads of the estradiol induced serum yolk but a non-glycoprotein fraction from these pituitaries with a molecular weight of 25000 to 35000 stimulated yolk incorporation.

17α-Hydroxy progesterone and 20β-dihydro-17α-Hydroxy progesterone were identified in plasma samples which had induced oocyte maturation *in vitro* bioassay, using a double isotope derivative assay. Cortisol, cortisone, deoxycorticosterone, 11-ketotestosterone and progesterone were

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identified in various plasma samples but not correlated to oocyte maturation.

Cortisone, deoxycorticosterone, 17α-hydroxy progesterone or 20β dihydro-17α-hydroxy progesterone induced maturation of oocytes incubated in plasma from male fish but appeared to have no conclusive effect upon oocytes incubated in artificial media. Injection of these steroids, estradiol-benzoate or testosterone into hypophysectomized fish did not induce oocyte maturation or ovulation.

A salmon gonadotropic preparation (SG-G100) or extracts of *P. americanus* pituitaries induced some oocyte maturation *in vitro*. Both a glycoprotein preparation from *H. platessoides* pituitaries and SG-G100 induced oocyte maturation and ovulation when injected into hypophysectomized fish.

ACKNOWLEDGEMENTS

I am grateful for the training and compatible supervision I have been given by Dr. D. R. Idler. Miss B. Truscott kindly put her knowledge of steroid biochemistry at my disposal. For this assistance, and the faith shown by these people and Drs. E. W. Crim, G. L. Fletcher and Mr. P. Woodhead, I will always be thankful.

I am deeply indebted to a large number of fellow graduate students and the diving staff at the Marine Sciences Research Laboratory for their assistance in the collection of the fish. The technical staff, research assistants and faculty of the Laboratory have offered assistance and a great deal of encouragement. I am grateful for the cooperation of Bonavista Cold Storage for facilitating pituitary collection and thank B. Bennett, J. Brown, R. Daggett, C. Emerson and J. Walsh for their aid in collecting the glands.

I thank Dr. Karen Nuti (University of Wisconsin), Dr. Ian Senciall and Miss Sheilagh Rahal (Faculty of Medicine, Memorial University of Newfoundland) for some preliminary steroid analyses. Dr. D. G. Copeman deserves special thanks for his helpful advice, particularly on the hypophysectomy technique.

The financial support of the Canadian Commonwealth Scholarship and Fellowship Administration and Memorial University of Newfoundland is gratefully acknowledged.

My wife must be acknowledged for her company, hard work, encouragement and faith.

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STEROID NOMENCLATURE

Common Names	Other Common Names	Scientific Names
Estradiol benzoate		1,3,5(10) Estratrien-3, 17β-diol, 3-Benzoate
Testosterone		4-Androsten-17β-ol-3-one
11-Ketotestosterone		4-Androsten-17β-ol-3, 11-dione
Progesterone		4-Pregnene-3, 20-dione
20β-Dihydroprogesterone	20β progesterone	4-Pregnene-3-one
17α-Hydroxy progesterone	17αOH progesterone	4-Pregnene-17α-ol-3, 20 dione
17α-Hydroxy, 20β-dihyro progesterone	17α 20β progesterone	4-Pregnene-17α-ol-3-one
Cortisol		4-Pregnene-11β, 17α, 21-triol-3, 20-dione
Cortisone		4-Pregnene-17α, 21-diol-3, 11, 20-trione
11-Deoxycortisol		4-Pregnene-17α, 21-diol-3, 20-dione
11-Deoxycorticosterone		4-Pregnene-21-ol-3, 20-dione
20β-Dihydro cortisone	20β cortisone	4-Pregnene-17α, 21 diol-3-one

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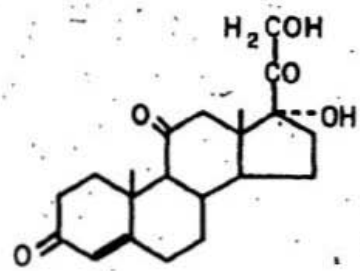
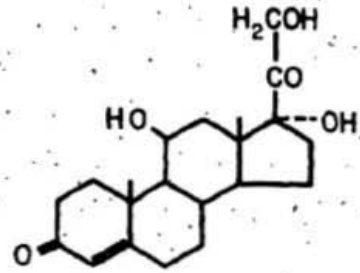
Figure 1

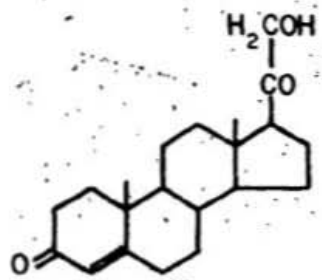
Structures of steroids identified in the plasma of female *P. americanus*.



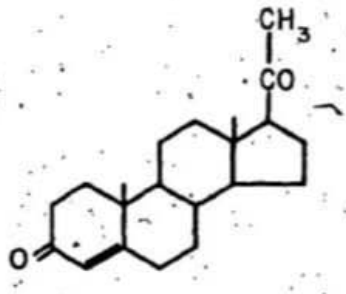
CORTISONE



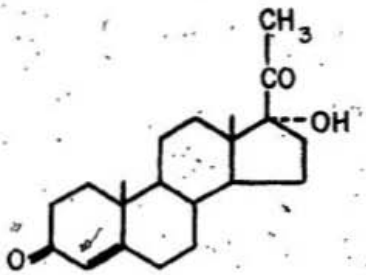
CORTISOL



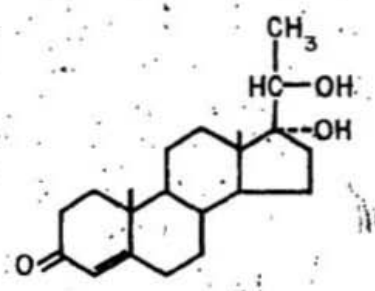
DEOXYCORTICOSTERONE



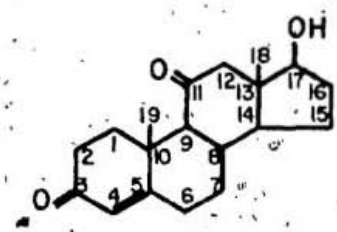
PROGESTERONE



17α OH PROGESTERONE



17α 20β PROGESTERONE



11-KETOTESTERONE

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GENERAL INTRODUCTION

One of the requirements for the development of intensive hatchery and fish culture practices is a knowledge of the physiological mechanisms involved in growth of yolky oocytes (vitellogenesis), and in the processes which complete the meiotic division (maturation) of the gamete prior to ovulation and spawning. These processes, which constitute oogenesis, have been reviewed by Schuetz (1969). Characteristically, major growth and accumulation of yolk reserves in oocytes occurs during an extended stage of meiotic prophase. When vitellogenesis is completed the meiotic division is resumed to produce a mature egg (maturation), capable of being fertilized after extrusion from the follicle (ovulation) and spawning.

Control of these mechanisms was investigated in the winter flounder *Pseudopleuronectes americanus* Walbaum as a model for the cold-temperature marine teleost. These fish are available throughout the year, undergo no major migrations and are restricted to shallow waters (Liem and Scott, 1966; Templeman, 1966). The annual reproductive cycle of the female flounder begins with the initiation of vitellogenesis in August. Oocyte growth appears to be completed by February and a period in which there is no further growth during the rest of winter ends at the time of spawning in May or June.

Vitellogenesis

A hypothesis developed for amphibia by Follett *et al.* (1968), Redshaw (1972), Wallace (1972) and Wallace and Bergink (1974), proposes

that vitellogenesis consists of at least two phases. A primary phase is the production of a serum lipophosphoprotein, vitellogenin, by the liver under estrogen stimulation. The secondary phase is a gonadotropin-stimulated incorporation of vitellogenin into the yolk of oocytes. Gonadotropins are assumed to be ultimately responsible for both phases by stimulating synthesis of estradiol by the ovary and by the direct effect on the gonad causing yolk incorporation.

Treatment with various mammalian and piscine gonadotropic preparations stimulated vitellogenesis in intact fish (Chistova, 1971; Ochai *et al.*, 1972; Shehadeh *et al.*, 1973). A homogenate of pituitaries from *Carassius auratus* L. caused an increase in gonad weight when injected into chemically hypophysectomised *Hypseleotris galii* (Mackay, 1973) and luteinising hormone induced gonadal growth in hypophysectomized *Heteropreustes fossilis* Bloch (Anand and Sundararaj, 1974).

Estrogens affect the teleost liver in ways which suggest that yolk synthesis does occur in a manner similar to that in amphibia. The most direct evidence is that of Plack and Frazer (1971) who showed that yolk proteins were produced in *in vitro* culture of liver slices from vitellogenic *Gadus morhua* L. Estrogen treatment has been shown to result in an increase in the size of livers of *Misgurnus anguillicaudatus* Cantor, *Oryzias latipes* Schlegel, *Gasterosteus aculeatus* L. and *Plecoglossus altivelis* Temminck and Schlegel (Kobayashi, 1953; Egami, 1955; Oguro, 1956; Aida *et al.*, 1973) and to induce in male livers the histological structure normally found in vitellogenic females. These effects may be explained as the results of a hormonal stimulation of protein synthesis by the liver.

Estradiol injection into *G. morhua* causes egg proteins to collect in plasma (Plack *et al.*, 1971) and induces changes in serum proteins of *Oncorhynchus nerka* Walbaum and *Salmo gairdnerii* Richardson (Ho and Vanstone, 1961; Takashima *et al.*, 1972). New plasma proteins are synthesised during vitellogenesis in *O. nerka*, *S. gairdnerii*, *Parophyrys vetulus* Girard, *P. altivelis* and *Brachydanio rerio* Ham. (Ho and Vanstone, 1961; Thurston, 1967; Utter and Ridgeway, 1967; Aida *et al.*, 1973; Heesen and Engels, 1973). These data suggest that in normal vitellogenesis proteins are transported in the blood and that estrogens are capable of stimulating their formation by the liver and/or cause their accumulation in the circulation by negative feedback (see below) to the pituitary.

Similar conclusions to those drawn from the protein work can be made after reviewing data on plasma calcium concentrations in teleosts. The concentration of this ion may act as an indicator for the presence of yolk protein since amphibian vitellogenin binds large amounts of calcium (Follett *et al.*, 1968; Wallace, 1970; and Ansari *et al.*, 1971). This might suggest that the high plasma calcium levels found in *G. morhua*, *C. auratus* and *Tilapia esculenta* during vitellogenesis may be an indicator of plasma transport of yolk (Hess *et al.*, 1928; Bailey, 1957; Garrod and Newall, 1958, and Oguri and Takada, 1967). Serum calcium levels are increased after injection of estradiol into various species (Bailey, 1957; Ho and Vanstone, 1961; Urist and Schjeide, 1961; Flemming *et al.*, 1964; Woodhead, 1969; Urist *et al.*, 1972) and this may be interpreted as further evidence for an estradiol stimulation of yolk synthesis by the teleost liver.

Much of the evidence for estrogen involvement in teleost

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vitellogenesis is circumstantial but the specific roles of gonadotropins and the hormonal control of yolk incorporation into oocytes have not been investigated. Estradiol injections inhibited vitellogenic growth in intact fish (Egami, 1954; Egami and Ishii, 1962; Sundararaj and Goswami, 1968) in a similar manner to hypophysectomy (Vivien, 1941; Barr, 1963; Yamazaki, 1965; Sundararaj and Goswami, 1968). This estrogen effect was explained by a negative feedback at the pituitary causing a reduction in the amount of gonadotropin released. In the work of Plack *et al.* (1971), intact *G. morhua* injected with estradiol synthesised yolk protein in the liver but, even though proteins accumulated in the serum, there was no incorporation into the ovary. If this case was similar to the negative feedback proposed above, the lack of gonadal incorporation of yolk may have resulted from the absence of a gonadotropin.

Hypophysectomised *P. americanus* were used to examine the separate roles of estrogen and pituitary hormone on vitellogenesis in the liver and in the gonad.

Oocyte Maturation.

The control of maturation of teleost oocytes has been attributed to an action of the pituitary gland. In hypophysectomised fish ovulation did not occur but if hypophysectomised *Carassius auratus* L. or *H. fossilis* were injected with luteinising hormone or human chorionic gonadotropin, ovulation occurred (Yamazaki, 1965; Sundararaj and Goswami, 1966). The use of gonadotropic and pituitary preparations to induce oocyte maturation and ovulation has been reviewed by Pickford and Atz (1957) and deVlaming (1974). Recently workers have used partially purified teleost gonadotropins

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to induce ovulation in teleosts (Sinha, 1971; Ishida, 1972; Kuo *et al.*, 1973; Sundararaj and Anand, 1972; Sundararaj *et al.*, 1972b) and Pandey and Hoar (1973) used clomiphene citrate to cause release of pituitary gonadotropin with subsequent ovulation in *C. auratus*.

When oocytes were incubated *in vitro*, gonadotropic preparations induced maturation or ovulation of oocytes from *Cobitis biwae* Jordan and Snyder, *M. fossilis*, *O. latipes*, *Acipenser stellatus* Pallas, *S. gairdnerii* and *Esox lucius* L. (Kawamura and Motonaga, 1950; Kirschenblat, 1959; Hirose, 1971; Goncharov, 1973; Jalabert *et al.*, 1973; Jalabert and Breton, 1974) but the effect on oocytes of *C. auratus* was slight and no effect on *H. fossilis* oocytes was seen (Jalabert *et al.*, 1973; Sundararaj and Goswami, 1972, 1974). The different reactions of oocytes of different species to this treatment suggests that the mediation of the maturation stimulus may vary from species to species.

The possibility that the gonadotropin induced ovulation is mediated by a steroid was raised by Kirschenblat (1959) and Ramaswami and Lakshman (1959) who found that cortisone, cortisol and deoxycorticosterone acetate induced ovulation in *Misgurnus fossilis* L. and *H. fossilis*. Later work by Sundararaj and Goswami (1966) showed that these steroids induced ovulation in *H. fossilis*, even after hypophysectomy. Several steroids induce oocyte maturation or ovulation in *in vitro* incubation experiments using oocytes from a number of species (Kirschenblat, 1959; Goswami and Sundararaj, 1971a, b, 1974; Hirose, 1972a; Fostier *et al.*, 1973; Jalabert *et al.*, 1973; Jalabert and Breton, 1974).

If oocyte maturation in all these species is mediated by a steroid, but the effect of direct action of gonadotropins on oocytes varies

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from species to species, a hypothesis of gonadotropin induction of a maturational steroid at some extra-gonadal source (such as the interrenal) accounts for the data for *C. auratus* and *H. fossilis* and in the other species maturational steroids appear to be synthesised by gonadal tissues.

Schuetz (1974) reviewed work on the control of oocyte maturation in amphibia and concluded that it supports a hypothesis in which pituitary gonadotropin stimulates follicle cells to synthesise a steroid which induces the maturational processes in the oocytes.

Maturation of amphibian oocytes *in vitro* has been induced using pituitary hormones (Schuetz, 1967a, 1967b; Brachet *et al.*, 1970; Thornton, 1971) and with steroids (Chang and Witshi, 1955; Schuetz, 1967a,b; Thornton, 1971; Alonzo-Bedate *et al.*, 1971; Merriam, 1971; Schorderet-Slatkine, 1972).

Masui (1967) attempted to demonstrate that the follicular cells produce the hormone responsible for maturation of oocytes from *Rana pipiens* Schreber. His approach (and that used by Schuetz, 1967; Dettlaff and Skoblina, 1969; Smith *et al.*, 1968) was to demonstrate that the *in vitro* effect of gonadotropins is no longer seen when the follicular layers are removed prior to incubation whereas progesterone maintained its action on such oocytes. Thornton (1972) detected a maturation inducing substance produced by *Bufo bufo* L. in response to a gonadotropin injection, using *in vitro* maturation bioassay (Thornton, 1971). Snyder and Schuetz (1973) demonstrated evidence for a follicular steroid synthesis associated with oocyte maturation and Wright (1971) showed that ovulation of *R. pipiens* oocytes in response to *in vitro* treatment with pituitary extract was

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abolished by injection of the donor fish with cyanoketone, a 3β hydroxysteroid dehydrogenase inhibitor. No inhibition of the progesterone-induced maturation was seen.

Because *H. fossilis* and *C. auratus* appear to mediate control of oocyte maturation via an extra-gonadal tissue, whilst in the other species tested the amphibian hypothesis of intra-ovarium maturation induction appears acceptable, it was desirable to evaluate the two hypotheses in *P. americanus* in order to help develop a theory on the control of maturation in teleosts.

GENERAL MATERIALS AND METHODS

Animals

Fish collected by divers, using hand nets, were transported from Chapel's Cove, Conception Bay, to the Marine Sciences Research Laboratory (60 km.) in at least 60 l of seawater. Precautions were taken to ensure adequate aeration in the warmer months and to avoid freezing in the winter.

At the laboratory female fish (300 - 1500 g) were selected by using a sealed beam lamp (U.S. Divers Co.) to demonstrate the gonadal silhouette and kept in small tanks of running seawater (approximately 0.5 m deep, 1 m^2) at a density of less than 20 fish/m^2. These tanks were maintained at ambient seawater temperatures (-1.5°C in winter up to 15°C in summer) unless specifically stated.

Blood Sampling

Blood samples were collected through 23 gauge needles in 1.5 - 5 ml. heparinized plastic syringes by a ventro-lateral approach midway along the body to a caudal blood vessel in the haemal arch. The iced blood was centrifuged within a few hours and plasma removed for freezing and transfer to storage at -70°C.

Anaesthesia

At temperatures above 2°C anaesthesia was accomplished by immersion of the fish in 4 l of seawater containing 0.5 gm (125 ppm) tricaine methane sulfonate (MS 222) (Kent Laboratories). At colder temperatures 1 gm

(250 ppm) was used. During operations anaesthesia was maintained by continuous irrigation of the gills using an ice chilled, recirculated solution of MS 222 (31 ppm).

Gonad Biopsy

Samples of gonad tissue could be removed for observation from the live animal after light anaesthesia using a specially modified pair of forceps or more usually using a Silverman Biopsy needle (14 ga). These instruments were inserted through the urinary papilla or the genital opening to gain access to the ovary.

Hypophysectomy

The anaesthetized fish was placed on its right side (pale side upwards) to expose the lower surface of the head. A 2-3 cm skin incision was made from the opercular hinge toward the angle of the jaws (Fig. 2). The fibres of the adductor mandibulae were retracted and scraped from the ventro-lateral surface of cranium where a facial nerve emerges.

A dental drill fitted with a number eight round burr was used to drill a hole 3-4 mm in diameter slightly anterior to the emergence of this nerve. A pasteur pipette attached to a water aspirator was used to remove bone debris and fluids from the cranial opening. An angled probe was inserted through this hole and the optic nerves moved dorsally; the pituitary gland was drawn into sight and could be aspirated. A check for hypophysectomy was made by noting the disappearance of the pituitary from the brain or by its appearance in the pipette.

The wound was filled with Aureomycin grease (Cyanamid of Canada Limited), when retraction was removed the muscle tissue resumed its

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Figure 2

1. Hypophysectomy technique. Completed operation, showing orientation of incision relative to opercular hinge and angle of the jaws (arrowed).
2. Open wound showing hole drilled in cranium through which the pituitary gland is aspirated.
3. Pituitary gland in aspirator pipette confirms success of operation.

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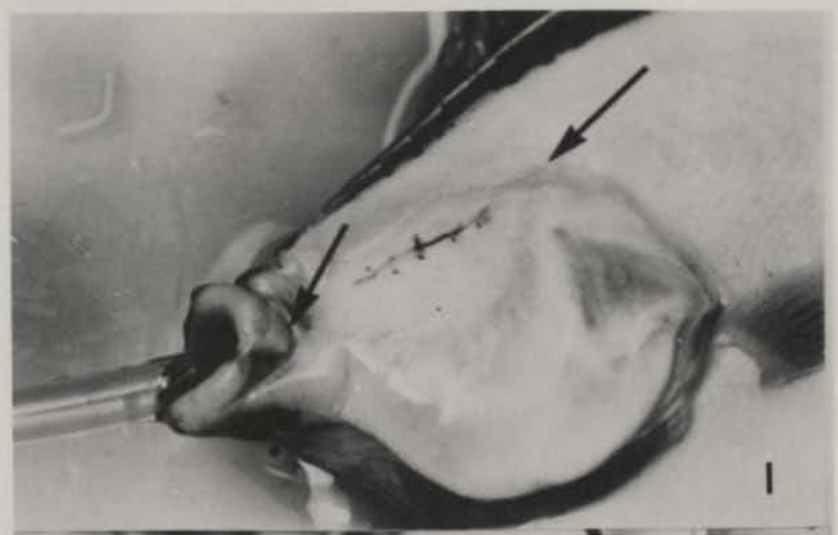
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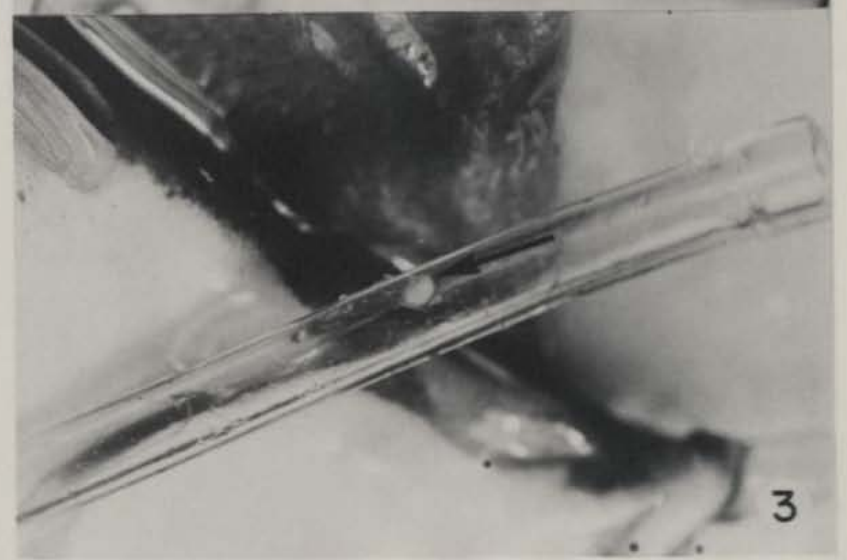
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normal position and Ethicon Plain Gut 000 was used to suture the skin wound.

Sham operated fish were subjected to identical procedures except that after the brain had been moved dorsally, only the debris and cranial fluids were aspirated, the pituitary remaining undisturbed.

Fish were tagged using spaghetti tags (Floy Tag and Manufacturing Co. Ltd.) which were marked with the fish's weight, and placed back in the seawater tank for recovery.

Preparation of Pituitary Fractions

Pituitary glands, collected from iced *H. platessoides* (up to 9 days post-mortem) at Bonavista Cold Storage, Fermeuse, Newfoundland were stored at -70°C till used. Purification was performed at 4°C.

Fractionation techniques were based on Idler *et al.* (1975). 28 gm of pituitaries collected in September 1973 were homogenized with 112 ml of a 40% ethanol, 0.5 M Tris-cl, 0.9% NaCl, 10^{-3}M EDTA, 10^{-4}M dithiothreitol (DTT) buffer, pH 7.8. After centrifuging at 7,500 g, the pellet was re-extracted and the final supernatant diluted with three volumes of ice cold ethanol, stirred and the precipitate allowed to settle. This precipitate was dissolved in pH 7.4, 0.05 M sodium phosphate, containing 10^{-4} M DTT and dialysed against two changes of pH 7.4 0.05 M sodium phosphate, 0.05 M NaCl, 10^{-4} M DTT. After application to a 2.6 X 35 cm column of Con A sepharose (Pharmacia Fine Chemicals) the column was flushed with the second phosphate buffer until the optical density (λ = 280 nm) of the eluate, collected by a fraction collector, was reduced to 0.01. Then the same buffer containing 0.2 M α-D methyl

glucoside was used to displace the glycoprotein fraction (PG1) which was concentrated by ultrafiltration (Amicon PM10) to 10 ml.

A second glycoprotein fraction (PG2) was prepared from 8.9 gm of pituitaries collected from sexually mature fish during January 1974. These pituitaries were extracted directly into 95 ml of pH 7.7 0.05 M Tris-cl, 0.5 M NaCl, 2 X 10^{-4} M DTT column buffer and 40 ml of the supernatant supplied to 0.9 X 25 cm column of Con-A sepharose, to separate the glycoprotein and non-glycoprotein fractions.

A third glycoprotein preparation (PG3) was prepared from 50 gm of pituitaries, collected during April 1972 and stored for two years at -70°C; the method was that used in preparing PG2 but the glycoprotein fraction was separated on a 26 X 35 cm Con-A sepharose column.

For a fourth preparation 56 gm of pituitaries from the same batch as those used to prepare PG 3 were extracted in a similar manner. Half of this extract was frozen at -70°C and the other half subjected to affinity chromatography on Con-A sepharose as described above. The glycoprotein (PG4) and non-glycoprotein (PNG4) fractions were concentrated by ultrafiltration (Amicon PM10) for use. After thawing, the remains of the extract was applied to Con-A sepharose and the concentrated non-glycoprotein fraction applied to a Sephadex G-75 column (80 X 5 cm) for elution with 0.5 M Tris-cl (pH 7.7 at 25°C), 0.9% NaCl, 1 mM EDTA, 10^{-4} M DTT. Part of the high molecular weight fraction of this eluate was separated, saved for recombination, and the remainder of the sample concentrated by ultrafiltration (Amicon PM10) for application to a pair of Sephadex G-75 columns (90 X 2.5 cm) arranged in series. The protein was eluted using the same buffer and 5 ml fractions collected. A protein

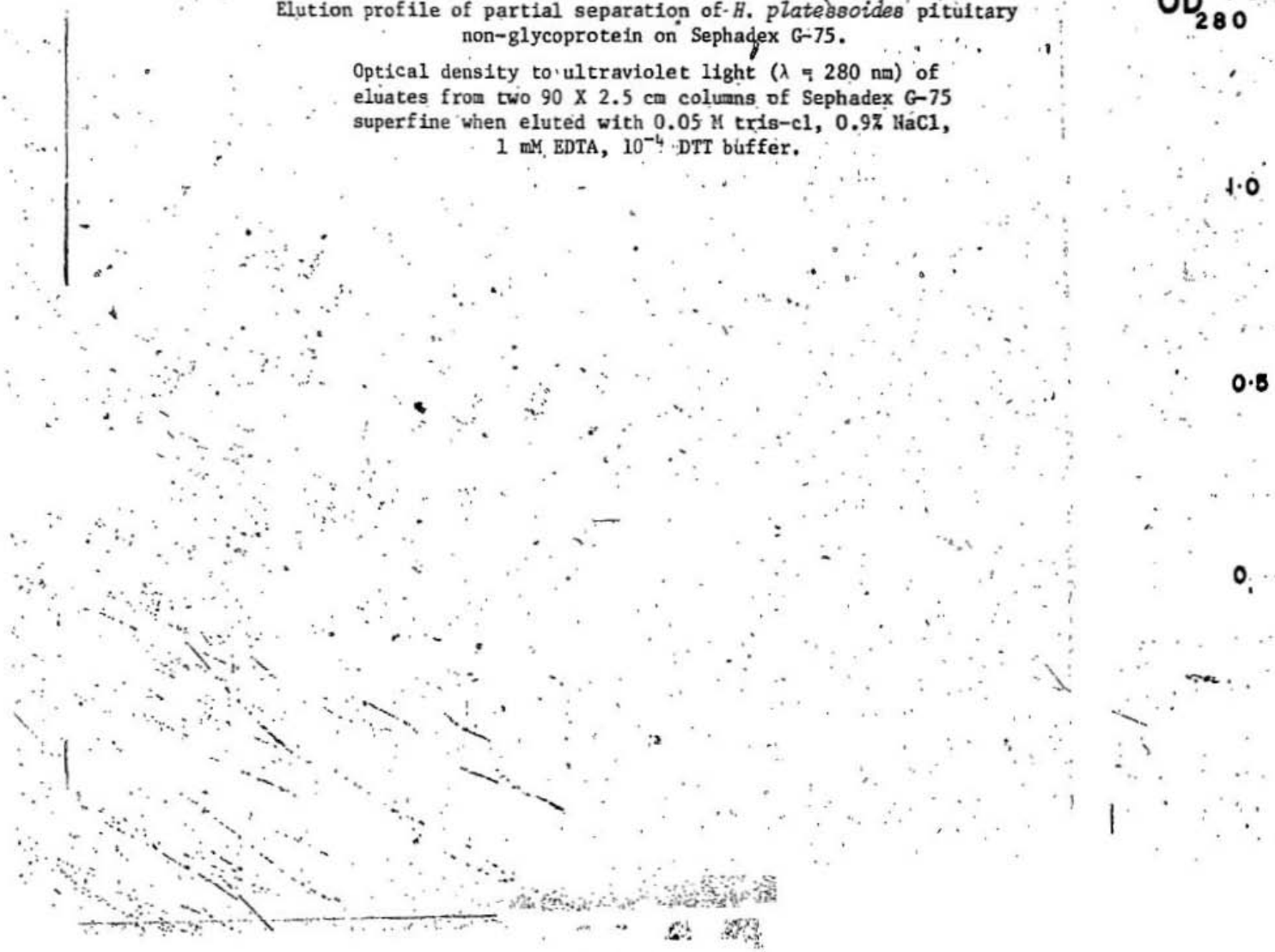
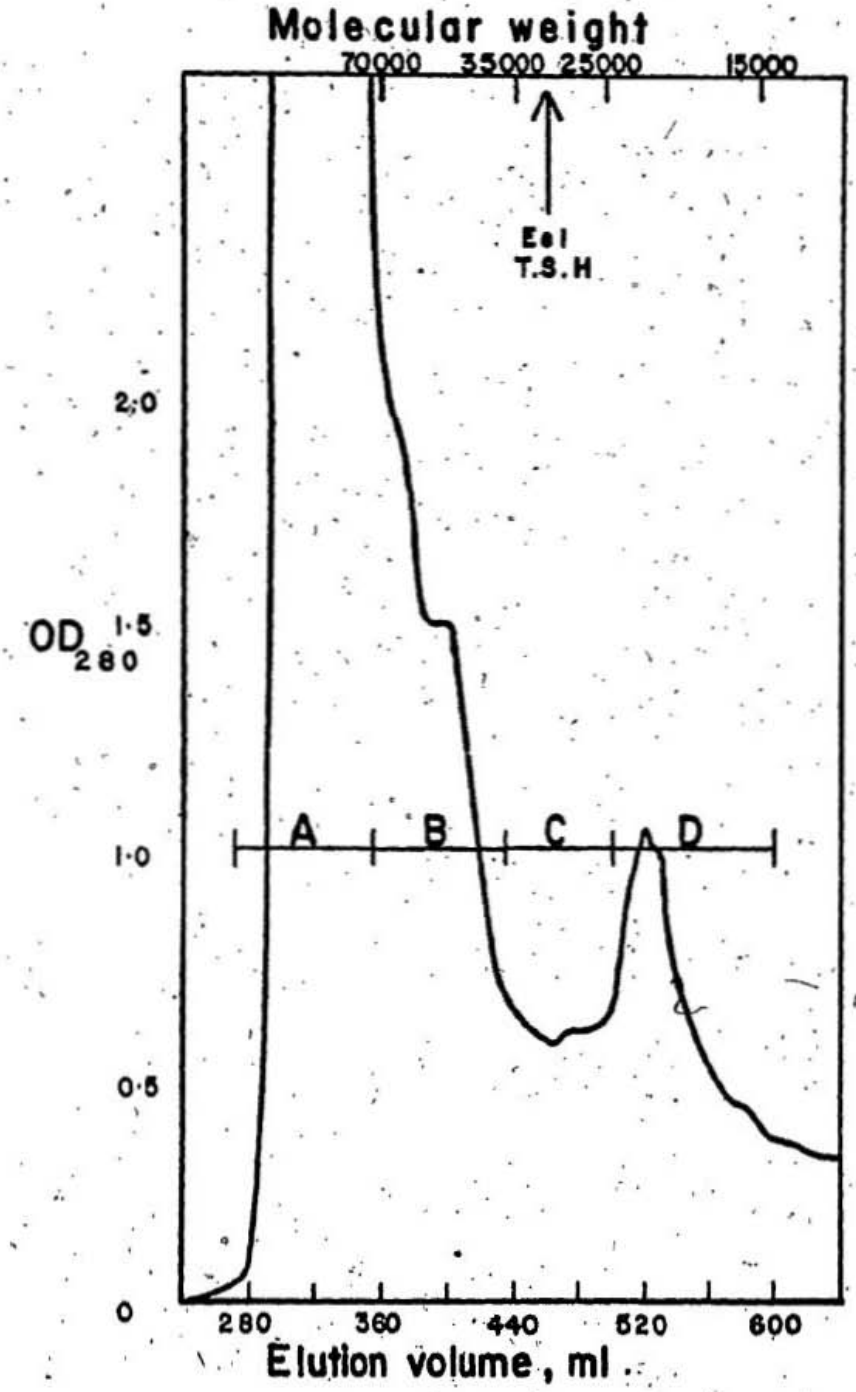


Figure 3

Elution profile of partial separation of *H. platessoides* pituitary non-glycoprotein on Sephadex G-75.

Optical density to ultraviolet light (λ = 280 nm) of eluates from two 90 X 2.5 cm columns of Sephadex G-75 superfine when eluted with 0.05 M tris-cl, 0.9% NaCl, 1 mM EDTA, 10^{-4} DTT buffer.



pituitary

) of
G-75
NaCl,

profile (Fig. 3) was prepared by measuring optical density of the fractions at a wavelength of 280 nm and tube contents combined to make four samples for injection.

Steroid Preparations

1. 11 Ketotestosterone.

^{14}C-Labelled steroid was prepared from ^{14}C-cortisone by the method used by Idler *et al.* (1971). Eight nM of cortisone were dissolved in 250 µl of 50% aqueous acetic acid to which 24 mg of sodium bismuthate was added. After 30 min shaking, the reaction mixture was filtered through a pasteur pipette plugged with glass wool and the residue washed with 1 ml of distilled water. Two volumes of dichloromethane (CH_2Cl_2) were used to extract the product from the filtrate. ^{14}C-Adrenosterone was purified by paper chromatography (washed, Whatman No. 1) in the heptane:benzene:70% methanol 1:1:1 (HBM 1:1:1) system.

^{14}C-11-Ketotestosterone was prepared by reduction of adrenosterone for 30 min in 2 µl of a 0.5 mg/ml solution of sodium borohydride in 80% aqueous tert-butanol. The reaction was stopped by addition of acetic acid and water and the steroid extracted with CH_2Cl_2. Purification was achieved by paper chromatography in HBM 1:1:1.

Radioinert 11-ketotesterone was prepared by sodium borohydride reduction of adrenosterone and partially purified by preparative thin layer chromatography in chloroform:methanol:water 90:10:1 on silica gel, spread to a thickness of 0.5 mm (PF 254 Brinkman Instruments). After desiccation over P_2O_5, 5% pyridine in acetic anhydride was used to form 11-ketotestosterone acetate. The acetate was purified by preparative

chromatography on silica gel using chloroform:95% ethanol 95:5.

2. 17α 20β progesterone-acetate.

500 mg of 17α-OH progesterone was shaken with 45 mg sodium borohydride in 60 ml of 80% aqueous tert-butanol for 50 min. 25 ml of 50% acetic acid was used to stop the reaction and the product was extracted into 100 ml of cold CH_2Cl_2. The extract was washed, evaporated under N_2 and the product crystallised from an ethanol solution by addition of water. 17α 20β progesterone was purified by preparative thin layer chromatography in chloroform:ethanol 10:90 (Fostier *et al.*, 1973). 17α, 20β Progesterone was acetylated for 17 hr and the acetate extracted with CH_2Cl_2. The acetate was purified by preparative chromatography in CH_2Cl_2:n-butyl acetate 70:30.

3. 20β Progesterone acetate.

25 µg of 20β-progesterone was acetylated with acetic anhydride and purified by thin layer chromatography in CH_2Cl_2:n butyl acetate 70:30.

PART A VITELLOGENESIS

INTRODUCTION

The production of yolky oocytes involves a large accumulation of material (including lipid) in the developing gonad with a utilization of stored materials. In an attempt to monitor this transfer, samples of fish were examined morphometrically and an analysis of total fat in liver, ovary and muscle were made at critical stages of the reproductive cycle.

Using an isotope labelling technique, the action of estradiol in vitellogenesis, either alone or together with pituitary fraction treatments in hypophysectomised female *P. americanus* was evaluated. In this approach the role of estradiol in vitellogenesis can be examined in a system where there are no interfering pituitary hormonal systems. The data of earlier workers, on fish and amphibia, have suggested that estradiol stimulation of yolk synthesis fails to induce the growth of oocytes in the absence of gonadotropin hormones. The hypophysectomy approach allows verification of the effects attributed to an estrogen suppression of gonadotropin release in the work on fish of Egami (1954), Egami and Ishii (1962), Sundararaj and Goswami (1968), Plack *et al.* (1971) and on amphibia by Follett *et al.* (1968) and Wallace and Dumont (1968).

Mammalian gonadotropic preparations were used by most of the earlier workers on maintenance or initiation of vitellogenesis in fish and amphibia. However, two partially purified fish gonadotropins showed

this activity when utilized by Sundararaj *et al.* (1972a, b) in hypophysectomised *H. fossilis*. Estradiol treated, hypophysectomised *P. americanus* were used to identify a teleost pituitary protein fraction which is capable of stimulating yolk incorporation into the gonad.

The pituitary preparations used were produced by methods currently in use for preparation of fish pituitary hormones (Idler *et al.*, 1975). The glycoprotein preparation had been shown to have biological gonadotropic effect when injected into hypophysectomised fish. An attempt was made to fractionate the non-glycoprotein fraction by gel-chromatography and the yolk incorporation assay used to identify the molecular weight range in which the factor, active in vitellogenesis, is found.

The comparative work of Wallace *et al.* (1966) and Jared and Wallace (1968) suggests that the yolk of fish eggs is basically similar to that isolated from amphibian or avian sources. Plack *et al.* (1971) used the extraction method developed by Jared and Wallace (1968) to prepare solutions of lipovitellin and phosvitin, the two major yolk proteins from teleost oocytes. This method was employed in the present study with the substitution of dialysis for the dilution-precipitation step. Amirante (1972) used dialysis to precipitate yolk from serum of *S. gairdnerii* but noted that precipitation was incomplete. In the present work it was found that serum vitellogenin could be precipitated by dialysis against distilled water only, in the presence of EDTA.

The yolk protein, phosvitin, when isolated from fish eggs has a phosphorus content of approximately 10% (Wallace *et al.*, 1966; Mano and Lipmann, 1966; Ito *et al.*, 1966 and Schmidt *et al.*, 1965). Mano and

Lipmann (1966) found that the total phosphoprotein phosphorus in roe of five species of fish ranged from 0.02% to 0.12% of wet weight. Wallace and Jared (1969) showed that 80% of the yolk protein phosphorus of *X. laevis* was in the yolk protein phosvitin. Because of the extremely high phosphorus content of this yolk material $H_3{}^{33}PO_4$ was chosen as the isotope for use in this work.

MATERIALS AND METHODS

In 1972 six samples of fish caught at locations within 60 km of the laboratory were autopsied after being held, unfed, at the laboratory for three or four days and in 1973 one further group was examined. The weights of whole fish, livers and gonads were taken and samples of these tissues and of upper mid-lateral muscle were weighed prior to drying in an oven at 90°C to determine tissue water content. Further samples were placed in cans, autoclaved, flushed with N_2 and sealed for storage at room temperature.

Fat assay. Prior to assay the canned tissues were frozen to facilitate handling. Three aliquots of 2-7 g weight of each finely chopped frozen tissue sample were added to 20 ml of freshly distilled tetrahydrofuran (THF) in 40 ml centrifuge tubes and placed on a Buchler oscillatory shaker (40-50 cpm) for two hr. After five min. of low speed centrifugation the supernatant was poured through glass wool into a tared vial and the pellet was extracted twice more with 20 ml of THF for one hr each time. Solvent was evaporated from the extract vial each time in a warm water bath under nitrogen. (N-evap, Organomation Association). The solvent free extract was held in an evacuated desiccator over P_2O_5

for at least three days prior to weighing.

Fat contents were calculated as percentage of wet and dry weights. The absolute amount of tissue lipid (mg/g of fish) after correction for the size of the fish is calculated from

% lipid of wet tissue X organ weight X 10/body weight.

Selected comparisons were made by t-test.

Role of Estradiol and Pituitary Preparations in Vitellogenesis

1. Experiment I, October 1973.

Three weeks after operation six sham and 14 hypophysectomised fish were given an intraperitoneal injection of $H_3{}^{33}PO_4$ and ^{3}H-leucine in saline at 2.0 µC/kg/0.5 ml on day 1. Six of the hypophysectomised and the six sham fish were injected once, intramuscularly, with peanut oil at 0.5 ml/kg, and the eight estradiol-3-monobenzoate treated fish received similar injections of oil containing the steroid dissolved at 3 mg/ml on day one and day three. On days three to six four estradiol treated fish received 0.5 ml/kg intraperitoneal injections of 5 mg/ml albumen in saline as a control, whilst four fish received an injection of plaice glycoprotein (= 13 glands/100 g body weight).

One week after the start of injections all the fish were bled with non-heparinized syringes. Samples of gonads and liver were frozen (and aliquots taken for determination of dry weights). Duplicate crude yolk protein preparations were made at 4°C by homogenizing 5-6 g of liver or gonad with four times its weight of cold 0.5 M NaCl containing 5 mM EDTA (Plack *et al.*, 1971). The homogenate was centrifuged at

100 000 g (IEC B-60) for 1.5 hr; a portion of the supernatant was re-centrifuged under the same conditions and a 15 ml aliquot taken for dialysis. About 10 mg of a crude yolk, prepared from *P. americanus* gonads, was added to sera to increase the size of the yolk precipitate. Sera were centrifuged at 100 000 g, then an aliquot was taken for dialysis.

Dialysis bags were prepared by boiling in distilled H_2O containing 10 mg EDTA/l followed by several rinses in cold distilled water. Tissue and serum preparations were dialysed against at least ten volumes of distilled water at 4°C for at least 24 hr; 0.2 gm EDTA/l was used when serum was dialysed. The precipitate of crude yolk was rinsed from the dialysis bag with distilled water into a scintillation vial. The vial was lightly centrifuged at 2 000 rpm for 15 min and the supernatant aspirated off. The precipitate was rinsed with distilled water, re-centrifuged and after aspiration of the washings, was subjected to Protosol (New England Nuclear) digestion at 50°C until the precipitate was completely dissolved. The solution in the vial was neutralised with glacial acetic acid, and Aquasol scintillator was added before counting in a Packard 2003, 2425 or 3375 Scintillation Counter.

The counts obtained were corrected for efficiency using external standardization and ^{33}P counts were adjusted to allow for radioactive decay since the death of the fish. Disintegrations per minute per gram wet weight were calculated and statistical comparisons between treatments were made by two level nested analysis of variance or by least significant difference (Sokal and Rohlf, 1969).

Experiment II, February 1974.

A plaice glycoprotein was prepared from pituitaries taken from fish which had been selected as adults. *P. americanus* pituitary extract as made in the Tris-NaCl buffer, from pituitaries collected from 75 vitellogenic fish brought to the laboratory. All fish received intraperitoneal injections of 2 uC/100 g $H_3{}^{33}PO_4$ 16 days after operations and further injections were made according to Table 1.

On the twenty-third day after operations the animals were killed and samples taken as in Experiment I. Sera were treated as before but the yolk precipitate was not washed after centrifugation. Two to three g of frozen livers or gonads were homogenised thoroughly with 10 ml of the yolk extraction buffer and centrifuged at 50 000 g for 45 min (Sorvall RC2B). About 9 ml of supernatants were decanted into ultracentrifuge tubes and the extracts centrifuged at 100 000 g for 1.5 hr. Six or eight ml of the supernatants were dialysed and the precipitates, centrifuged in scintillation vials, were redissolved in 3 ml of yolk extraction buffer. Fifteen ml of Aquasol were added and after thorough mixing ^{33}P was counted.

Experiment III, September 1974.

After operations the fish were held at 9°C (3°C below ambient) for seven days to aid survival of post operational stress. For the 23 days between operation and injection (Table 2) the fish were maintained in an indoor tank with constant illumination.

During preparation, the yolk extracts were dialysed against two changes of at least ten volumes of distilled water.

TABLE 1

Treatments Experiment II, daily injections, days 16, 17, 18 and 19.

n	Surgery	Daily Intraperitoneal Injections	Protein (µg)/100 g injected
7	Sham	(α-D methyl glucoside buffer)	
5	Hypophysectomised	" " " "	
4	"	Estradiol + plaice pituitary extract	640
6	"	Plaice pituitary extract (= 2 glands/100 gm)	640
5	"	Flounder pituitary extract (= 0.34 glands /100 gm)	125
5	"	Plaice pituitary glycoprotein fraction (= 4 glands/100 g)	12
5	"	Plaice pituitary non-glycoprotein fraction (= 4 glands/100 gm)	500

Treatments, Experiment III, injections on days 22, 23, 24, 25, 26 and 27 after operations.

n	Surgery	Initial Injection	Daily Injections	Protein/100 gm
8	Sham	oil	α-D methyl glucoside buffer	
4	Hypophysectomised	estradiol in oil (1.5 mg/kg)	" " " "	
6	"	" " " " "	plaice pituitary glycoprotein fraction (≈ 5 glands/100 gm)	6 µg
6	"	" " " " "	plaice pituitary non-glycoprotein fraction (≈ 5 glands/100 gm)	640 µg

Experiment IV, November 1974.

Fish were allowed to recover after hypophysectomy at ambient sea water temperatures (6-6.5°C) for 10 days, then the temperature was raised to 8-9°C for the remaining 14 days and during treatments to stimulate metabolic activity. Preparation of the pituitary fractions for injection took ten days. The elution profile (OD_{280}) of the non-glycoprotein fractionation from Sephadex G-75 is shown in Figure 3. The extract in the fraction collector tubes was pooled to give four fractions with molecular weights of approximately; > 80 000 (A), 35 000 to 80 000 (B), 25 000 to 35 000 (C) and 15 000 to 25 000 (D). The volume of each fraction was adjusted by concentration or dilution with buffer to 100 ml so that 1 ml of each fraction represented an extraction of approximately 28 pituitary glands. All fish received a single intramuscular injection of 1.5 mg estradiol benzoate/kg and six daily injections of the pituitary fractions (= 6 glands/100 gm) or an equivalent amount of buffer as control.

Preparation of yolk extracts, counting and data processing was similar to that already described.

RESULTS

The gonad weights of fish in the August sample were significantly ($P < 0.01$) greater than in the spent fish sampled in July. This difference is not due to an incorporation of fat since there is no significant difference in gonad total fat/gm between these groups (Figures 4 and 5). A significant difference ($P < 0.01$) between liver weights in fish of these groups may be associated with the initiation of vitellogenesis since

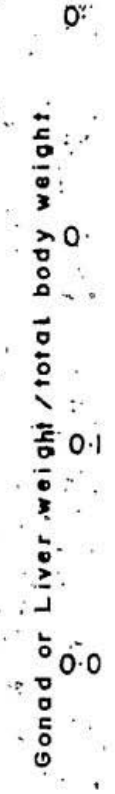


Figure 4

Mean gonadosomatic (gonad weight/total body weight) and mean hepatosomatic (liver weight/total body weight) indices. The relative sizes of gonads and liver in groups of fish examined during various phases of reproduction. (Numbers in parentheses = number of fish, confidence intervals = one standard error).

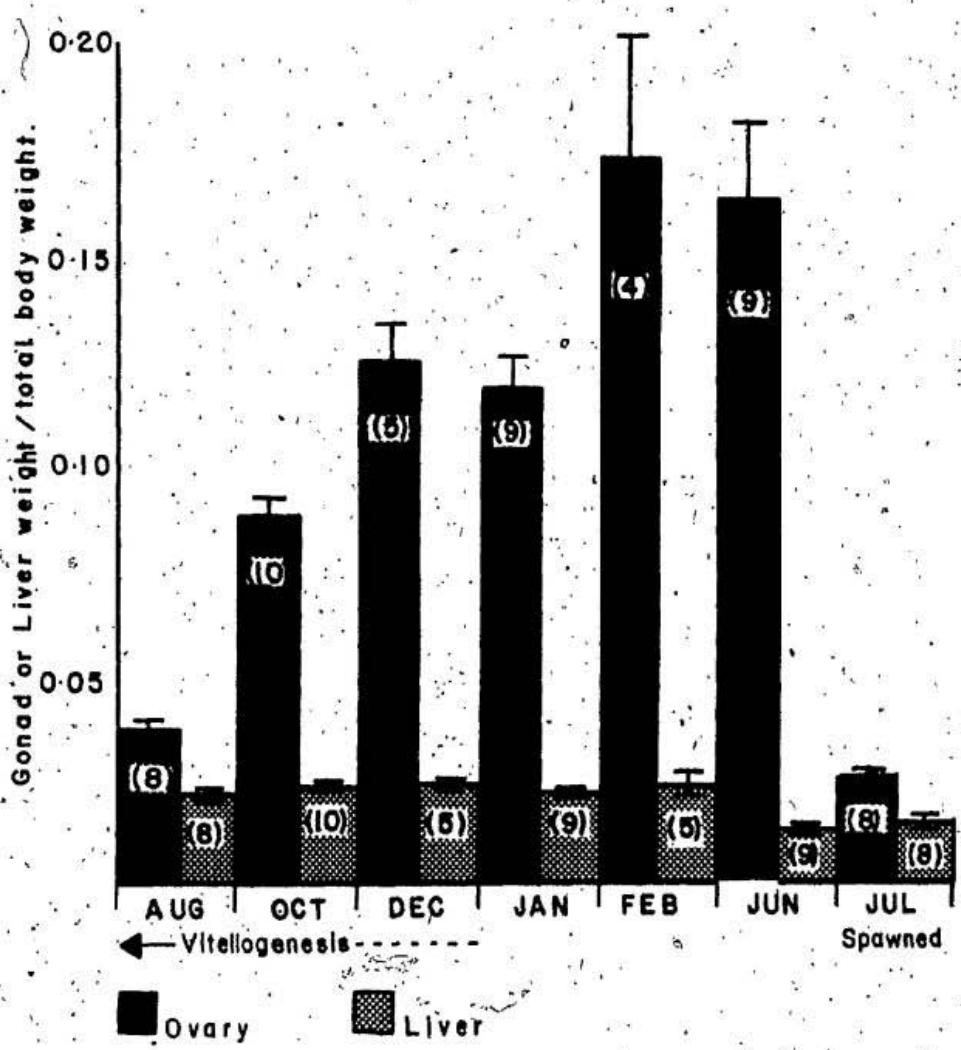


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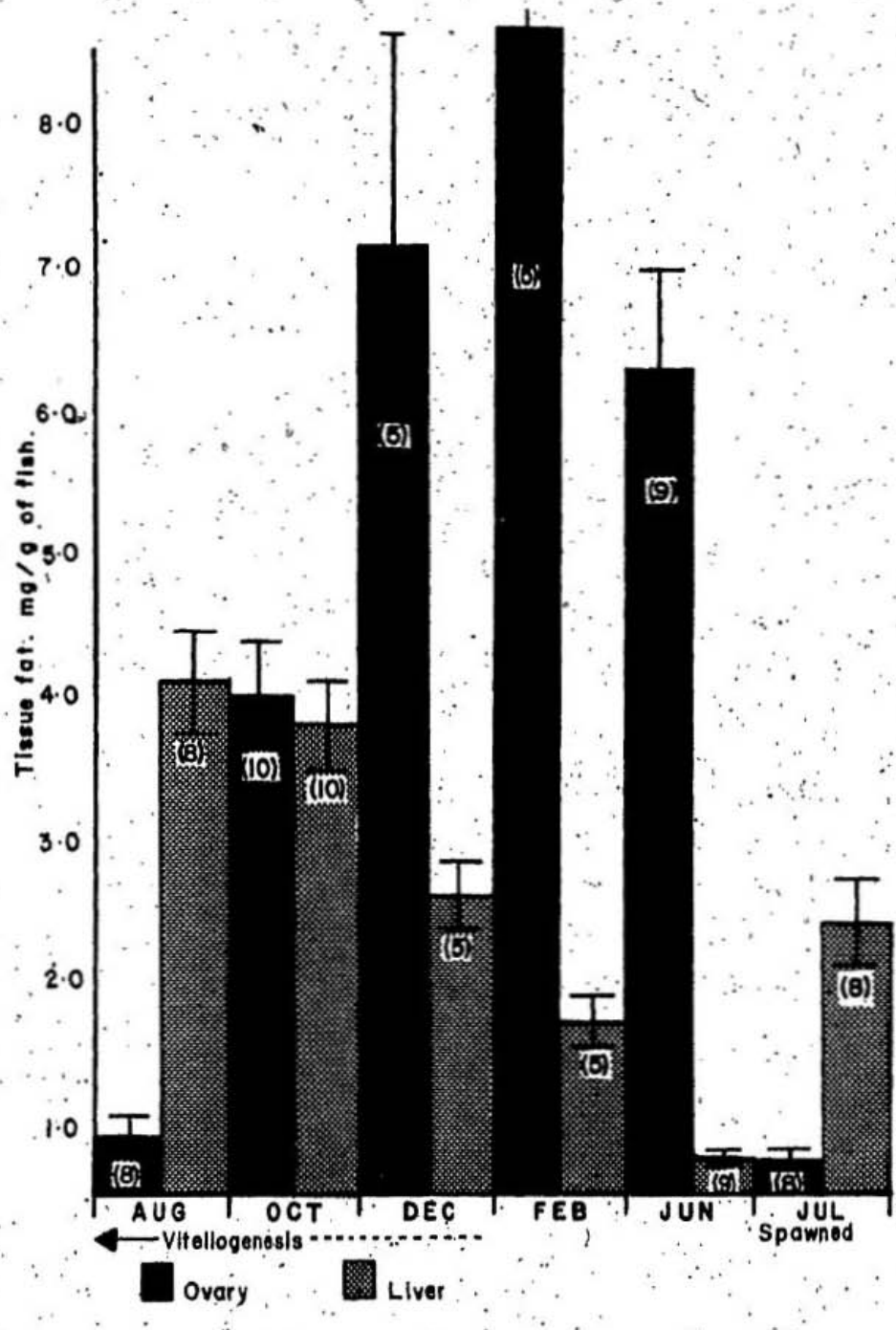
Figure 5

Mean lipid content of tissues: mg/g total body weight. The relative concentration of lipid within the gonads and liver of fish examined during various phases of reproduction. (Numbers in parentheses = number of fish, confidence intervals = one standard error).



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the difference is greater than that between fish in the June and July samples. A sample of eleven fish caught in Chapel's Cove in mid-August contained ten animals from which a biopsy sample of eggs showed that vitellogenesis has started.

Insufficient data is presented here to clarify the changes which may occur during an annual cycle within a single population of fish. However there is a significant difference ($P < 0.01$) in muscle fat (Table 3) between February and June, in fish captured at Chapel's Cove, which may represent utilization of fat during winter starvation.

The 2.1 mg/g difference ($P < 0.05$) in total liver fat (Figure 5) between fish in October and February samples is not sufficient to account for the 4.7mg/g difference between the gonads of these fish. *P. americanus* begins winter starvation during October so that the material deposited in the gonad during early winter is produced by mobilization of reserves from some storage tissue. The present results do not indicate that the muscle represents this store.

An increase ($P < 0.001$) in incorporation of $H_3{}^{33}PO_4$ into a yolk fraction of liver was seen when hypophysectomised fish were treated with two injections of 1.5 mg/kg estradiol benzoate at the start of the vitellogenic season (October/November, Figure 6, Experiment I). No statistically significant effect could be demonstrated by a single such injection at an earlier (September) or later (February) part of the season. Because of experimental variability the incorporation of ^{3}H-leucine into this fraction did not demonstrate any significant effects due to estradiol treatment. No effects, on $H_3{}^{33}PO_4$ incorporation into liver yolk extract, due to treatment of hypophysectomised fish with

TABLE 3

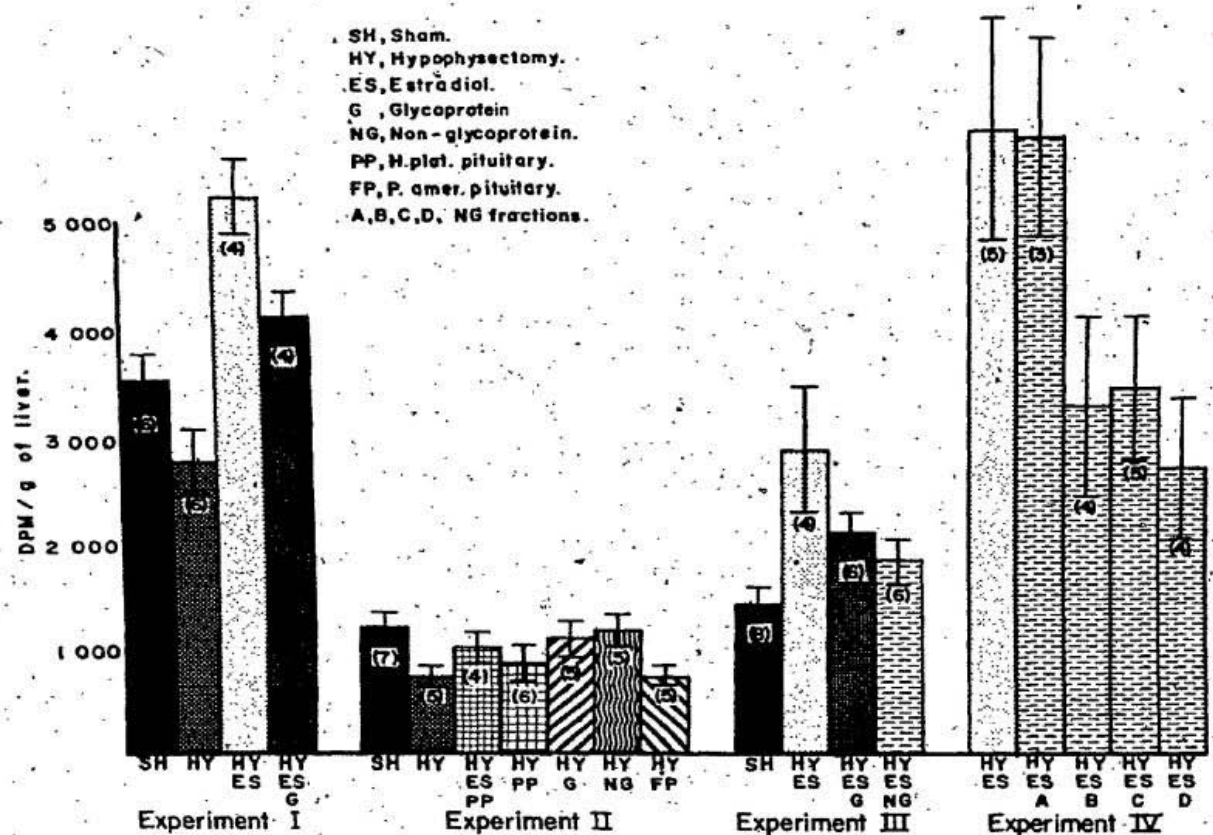
mg Lipid/g of muscle (dried).
Mean ± standard error.
(n = number of fish).

August	October	December	February	June	July
144 ± 13 (8)	82 ± 74 (9)	141 ± 24 (5)	135 ± 6 (5)	87 ± 5 (8)	135 ± 11 (6)

Figure 6

Mean disintegrations per minute $H_3{}^{33}PO_4$ incorporated into yolk fraction/g of liver.
Radioactivity incorporated into the material extracted as yolk from 1 g of liver from fish under experimental treatments.
(Number in parentheses = number of fish, confidence interval = one standard error).

SH, Sham.
HY, Hypophysectomy.
ES, Estradiol


SH, Sham.
HY, Hypophysectomy.
ES, Estradiol.
G , Glycoprotein
NG, Non-glycoprotein.
PP, H.plat. pituitary.
FP, P. amer. pituitary.
A,B,C,D, NG fractions.
DPM / g of liver.
5 000
4 000
3 000
2 000
1 000
SH HY HY ES HY ES G
Experiment I
SH HY HY ES PP HY PP HY G HY NG HY FP
Experiment II
SH HY ES HY ES G HY ES NG
Experiment III
HY ES HY ES A HY ES B HY ES C HY ES D
Experiment IV

pituitary fractions have been seen (Figure 6) even if the fish received an estradiol injection in addition.

Analysis of a serum labelled yolk precipitate has demonstrated that estradiol treated, hypophysectomised, fish carry more of this material in the serum than do sham injected fish ($P < 0.01$, Figure 7, Experiment III). The content of labelled serum yolk protein of estradiol injected fish after treatment with non-glycoprotein pituitary extract, was significantly lower ($P < 0.01$) than that seen after glycoprotein extract treatment. In experiment IV it was demonstrated that this serum yolk decreasing property was contained in fraction C of the pituitary non-glycoprotein (M.W. 25-35 000).

At the gonad, hypophysectomy results in a reduced isotope incorporation into yolk (Figure 8, Experiment I). This effect is partially ($P < 0.05$) corrected by treatment with various pituitary preparations (Figure 8, Experiment II). Some increase in the incorporation of $H_3{}^{33}PO_4$ has been seen due to estradiol treatment of hypophysectomised fish but this incorporation was stimulated further by pituitary glycoprotein and non-glycoprotein fractions ($P < 0.05$, Figure 8, Experiment III). The increased incorporation was greater ($P < 0.01$) into the gonad yolk fraction of the fish which had been injected with the pituitary non-glycoprotein preparation than the glycoprotein treated fish. When the non-glycoprotein pituitary extract was subjected to chromatography, fraction C was the only fraction found to stimulate ($P < 0.05$) this yolk incorporation (Figure 8, Experiment IV).

The rate of $H_3{}^{33}PO_4$ incorporation into the gonad yolk extract of sham operated fish was less in experiment III than in experiment I, even

Figure 7

Mean disintegrations per minute $H_3{}^{33}PO_4$ incorporated into yolk fraction/ml of serum.
Radioactivity incorporated into the material extracted as yolk from 1 ml of serum from fish under experimental treatments.
(Numbers in parentheses = number of fish, confidence intervals = one standard error).

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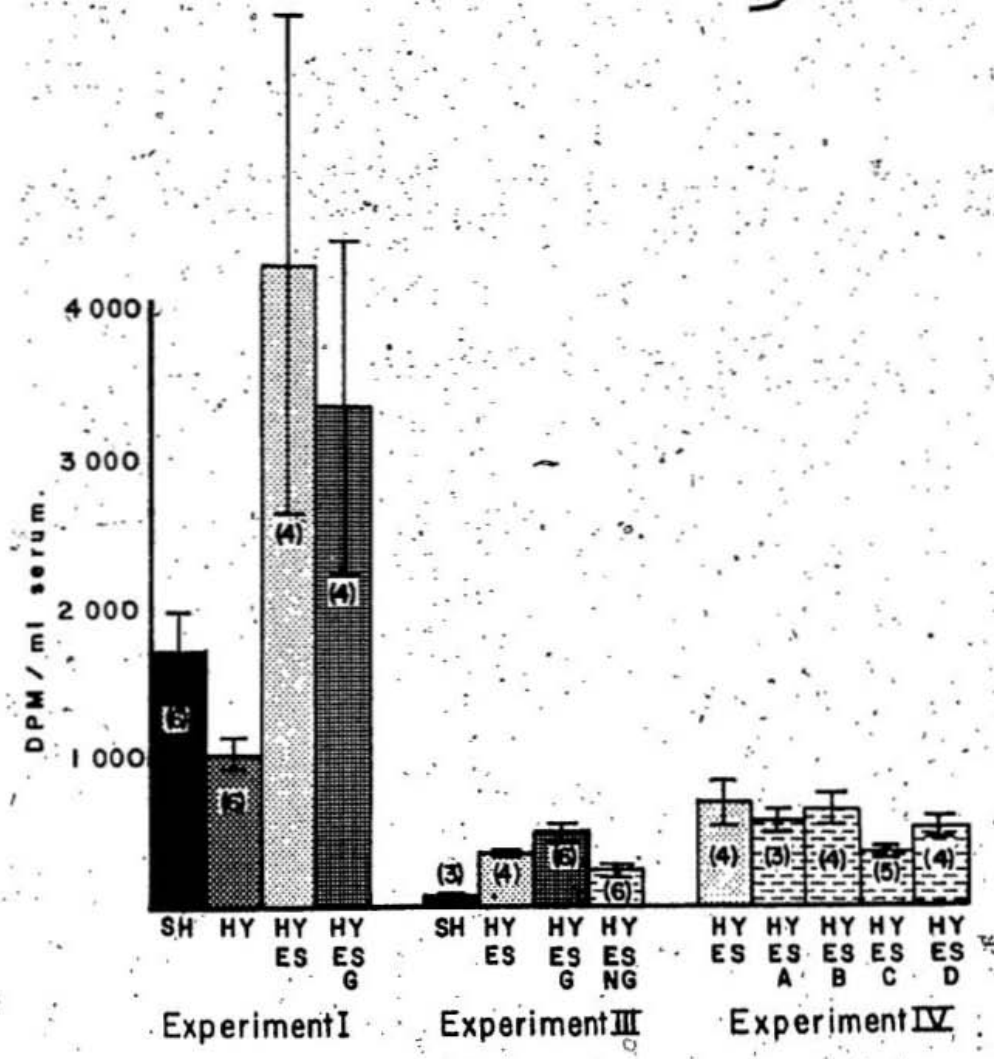

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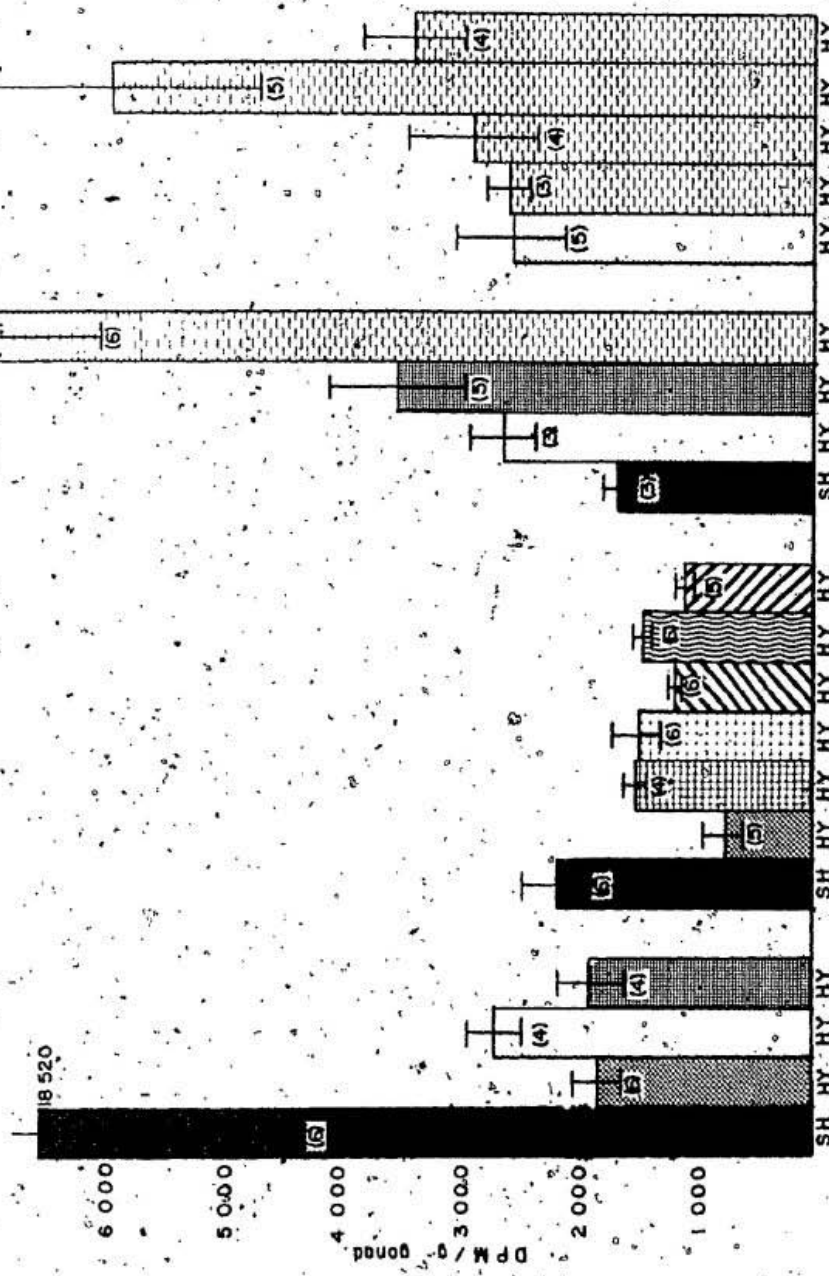
Figure 8

Mean disintegrations per minute $H_3{}^{33}PO_4$ incorporated into yolk fraction/g of gonad. Radioactivity incorporated into material extracted as yolk from 1 g of ovary from fish under various experimental treatments. (Numbers in parentheses = number of fish, confidence intervals = one standard error).


DPM / g gonad
6 000
5 000
4 000
3 000
2 000
1 000
18 520
SH HY HY ES ES G
SH HY HY ES PP PP
SH HY HY HY ES PP G NG FP
SH HY HY HY ES G NG
SH HY ES ES G
HY HY ES ES A B C D

though the responses to estradiol and pituitary fraction treatments are similar. Incorporation of $H_3{}^{33}PO_4$ into gonad yolk in response to estradiol stimulation is similar in the experiments performed in early vitellogenesis (I, III and IV), however in the liver there is considerable difference in response between different experiments.

In experiment I ^{3}H-leucine was incorporated into yolk fractions 1.7 times more than $H_3{}^{33}PO_4$, this ratio being approximately constant throughout the experiment. The ratio of isotope incorporation seems to be more than twice as high in the gonad of sham operated fish (Table 4).

At the conclusion of the experiments the gonadosomatic indices (Figure 9) of hypophysectomised fish were lower than in sham operated fish. Treatment of hypophysectomised fish with estradiol (Experiment I and III) fails to increase the G.S.I. significantly but an effect was seen after treatment with the non-glycoprotein pituitary extract of fraction C of this extract (Experiments III and IV) to restore G.S.I. to values nearer those shown by sham operated fish.

Data from the experiments early in the vitellogenic phase shows lower hepatosomatic indices in sham operated animals than in hypophysectomised fish ($P \leq 0.05$, $P \leq 0.001$, Figure 10, Experiments I and III). The effect of estradiol appeared to increase the weight of the liver ($P \leq 0.001$, Experiment I) and no effects due to treatment with the pituitary fractions, alone or in addition to estradiol, were seen.

No significant effects of treatment on water content of livers or gonads were observed; no check was made in the case of PNG4 treatments.

TABLE 4

Isotope ratios in gonadal yolk extract. Experiment I.

$^{3}H/^{33}P$ D.P.M. ± Standard Error

	Sham (n = 4)	Hypophysectomised (n = 4)	Hypophysectomised/ Estradiol (n = 4)	Hypophysectomised/ Estradiol/ Glycoprotein (n = 4)
Liver	1.60 (0.20)	1.96 (0.20)	1.36 (0.20)	1.25 (0.15)
Serum	1.45 (0.10)	2.66 (0.16)	1.33 (0.16)	1.39 (0.17)
Gonad	4.20 (0.48)	1.78 (0.11)	1.36 (0.19)	2.10 (0.58)

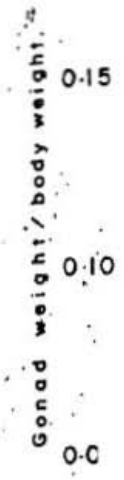
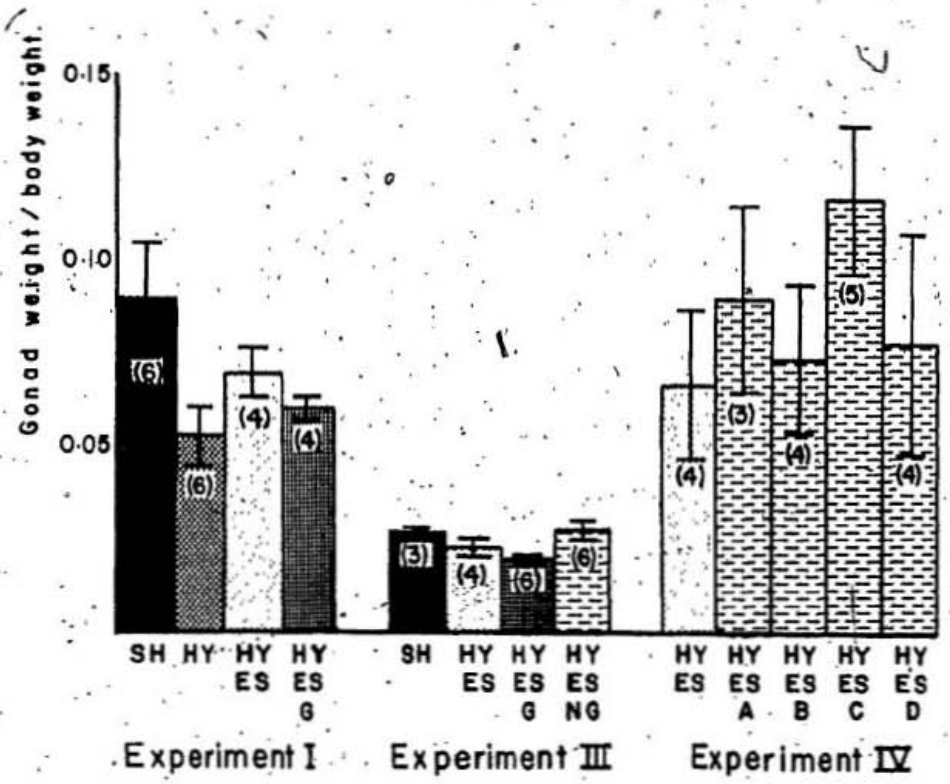


Figure 9

Gonadosomatic index (gonad weight/total body weight).
The relative gonad weights of fish under various
experimental treatments.
(Numbers in parentheses = number of fish,
confidence intervals = one standard error).


Gonad weight / body weight
0·15
0·10
0·05
(6)
(6)
(4)
(4)
(3)
(4)
(6)
(6)
(4)
(3)
(4)
(5)
(4)
SH
HY
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Experiment I
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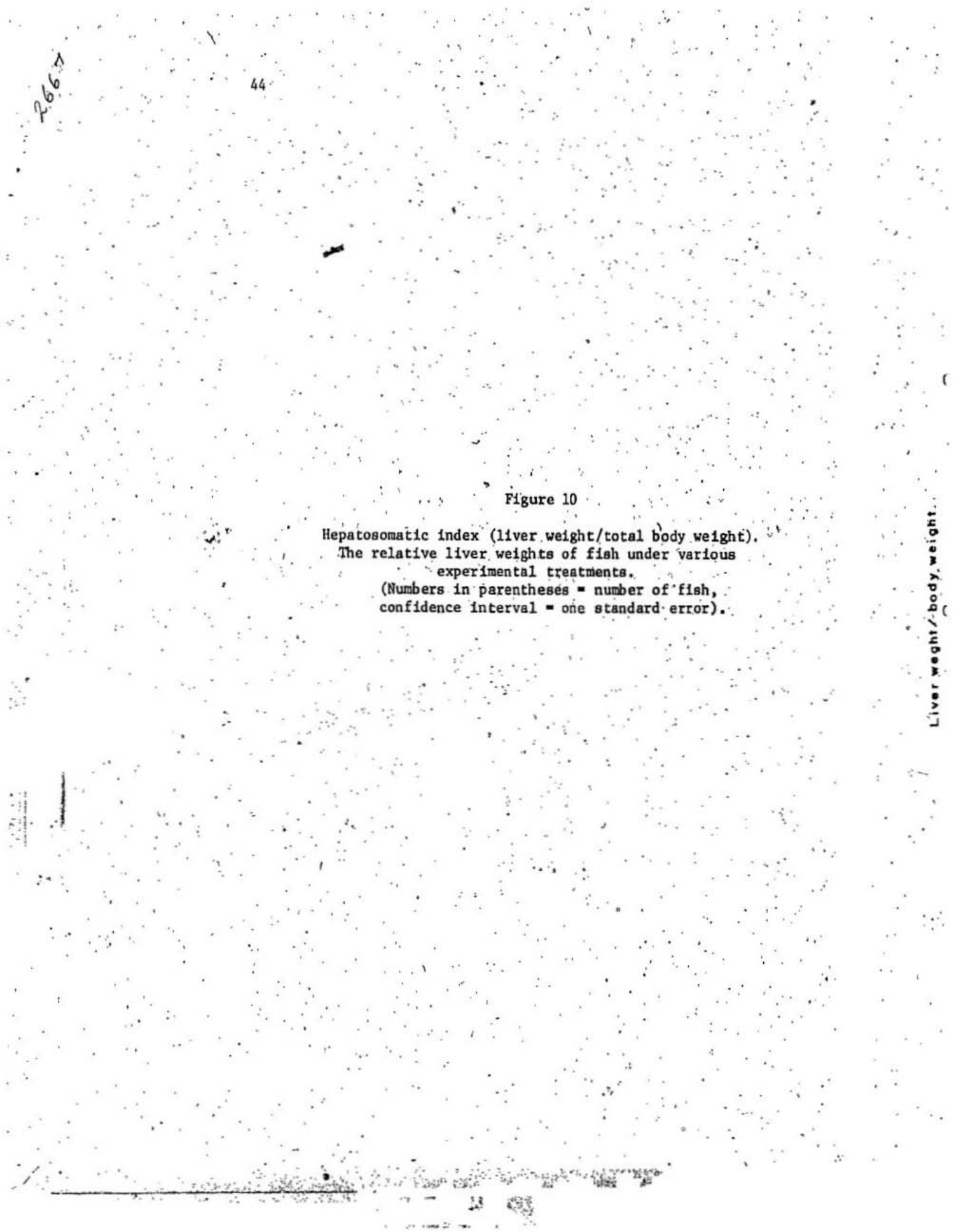
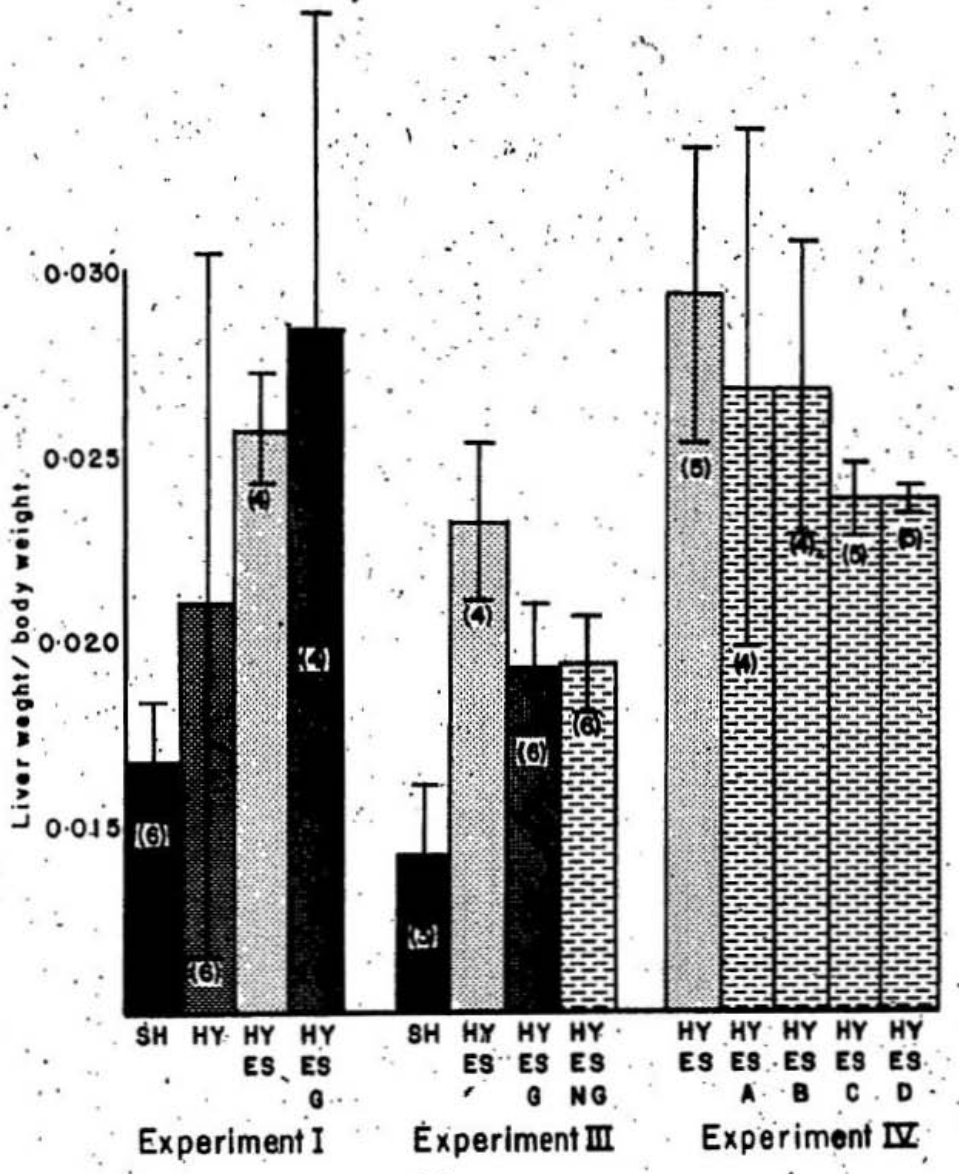


Figure 10

Hepatosomatic index (liver weight/total body weight). The relative liver weights of fish under various experimental treatments. (Numbers in parentheses = number of fish, confidence interval = one standard error).


Liver weight/ body weight
0·030
0·025
0·020
0·015
(6)
(6)
(4)
(7)
(5)
(4)
(6)
(6)
(5)
(4)
(4)
(5)
(5)
SH
HY
HY ES
HY ES G
SH
HY ES'
HY ES G
HY ES NG
HY ES
HY ES A
HY ES B
HY ES C
HY ES D
Experiment I
Experiment III
Experiment IV

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DISCUSSION

Physiological Changes During Vitellogenesis

Lipid analysis of ovaries from *P. americanus* shows little concentration change during vitellogenesis (Table 3), however due to the growth of the ovaries during this period there is an accumulation of a large amount of fat in the organ (Figure 5). Similarly Lusk (1969) and Shatunovsky (1971) found that there was little change in the percentage content of fat in the gonads of *Salmo trutta fario* L. and *Gadus morhua callarias* (L.) during vitellogenesis. However Jafri (1968) and Lapin (1973) showed considerable changes in this concentration at the start of vitellogenesis in *Cirrhina mrigala* (Ham) and *Platichthys flesus bogdanovi* (Sandberg) ovaries. Shatunovsky (1971) developed an energy model for female *G. morhua* in which 60% of liver lipid reserves and 53% of body protein are mobilised during the period of starvation and vitellogenesis. Yolk deposition in the gonad led to an increase in their calorific value from 4% to 36% of the total body value. This mass accumulation is similar to that demonstrated in *P. americanus* when gonadosomatic indices increase from 4% to 17% during vitellogenesis.

Whereas in male *Gobius paganellus* Gmelin liver lipid concentrations decreased slightly in association with reproductive development but the large lipid accumulation during the rest of the annual cycle resulted in a net increase in liver lipid during the life of the fish, in females the decrease in liver lipid associated with reproduction is much greater and no net increase with age was seen (Schul'man, 1967). This difference between the sexes probably represents the utilization of liver stores during

vitellogenesis. This work and the investigations on *C. mrigala* and *P. flesus* and the data on *P. americanus* all provide evidence that there is a marked depletion of liver lipid reserves during the period of vitellogenesis.

The role of muscle lipid stores is not so clear. Jafri (1968) noted that the fat stores in visceral mesenteries of *C. mrigala* were utilized during this period but that the fat content of the body musculature appeared to increase; also, the decrease in muscle fat shown by Lusk (1969) for *S. trutta* occurs after the spawning period and is more likely to be due to winter starvation. The fluctuations in muscle fat content in *Platichthys flesus* (Lapin, 1973) are extremely small as are those seen in *Pseudopleuronectes americanus*. However, the fat reserves demonstrated in muscle of *P. flesus* are only one quarter of those seen in *P. americanus*, so that a slight decrease in the concentration of this lipid reserve in the latter fish could provide large amounts of material for the vitellogenic processes. No changes in muscle lipid concentration which can be associated with vitellogenesis have been seen but catabolism of muscle tissues would also provide material for use in vitellogenesis. McKinnon (1972) showed a depletion of energy reserves and a loss of body weight in *H. platessoides* during winter starvation. Approximately 20% of this energy was accumulated in the gonads. Starved *P. americanus* held at this laboratory from November until April exhibit a 3 to 12% loss of body weight. If these changes were due to catabolism of muscle tissues considerable amounts of energy reserves would be made available for metabolic processes including vitellogenesis. Field observations show that the eviscerated body weight of fish of the same length is approximately 10% lower in February than September (Fletcher, pers. comm.).

Physiological changes in plasma constituents have often been sociated with the vitellogenic process. Takashima *et al.* (1971) uggested that elevated levels of triglycerides and free fatty acids in ver and plasma were part of a mechanism to mobilize these products nto the blood for transport to the growing oocytes of *S. gairdnerii.* ese findings could be accounted for if body lipid reserves were bilized to the liver for synthesis into yolk material. Estrogens, hich have been implicated in a central role in vitellogenesis, have een used to induce similar effects. Ho and Vanstone (1961) stimulated n increase in plasma lipid using estradiol-benzoate in *Oncorhynchus nerka* albaum and Takashima *et al.* (1972) used diethyl stilbesterol to induce an ncrease in plasma lipid and lipoprotein in *S. gairdnerii.* This latter increase was suppressed when fish were treated with mammalian thyroid powder. The contrasting work of Minick (1971) was unable to show any effect on serum free fatty acids after treatment of *C. auratus* with estradiol, thyroxine, thyroid stimulating hormone or mammalian gonadotropin.

It is not known whether lipid materials are directly accumulated in the oocytes or whether the gonad lipid accumulation is in the form of lipoprotein yolk. It would appear that estrogens may be capable of mobilizing the body lipid reserves as triglycerides or free fatty acids and inducing the synthesis of this material as lipoprotein for transfer to oocytes.

Effect of Hypophysectomy on Vitellogenesis

The present work has shown (Figure 8) a dramatic effect due to hypophysectomy on the concentration of labelled yolk fraction in the gonads. The effect of hypophysectomy on vitellogenesis of *P. platessa*

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(Barr, 1963). *C. auratus* (Yamazaki, 1965), *Couesius plumbeus* Agassiz (Ahsan, 1966), and *H. fossilis* (Sundararaj and Goswami, 1968) was to arrest development of yolky oocytes and induce gonadal regression. No significant effects of hypophysectomy on liver or serum labelled yolk fractions was seen in *P. americanus*. The data suggest (Figures 6 and 7) however that smaller amounts of radioactivity are found in both these fractions in hypophysectomised animals; this would occur if hypophysectomy resulted in cessation of synthesis of yolk material in the liver.

In the first experiment, a higher $^{3}H/^{33}P$ isotope ratio was found in the yolk extracted from the gonads of sham operated fish than in those of fish which were hypophysectomised and treated with hormones. Wallace *et al.* (1972) found no evidence for direct incorporation of $Na_2H^{33}PO_4$ or ^{3}H-leucine into yolk material of *X. laevis* oocytes. They demonstrated *in vitro* uptake of isotope labelled yolk into oocytes and propose that partial dephosphorylation occurs during the rearrangement of vitellogenin to form lipovitellin and phosvitin. The approximately constant ratio seen in all extracts in this experiment (Table 4) suggest that the material found in the three body compartments of hypophysectomised *P. americanus* is similar but, in the intact fish during active absorption of yolk material from the blood stream, some dephosphorylation may occur. No double isotope labelling experiments have been attempted using the non-glycoprotein pituitary preparations.

Function of Estradiol in Vitellogenesis

The liver of hypophysectomised *P. americanus* females responded to estradiol treatment by an elevated incorporation of radioactivity into the yolk containing preparation, except in the experiment performed at

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the end of vitellogenesis at cold water temperatures. The labelled yolk fraction of serum was increased by this treatment but no significant effect of estradiol treatment on the concentration of this labelled fraction in gonads was shown. These data support a hypothesis that yolk material is synthesised by the liver under the influence of estradiol, and that this material is passed into the blood stream where it accumulates.

Estrogens have been shown to have functions in fish which are probably related to vitellogenesis (p. 2). Plack *et al.* (1971) assayed the estradiol-induced appearance of yolk protein in the plasma and tissues of *G. morhua*. Their data, and earlier work (Plack and Pritchard, 1968; Woodhead, 1969 and Plack and Frazer, 1971), established an involvement of estradiol in yolk formation. No incorporation of yolk into ovaries was seen in estradiol stimulated immature *G. morhua* which is similar to the results in hypophysectomised *P. americanus*. Egami (1954) and Egami and Ishii (1962) found that estradiol injection into maturing *O. latipes* or *M. anguillicaudatus* inhibited gonadal growth. This inhibitory effect could be removed by simultaneous injection of frog pituitary extract which suggests that the estradiol inhibition of gonadal growth occurred because of a negative feedback inhibition of release of pituitary gonadotropin. Sundararaj and Goswami (1968) found that estradiol was effective at slowing the onset of gonadal atresia after hypophysectomy in *H. fossilis* but in an intact fish the results were similar to those of Egami (1954) and Egami and Ishii (1962), and histological examination of the pituitary glands of these fish showed a decrease in basophilic cells which may be evidence for negative feedback. Anand and Sundararaj (1974) propose that the partial maintenance effected by estradiol may be due to inhibition of

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lysosomal activity. There was no direct evidence for this, but in the present study no direct stimulatory effects of estradiol on gonad growth were demonstrated.

Mackay (1973) showed an inhibition of vitellogenesis, as indicated by gonadosomatic index, by several steroids including estrone in *H. galii*, which was similar to that found when the fish were treated with Methallibure, a substance which is presumed to block release of pituitary gonadotropins.

Estrogens have been tentatively identified in the ovaries or body fluids of teleosts on several occasions (Ozon, 1972). Donahue (1941) prepared extracts of the vitellogenic ovaries of *P. americanus* which were shown, by mouse bioassay, to contain estrogens.

Extensive work on estrogen stimulation of liver yolk synthesis has been performed in amphibia. Follett and Redshaw (1968) found that estradiol treated *X. laevis* accumulated vast quantities of vitellogenin in their plasma. Estradiol induction of vitellogenin synthesis has been found by Wallace and Jared (1968, 1969), Dolphin *et al.* (1971), Clemens and Lofthouse (1972), Wittliff and Kenney (1972) and Zelson and Wittliff (1973). Bergink *et al.* (1974) reviewed and examined a similar system in birds. Redshaw *et al.* (1969) examined the specificity of steroids on the liver vitellogenic synthesis and found that estradiol and estrone were powerful vitellogenic agents, but testosterone, progesterone, or cortisone were ineffective. Wittliff and Zelson (1974) found that cortisol, progesterone and testosterone were not effective. The estradiol-induced hepatic formation of yolk in *P. americanus* appears to be similar to that which these workers have shown in amphibia.

The hepatosomatic index of sham operated fish was lower at the end

f the experiments which might be expected under conditions of starvation hen the sham fish are the only ones in which vitellogenesis continues o utilize liver stores. The estradiol-induced increase in the hepatosomatic index seen in hypophysectomised *P. americanus* has been found by ther workers. Kobayashi (1953) found that estrone pellet implantation esulted in an increase in the weight of livers in *M. anguillicaudatus*, Egami (1955) used estrone to demonstrate an increase in liver weight in le and female *O. latipes* and Oguro (1956) obtained similar results for oth sexes of *G. aculeatus*. Such an effect could be the result of rphological changes which are necessary to facilitate yolk protein synthesis. Kobayashi (1953) and Egami (1955) showed an estrogen induced structural feminization of male livers and Ishii (1971) demonstrated higher hepatosomatic indices in vitellogenic *O. nerka* than those found in immature females or males. This morphological difference was associated with histological and histochemical differences which demonstrated the vitellogenic liver to be an active organ of protein synthesis.

Effect of Pituitary and Other Gonadotropic Preparations on Vitellogenesis

Extensive work in amphibia on the role of gonadotropins, alone or in addition to estradiol treatment, in vitellogenesis suggests that oocyte yolk incorporation occurs independently of the estrogen-stimulated hepatic synthesis and responds to gonadotropic stimulation. Wallace and Dumont (1968) and Follett *et al.* (1968) suggested that the high serum vitellogenin levels resulting from estradiol stimulation in *X. laevis*, occur because negative feedback by estradiol inhibits release of pituitary gonadotropin which in turn leads to a cessation of yolk uptake by the oocyte. Similar

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effects of estradiol treatment in the absence of pituitary hormones have been reviewed in the previous section and are seen in this work with *P. americanus* (Figure 8). Human chorionic gonadotropin (HCG) stimulates complete vitellogenesis with development of yolky oocytes in female *X. laevis* (Wallace and Jared, 1968), and treatment of animals with estradiol and follicle stimulating hormone (FSH) results in accumulation of vitellogenic material into oocytes but no accumulation was seen in plasma (Follett *et al.*, 1968). Wallace and Dumont (1968) found that estradiol plus HCG produced a similar effect. Emmersen and Kjaer (1974) used hypophysectomised female *Bufo bufo bufo* L. to show that estradiol treatment caused an accumulation of vitellogenin in serum; additional HCG administration depressed these levels and a group of vitellogenic oocytes appeared in the gonad. Treatment with HCG in the absence of estradiol was capable of stimulating growth of vitellogenic oocytes without inducing the extremely high serum levels of vitellogenin seen after estradiol stimulation, presumably since the HCG-stimulated oocytes are capable of clearing it from the circulation. *In vitro* studies (Jared and Wallace, 1969; Wallace *et al.*, 1970; Wallace, 1972; Wallace and Bergink, 1974) have shown that oocytes taken from an animal which received a HCG injection 24 hr earlier will actively accumulate labelled vitellogenin in culture. No direct effect of HCG treatment *in vitro* can be induced so the direct action of these treatments is not clearly understood.

In *P. americanus* whole pituitary extracts and pituitary fractions stimulated incorporation of label into the gonad yolk fraction. When the pituitary glycoprotein and non-glycoprotein fractions were tested in

estradiol injected fish, no significant effect on labelled gonad yolk concentration was produced with the glycoprotein. However, treatment with the non-glycoprotein fraction resulted in higher concentrations of labelled yolk in the gonads which suggest that the fraction stimulates uptake of the estradiol-induced, liver-synthesized yolk into the oocytes.

The glycoprotein fraction used in this experiment was prepared from the same batch of pituitaries that was used for preparation of the glycoprotein fraction which was effective as a gonadotropin in inducing *in vivo* ovulation when used at an equivalent dose (Table 19, p. 95). It would appear that these data do not implicate this fish pituitary fraction, which should contain any classical glycoprotein, gonadotropin(s), in the vitellogenic role that has been demonstrated for mammalian gonadotropins in amphibia, but that another pituitary fraction is implicated.

In several teleost species the role of mammalian gonadotropic preparations in stimulating vitellogenesis has been tested with varying results. Moller-Christensen *et al.* (1958) induced precocious vitellogenic growth in *Anguilla anguilla* L. using HCG together with an injection of either hexoestrol (a synthetic estrogen) or estradiol dipropionate. No such effect was seen after treatment with hexoestrol and antex leo, a non-chorionic mammalian gonadotropin. Similar results were found when Ochai *et al.* (1974) used estradiol dipropionate injection with an amount of mammalian gonadotropin (Synahorin = human chorionic gonadotropin plus anterior pituitary extract), which in itself was not stimulatory, in *Anguilla japonica* Temminck and Schlegel. The hypophysectomy-induced oocyte atresia of *C. auratus* was not affected by treatment with HCG, pregnant mare serum or mammalian anterior pituitary extract (Yamazaki, 1965). Shehadeh

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(1973) used HCG to maintain vitellogenesis in *Mugil cephalus* L. but was unable to demonstrate these effects with mammalian or fish pituitary preparations. Mackay (1973) was unable to maintain vitellogenesis in "gonadotropin deprived" *H. galii* using HCG however Anand and Sundararaj (1974) found that luteinizing hormone and HCG would successfully maintain vitellogenic oocytes in hypophysectomised *H. fossilis*, but other mammalian hormones including thyroid stimulating hormone were ineffective. When teleost pituitary preparations have been used, a stimulation of vitellogenesis has consistently been seen. Barranickova (1973) quotes Sakun (1967, 1970) as having found a stimulation of vitellogenesis after administration of *C. auratus* pituitaries to intact *Coregonus lavaretus pidschian* Gmelin. Chistova (1971) found a species and sex specific response to pituitary treatment in *Tilapia mossambica* Peters; pituitaries from *Oncorhyncus keta* Walbaum, *Oncorhyncus gorbuscha* Walbaum or female *T. mossambica* accelerated vitellogenesis, as assessed by an increase in gonad weight and oocyte diameter, but no such effect was produced by treatment with carp, pike-perch or male *T. mossambica* glands. Yamamoto *et al.* (1974) found that injection of pituitary extracts (*O. gorbuscha* and *O. keta*) effectively induced premature vitellogenesis in *A. japonica*. Pituitary extracts and partially purified gonadotropins have been shown to replace the pituitary in hypophysectomised fish and abolish the post-hypophysectomy gonadal atresia. Yamazaki (1965) found that vitellogenesis of hypophysectomised *C. auratus* was maintained by treatment with *O. keta* or *O. gorbuscha* pituitaries and Sundararaj *et al.* (1972a, b) found that a carp pituitary fraction and the salmon pituitary gonadotropin (SG-G100) were able to induce vitellogenesis in regressed ovaries and maintain

vitellogenesis in hypophysectomised *H. fossilis*.

These workers have demonstrated that generally fish pituitary preparations stimulate vitellogenesis in fish; in some species placental gonadotropins are effective and in only one case mammalian pituitary gonadotropin is effective. The situation is not so clear in *P. americanus*.

When the non-glycoprotein fraction of *H. platessoides* pituitary was partially separated by Sephadex G-75 to yield subfractions, the vitellogenic activity was found to be restricted to the subfraction which contains proteins in an approximate molecular weight range of 25 000 to 35 000. This fraction increased the gonadosomatic index of estradiol treated hypophysectomised fish and the content of labelled yolk in the ovary.

This fraction does not contain the glycoprotein, gonadotropin of the *H. platessoides* pituitary. Yamajaki's results are compatible with those seen in *P. americanus* but the results of Sundararaj *et al.* (1972a, b) can only be compared if the two fish "gonadotropic" fractions which stimulated vitellogenesis are likely to contain the pituitary protein which has been included in fraction C of the present work. The preparation from carp pituitaries involved a saline extraction followed by chromatography on Sephadex G-100 of the soluble fraction (Sundararaj *et al.* 1972b). Donaldson *et al.* (1972) prepared SG-G100 from *Oncorhynchus tshawytsha* Walbaum pituitaries by an alcoholic extraction followed by chromatography on Sephadex G-100. In neither case can it be certain that the selected fraction did not contain the equivalent of the non-glycoprotein fraction C. The data of Idler *et al.* (1975) suggests that a gonadotropin with up to ten times the potency of SG-G100 can be purified and Donaldson (1972)

found that further purification of SG-G100 appeared to increase the potency of this preparation. If these fractions were only partially purified, the results of Sundararaj *et al.* (1972a, b) could be due to the presence of a non-glycoprotein similar to the fraction C of this work. The gonadotropin prepared from pituitaries of *Cyprinus carpio* L. by Burzawa-Gerard (1971) was purified by gel chromatography, ion exchange chromatography and electrophoresis. The gonadotropin of *O. kata* prepared by Idler *et al.* (1975) involved an affinity chromatography step to remove non-glycoproteins followed by gel chromatography and electrophoresis. These highly purified preparations have not been assayed for vitellogenic activity and the only action demonstrated in female fish has been the stimulation of adenyl cyclase activity in pre-vitellogenic *C. auratus* (Fontaine *et al.*, 1970) and in immature *S. gairdnerii* (Idler *et al.*, 1975), a stimulation which has not been shown to be related to vitellogenesis. It is worth noting that none of the work on amphibian vitellogenesis has used purified amphibian hormones at all.

The inconsistency in the effect of mammalian gonadotropins on teleost vitellogenesis could arise because of species specificity and possibly if these preparations were not acting in a normal gonadotropic manner. The *in vitro* uptake of yolk by oocytes of *X. laevis* can only be induced by treatment of the donor animal with HCG. a direct effect of HCG on the oocytes cannot be seen (Wallace and Benquick, 1974). Fontaine (1969a) has demonstrated that mammalian pituitary gonadotropins are capable of stimulating the thyroid of *A. anguilla* and *S. gairdnerii* but the effects of placental gonadotropins have not been evaluated in this

system. Evidence that the thyroid may be involved in vitellogenesis, and suggesting that these gonadotropins could act through stimulation of this gland, comes from the histological demonstration of cycles of activity in thyroid tissue of several teleosts, which are associated with gonadal development (Pickford and Atz, 1957, p. 154). Honma and Tamura (1963) found an active thyroid in *P. altivelis*, after completion of their migration, during gonadal development and Woodhead and Woodhead (1965) showed that the thyroid tissue of adult *G. morhua* appeared more active than in immatures when the gonads of the older fish were undergoing vitellogenesis.

Fontaine (1961) found that treatment of migrating *A. anguilla* with mammalian thyroid-stimulating hormone for four weeks induced a slight increase in gonadosomatic index. The abolishment of the estradiol induction of plasma lipoprotein in *S. gairdnerii* (Takashima *et al.*, 1972) by treatment with mammalian thyroid powder could be due to the stimulation of incorporation by oocytes. However, Woodhead (1969) showed a similar thyroxin effect on plasma calcium to occur in male and female *G. morhua*.

There appears to be enough evidence to suggest that the thyroid system could be involved in teleost vitellogenesis. That the stimulus is obviously not an effect of the classical glycoprotein gonadotropin in *P. americanus* is clear because the *H. platessoides* pituitary glycoprotein fraction contains the gonadotropin responsible for maturation and ovulation (Table 19, p. 95). The non-glycoprotein fraction C which is effective in stimulating yolk incorporation into the gonad of *P. americanus* (Figure 8, p. 39) contains proteins within a molecular weight range of approximately 25 000 to 35 000. Mammalian prolactins and growth hormones are found to

ave molecular weights slightly smaller than this (20 000 to 23 000, orsyth, 1967; Greenwood, 1967).

Thyrotropins (TSH) have molecular weights of 20 000 to 30 000 Condliffe and Robbins, 1967). Fontaine and Condliffe (1963) purified hyroid stimulating hormone from pituitaries of *A. anguilla* in four hromatography procedures. The purified TSH had a molecular weight of 1 000 and apparently contained a much smaller proportion of amino sugars han does beef thyrotropin. No information is available to suggest ether such a "glycoprotein" would be bound to the concanavlin-A system ed in the present work. It would appear that the vitellogenic factor found in fraction C could be a thyrotropin.

The possibility that the teleost pituitary synthesises two nadotropins has been extensively discussed (Burzawa-Gerard and Fontaine, 1972; Reinboth, 1972; Donaldson, 1973; De Vlaming, 1974). The suggestion by some of these authors that the gonadotropic preparations isolated are re and affect both vitellogenic and maturational processes has been criticized above. Reinboth (1972) suggests that the histological demonstration of two gonadotrops in the teleost pituitary may have been limited by technical problems or due to specific differences. Another problem for this histological approach may occur if the two gonadotrops were active during separate phases of reproduction as might be the case if two different hormones controlled vitellogenesis and oocyte maturation.

No direct action of fraction C on the gonads has been shown in this work but if it contained the second gonadotropin, a non-glycoprotein which stimulated vitellogenesis in *P. americanus*, the varied effects of mammalian gonadotropins in other teleosts would have to be explained as

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to species specificity alone. The heterothyrotropic activity shown Fontaine (1969a) could occur because the teleost thyroid receptors e not sufficiently specific to distinguish the gonadotropin from yrotropins. If this was the case then the cycles of thyroid activity n in fish would suggest that the teleost thyroid even responds to the genous vitellogenic gonadotropin or possibly by an effect on the yroid of the estrogens involved in vitellogenesis (Sage, 1973).

SUMMARY

1. Between October and February, vitellogenic fish add 4.7 mg of ipid per g of fish to the ovaries. Part of this accumulation may occur a result of mobilization of 2.1 mg of liver lipid per g of fish; the est may be due to mobilization of muscle lipid reserves.

2. Hypophysectomy of vitellogenic fish results in the cessation of itellogenesis. Primarily this is due to a failure of the system of yolk ıcorporation into oocytes but also due to a cessation of yolk synthesis in the liver.

3. Estradiol treatment of hypophysectomised fish stimulates hepatic synthesis of yolk and leads to yolk accumulation in serum. There is also a concomitant estradiol-induced increase in liver weight which may result from the activity of protein synthetic mechanisms.

4. A fish pituitary glycoprotein gonadotropic preparation did not induce yolk uptake into the oocyte.

5. A non-glycoprotein fish pituitary fraction, with a molecular weight range of 25 000 to 35 000, induced active incorporation of yolk into oocytes and an increase in gonad weights.

6. Speculation that the pituitary fraction stimulating vitellogenesis might be a thyrotropin could account for observations in studies on the teleost thyroid and may be compatible with knowledge on the only teleost thyrotropin purified.

PART B OOCYTE MATURATION

INTRODUCTION

The results of Goswami and Sundraraj (1971), showing no oocyte turational activity of gonadotropin on *H. fossilis* oocytes *in vitro*, contrasts with work on other species where such preparations were found to be effectiye *in vitro* (Kawamura and Motonaga, 1950; Utsonomiya, 1954; Nadamitsu, 1961; Dettlaff and Skoblina, 1969; Davydova, 1972; Goncharov, 973).

The hypothesis for species other than *H. fossilis* is similar to that held for amphibians; a gonadotropin induction of an ovarian steroid results in maturation. In *H. fossilis* it is proposed that a steroid is again involved but in this case it is produced by the interrenal gland in response to gonadotropin. Several approaches to evaluate the pituitary-interrenal-gonad hypothesis proposed for *H. fossilis* were possible in this work using *P. americanus*. *In vitro* incubation of oocytes and perfusion of isolated ovaries with gonadotropic preparations was performed in order to investigate the maturational effects of these hormones in a system isolated from the influence of interrenal tissue. The incubation technique was also used to investigate the role of steroids which have been implicated in the process of oocyte maturation.

Surgery was performed to allow collection of venous effluent from the gonads of fish at various stages during the maturation process and isolated gonads were perfused *in vitro* with media containing pituitary and gonadotropic preparations. Both approaches yielded fluids to be

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subjected to chemical assays for steroids implicated by other workers or shown to have activity in the *in vitro* bioassay. This approach would demonstrate whether any of these maturational steroids could be synthesised by the ovary of *P. americanus*.

Jalabert *et al.* (1972) found that the coelomic fluid which was collected when *S. gairdnerii* were manually stripped at a hatchery sometimes had ovulatory activity. Egg fluids were collected from *P. americanus* in an attempt to show accumulation of maturational or ovulatory substances in these fluids.

One previous report on the occurrence of cortisol in the plasma of *P. americanus* (Bondy *et al.*, 1957) represented the entire knowledge of the non-estrogen, steroid physiology of this fish. In fact, steroids have been conclusively identified in only a few marine species (*Clupea harengus* L., *G. morhua*, Table A VIII, Idler and Truscott, 1972; *Hemitripterus americanus* Gmelin, Owen and Idler, 1972; *Hippoglossus hippoglossus* L., Weisbart and Idler, 1971), the majority of such work being performed in *A. anguilla*, *Oncorhynchus* spp. and *Salmo salar* L. This latter work is quite extensive but, in the case of *A. anguilla* it is related to osmoregulation, and in the salmonid species the concurrent migration and sexual development mean that no conclusive indications of steroid involvement in gonadal changes can be made. In *P. americanus* there are no apparent migrations imposing osmotic stresses so that chemical analysis of selected samples should identify steroids which may be involved with oocyte maturation in this species. The double isotope derivative assay was used in this work to ensure proof of identity as well as quantification of the steroids assayed for.

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With the growing amount of data available on steroidal induction of teleost oocyte maturation and a growing list of implicated steroids, it became clear that identification of the steroids active in *P. americanus* should be made by *in vitro* incubation assay and *in vivo* injection experiments. With these data, that of Sundararaj and Goswami (1972, 1974), Hirose (1972a), the work of Colombo *et al.* (1973) showing ovarian steroid synthesis and Jalabert *et al.* (1972, 1973), Jalabert and Breton (1974) showing that some progestins were powerful maturational agents, analyses for several maturational steroids could be performed in plasma and ovarian fluids. In order to be certain that analyses were performed on fluids which did have maturational activity, an *in vitro* oocyte maturation bioassay was used to identify samples which could then be pooled for analysis and comparison as active or inactive plasma.

MATERIALS AND METHODS

In Vitro Incubation of Oocytes

(a) Gonads were excised from a fish freshly killed by a blow on the head. A thin strip of ovarian wall was cut in the median surface of the gonad so that it could be pulled out of the organ with oocyte-covered ovarian lamellae attached. This strip was cut into approximately ¼ cm cubes and placed in scintillation vials containing incubation media (2, 5 or 10 ml). Head kidney tissues used in some experiments were taken from fish, dead for less than eight hours, or from fish donating eggs or plasma for incubation.

(b) Incubation was accomplished by placing caps, through which a small hole had been drilled, on the vials and installing the vials in an

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N-evap used to distribute a gentle flow of compressed air. Later 1974 experiments used air which was bubbled through a water column to ensure water saturation. The whole apparatus was kept in a 10°C cold room and the water baths helped maintain constancy of temperature.

For 1973, attempts were made to increase the osmotic pressure (O.P.) of Forster and Hong's (1958) medium to that found in fish plasma in April:

1. Forster and Hong's, but 1.5 X NaCl, 0.1% glucose and Tris 7.4 to bring O.P. to 695 mOsm.
2. Forster and Hong's but 1.5 X NaCl, 0.1% glucose and 0.05 M Tris 7.4, Glycerol ca. 20 ml, O.P. of 690 mOsm.

In 1974 attempts were restricted to May and June and media with osmotic pressures of about 460 mOsm (Appendix B). Later experiments used Hepes (N-2 Hydroxyethylpiperazine-N'-2-ethane sulphonic acid, Calbiochem) buffer at 7.2 - 7.4 in place of Tris.

In 1973 some experiments were performed using for incubation dilutions of the fluids released from a thawing ovary for incubation; others using the fluids collected upon centrifugation of whole, fresh ovaries at 1,000 g for 16 hr or plasma collected from maturing fish. In 1974 *in vitro* incubations were performed in plasma from fish at various stages of the maturation process, male plasma or ovarian fluids. For these experiments the fluid was added to a vial containing sufficient dried Hepes to give a 0.05 M solution of buffer pH 7.3 (at 5°C).

Stock solutions of 1 mg/ml steroids were prepared in ethanol and stored at -5°C; 10 µg/ml was used in most incubates. Partially purified salmon gonadotropin (SG-G100, Donaldson *et al.*, 1972) was kept at 4°C in

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flounder saline (1 mg/ml) for a few days or frozen for up to one month. ACTH dilute stock solution of 260 mg/ml was kept frozen between use. Freshly prepared stocks of dibutyryl cyclic AMP (Sigma Chemical Co.) were made for each incubation. Fresh preparations of flounder pituitary extract were made by homogenizing thawed pituitaries in a chilled glass homogenizer, with distilled water or incubation medium. Plaice pituitary glycoprotein fraction (PG3), prepared from glands collected from sexually mature fish caught during the spawning season (Pitt, 1966), was used for incubation or injection in the undialysed α-D-methyl glucoside containing buffer and frozen between experiments.

(c) At intervals pieces of ovarian tissue were removed from the incubate and examined using a dissecting microscope to evaluate the percentage of mature oocytes. Statistical comparisons were made by analysis of variance using an arcsine transformation of the percentage data.

In Vivo Induction of Oocyte Maturation

Freshly caught fish were hypophysectomised during the natural spawning season and subjected to steroid and protein hormone treatment by injection. Water temperatures in the tanks rose from 4°C to 6°C during the period of the experiments.

Steroids were dissolved in a minimal amount of ethanol, diluted with peanut oil to a concentration of 1 mg/ml, and injected intraperitoneally at a dose of 1 mg/kg on the day of hypophysectomy and on the three following days. On each occasion a biopsy sample of oocytes was taken for microscopical examination to ascertain their maturational state. Salmon gonadotropin (SG-G100), dissolved in flounder saline at

1 mg/ml, was injected in the same manner as steroids. Plaice pituitary glycoprotein PG3 was injected intraperitoneally at 1.1 mg protein/3 ml of total glycoprotein fraction/kg of fish. Peanut oil and flounder saline injections were made as controls for the steroid and protein experiments, respectively.

Fish were randomly selected for the initial injections and two days after the last injection fish which had not matured were distributed equally into groups for further injection regimes (Table 5).

Collection of Ovarian Venous Effluent

Samples of a few oocytes were obtained by biopsy and examined to select fish whose oocytes could be classed as non-mature, maturing or matured.

The body wall of the anaesthetised fish was cut from the vent area towards the posterior end of the kidney region and along the dorsal edge of the body cavity. When the gonad was lifted slightly and pulled ventrally the artery and vein were exposed; the mesentery holding them together was slit and the arterial flow reduced by a hemostatic clamp. The vein was ligated close to the body and a cannula (Intramedic, Clay Adams, P.E. 90 or 60) filled with heparin inserted through a slit in the wall of the vein towards the gonad. The cannula was held in place by tying surgical silk around the vein containing it and attached to a T-joint through which more heparinized saline could be introduced. After unclamping the artery and starting the flow by gentle suction, the effluent was collected into an iced, heparinized tube. The blood was treated in the usual manner.

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TABLE 5

Injection regimes: *In vivo* ovulation experiments.

	Experiment I		Experiment II	
	Number of Fish	Treatment	Number of Fish	Treatment
First regime	5	peanut oil	4	peanut oil
	4	17α20β progesterone	4	deoxycorticosterone
			5	cortisone
Second regime	1	peanut oil	3	peanut oil
	4	testosterone	5	SG-G100
	4	17αOH progesterone	5	estradiol
Third regime			3	Saline
			4	PG3

In a few experiments the ovarian artery of the lower gonad was cannulated by inserting a 26 gauge needle, attached to a cannula tube into the vessel. In all experiments an additional blood sample was taken from a caudal blood vessel after completion of effluent collection.

Ovarian Perfusion

Pairs of ovaries were excised from fish killed by a blow on the head after the ovarian vein and artery had been ligated. The gonads were excised and transferred to a 4°C cold room for perfusion. The blood vessels were carefully separated, a 26 gauge needle attached to a cannula (PE20) inserted into the artery, and a cannula inserted into the vein. Gentle suction was applied to the vein and a 5 ml syringe was used to force 1% heparinized saline into the artery to check for leaks and to flush the erythrocytes from the gonadal blood vessels. The arteries of the gonads were connected to a syringe type perfusion pump (Harvard Apparatus) and perfused at a rate of 2.5 ml per hour for 45-50 ml. After 20 hr the perfusates were centrifuged to remove any erythrocytes and frozen for storage at -70°C. One gonad from each fish was taken as control, the other as an experimental perfusion.

Extracts of flounder pituitaries (male + female, 0.04-0.08 glands/ml, five experiments), 8.2 IU/ml HCG (Sigma Chemical Co. two experiments), and 3 µg/ml luteinizing hormone (Sigma Chemical Co., three experiments) were used for perfusion in 600 mOsmole flounder saline containing a 50 µg TPNH/ml. No hormone was used for the second 20 hr of perfusion.

In an attempt to induce maturation using cortisone the ovaries of three fish were perfused with 0.1 µg/ml of the steroid prior to incubation

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in medium containing 10 µg/ml of cortisone.

Double Isotope Derivative Assay

^{14}C Labelled tracer steroids were purified shortly before use by paper chromatography; then 5,000 to 10,000 disintegrations per minute of each of the steroids to be analysed, were evaporated to dryness in centrifuge tubes. These tracers served to estimate recovery through the assay after the samples were quantitatively transferred to the tubes and equilibrated with the tracers for at least three hours. NaOH (1.0 M) was added to adjust the pH to greater than 9 and the samples transferred with cold (4°C) dichloromethane to a separating funnel for extraction with ten volumes of the organic solvent. The aqueous phase was washed with one further volume of CH_2Cl_2 and the combined organic phases washed with one twentieth volumes of 0.05 M acetic acid, 0.05 M NaOH and two washes of distilled water before flash evaporation. The dried extract was transferred to a centrifuge tube using CH_2Cl_2 methanol (9:1), and the solvent evaporated by a stream of nitrogen in an N-evap (Organomation Assoc.). The extract was redissolved in benzene/ethanol (9:1) for storage under refrigeration.

Thin layer chromatography (silica gel GF254 Brinkman Instruments 0.3 mm thickness was performed in the benzene:ethyl acetate 2:1 system for all samples in Assays 2, 3 and 4 by developing the chromatogram twice or three times. The areas of chromatograms for elution of sample steroids were identified by using the relative mobilities of the assay steroids to marker steroids run on the same plates. The gel in the sample areas was removed by vacuum elutor onto a sintered glass disc and the steroid eluted from the gel into a centrifuge tube using CH_2Cl_2 ethanol 9:1;

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the solvent was evaporated and the sample transferred to a 3 ml conical centrifuge tube, redissolved in benzene:ethanol 9:1 and stored in a refrigerator. In Assay 2 the three eluted areas contained cortisol + cortisone + 11-ketotestosterone, deoxycorticosterone + 11-deoxycortisol, 17α OH progesterone + progesterone; in Assay 3, progesterone, 17α OH progesterone and 17α 20β progesterone were separated and in Assay 4, deoxycorticosterone, 17α OH progesterone, 17α 20β progesterone and cortisone were all separated.

17α OH Progesterone and progesterone were reduced with 20β OH steroid dehydrogenase (Sigma Chemical Co.). These samples were dried into the tip of a centrifuge tube and dissolved in a drop of propylene glycol; 1.5 ml of 0.05 M Tris 7.4 (Sigma Chemical Co.) containing 0.23 units of the enzyme and 1.5 µg DPNA (Boehringer-Mannheim) was added and the mixture incubated at 37°C. After 2-5 hr incubation the reduced steroid was extracted into 12 ml of cold CH_2Cl_2 and the extract washed twice with water. The sample was then transferred to a 3 ml centrifuge tube for acetylation.

Four to six hours before acetylation the solvents were evaporated from the tubes containing the sample extracts, those containing aliquots of ^{14}C tracers, one containing 50 µg radioinert corticosterone, and then all were placed under vacuum in a P_2O_5 dessicator. After drying, the tubes were stoppered and removed to a fume hood where flame dried pipettes were used to add double distilled pyridine to the ^{3}H-acetic anhydride (New England Nuclear, 50 or 100 mC/mM) in a ratio of 1.4 or 2:1 and to place aliquots of this acetylating mixture into each assay tube. The tubes were tightly stoppered and sealed with tape for incubation at 37°C

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for 16 hr after which the reaction was stopped by addition of 1.5 ml of 25% ethanol. The aqueous ethanol was extracted with 10 ml CH_2Cl_2 and extracts were washed twice with 1.5 ml water. The solvent was evaporated under nitrogen and the sample stored in benzene/ ethanol (9:1). Approximately 10 µg of radioinert steroid acetate was added as a marker to permit visualisation during chromatographic purification. Thin layer chromatography and descending paper chromatographic systems are listed in Table 6; the areas for elution were determined by visualisation of steroids under ultraviolet illumination (wavelength = 240 nm). Steroid acetates were eluted from paper with ethanol. Aliquots from the last two or three chromatograms were placed in scintillation vials and evaporated to dryness.

Samples to be crystallised were dissolved together with 5-10 mg steroid acetate in a minimal amount of hot ethanol. A few drops of water were added after cooling and crystal formation induced by vigorous shaking. Cortisol and cortisone were crystallized from acetone-pentane. The samples were placed in a freezer for at least five minutes, a couple more drops of water (or pentane) added, then the crystals were centrifuged to enable aspiration of the supernatant. The crystals were partially dried under nitrogen and an aliquot placed in a scintillation vial. Crystallisation was repeated three or four times for each sample to ensure isomorphicity.

Dried aliquots or crystal samples in scintillation vials were dissolved in 150 to 300 µl ethanol using heat if necessary; 15 ml of Liquifluor scintillant (New England Nuclear) was added, mixed, and the sample placed in a liquid scintillation counter (Packard 2003, 2425 or

TABLE 6

Steroid acetate

Chromatography systems in sequence

Assay 1							
Cortisol	1	5	3	E	A	B	
Cortisone	1	5	3	E	A	B	
Corticosterone	1	5	3(3X)		E	F	
Assay 2							
Cortisol	1	2	B				
Cortisone	1	2	B				
11-deoxycorticosterone	1	3	4	B	C		
11-deoxycortisol	1	4	5				
11-keto testosterone	1	2	C				
20β OH progesterone	5	6	3	C	D		
17α20β progesterone	5	3	5	B			
cortiçosterone	1	3	4	B	C	G	
Assay 3							
20β progesterone							
17α20β progesterone	5	3	A	C			
corticosterone	1	5	7	2	A	E	
Assay 4							
cortisone	2	5	A	E	F		
deoxycorticosterone	8	5	A	3	C	E	
17α20β progesterone	5	3	A	D	C	D	(repeated on some)

Thin layer chromatography systems.

1. chloroform:methanol (96:4).
2. ethyl acetate:chloroform:water (90:10:1).
3. dichloromethane:n-butyl acetate (70:30).
4. t-butanol:hexane (25:75).
5. cyclohexane:isopropanol (70:30).
6. benzene:ethyl acetate (80:20).
7. hexane:ethyl acetate (60:40).
8. cyclohexane:ethyl acetate (50:50).

Paper chromatography systems.

A. mesitylene:methanol:water (50:33:16) (reversed phase).
B. cyclohexane:benzene:methanol:water (100:40:100:20).
C. heptane:80% methanol (50:50).
D. benzene:hexane:methanol:water (33:66:80:20).
E. cyclohexane:dioxane:methanol:water (100:100:50:25).
F. cyclohexane:benzene:methanol:water (100:70:100:25).
G. heptane:benzene:70% methanol (33:33:33).

tight

3375) for double label counting. An efficiency curve, set up using prepared standards, was used to calculate disintegrations per minute for ^{3}H and ^{14}C in each sample to establish an isotope ratio.

The concentration of an ethanol solution of the purified corticosterone acetate was measured by extinction of ultraviolet light (SP 500 Spectrophotometer, Pye Unicam Ltd.) and an aliquot of this solution was counted to determine the specific activity of acetic anhydride used for acetylation.

Steroid concentrations (µg/100 ml) were calculated according to the equation:

$$\frac{\text{Sample isotope ratio} - {}^{14}\text{C tracer ratio} \times \text{DPM tracer}}{\text{Acetic anhydride specific activity (DPM/µM)}} \times \frac{\text{MW of steroid}}{} \times \frac{100}{\text{Sample volume}}$$

Steroid-Protein Binding

Multiple equilibrium dialysis (Westphal, 1971) was used to compare the protein binding activity of 24 plasmas for 17α 20β progesterone. The technique, based on that used by Westphal *et al.* (1961), employed 0.5 ml aliquots of plasma diluted with 0.5 ml glucose free flounder saline. Duplicate dilutions of each plasma were placed in washed dialysis tubing (0.6 cm diameter, pore/size < 12,000 m.w.) and the knotted bags placed in an Erlenmeyer flask with three volumes of glucose free flounder saline containing approximately 10,000 DPM ^{14}C - 17α 20β progesterone (specific activity 59.1 mC/mM) per dialysis. The flask was attached to a Multipurpose Rotator (Scientific Industries Inc.) which inverted it approximately every 10 seconds. After 87 hr the bags were removed from the flask, rinsed with distilled water, blotted dry and drained and flushed with a few drops of

tight binding

distilled water into scintillation vials; ten ml of Aquasol was added for scintillation counting.

Two bags containing flounder saline acting as blanks and samples of the dialysing medium were used to estimate the amount of tracer in 1.0 ml of protein free solution (F). The amount of tracer bound (B) by 0.5 ml of plasma is calculated by subtraction of quantity F from the value obtained for radio-activity in the dialysed plasma sample. The quantity of steroid bound to the protein of one ml of each plasma is 2B/specific activity. Percent bound was calculated as $\frac{B}{B+F} \times 100$ for each sample.

RESULTS

Steroid Assays

Full details of these assays are supplied in Appendix A. It would appear that the high ^{3}H counts seen in Assay 2 (Table 7), before it was abandoned because of low ^{14}C recovery, indicated 17α OH progesterone activity since this steroid was found by both subsequent assays. The results of Assay 3 are very variable for 17α 20β progesterone, they are however the end result of isopolar and isomorphic purifications. Assay 4 would appear to demonstrate clear differences between 17α 20β progesterone found in pools of plasma which have maturational activity and those which have no such activity (Table 8). Such differences were not obvious in the analysis of ovarian fluids.

11-Ketotestosterone, progesterone, 11-deoxycortisol and deoxycorticosterone were not detected or were demonstrated in very small quantities. Cortisol levels demonstrated in plasma were within the

TABLE 7

Assay 2. Double isotope derivative assay results for plasmas of two fish and two gonadal perfusates.

(µg/100 ml)

	Non-mature		Fully mature		Control, gonad perfusion (11-1-72)	Flounder pituitary gonad perfusion (11-1-72)
	Peripheral plasma (20-3-72)	Gonadal plasma (20-3-72)	Peripheral plasma (9-6-72)	Gonadal plasma (9-6-72)		
progesterone	X	X	pooled XS crystal-lized 0.2µg/100ml			X
17α OH progesterone	Assay terminated due to low ^{14}C recovery. ^{3}H indicates may be present					
11-deoxycortisol	Two TLC purifies to tracer ratio					
deoxycorticosterone	* 0.3	0.15	0.19	*0.5	0.07	0.03
cortisone	* 1.03	*0.6	*0.7	*1.3	*0.03	*0.03
cortisol	*13.9	*7.8	*3.1	*4.0	*0.03	*0.03
11-ketotestosterone	*.0.8	*0.6	not pure	*0.6	not pure	not pure

*crystallised.

TABLE 8.

Assay 4. Double isotope derivative assay results for pools of fluids which had been characterized by *in vitro* maturation bioassays.

	n	cortisone	deoxycorticosterone	17α-OH progesterone	17α20β di-OH progesterone
Plasma (+ve in bioassay)	4	*0.88	*0.02	9.3	1.4
Plasma (-ve in bioassay)	7	*0.78	*0.09	≤ 0.44	≤ 0.4
Ovarian fluid (+ve in bioassay)	2	*0.18	*N.D.	≤ 0.40	≤ 0.4
Ovarian fluid (-ve in bioassay)	3	*0.13	*0.03	≤ 1.80	≤ 0.3

µg/100 ml; *crystallised.
+ve; positive.
-ve;, negative.

physiological range expected (16 µg/100 ml; Bondy *et al.*, 1957)(Table 7, Table 10). Cortisone levels were low in all cases and differences between the samples which were active and inactive *in vitro* maturation bioassay were extremely small.

Plasma Protein Binding

The quantity of 17α 20β progesterone bound to female plasma does not appear to undergo any major change during the spawning season (Table 11). No difference in plasma protein binding between groups of maturing and non-mature fish or fish sampled one month prior to spawning was seen. However, it seems that the plasma used in the second *in vitro* experiment using male plasma bound this steroid almost twice as actively as the mean of all female plasmas.

In vitro Oocyte Incubation

When portions of incubated ovarian tissue were teased apart on a microscope slide a quantitative assessment of the state of maturity of 50-150 eggs could be made with the use of a dissecting microscope (Figure 11). Displacement of the germinal vesicle (nucleus), as described by Goswami and Sundararaj (1971a) was noted but the stage of germinal vesicle breakdown was difficult to assess. The stage of maturation was recorded only when yolk granules had coalesced to produce a homogeneous, transparent cytoplasmic mass from which no intact nucleus could be isolated upon mechanical rupture of the oocyte. In order to reduce the risk of contamination, daily observations were made on different portions removed from the incubate on each occasion. These were then discarded.

Experiments in which multiple control incubations were made clearly

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TABLE 9

Assay 3. Progestin levels in plasma of fish selected as maturing or non-mature.

		Progesterone	17α OH progesterone	17α 20β progesterone
non-mature 25-5-72	peripheral	0.15	1.6	4.3
	gonadal	0.35	0.7	0.5
non-mature 22-3-72	peripheral	0.1	2.1	0.9
	gonadal	0.08	9.7	8.1
maturing 13-6-72 (20% of eggs mature)	peripheral	0.06	0.8	1.6
	gonadal	0.04	15.0	5.6
maturing 16-6-72 (50% of eggs mature)	peripheral	0.08	108.0	4,67
	gonadal	0.39	88	0.3

ug/100 ml plasma
all assays crystallised.

TABLE 10

Assay 1. Cortisol and cortisone (µg/100 ml) determined in pooled plasma samples.

Stage of fish	Date of sampling	n	Cortisol	Cortisone
Ripe	23/4/71	1	1.6	Not detected
Spent	23/4/71 to 11/5/71	4	1.7	Not detected
Resting	30/6/71	3	1.0	Not detected

TABLE 11

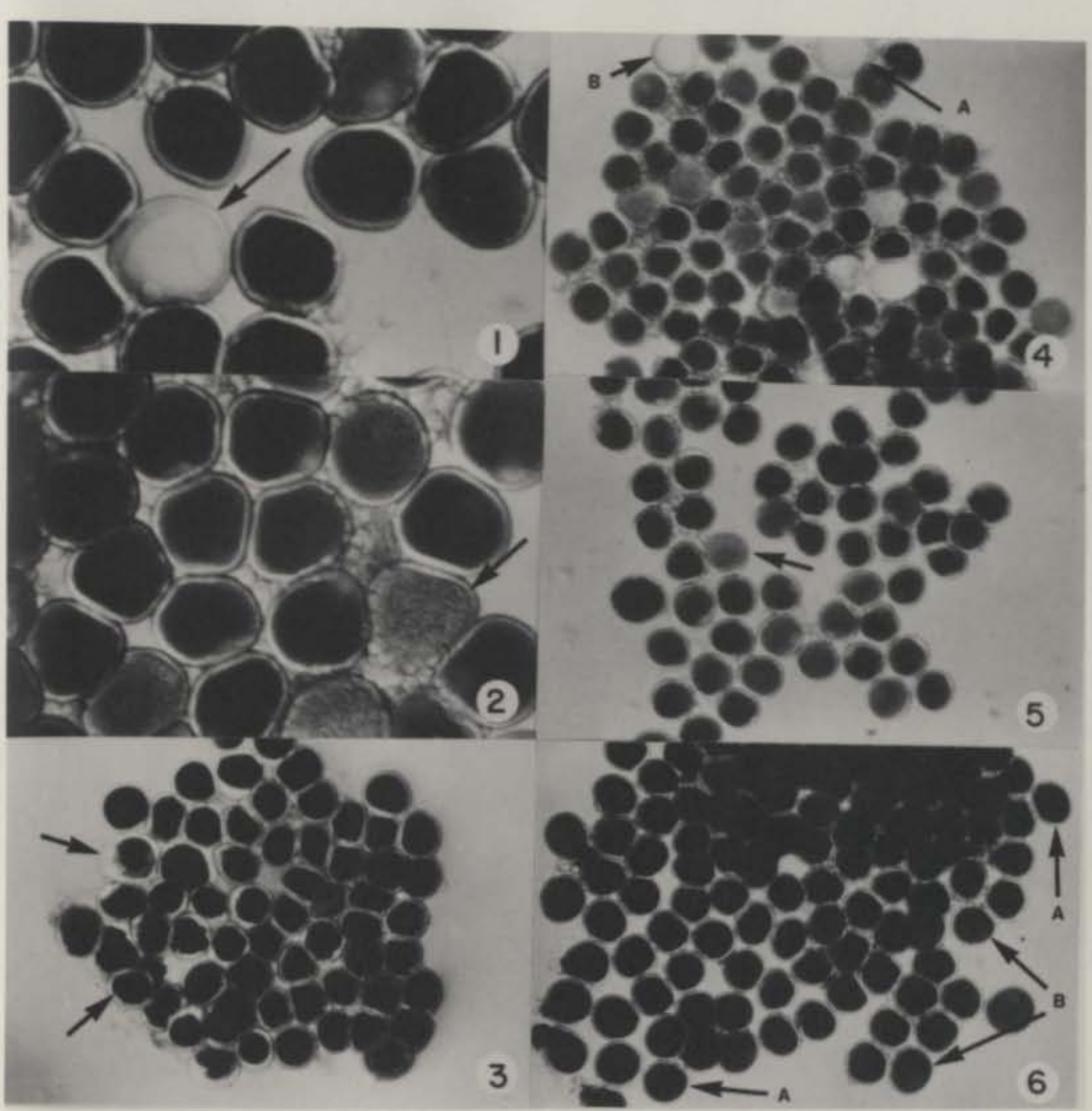
Binding of 17α 20β progesterone to plasma proteins determined by multiple equilibrium dialysis.

	weak positive in bioassay	sampled 24 hr before maturation	samples collected in March	negative in bioassay (June)	male plasma
% label bound ± 1 S.E. (n = number of plasma samples)	83.5 ± 8.3 (2)	77.6 ± 5.5 (5)	77.3 ± 6.7 (6)	78.2 ± 6.1 (7)	86.7 (1)
mass of tracer bound µg/100 ml plasma	2.5	1.7	1.7	1.8	3.3

Figure 11

Oocyte maturation stages

1. Mature but unovulated oocyte.
2. Maturing oocyte showing partial coalescence of yolk.
3. Sample of oocytes after six days of *in vitro* culture. Some abnormal but the majority are normal.
4. Several stages of oocyte maturation.
   A. Ovulated egg. B. Mature oocyte within follicle.
5. Maturing oocyte with no distinguishable nucleus. Many other oocytes have displaced germinal vesicles.
6. A. Normal pre-mature oocytes. B. Displaced germinal vesicle.


1
2
3
B
A
4
5
A
B
A
6

demonstrate a heterogeneity of response to incubation or within the ovarian pieces. Two way analysis of variance of an experiment using multiple replicates demonstrates no significant variation amongst six incubates but there was significant ($P < 0.05$) increase in maturity of these incubations over a 96 hr time period (Table 12).

Effect of Stage of Oocyte Maturity at Start of Incubation

Twenty-one fish were killed to supply oocytes in which the nucleus was not displaced and the oocytes could be described as non-mature; on incubation some maturation activity was seen in nine of these *in vitro* experiments. Four out of five experiments using oocytes which had displaced nuclei produced some maturational activity. It would seem that the demonstration of some maturational activity was possible in oocytes at either of these stages and also, that those which had displaced nuclei could not always be stimulated, even by treatments which have produced some activity in non-mature oocytes. Six experiments utilizing oocytes from gonads in which there were already some mature oocytes, failed to show any additional effect due to incubation with exogenous hormones (Table 13).

Incubation in Media

Incubates of partially mature ovarian pieces as controls never completed the maturation processes even after five or six days. Evaluation of the effect of buffers is inconclusive; when Tris was used, maturation did not continue in three experiments but did in others. All these early experiments were performed in a medium of 700 mOsmoles, but in an experiment where a 470 mOsmole medium was used, some increase in oocyte

tight

TABLE 1

Influence of *in vitro* culture on replicate pieces of ovarian tissue. Percent mature oocytes in a sample.

Replicate	24 hr	48 hr	72 hr	96 hr
a	1.5	12.9	8.9	9.2
b	7.0	5.9	26.1	30.1
c	12.0	4.5	10.1	19.8
d	7.8	25.5	28.1	10.8
e	4.7	19.7	18.7	20.6
f	3.4	1.7	32.2	10.0

F Replicates = 0.93, N.S.
F Time = 3.50, $P < 0.05$

TABLE 13

Effect of hormones on *in vitro* incubation of oocytes in artificial media: Inability to add to endogenous stimulus. Percent matured oocytes in a sample. (Arcsine transformed data in brackets)

Treatment	48 hr	72 hr	96 hr	144 hr
Control	33 (35.1)	16 (23.6)	31 (33.7)	29 (32.8)
Cortisone	37 (37.5)	13 (21.1)	49 (44.5)	49 (44.4)
Deoxycorticosterone	25 (30.0)	24 (29.0)	31 (34.0)	33 (34.9)
Progesterone	39 (38.7)	20 (26.9)	4 (11.5)	29 (35.5)
Pituitary	0	21 (27.1)	0	41 (39.5)
SG-G100	33 (35.1)	34 (35.7)	56 (48.2)	34 (35.6)

LSD ($P < 0.05$) = 20.1 (Arcsine).

aturation was shown. Any differences observed between oocytes incubated n media at either of the osmolalities could not be attributed to the smotic difference. Hepes buffer was used for the remaining experiments f 1974 after the control oocytes in an experiment using this buffer had hown increased incidence of maturation from 6% to 21% in three days, hile Tris buffered controls in the same experiment showed no such ncrease. In experiment 74-5 (Table 16), considerable oocyte maturity was een in control incubates in Hepes buffered plasmas and ovarian fluids.

The incubation of eggs in salines did not demonstrate conclusive ffects due to hormone treatment. The variability in experiment 73-9 F2 sked a slight effect due to cortisone, deoxycorticosterone and rogesterone (Table 14). In experiments involving eggs from donors which ad not begun the maturation process any effects due to hormone treatment ere exceedingly small. This insensitivity, combined with the experimental ariability, means that it was not possible to quantify any effects in ive other experiments. Slight qualitative effects demonstrated in other xperiments in which control incubates were unchanging, yielded further vidence of some role in oocyte maturation for cortisone (four out of ive occasions), deoxycorticosterone (one out of three), 17α OH rogesterone (one out of five), 17α 20β progesterone (two out of three) 0β progesterone (one out of two) and progesterone (one out of two). lbutyryl cyclic AMP was tested at 2 mg/ml, 500 µg/ml, 100 µg/ml and light *in vitro* oocyte maturational activity was seen with all three ses.

Pieces of one of three ovaries perfused with 1 µg/ml of cortisone an attempt to obviate permeability problems, developed significantly

Light Binding

The problem of exp rimental variability.
Percent matured oocytes in a sample.
(Arcsine transformed data in brackets)

Treatment	24 hr	48 hr	72 hr	96 hr	Mean
Control	2 (7.7)	6 (14.5)	19 (26.0)	7 (14.8)	8.5 (15.9)
Cortisone	15 (23.0)	23 (28.8)	21 (27.1)	1 (6.8)	15 (21.4)
Deoxycorticosterone	43 (40.8)	11 (20.1)	7 (15.7)	17 (24.3)	19.5 (25.2)
Progesterone	27 (31.2)	25 (30.0)	28 (31.9)	7 (15.7)	21.5 (27.2)
Pituitary	9 (17.2)	18 (25.0)	11 (19.5)	0	9.5 (15.4)

LSD ($P < 0.05$) = 12.3 (Arcsine).

ater ($P < 0.025$) oocyte maturation than controls upon subsequent
ubation for 120 hr (Table 15).

ubation in Ovarian Fluids and Plasmas

When ovarian fluids were used as incubation media it was found
t some were active while others were not. In one experiment fluids
m ovaries of fish classified as non-mature or ovulated induced some
urational activity but those from maturing fish were inactive. It
interesting to note that additional hormone treatment in the non-
ture ovarian fluid did not add to the endogenous stimulation of oocyte
turation.

Of a total of eleven ovarian fluid samples bioassayed in this
k, four induced some maturational activity. Pools of two of the
ightly active fluids and three of the inactive fluids were taken for
arison in double isotope derivative Assay 4, but showed no differences
concentrations of cortisone, deoxycorticosterone, 17α OH progesterone
l 17α 20β progesterone.

Plasma samples collected from fish at the three stages of final
cyte maturation (pre-mature, maturing and mature) were tested and all
ee induced some maturational activity. Addition of cortisone, deoxy-
ticosterone, 17α OH progesterone and 17α 20β progesterone, to the
sma from the non-mature fish induced a statistically significant
< 0.05) stimulation of maturation, but testosterone had no effect
ble 16).

To test whether this *in vitro* maturational activity by plasma was
sent at times other than immediately before ovulation, two samples
m non-mature females, one from March, the other from June, were also

tight

TABLE 15

Effect of perfusion with cortisone prior to *in vitro* incubation. Percent matured oocytes in a sample.

	Replicate			
	a	b	c	d
Control perfused				
72 hr	3	0	0	0
96 hr	0	0	0	0
120 hr	0	0	0	0
Cortisone perfused				
72 hr	9	3	0	5
96 hr	0	1	0	0
120 hr	2	5	0	0

$F(\text{treatment}) = 6.28$ $\quad F_{0.025[1,18]} = 5.98$

$F_{0.01\,[1,18]} = 8.28$

Effect of hormones on *in vitro* oocyte incubation in plasma from a female fish.
Percent mature oocytes in a sample.
(Arcsine transformed data in brackets)

Treatment	72 hr	96 hr	120 hr	144 hr	Mean
Control	3 (7.0)	7 (15.0)	14 (21.6)	8 (16.4)	8 (16.4)
Cortisone	12 (20.2)	8 (16.2)	41 (39.8)	20 (26.8)	19 (25.7)
Deoxycorticosterone	5 (13.3)	9 (17.0)	11 (19.7)	31 (33.9)	13 (21.0)
17α OH progesterone	2 (7.3)	23 (28.8)	25 (29.9)	31 (34.0)	18 (25.0)
17α 20β progesterone	4 (12.7)	29 (32.3)	24 (29.0)	19 (25.8)	17 (24.7)
Testosterone	0	6 (14.3)	4 (10.9)	12 (20.4)	4 (11.4)

LSD ($P < 0.05$) = 9.04 (Arcsine)
LSD ($P < 0.02$) = 10.94 (Arcsine).

yed in experiment 74-6. Neither of these plasma showed any capacity stimulate maturation even though the oocytes used in this experiment shown to be capable of maturation (Table 17).

Twenty-eight plasmas from non-mature and maturing fish were bio-ayed by *in vitro* oocyte incubation. Seven of these induced up to 10% uration. Two of the seven donor fish had been classified as pre-ure at the time of blood collection. However, one of these pre-mature h had been subject to repeated biopsy and blood sampling and the active had been collected 24 hr before the onset of maturation was noted. enteen samples from matured fish bioassayed in another experiment lded only two which showed traces of activity. For the fourth steroid ay, four of these seven active plasmas and seven of the original 21 -active plasmas were pooled and analysed to show a difference in 17α OH gesterone and 17α 20β progesterone concentrations (Table 8).

Two experiments using buffered plasma from male fish as the bation medium produced varying results. Plasma collected from male h immediately before use did not itself stimulate any maturation, but used for incubation of oocytes with cortisone, deoxycorticosterone, OH progesterone, 17α 20β progesterone or SG-G100 maturation of some ytes did occur (Table 17). In the second experiment the plasma been collected from a spent or recovering fish the previous July and maturational activity was seen in controls or steroid treated incubations. ever, some maturation was induced in oocytes incubated with an extract pituitary glands (3.5/ml of plasma).

tight

TABLE 17

Effect of hormones on *in vitro* oocyte incubation in plasma from male fish.
Percent mature oocytes in a sample.

Treatment	111 hr	140 hr	164 hr	188 hr	212 hr
Control	0	0	0	0	0
Cortisone	2.4	15.0	9.4	25.7	15.3
Deoxycorticosterone	2.6	3.2	17.1	1.6	3.9
Estradiol	0	0	0	0	0
17α progesterone	1.3	13.3	3.3	13.2	34.5
17α 20β progesterone	1.3	1.9	6.3	22.8	25.4
Testosterone	0	0	0	0	0
SG-G100	0	+	2.2	2.2	2.1

+ = maturation not quantified.

vo Injections.

Pituitary extract, HCG, SG-G100, cortisone and progesterone ected intraperitoneally into intact fish yielded no meaningful results. riments performed early in the season produced no maturation, and r in the season natural maturation obscured any experimental effects. e 18 presents the results of one experiment using this procedure.

In 1974 fish were hypophysectomised to avoid the onset of natural ıration during an experiment (Table 19). No response to four daily ctions at 1 mg/kg was evident upon treatment with cortisone, deoxy- .icosterone, 17α OH progesterone, 17α 20β progesterone, estradiol- oate or testosterone. One month after the start of these experiments .e were still no signs of maturation when biopsy samples were examined oscopically. In the fishes treated with SG-G100, maturation was ıced; the three healthy animals proceeded to become fully ovulated st the other died prior to reaching this state. Plaice pituitary coprotein (PG3) was effective in inducing maturation in three treated .es whilst a fourth fish which was dying was not stimulated.

i Perfusion

None of the oocytes in gonads perfused with HCG, LH or pituitary tacts exhibited any maturation, even when ovarian pieces from two LH- fused gonads were maintained in *in vitro* incubation for more than ten . No ovarian pieces from pituitary perfused ovaries were maintained incubation so it can only be stated that perfusion of gonads with uitary extracts or HCG was not effective at inducing any maturation 40 hr.

tight

TABLE 18

Biopsy observations of oocytes from injected fish.

Fish	Injection treatment	Date of Injection					
		7-VI	9-VI	11-VI	13-VI	15-VI	18-VI
1275	pituitary	-	N	N	D	Dead	
1280	< 2 glands/fish	-	D	M	O	O	O
1276	cortisone	-	N	N	N	N	N
1281	5 mg/fish	-	few M	few M	17% M	45% M	75% M
1278	sham	-	N	N	N	D	D
1282	0.5 ml/fish	-	N	N	N	D	D
1279	Urophysis	-	N	Dead			
1283	< 2 glands/fish	-	N	N	Dead		

- = no observation
N = normal
D = displaced nucleus
M = mature
O = ovulated fully.

TABLE 19

Effect of steroid and protein hormone injections on oocytes of hypophysectomised fish.

Treatment	Previous treatment(s)	Days to nuclear displacement	Days to mature	Maximum percent mature	Days to ovulated
SG-G100	deoxycorticosterone	4	5	3	8
	cortisone	4	5	20	7
	control	4	11	50 (fish dying)	
	control	4	5	100	8
plaice glycoprotein PG3	deoxycorticosterone estradiol benzoate	–	8	20	10
	cortisone estradiol benzoate	5	–	–	8
	cortisone estradiol benzoate	–	4	10	–
	control estradiol benzoate	11	–	–	–

DISCUSSION

oid Analysis

Double isotope derivative assays, for steroids implicated in te maturation, were made on plasma samples taken from fish in the opriate stage of sexual development. Cortisol had been implicated by ami and Sundararaj (1971) as an *in vitro* maturation inducing agent in *ossilis* and by Hirose (1972a) for *O. latipes*. Assay 1 confirmed the ence of cortisol in the plasma of *P. americanus* during the period before spawning (April-May) until the resting and recovery phase y-August). The values obtained in this work (< 2 µg/100 ml) are iderably lower than those reported by Bondy *et al.* (1957) for a ed sample of 25 male and female *P. americanus*. No details were given heir work of the method of blood sampling and the analytical techniques inadequate to make a conclusive identification or purify the steroid quantification. The values obtained for the two fish in Assay 2 were rer to those obtained by these workers. This difference is probably to a reaction by the animal to the stress of prolonged blood collection the ovarian vein. (For a review of "stress" effects on corti-teroidogenesis see Idler and Truscott, 1972). The high concentrations cortisol in plasma of these two fish of different maturational states ests that this steroid is unlikely to be involved in oocyte maturation *P. americanus*. Kirshenblat (1959) demonstrated maturational activity to cortisone treatment in *M. fossilis*. Moderate amounts of cortisone the insignificant differences in concentration demonstrated by an ay on pooled plasma samples (Assay 1), and on gonadal and peripheral ma of two selected fish (Assay 2), suggests that it is unlikely that

tight Binding

this steroid is involved in oocyte maturation.

Deoxycorticosterone was found to be present at similar low levels in the plasma of a ripening female *P. americanus* prior to spawning, in a fully mature fish, and at extremely low levels in pooled plasmas which had been collected through this period of the reproductive cycle. Deoxycorticosterone, reported to be the principal maturation inducing steroid for *H. fossilis* (Goswami and Sundararaj, 1971); has recently been implicated in maturation of *C. auratus* oocytes *in vitro* (Jalabert *et al.*, 1973), and was found by Colombo *et al.* (1973) to be synthesised *in vitro* by the ovaries of *Leptocottus armatus* Girard, *Gillichthys mirabilis* Cooper and *Microgadus proximus* Girard. The low plasma levels detected suggest deoxycorticosterone is unlikely to be directly involved in oocyte maturation of *P. americanus*. Analysis of the peripheral and gonadal plasma of two fish failed to demonstrate the presence of 11-deoxycortisol. This steroid has recently been demonstrated to have some *in vitro* maturational activity with *H. fossilis* oocytes (Sundararaj, 1974) and *C. auratus* oocytes (Jalabert *et al.*, 1974) and was one of the steroids synthesised by ovaries in the work of Colombo *et al.* (1973).

Schmidt and Idler (1962) found 11-ketotestosterone in *O. nerka* during the migration up river for spawning. Double isotope derivative assay of plasma from two fish in the present work showed moderate levels only. More extensive work (Idler, unpublished) has established that 11-ketotestosterone is not an important plasma steroid in female *P. americanus* and significant concentrations occur only in males.

Progesterone has been implicated in induction of maturation of amphibian oocytes (Schuetz, 1974), and has *in vitro* maturational activity

in *H. fossilis* (Goswami and Sundararaj, 1971), *S. gairdnerii*, *E. lucius*, and *C. auratus* oocytes (Jalabert *et al.*, 1973; Jalabert and Breton, 1974). Progesterone was assayed in plasma from six fish in the March to July period. The low values obtained, which do not suggest an involvement in oocyte maturation, were in the range which had previously been demonstrated in these samples by Nutt (pers. comm.) using radioimmunoassay. Of these steroids deoxycorticosterone and cortisone are the only ones which have been assayed in the plasmas which were positive in the *in vitro* oocyte maturation bioassay so these are the only steroids for which it can conclusively be stated that there is no correlation between steroid concentration and maturational activity.

17α OH Progesterone appears to be a major steroid in the circulation of some female *P. americanus*. Schmidt and Idler (1962) reported high levels of this steroid in *O. nerka* captured during migration near the time of spawning. The synthetic mechanism for formation of this steroid has been demonstrated in ovaries (Colombo *et al.*, 1973) and in interrenal tissues (Table A VII, Idler and Truscott, 1972) of several fish species. 17α OH Progesterone has been shown to be an active *in vitro* oocyte maturation inducing agent in *S. gairdnerii* (Jalabert *et al.*, 1972), *E. lucius* (Jalabert and Breton, 1974), and to have some activity in *C. auratus* (Jalabert *et al.*, 1973). The *in vitro* oocyte incubation data presented here suggests that this steroid and 17α 20β progesterone may have similar roles in *P. americanus*. Significant plasma concentrations of 17α 20β progesterone were found in four fish sampled between March and July. A pool of plasmas collected from female *P. americanus* and which had been shown to have maturation-inducing activity, had a greater

oncentration of 17α OH progesterone and 17α 20β progesterone than a pool f inactive plasmas. In *S. gairdneri* 17α 20β progesterone was the most otent maturation-inducing steroid identified (Fostier *et al.*, 1973).

n vitro Incubation - Technical Limitations

The variability manifested by the experimental technique used in he present work has made it difficult to conclusively evaluate the apability of the hormonal systems to induce oocyte maturation. This ariability appears to be a product of the sampling method of assessment uggesting that during incubation, heterogeneity is developed within the lump of incubated oocytes since no significant differences were seen between eplicate incubations of the same tissue (Table 12). This was to be xpected since observation of the maturation processes *in vivo* has never stablished any differences between different parts of the ovary.

Complete *in vivo* maturation occurs during a period of less than 8 hr. *In vitro* incubation, even in plasma, has been unable to induce ore than 30% maturation in periods of incubation of up to 212 hr. This nsensitivity of response, even in experiments in which some oocyte aturation was induced by hormone treatment, suggests that the technical esign of these experiments is not adequate to positively evaluate the ffects of hormones on oocyte maturation.

Culture of clumps of oocytes *in vitro* with successful hormonal nduction of oocyte maturation has been shown for several teleost species Kawamura and Motonaga, 1950; Kirschenblat, 1959; Nadamitsu, 1961; Goswami nd Sundararaj, 1971b and Jalabert *et al.*, 1972). However, Hirose (1971) ound that cultures of ovarian fragments were considerably less sensitive o hormonal stimulation than cultures of separated oocytes. The perfusion

of gonads from *P. americanus* with physiological doses of cortisone prior to *in vitro* incubation did not induce significant oocyte maturation in two of the three ovaries tested; a slight effect was seen in the other. Cortisone has been shown to have some maturational activity when oocytes are incubated in plasma (Table 16), but even when gonads were perfused, and presumably the hormones delivered to the target tissues, no major oocyte maturation was induced. This experiment together with the data discussed below on hormonal induction of oocyte maturation in plasma, suggests that there were no problems of permeability to the hormones within the oocyte clumps.

Observation of oocytes of *P. americanus* in incubation for up to 212 hr has not shown any evidence that those in the clump centres were killed; however, the experience of Fostier (pers. comm.) in *S. gairdneri* was that the ability of oocytes to respond to hormonal stimulation, even though they are still viable, might be critically limited if oxygen diffusion was a problem. A lack of sufficient oxygen diffusion into clump centres might lead to the development of a heterogeneity of response to treatment within the clumps which, due to the sampling method of assessment employed in the present work, could account for the experimental variability and insensitivity demonstrated.

Jalabert *et al.* (1972) stress that oocytes in which the germinal vesicle has migrated to a peripheral position were essential for successful *in vitro* studies in *S. gairdneri*. The present work would suggest that clear demonstration of maturational activity was not possible in any incubation performed in artificial media. Some maturational activity was nonetheless seen even in experiments using oocytes prior to the stage of

migrated germinal vesicle.

Only one experiment (Table 13) using oocytes from the ovary of a fish which had started to mature naturally gave an indication that additional hormone administration may be able to augment the natural stimulation. It would seem that using an assay system of this sensitivity it is not possible to evaluate the effects of hormone treatments in addition to an earlier *in vivo* stimulation.

Maturation and Ovulation

At no time during the present work has ovulation been noted in *in vitro* experiments. In *S. gairdneri* ovulation does not occur *in vitro* after stimulation with gonadotropic or steroidal preparations (Jalabert *et al.*, 1973), but ovulation did occur when oocytes were incubated in some egg fluids which had been collected after forced stripping of ripe fish. In an enzymatic analysis of these fluids, Breton *et al.* (1974) demonstrated higher activities of trypsin and chymotrypsin in fluids which stimulated ovulation. Hirose (1972) claims that in *O. latipes* gonadotropic or steroidal treatment of oocytes *in vitro* resulted in maturation and ovulation. His description of ovulation is a little unclear (Hirose, 1971) but his ovulated oocytes were capable of being fertilized. The hormonally induced changes seen after treatment of oocytes, from which the follicular layers had been digested, were described as ovulation (Hirose, 1972b) which suggests he does not distinguish between maturation and ovulation. Sundararaj and Goswami (1972) did not separate the induction of maturation and ovulation in evaluation of hormone treatments.

Kawamura and Motonaga (1950) and Utsonomiya (1954) showed that frog pituitary suspensions were capable of inducing ovulation *in vitro* in *C. biwae*. In *M. fossilis*, Kirshenbalt (1959) was unable to induce *in vitro* ovulation. Goncharov (1973) did not distinguish maturation from ovulation *in vitro* incubation of oocytes from Caspian sturgeon. Schuetz (1967b, 1969) suggests that in *R. Pipiens* a progesterone-like ovulation inducing substance is capable of inducing ovulation but Alonso-Bedate *et al.* (1971) found that progesterone was not effective at inducing ovulation *in vitro* in *Dicoglossus pictus*.

It seems that considerable confusion exists in the literature about ovulation *in vitro*. It is possible that *S. gairdnerii* with its "naked" gonad structure would employ different mechanisms than the other fish whose oocytes do ovulate *in vitro*. The negative results in *P. americanus* may reflect the insensitivity of the assay system or the absence of mechanical effects due to action of ovarian and body musculature around the mature eggs. It is possible that normal ovulation occurs by mechanical rupture of follicles after the swelling of oocytes during maturation. Hirose *et al.* (1974) showed a 9% increase in the diameter of oocytes from *Plecoglossus altivelis* and the present work has shown a 37% increase in the diameter of *Pseudopleuronectes americanus* oocytes during maturation and ovulation.

Nadamitsu (1961) investigated the processes of ovulation of *M. fossilis* eggs *in vitro* after *in vivo* stimulation with frog pituitary extracts. He hypothesises that agents such as potassium fluoride or EDTA, by removing Ca^{2+} from the follicular membranes, bring about ovulation of mature oocytes. The contribution of ions to maturation and ovulation are

ot understood. Failure to induce either of the processes in *P. americanus* ocytes *in vitro* could be the result of insufficient attention to the onic composition of the media. In the present work no ovulation was emonstrated under the hormone treatments tested even when oocytes were ncubated in plasma.

ncubation Media

The Forster and Hong flounder saline was modified for this work n order to match its constituents as closely as possible to the physico-hemical properties of *P. americanus* body fluids. It was observed that he pH of a few plasmas tested ranged from pH 7.0 to 7.4; the Na^+ con-entration of gonad fluids was 150-200 meq/l and K^+ in these fluids was 0-12 meq/l. The osmotic pressure of gonad fluids was similar to that een in plasma during the March to July season (470-720 mOsmoles: letcher, in press).

The components of media used for *in vitro* oocyte incubation in everal investigations are listed in Appendix B. Skoblina's (1974) loach ringer is notable for the high pH optimum found in test experiments, hilst all the other media are used at pH 7.0-7.5. Ionic calcium is nigher in the Forster and Hong based media than the other marine teleost edium (Cobb, 1973), or any of the media used for fresh water species. If the hypothesis of inhibition of ovulation by cations (Nadamitsu, 1961) is applicable, this higher concentration could account for the inability to demonstrate *in vitro* ovulation of *P. americanus* oocytes in the present ork.

Iwamatsu (1973) found that bovine serum albumin (fraction V) was necessary for successful *in vitro* maturation of oocytes from *O. latipes*

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il he optimised the ionic concentration; no effect could be attributed the presence of the protein in the final balanced salts solution. ever Skoblina (1974) demonstrated that the use of a 0.1% solution of albumin in the Ringer's solution facilitated the *in vitro* maturation ponse of *M. fossilis* oocytes to pituitary homogenates. The use of a ium containing 20% calf serum by Hirose (1971) raises the possibility t response to hormone treatments seen in his experiments could be due action of some serum constituent in conjunction with the treatment. importance of protein additives during *in vitro* maturation is unclear ce in the extensive work of Jalabert and Sundararaj *in vitro* maturation induced in the absence of such additives. It would seem that the bility to demonstrate significant maturation of *P. americanus* oocytes ubated with flounder saline is unlikely to be the result of the otein free nature of this medium.

e Effect of Steroid Hormones on Oocyte Maturation

Some maturation inducing activity by 17α OH progesterone and 17α β progesterone has been seen when *P. americanus* oocytes were incubated plasma from male or female fish (Table 16, 17); slight effects were n upon incubation in saline Table 14). 20β progesterone and progesterone fects on oocytes incubated in saline were small but these steroids were t tested in plasma incubation.

Fostier *et al.* (1973) found that 17α 20β progesterone was more tive as an *in vitro* maturation inducing agent in *S. gairdnerii* than ther 17α OH progesterone or 20β progesterone. Jalabert and Breton (1974) owed that 17α OH progesterone and 20β OH progesterone were eight times powerful maturation inducers as progesterone or deoxycorticosterone

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with *in vitro* culture of *E. lucius* oocytes. *In vitro* maturation experiments in *C. auratus* (Jalabert *et al.*, 1973) showed slight effects due to progestins while progesterone has maturational action on oocytes of *H. fossilis* and *O. latipes* incubated *in vitro* (Goswami and Sundararaj, 1971; Hirose, 1972).

17α OH Progesterone and 17α 20β progesterone were present at high levels in the pool of biologically active *P. americanus* plasma and also in plasma from three out of four individual fish analysed. One of these fish had been sampled one month before any fish undergoing maturation were seen in the laboratory, one other had just begun oocyte maturation (20% mature) while the other was only beginning maturation (5% mature). The appearance of the progestins in biologically active plasma is evidence, when taken together with the *in vitro* incubation data, that these steroids may be involved *in vivo* maturation. It is difficult to reconcile the findings of significant amounts of these hormones in a fish which was unlikely to be undergoing maturation, with an hypothesis involving 17α OH progesterone and 17α 20β progesterone as maturation inducing substances.

One possible explanation of the appearance of detectable amounts of maturation inducing steroids in plasma prior to the onset of natural maturation could be that a change in protein binding for these hormones, together with increased synthesis, is part of the maturation stimulus. Fostier (pers. comm.) has found that use of plasma for *in vitro* incubation of oocytes does not always result in steroid induced maturation if their normal experimental dosages are used. He explains this anomaly by saying that binding capacity maintains a sub-minimal dose of unbound hormone in-culture. However, in the present work no difference in binding of 17α 20β

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progesterone to plasma proteins, which could be correlated to maturation, has been demonstrated. This analysis was not performed on any of the plasmas assayed by double isotope derivative assay, but aliquots of two plasmas which had demonstrated slight *in vitro* maturation were included. The amount of tracer steroid bound to the plasma in all samples tested exceeds the value of 1.4 µg 17α 20β progesterone/100 ml determined by DIDA of pooled plasmas with maturational activity. The mass of labelled steroid added in this experiment represents 3.6 times this endogenous level. The maximum concentration bound would be equivalent to 4.2 µg/100 ml of the male plasma which is less than half of the concentration measured in some plasmas from *P. americanus*. Idler and Freeman (1969) found that a dose of 25 µg testosterone/100 ml was necessary to saturate the plasma binding system for this hormone in *Raja radiata* Donovan, a dose which was 2.5 - 5 times higher than the physiological plasma concentrations demonstrated by Fletcher *et al.* (1969). It would seem unlikely that the amount of radioactive steroid added was enough to saturate the binding system in *P. americanus* plasma which means that the value for binding is probably accurate for this steroid concentration.

The steroid binding sites in the male plasma tested would likely be saturated under the conditions of the *in vitro* incubation experiment (equivalent to 2500 µg steroid/100 ml plasma), thus the difference in protein binding between the two male plasmas used for *in vitro* incubation would be unlikely to account for the failure of steroids to induce oocyte maturation in one of them.

Deoxycorticosterone and cortisone induce maturation of *P. americanus* oocytes *in vitro*. However, the analysis of plasma steroids suggests that

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the *in vitro* action of these steroids is not the normal *in vivo* system. Deoxycorticosterone is a powerful oocyte maturational agent in *H. fossilis* (Goswami and Sundararaj, 1971) and has been shown to be active on oocytes of *C. auratus* (Jalabert *et al.*, 1973) but has not been conclusively identified as a normal *in vivo* maturational agent in either species. Kirschenblat (1959) showed that cortisone induced maturation of oocytes of *M. fossilis* cultured *in vitro* but there is no evidence for a direct action of this steroid in normal *in vivo* oocyte maturation of any species.

Cortisol is an active *in vitro* maturational agent on oocytes of *H. fossilis* and *O. latipes* (Goswami and Sundararaj, 1971; Hirose, 1972) but no action on *P. americanus* oocytes was seen *in vitro* and analysis of plasma concentrations did not support any hypothesis of involvement of this steroid in maturation.

Intraperitoneal injections of steroids into hypophysectomised *P. americanus* at 1 mg/kg (on each of four days) did not induce maturation or ovulation. Though one intra-muscular injection of 1.5 mg/kg was adequate to stimulate vitellogenesis with estradiol (see Part A) this dose was much lower than that used by other workers to stimulate maturation and ovulation (Sundararaj and Goswami, 1966). Their work involved single intraperitoneal injections of up to 300 mg/kg and resulted in maturation and ovulation of *H. fossilis* oocytes with cortisol acetate, deoxycorticosterone acetate and cortisone acetate but no effect was observed with progesterone, testosterone propionate or estradiol benzoate. These data suggest that the amounts of steroids injected may have been insufficient to induce *in vivo* maturation of oocytes in *P. americanus* so that the *in vivo* effects of steroids may not have been demonstrated

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conclusively.

The Effect of Protein Hormones on Maturation

Salmon gonadotropin (5 µg/ml) was slightly effective as a maturation inducing substance when used for oocyte incubation in one male plasma (Table 17, p. 92) but was unable to induce any activity in another experiment (50 µg/ml). Two *H. platessoides* glycoprotein extracts demonstrated no maturational activity at the doses used for incubation (PG2, 0.2 pituitaries/ml; PG3, 0.8 pituitaries/ml) even when PG3 was used in a co-culture of oocytes with head kidney tissue. *P. americanus* pituitary extracts containing 0.1, 0.2, or 3.5 glands/ml induced very slight activity on three occasions but nine other experiments showed no effect due to treatment with 0.1 up to 1 gland/ml.

The salmon gonadotropin (SG-G100) has been used to induce *in vitro* maturation in *S. gairdneri* (0.5 µg/ml, Jalabert *et al.*, 1972), *C. auratus* (2 µg/ml, Jalabert and Breton, 1974). In *H. fossilis*, Sundararaj *et al.* (1972) found that up to 40 µg/ml of SG-G100 was incapable of inducing ovulation. Goswami *et al.* (1974) showed some *in vitro* maturation when *H. fossilis* oocytes were incubated with 20 µg/ml SG-G100 and that this response was increased by co-culture of the oocytes with head kidney tissue. In the present work no clear effect due to SG-G100 treatment of oocytes, incubated in saline, was demonstrated with or without head kidney co-culture.

Treatment of hypophysectomised *P. americanus* with SG-G100 or pituitary glycoprotein fraction (PG3) resulted in maturation of oocytes. This salmon preparation has been used by Yamazaki and Donaldson (1968), Sundararaj and Anand (1972), Ishida *et al.* (1972) and Shehadeh *et al.*

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(1973) to induce ovulation in *C. auratus*, *H. fossilis* (1.2 mg/kg), *P. altivelis* (10 mg/kg) and *Mugil cephalus* L. (20 mg/kg). The fraction PG3 is the glycoprotein material of *H. platessoides* pituitary extract and should contain any classical gonadotropins. In this *in vivo* work it has behaved in a similar manner to the salmon gonadotropin but no *in vitro* maturational activity has been demonstrated.

Perfusion of ovaries with human chorionic gonadotropin (HCG), luteinizing hormone (LH) or *P. americanus* pituitary brei did not result in an induction of maturation. This might suggest that gonadotropins cannot stimulate oocyte maturation directly. Jalabert *et al.* (1972) found that whereas 0.5 µg/ml SG-G100 was effective in *in vitro* maturation in *S. gairdnerii*, doses of up to 10 µg/ml LH or (FSH) and 40 I.U. HGG were ineffective. *In vitro* induction of maturation of *C. auratus* clearly demonstrated a specificity of action even among fish pituitary preparations and a clear insensitivity to mammalian preparations; LH or FSH at 100 µg/ml was totally ineffective and 200 IU HCG produced a very slight effect (Jalabert *et al.*, 1973). In *E. lucius* LH and FSH (1 mg/ml) and HCG (250 IU/ml) were totally without maturational effect *in vitro*. Piscine preparations however, induced *in vitro* maturation in *E. lucius* (Jalabert and Breton, 1974). Sundararaj and Anand (1972) showed that SG-G100 was 2.5 times as effective as LH in stimulating maturation of *H. fossilis* oocytes *in vitro*. Hirose (1971) showed that *in vitro* maturation of *O. latipes* oocytes resulted from treatment with low doses (25 IU, 0.4 µg/ml) of HCG or LH; much higher levels were needed using pregnant mare serum or FSH but some effect was seen with these. Further work (Hirose, 1972) showed SG-G100 to be ten times as effective as LH.

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se data suggest that the specificity and sensitivity of the response gonadotropic preparations varies between fish species. Insensitivity the gonad of *P. americanus* to mammalian preparations may account for inability of perfused gonadotropins (8.2 IU HCG, 3 µg LH/ml) to uce bocyte maturation.

Ovulation was induced in hypophysectomised *P. americanus* using r injections of PG3 equivalent to five pituitary glands/100 gm of fish reas perfusion was performed using an extract of two to four *P. ricanus* pituitaries extracted in 50 ml of medium. It seems likely, the light of the later experiments, that this amount of extract was ufficient to stimulate maturation, and that these results do not add evidence to the argument over the site of formation of maturation ducing steroids.

me Course of Oocyte Maturation

The time lag between stimulation and appearance of maturation is ot yet known. When 38 plasma samples were bioassayed *in vitro* only seven howed maturational activity. Of these seven, five samples had been ollected from fish whose gonads were partially mature and undergoing vulation whilst the other two showed no sign of maturation. One plasma s part of a series of blood samples taken from the same fish at daily ntervals; the sample which showed biological activity was collected 22 hr efore the onset of maturation was confirmed. No biological activity was een in any of three more daily samples even though ovulation was not omplete on the fourth day. These data suggest that the maturation timulus precedes morphological changes by at least 22 hr. Treatment with onadotropic preparations *in vivo* induced maturation five days after the

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rst injection and *in vitro* incubation in plasma demonstrated maturation
ter four days. This delay means that the pre-mature, maturing and
ture classification used when samples were collected in this work are
t accurate enough to predict the sampling necessary for analysis of
turation inducing substances. This could explain the high levels of
α OH progesterone and 17α 20β progesterone seen in a sample from a non-
ture fish in Assay 2. The lack of oocyte maturational activity in many
the plasmas which had been designated as maturing or mature, and the
t that in the serially sampled fish only the first sample had this
tivity, suggests that any hormonal maturation-inducing stimulus must be
pidly removed from the blood.

thods of Control of Oocyte Maturation

The finding of significant concentrations of 17α OH progesterone
d 17α 20β progesterone in plasmas having biological activity is not
nclusive proof that these steroids are involved in *P. americanus* oocyte
turation. The anomalous finding of these steroids in the plasma of one
ish a month before the spawning season is hard to explain.

The demonstration of low levels of these steroids in ovarian fluid
oes not contradict a hypothesis involving them in maturation since in the
ioassay these two samples developed only one or two percent maturation
fter ten days and the yolk mass remained heterogeneous even then. In
ontrast, three of the four active plasmas which induced fully transparent
ture oocytes, did so within four days.

It is possible that the maturational activity of these fluids
nstrated in the bioassay is due to the presence of gonadotropin. It
not possible to assess the amount of this hormone present, but the

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periments involving incubation in male plasma could be explained by hypothesis of joint action of gonadotropic hormones with steroids r maturation. When fresh plasma from ripe males was used, no turational activity was seen in control incubation and only a little th testosterone, but the other steroids induced up to 30% maturation. en frozen plasma from a spent fish was used for incubation, slight ocyte maturational activity was seen when it was used with a flounder ituitary homogenate, but no effects due to steroids could be shown. rr (1963) showed that *Pleuronectes platessa* L. pituitaries collected rom fish in the summer demonstrated less gonadotropic activity when jected into hypophysectomised fish than do glands from winter fish hich suggests that the plasma of a spent fish would contain little onadotropin. It would seem possible that this present work may have hown maturational activity due to steroids in the presence of factors, ossibly gonadotropins, which are not stimulatory by themselves. In emale plasma, which has an endogenous maturational activity, treatment f oocytes with steroids produces additional effects. Ovarian fluid, o thirds of which was collected from the same fish as the active plasmas ad maturational activity on incubated oocytes but did not demonstrate any dditional stimulation due to steroid treatment. It would seem unlikely. hat the extracellular fluids contained in the gonad would be rich in onadotropin since, as a target tissue, the gonad follicles should bind he hormone actively to cellular receptors.

In this work no maturational activity of steroids in the presence f *H. platessoides* glycoprotein was seen when oocytes were incubated using saline. Hirose (1972) failed to demonstrate any additional effect due

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the presence of HCG in incubation of *O. latipes* oocytes with cortisol r progesterone. The data of Jalabert *et al.* (1973) shows that for *C. ratus* only deoxycorticosterone induces maturation in the presence of etapirone and in *S. gairdnerii* 20β progesterone was the only steroid ested (17α 20β progesterone was not tested) which effectively induced aturation in oocytes from which the follicle cells had been digested. everal steroids have now been shown to have some maturational activity n *P. americanus*. Similar demonstrations have been given by Sundararaj .d Goswami (1971, 1974) for *H. fossilis*; and by Jalabert *et al.* (1973) or *S. gairdnerii* and *C. auratus*. However the work with Metapirone and follicular oocytes (Jalabert *et al.*, 1973) suggests that some of these ormones may not be directly active, but gain their activity after nzymatic transformation in the follicle cells to the actual maturation nducing substance.

If a gonadotropic factor is necessary, in addition to any of the ested steroids, for the induction of maturation in *P. americanus* oocytes, his could explain the lack of effect of steroid injections into hypophyectomised fish and the different results obtained by *in vitro* culture n different plasmas. However culture of oocytes with steroids and PG3 = 0.8 glands/incubate) in saline did not induce oocyte maturation.

A slight (2%) *in vitro* maturation-inducing effect was seen due to ncubation of oocytes with salmon gonadotropin in male plasma. A similar xperiment demonstrated slight effects due to treatment with a brei f 3.5 female *P. americanus* pituitary glands per ml of plasma. In *H. ossilis* (Sundararaj *et al.*, 1972) doses of up to 40 µg/ml of SG-G100 ere ineffective and *H. fossilis* pituitary homogenate (Goswami and

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Sundararaj, 1971) was ineffective at doses up to 800 µm/ml, a dose which probably represents 1 gland/ml.

This slight activity of *in vitro* gonadotropic preparations could indicate that *P. americanus* unlike *H. fossilis*, but similar to *A. stellatus*, *C. auratus*, *E. lucius*, *M. fossilis*, *O. latipes* and *S. gairdnerii* is able to synthesise maturation inducing substances within the gonad in response to gonadotropins. One limitation of this data on *P. americanus* oocytes must be that the composition of the incubation plasma is unknown and any of its constituents could have reacted with the test preparations to produce these results.

Attempts to identify the source of these proposed maturation inducing steroids by quantitative analyses of gonadal and peripheral plasmas have not demonstrated this source. Collections of the peripheral blood samples were always made after gonadal venous effluent had been collected. Since the duration of gonadal effluent collection took from one to six hours, the basis for comparison of these plasmas is limited because of the time difference of collection between the two samples.

SUMMARY

1. Steroids identified as major plasma constituents are cortisol, 17α OH progesterone and 17α 20β progesterone.

2. Steroids identified as minor plasma constituents are cortisone, deoxycorticosterone, 11-Ketotestosterone and progesterone.

3. Plasma samples, which demonstrated maturation-inducing activity in an *in vitro* bioassay, were found to contain more 17α OH progesterone and 17α 20β progesterone than samples which did not demonstrate maturational activity.

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4. Treatment of oocytes, incubated in some fish plasmas, with cortisone, deoxycorticosterone, 17α OH progesterone or 17α 20β progesterone resulted in some maturation; this could be due to joint action with a gonadotropic constituent of some plasmas.

5. A slight *in vitro* maturational effect of gonadotropic preparations suggests that the gonad of *P. americanus* may respond directly to pituitary stimulation.

6. Treatment of hypophysectomised fish with a salmon gonadotropin preparation or fish pituitary glycoprotein preparations induced maturation of oocytes and ovulation.

7. Injection of 17α 20β progesterone, 17α OH progesterone, testosterone, estradiol, deoxycorticosterone or cortisone into hypophysectomised fish did not induce oocyte maturation or ovulation.

8. No conclusive oocyte maturational activity was detected for any steroids tested in *in vitro* incubations using synthetic media.

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APPENDIX A

Double isotope derivative assay results

Steroid and Sample Description		Volume of Sample	D.P.M. Tracer	Paper eluate		Crystals			
				1	2	1	2	3	4
Assay 1									
Cortisol	^{14}C		60 000	0.39	0.46	0.67	0.78	0.69	
(Ripe)	A	2.76	9 580	2.19	1.85	1.40	1.15	1.37	
(Spent)	B	4.98	9 580	2.26	2.07	2.13	2.00	1.99	
(Resting)	C	5.34	9 580	1.65	1.53	1.53	1.46	1.46	
Cortisone	^{14}C		60 000	0.44	0.45	0.56	0.78	0.90	
	A	2.76	10 000	0.59	0.54	0.39	0.26	0.17	
	B	4.98	10 000		1.05	0.66	0.42	0.58	
	C	5.34	10 000		0.58	0.61	0.63	0.84	
Assay 2									
Progesterone	^{14}C		19 500		1.14				
(Non-mature-G)	1	3.30	3 860						
(Non-mature-P)	2	4.24			1				
(Maturing-G)	3	5.00			8				
(Maturing-P)	4	4.83			4	Recovery at this stage, 4%			
Control perfusion	5	10.00			2				
Pituitary "	6	10.00			1				
1+2+3+6					5.3	2.04	2.4	2.4	

APPENDIX A (CONTINUED)

Steroid and Sample Description		Volume of Sample	D.P.M. Tracer	Paper eluate		Crystals			
				1	2	1	2	3	4
17α OH progesterone	1	3.30	3 650		135				.1 %
	2	4.24	3 650		25				.3 %
	3	5.00	3 650		230				.1 %
	4	4.83	3 650		335	inadequate ^{14}C recovery to quantify			.01%
	5	10.00	3 650		72				.05%
	6	10.00	3 650		68				
Deoxycorticosterone ^{14}C			"27 000"		1.2	(Ratio 0.96 obtained Assay 3)			
	1	3.30	5 421	3.4	2.8	1.5	1.24	1.6	
	2	4.24	5 421		1.3				
	3	5.00	5 421		1.46				
	4	4.83	5 421		6.1		2	2.2	
	5	10.00	5 421		1.5				
	6	10.00	5 421		1.2				
11 deoxycortisol ^{14}C			"27 000"		1.2				
	1	3.30	"5 000"		1.118				
	2	4.24	"5 000"		1.3				
	3	5.00	"5 000"		0.8				
	4	4.83	"5 000"		0.97				
	5	10.00	"5 000"		1.6				
	6	10.00	"5 000"		0.74				

APPENDIX A (Continued)

Steroid and Sample Description	Volume of Sample	D.P.M. Tracer	Paper eluate		Crystals			
			1	2	1	2	3	4
11 ketotestosterone ^{14}C		"27 000"		4.04 (Ratio 0,8 calculated from suppliers data)				
1	3.30	5 444		3.8	2.05	2.7	1.95	
2	4.24	5 444		3.22	2.1	2.4		
3	5.00	5 444		5.3				
4	4.83	5 444		3.7	2.3	2.45	2.2	
5	10.00	5 444		1.3				
6	10.00	5 444		3.8				
Cortisol ^{14}C		"25 500"		0.9				
1	3.30	5 065		18	23.6	24.3	24.1	24.1
2	4.24	5 065		17.2	17.4	18.2	17.4	17.2
3	5.00	5 065		8.0	8.96	8.3	8.8	9.1
4	4.83	5 065		5.6	10.8	10.6	10.8	10.9
5	10.00	5 065		1.1	1.2	1.1	1.1	1.1
6	10.0	5 065		1.1	1.3	1.1	1.1	1.2
Cortisone ^{14}C				(Ratio 0.8 calculated from suppliers data)				
1	3.30	4 981		2.2	2.56	2.42	2.55	2.65
2	4.24	4 981		2.3	2.1	2.03	1.96	2.02
3	5.00	4 981		2.8		2.5	2.6	2.54
4	4.83	4 981		4.3	4.1	3.95	3.96	3.8
5	10.00	4 981		1.1	1.05	1.03	1.08	0.96
6	10.00	4 981		1.1	1.0	1.1	1.05	1.07

APPENDIX A (Continued)

Steroid and Sample Description	Volume of Sample	D.P.M. Tracer	Paper eluate		Crystals			
			1	2	1.	2	3	4
Assay 3								
Progesterone ^{14}C		11 440			0.91	0.85	0.90	
1	8.30	11.440	9.25	2.69	1.36	1.01	1.03	1.07
2	5.60	11 440	6.54	2.01	1.06	0.97	0.95	1.04
3	6.80	11 440	5.95	2.83	1.15	1.02	1.04	1.04
4	4.90	11 440	44	12.6	5.06	1.94	1.51	1.61
5	3.50	11 440	2.95	2.99	1.07	0.96	0.97	0.93
6	3.65	11 440	2.25	1.50	1.08	0.93	0.98	0.99
7	3.10	11 440	3.95	2.55	1.68	0.93	0.99	0.99
8	3.50	11 440	98.2	19.9	1.98	0.95	1.04	0.99
17α OH progesterone ^{14}C		12 009			0.82	0.83	0.86	
1	8.30	12 009	11.88	3.28	2.51	2.64	2.51	2.45
2	5.60	12 009	22.8	22.99	22.1	22.4	22.3	22.4
3	6.80	12 009	172.9	151.9	163.1	166.2	166.0	164.4
4	4.90	12 009				142		
5	3.50	12 009	13.1	2.29	1.45	1.47	1.52	1.64
6	3.65	12 009	4.46	1.15	0.97	0.93	0.91	0.90
7	3.10	12 009	7.58	2.67	2.48	2.38	2.30	2.49
8	3.50	12 009	12.76	13.04	12.09	12.16	11.82	12.04

APPENDIX A (Continued)

Steroid and Sample Description		Volume of Sample	D.P.M. Tracer	Paper eluate		Crystals			
				1	2	1	2	3	4
17α 20β progesterone ^{14}C			5,424			0.81	0.79	0.82	0.82
	1	8.30	5,424	8.44	8.73	7.91	7.84	7.98	8.01
	2	5.60	5,424	20.07	18.82	18.49	18.55	18.69	18.93
	3	6.80	5,424	18.19	16.23	16.84	16.12	16.56	16.89
	4	4.90	5,424	4.51	2:33	1.91	1.85	1.99	2.11
	5	3.50	5,424	6.47	5.10	4.72	4.66	4.66	4.85
	6	3.65	5,424	1.22	1.08	1.07	1.01	1.08	1.12
	7	3.10	5,424	2.55	2.34	2.36	2.16	2.19	2.21
	8	3.50	5,424	23.5	22.5	21.0	21.6	21.5	21.3
Assay 4									
Deoxycorticosterone ^{14}C			6,960			(0.53 calculated from assay, J. Walsh and B. Truscott)			
(Plasma +ve)	1	12.25	6,960	4.3	0.9	0.67	0.56	0.59	
(Plasma -ve)	2	14.15	6,960	5	1	0.56	0.8	0.76	
(Ov. fluid +ve)	3	4.67	6,960	8.4	1.4	0.81	0.53	0.5	
(Ov. fluid -ve)	4	8.45	6,960	8.2	1.4	0.59	0.91	1.6	0.57

APPENDIX A (Continued)

Steroid and Sample Description	Volume of Sample	D.P.M. Tracer	Paper eluate		Crystals			
			1	2	1	2	3	4
Cortisone ^{14}C		7,210			(0.39 calculated from assay, B. Truscott)			
1	12.25	7,210	6.5	3.1	2.36	2.46	2.34	
2	14.15	7,210	12.5	4.2	2.42	2.75	2.35	
3	4.67	7,210	3.5	1.6	0.54	0.55	0.86	
4	8.45	7,210	1.8	1.8	0.61	0.75	0.58	
17α 20β progesterone ^{14}C		6,170			(0.36 calculated from assay 3)			
1	12.25	6,170	10.0	4.5	*4.02	*2.13	**4.86	
2	14.15	6,170	17	3.4	*2.6	*1.73		
3	4.67	6,170	10	2.9	*3.7	*0.83		
4	8.45	6,170	3.8	1.7	*1.6	*0.93		
17α OH progesterone ^{14}C		6,946						
1	12.25	6,946		26	*23.3			
2	14.15	6,946	7.5	2.12	*2.6	*1.63		
3	4.67	6,946	9	2.18	*1.19	**0.74		
4	8.45	6,946	21	4.3	*7	*3.4		

* Paper.
** Sample recovered from Scintillator (Coghlan & Scoggins 1967) and rechromatographed (2 TLC).

APPENDIX B

Fish media composition (gm/l)

	Forster and Hong (1957)	Cobb (1973)	Flounder I	Flounder II	Jalabert *et al.* (1973)		Goswami and Sundararaj (1971)	Hirose (1971)	Iwamatsu (1973)	Kirshenblat (1959)	Skoblina (1974)	Goncharov (1973)
NaCl	7.9	8.22	12.0	12.0	7.3	8.6	3.7	8.0	6.5			
Buffer			TRIS 7.6 HEPES 16.8								pH 8.5	pH 7.0
KCl	0.19	0.39	0.75	0.75	0.18	0.23	0.32		0.4			
$CaCl_2$	0.17	0.54	0.17	0.17	0.26	0.38	0.16		0.2			
$MgCl_2 \cdot 6H_2O$	0.09		0.09	0.09	0.18	0.2						
$MgSO_4 \cdot 7H_2O$					0.07	0.07	0.16					
NaH_2PO_4	0.07	0.28	0.07	0.07	0.08	0.12	0.1					
$NaHCO_3$	0.84	0.2	0.84	0.84	0.57	0.98	1.76		to pH 7.3			
Glycerol				ca 20 ml								
Glucose		1.0	1.0	1.0	1.0	1.0	0.8					
Other		(*P. platessa*)	(*P. americanus*)	(*P. americanus*)	(*S. gairdneri*)	(*C. auratus*)		20% calf serum Medium "199" (*O. latipes*)	5.0 bovine serum albumen (*O. latipes*)	"Loach ringer" (*M. fossilis*)	"Loach ringer" 1.0 egg albumen (*M. fossilis*)	"Loach ringer" 1.0 egg albumen