THE CONTROL OF CYCLICAL CHANGES IN THE TESTICULAR ACTIVITY OF THE LAKE CHUB COUESIUS PLUMBEUS (AGASSIZ)

by

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in the Department of

Zoology

We accept this thesis as conforming to the required standard

The University of British Columbia
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ABSTRACT

The annual testicular cycle of a teleost the lake chub (Coulosia plumbeus) from a north temperate latitude (51°N) has been studied by histological and histochemical methods with a planimetric evaluation of the different spermatogenetic stages. The annual cycle is divided into five different stages and the cyclical testicular changes have been correlated with changing environmental conditions. Lobule boundary cells, considered to be homologue of the Leydig cells of higher vertebrates, have been identified, and the changes in their secretory activity have been reported.

Temperature is the major environmental factor controlling the testicular cycle. The pituitary gland, through its gonadotropin(s), mediates between environmental changes and developments in the testes. Higher temperatures (16°-22°C) promote spermiogenesis and spermiation, whereas low temperatures (5°-12°C) are more conducive to gonial proliferation and the initial phase of spermatogenesis.

Hypophysectomy affects the mitotic ability of the spermatogonia, completely blocks their transformation into spermatocytes and suppresses the secretory activity of the lobule boundary cells.

Replacement therapy with fish gonadotropin and mammalian LH restores spermatogenesis to a large extent and maximum response is elicited with whole fish pituitary extract. It is proposed that the fish pituitary gonadotropin is similar to mammalian LH and in this species mammalian FSH is
(iii)

physiologically inactive in the restoration of testicular activity.

Since whole fish pituitary produces a maximum response it is suggested that factors such as TSH and STH have a probable synergistic role in the testicular maturation in *Couesius plumbeus*. Evidence is presented that a weak endogenous rhythm of activity is partly responsible for the timing of various testicular changes.
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\[
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INTRODUCTION

Reproductive activities in most animals undergo cyclical changes which are characteristic of each species (Bullough 1961). These cyclical changes have been carefully studied, in both vertebrates and invertebrates, and are governed by a number of environmental and neuroendocrine factors.

In several fishes the gonadal controls have now been worked out in considerable detail. It is well established that the pituitary gland plays an essential part (Vivien, 1938, 1941, 1939; Matthews, 1939; Burger, 1941; Pickford, 1953; Tavolga, 1955; Barr, 1963; and reviews by Hoar, 1955, 1957, 1962; Ball, 1960; Marshall, 1960; and Dodd, 1960). Correlative cyclical changes in the histological appearances of the pituitary gland and gonads have frequently been demonstrated (Verhoeven and Van Oordt, 1955; Sokol, 1955). Among environmental factors light and temperature are particularly most significant in north temperate latitudes (Van de Eeckhoudt, 1947; Harrington, 1950, 1956, 1957, 1959; Baggerman, 1957; Hoar, 1961; Henderson, 1963). Harrington (1959b) and Atz (1957) have critically reviewed the literature.

Investigations of these controls in fishes have concentrated on the female chiefly because the influences of the controlling factors on the gonads is reported to be reflected with greater dependability and specificity in the ovaries than in the testes. Oogenetic stages in fishes are easy to identify, demarcate, and quantify (Harrington, 1959a). In the few studies
involving males, the changes noted were of a gross nature and usually did not include detailed histological examination (Medlen, 1951; Baggerman, 1957; Harrington, 1956; Henderson, 1963). Subtle cyclical changes in the testes paralleling those in the ovaries may occur but have escaped notice because of a lack of accurate methods of quantifying the level of maturation of the testes.

The endocrinological studies of the controlling mechanism also have been rather inconclusive because (a) fish have usually been treated with hormones without previous hypophysectomy to eliminate the effects of endogenous gonadotropins and (b) only mammalian gonadotropins or crude fish pituitary extracts have been used in attempts to demonstrate the pituitary control.

There are indications that in fishes the gonadotropin responsible for gonadal maturation is physiologically similar in action to the mammalian Luteinizing hormone (LH). There have also been reports of a factor in fishes similar to mammalian follicle-stimulating hormone (FSH) (Kirshenblat, 1949; Witschi, 1955; Otsuka, 1956). Witschi (1955) reported a preponderence of an LH-like factor over an FSH-like factor in fishes on the basis of standard assay methods. Ramaswami (1962) pointed to the presence of LH in the Indian catfish, *Heteropnuestes fossilis*, but made no specific tests for the presence of FSH. Ahsan and Hoar (1963) showed that mammalian FSH failed to produce any substantial stimulation in *Gasterosteus aculeatus*. The literature has been reviewed (Hoar, 1964b) and uncertainties still exist concerning the number of gonadotropins and their physiological
There are several unresolved problems associated with the reproductive physiology of the male teleost. The environmental regulation of gonadal activity varies in the different teleosts. It might be light controlled, temperature controlled, synergistic control by light and temperature or by an endogenous rhythm modified by these factors (Bullough, 1940). Differences have been found in fishes belonging to different families and even in closely related species living in widely different latitudes (Harrington, 1959a). Moreover, there are few long term studies of the pituitary control of the fish testes. Critical investigation of pituitary function requires hypophysectomised fish and replacement therapy with purified fish gonadatropin (preferably from the same or closely related fish).

With these points in mind, male Couesius plumbeus (Agassiz) (= Hybopsis plumbea of Bailey) were selected for a comprehensive analysis of control of the fish testicular cycle. This fish is particularly suitable because of its abundance, hardiness, size, and suitability for hypophysectomy. The investigations were conducted along four different but interrelated lines:

i) Study of the annual testicular cycle of C. plumbeus with reference to the environmental temperature and photoperiod.

ii) Study of the effect of hypophysectomy on the testes and the seasonal variations in the response to pituitary ablation.
iii) Study of the effect of replacement therapy on the testes of hypophysectomised fish using:
   a) Fractionated salmon pituitary gonadotropin.
   b) Crude salmon pituitary extract.
   c) Purified mammalian FSH.
   d) Purified mammalian LH.

iv) Experimental analysis of the effect of photoperiod and temperature on stocks of wild fish captured in different phases of their annual cycle.
MATERIALS AND METHODS

Samples of male Couesius plumbeus (Agassiz) (Family: Cyprinidae) were captured each month, usually in the first week, from "No fish" Lake and the adjacent 3-mile Lake near Clinton, British Columbia, at approximately 51°8'N and 121°40'W. They were held under standard aquarial conditions.

Collections were usually made during the first week of the month, beginning in June 1962 and ending in November 1962. Monthly collections were started again in late April 1963, the last collection being in the month of November. Surplus fish from 1962 collections were kept outdoors in troughs of running water under as near natural conditions as possible and sampled monthly from January to April in 1963.

Each set of experiments were identified by the month and the year in which the fish were hypophysectomised. Regular replacement therapy began with fish hypophysectomised in July 1962. Each experiment lasted about 50 - 60 days.

A batch of 20 - 30 fish was killed immediately after arrival at the laboratory for the study of the annual testicular cycle. The remaining fish were used for pituitary removal procedures and for photoperiod and temperature experiments. Hypophysectomy

Fish were acclimated to laboratory water and temperature conditions for at least 36 hours before hypophysectomy.

The pituitary gland of Couesius plumbeus is oval anteroposteriorily and rounded dorsventrally in cross section. It is situated in the cranial floor immediately above the parasphenoid. Seen from the roof of the buccal cavity after the
removal of the buccal epithelium it appears as a white oval body about 1 mm in its longest diameter, situated at the level of the first gill cleft. The general topography of the pituitary gland is shown in Figure 1.

The pituitary is connected to the hypothalamus by a narrow vertical stalk. Like all the fishes which possess a stalk, the hypophysial recess in *C. plumbeus* is almost obliterated (Kerr, 1942). The relationship of the pituitary to the brain is shown in Figure 3A. It is the presence of a narrow stalk, its vertical disposition and the generally anteriad position of the gland itself which make *C. plumbeus* an ideal fish for hypophysectomy.

**Procedure of hypophysectomy.** Fish ranging from about 57 mm to 70 mm forklength were hypophysectomized by a modification of the opercular approach described by Abramowitz (1937). They were anesthetised with 1:2000 MS 222 (Tricaine Methane Sulfonate - Sandoz) dissolved in aquarium water, by placing them in the anesthetic solution until swimming movements ceased and the opercular movements were appreciably slowed down.

The fish was then placed belly upwards in a groove made in a soft sponge fitted into a small rectangular plastic tray (18 x 12 x 3 cm), which constituted the 'operation table'. The tray had an opening at either end, the opening farther from the operator serving as an inlet for fish physiological saline (Young, 1933). This was led into the groove through a 2 mm diameter polyethylene tube projecting about 5 - 8 mm into the groove. The other opening served as an outlet for waste saline and blood.
Fig. 1. Camera lucida diagram of the topography of the Pituitary Gland (P) of Couesius plumbeus. Only the mucus membrane covering the roof of the buccal cavity is removed.

1. Internal carotid artery
2. Branch from the internal carotid artery
3. Circulus cephalicus
4. Maxillary nerve
5. Optic nerve
6. First gill cleft \( (X \text{ circa } 30) \)
7. Parasphenoid
The fish was held in position by passing over the body a pair of rubber bands held on either side of the groove by pins. A beam of light from a 100-watt lamp cooled through a round-bottomed litre flask of cold water was directed towards the inlet half of the groove. The fish was slightly tilted to its right on its back. The saline inlet tube was then passed into the buccal cavity of the anesthetised fish through the mouth.

The operculum was raised and the cleft between the branchiostegal membrane and the gular region of the lower jaw was extended by a forwardly directed incision with a fine pair of scissors. This provided a much larger opening of the gill cleft into the roof of the buccal cavity. The operculum was lifted and kept parted by a retractor fashioned from a bent pin. The mucus membrane covering the palate was cut by a slightly oblique transverse incision with an iridectomy scissor. The cut membrane was flushed with saline and when necessary excess fluid was removed with a fine tube attached to a suction tap. Usually there was little bleeding up to this point in the operation.

The parasphenoid was now bared, thus exposing the oval whitish pituitary (Fig. 1). It was cut obliquely immediately behind its widest part by an iridectomy scissor. A Morse Scaler was then inserted above the bone and the pituitary was loosened. The flow of saline which was kept at a minimum to this point and even stopped occasionally, was now increased. Blood was flushed and the pituitary was sucked out with the fine tube referred to above. The flow of saline was gradually decreased over a period of 2 minutes, and the fish was allowed to recover (on the operation table) for 5 - 10 minutes with constant saline flow.
Fig.2A. A general view of the set up for hypophysectomy

B. Some of the instruments used in the operation
over its gills before being transferred to a 0.2% bath of equal parts of sodium and calcium chloride prepared in aquarium water (Chavin, 1956). The fish were left in this postoperative bath for about 6 hours. They were then transferred to 5½ or 10 gallon aquaria in which they were kept until the autopsy. A head magnifier (about 6 times) was used during the operation. The general set-up used for hypophysectomy is shown in Figure 2.

The survival rate in the operative procedure was about 50%. This low survival was due to the care taken to see that the pituitary was completely removed. Causes of casualty were either excessive bleeding or damage to the brain.

The hypophysectomised fish were kept for 30 – 40 days before any replacement therapy was initiated. During this period they fed well and appeared to be healthy; feeding was not started until two days after hypophysectomy.

They were kept in lots of about 6 in aerated aquaria, fitted with "outside filters" (Fig. 4); water was usually changed once a week. The temperature ranged from 18°C to 21°C.

The fish were fed daily with frozen brine shrimp (Wardley's, Saskatchewan). Feeding was curtailed to alternate days in experimental fish during the subsequent fortnight of injections.

**Injections**

Both mammalian and fish pituitary hormones were used as follows:

1. Fractionated salmon pituitary gonadatropin.
2. Crude salmon pituitary extract.
3. Pure mammalian FSH (Armour Porcine Lot #216-175-6 and #216-177-6)
Fig. 3A. Sagittal section of head through the pituitary gland and adjacent brain tissue of *C. plumbeus* (X 750)

B. Sagittal section of the pituitary region of the brain after hypophysectomy (X 700)
4. Pure mammalian LH (Armour Ovine Lot #216-176-5 and #216-178-5)

In replacement therapy administered during May 1963 - October 1963, fish that were injected with fractionated fish gonadotropin(s) and mammalian LH were kept during the duration of the injection at two temperature conditions, viz: one batch at 9°C ± 1.5°C and the other at the usual 19.0 ± 1.5°C.

Each hypophysectomised fish to be treated with fractionated salmon gonadotropins received a total of 0.6 ml of the fractionated preparation. This total amount was given in 6 equal injections of 0.1 ml/fish administered on alternate days spread over two weeks. Fish treated with crude salmon pituitary extract received a total of 0.3 ml of the preparation also divided into 6 equal doses.

Fish receiving mammalian FSH or LH each received 0.3 mgm - 0.35 mgm of purified powder dissolved in fish saline. Each injection carried 0.06 mgm of the hormone in 0.05 ml of solution. Normal controls as well as hypophysectomised controls were injected with a total of 0.6 ml of saline per fish.

Fish were lightly anesthetised and injected intraperitoneally with a 0.25 ml syringe and a gauge #30 needle so that the needle pierced the belly ahead of the anal opening. Some time was allowed before the needle was withdrawn from the body. This precaution, coupled with light anesthesia, ensured that little or no hormone was lost from the fish after the injections. All the above hormonal treatments were started on the same date and fish were autopsied one or two days after the last injection.
Fig. 4. A general view of the aquaria used to keep experimental fish and controls
Preparation of Salmon Pituitary Extracts

The salmon extracts were prepared from Spring Salmon (Oncorhynchus tshawytscha) and Coho Salmon (Oncorhynchus kisutch) pituitaries by Miss Barbara Findley and Mr. Peter Schmidt at the Technological Research Laboratory of the Fisheries Research Board of Canada, Vancouver, B.C. Separation of the gonadotropin was by starch gel ion exchange electrophoresis. The following outline of the procedure of fractionation has been provided by Dr. M. Smith.

The crude pituitary extract was prepared by grinding 1 gram of frozen salmon glands, obtained from mature spawning fish of either sex in equal numbers, with 1.5 volumes of 1.25% NaCl solution, using a glass homogenizer. After centrifuging, the extract was dialysed in cold glycine buffer for 6 hours. Then, 0.2 ml of this extract was applied to each of the 4 slots of the starch gel. The starch solution consisted of 11% hydrolysed starch in 0.004 M glycine solution at pH 9.5. The electrophoresis was carried out for 16 hours at 300 volts using 0.3 M borate buffer in 10% NaCl solution. On completion of the electrophoresis the gels were cut on the positive side 1.2 cm and 5.0 cm from the origin. This strip 3.8 cm long contained the desired gonadotropic fraction as evidenced by spermatokinetic tests on frogs and earlier experiments on steelhead trout. It was cut into small pieces and put into a dialysing bag filled with glycine buffer and suspended in a beaker also containing glycine buffer.

Two strips of filter paper were run from the borate buffer and the NaCl solution of the electrophoresis apparatus
into the beaker. A three hundred volts DC was applied for 16 hours after which the glycine solution in the bag was removed, centrifuged and evaporated to approximately 0.25 ml by freeze drying. The residue was then made up to volume of 1 ml with saline solution.

The crude whole salmon pituitary extract was prepared by grinding 1 gm of frozen spring salmon gland with 2 volumes of 1.25% NaCl. All these hormone preparations were stored under refrigeration.

The total amount of salmon extracts injected into each fish amounted to an equivalent of 2.5 fresh fish glands weighing a total of 200 mgm.

Controls were of two kinds. Sham operated fish were kept in conditions identical to those of the operated ones and were called "sham operated controls". Some hypophysectomised fish were also kept under similar conditions. They constituted the "operated controls". Both these control groups were injected with equivalent amounts of fish saline and autopsied at the same time as the experimental fish. The batch of fish autopsied at the beginning of each experiment composed the normals or initial controls. They provide the picture of the annual testicular cycle and also give an idea of the state of the testis in the fish at the start of each experiment. Sham operation included extension of the opercular opening and the incision of the mucus epithelium of the buccal roof in a manner similar to that employed for complete pituitary ablation.

Photoperiod Experiment

In January 1963 fish from the outdoor tanks were put
under controlled photoperiods of 16 hours light alternating with 8 hours darkness and 8 hours light alternating with 16 hours of darkness. The tanks were provided with light-proof tops carrying a light source of 8 watts equivalent to 27 ft.c. Temperatures ranged from 5°C in January to 6.5°C in April. Fish were sampled after 45 and 75 days.

Later it was decided to subject fishes brought directly from the lake at various periods of their annual cycle to a combination of light and temperature control.

On April 30, 1963, fish constituting the prespawning stock were placed in equal numbers under each of the following regimes:

1. Long photoperiod (16 hr light) and low temperature (8°C ± 2°C).
2. Short photoperiod (8 hr light) and low temperature (8°C ± 2°C).
3. Long photoperiod (16 hr light) and high temperature (19°C ± 1.5°C).
4. Short photoperiod (8 hr light) and high temperature (19°C ± 1.5°C).

The fish in this temperature-photoperiod experiment were housed in four separate fibreglass tanks measuring 63 x 46 x 36 cm each of which was provided with a light-proof top containing lamps which gave 27 ft.c at water surface. The lower temperatures were those in the gently flowing tap water; higher temperatures were achieved by thermostatically controlled heaters.

Postspawning fish brought from the lake on August 9,
1963 were likewise put under the above photoperiod-temperature combination. The final stocking of fish under these conditions was made with the "Pre freeze up" batch collected on November 1, 1963. Care and feeding of these fish were similar to those used for replacement therapy study. These three stocks of fish were marked distinctively and sampled at regular intervals.

**Autopsy**

Fish were killed by overanesthetising. The body weight and the fork length of each fish were recorded. The abdomen was opened and both testes were dissected out, freed from adhering adipose tissue and weighed to the nearest of 2.5 milligrams (on a Mettler Type H3 Balance). Usually the right testis was fixed in Bouin's fluid for routine histology and the left fixed in Baker's Formol-Calcium solution for the cholesterol test and lipid staining. Cholesterol and lipid staining was restricted to the testes belonging to the operated fish.

The Bouin fixed testes were processed in the usual way, embedded in paraffin wax (M.P. 56-58) and representative serial longitudinal sections cut at 6/μ and usually stained with Ehrlich's Hematoxylin and Eosin.

The formal-calcium fixed testis was cut into two pieces. One half was frozen sectioned with an International Harris Cryostat. Sections were made at 25 - 30/μ and tested by Schultz's process for cholesterol and its esters. Adam's (1961) method was tried but was discarded in favour of Schultz's.

The other half of the testis was embedded in gelatin (Gurr, 1953), sectioned at 8/μ and stained with Sudan Black B following the procedure given by Humason (1962). The sections
were sometimes counterstained with Carmalum. In some instances thick (25 μ) gelatine-embedded testis sections also were subjected to Schultz test for cholesterol. Testes of the photoperiod experiment were subjected to usual histology only.

**Evaluation of Testicular Maturation**

Each testis was carefully examined under the microscope and gross observations were noted using the high power and oil immersion lenses. Ten lobules from each testis were selected at random, and with a Spencer Camera lucida attached to a Leitz-Wetzlar microscope with monocular attachment, the outlines of the selected lobules were charted on a white sheet of paper. The spermatogenetic nests inside each cyst were identified and their outlines made within the lobule walls (Magnification C.X. 270). The names of the stages in a cyst and the state of activity, viz., resting or dividing were all noted on the projection. The areas of the various cell nests were then measured with an Aristo Planimeter (Dennert & Pape, Hamburg) in planimetric units and tabulated. The same planimetric settings were used for all measurements.

The percentage composition in terms of the relative abundance of the various types of spermatogenetic stages was thus obtained. This process also gave an indication of the relative activity of a fish testis by giving the ratio of the dividing and resting stages in a cyst. The condition of the spermatic ducts, its contents (if any) and its cellular lining were studied and records maintained.

**Meteorological data**

Temperatures of the lake from which most of the fish
were obtained were regularly recorded by Mr. J.A. McCabe and are reliable within the limits of field data. The daylight period at 51°N latitude was calculated from the tables of Sunrise and Sunset times in 'The Observer's Handbook' published by the Royal Astronomical Society of Canada, Ottawa.

Gonosomatic Index

The gonosomatic index for each fish was calculated in the usual manner:

\[
\text{Gonosomatic index (GSI)} = \frac{\text{weight of gonads}}{\text{weight of body}} \times 100
\]

To find whether the pituitary had been successfully removed, the head was dissected by cutting the healed incision in the mucus membrane and removing the parasphenoid bone. The pituitary area was checked for the gland. In cases of doubt the hypothalamic area was checked histologically.
RESULTS

ANNUAL TESTICULAR CYCLE

General Structure of the Testis

The testes in *Couesius plumbeus* Agassiz are a pair of elongated structures lying above the intestine laterodorsally on either side of the swimbladder. They extend from the genital opening to almost the anterior end of the abdominal cavity. At the posterior end of each testis, a vas deferens arises and opens outside by a single genital opening after meeting with its opposite number a very short distance from the genital pore. The testis is slightly triangular in cross section with the genital artery and vein lying on its ventral face. Each testis is enveloped in a very thin coelomic epithelium overlying the delicate tunica albuginea. The tunica albuginea remains uniform in its thickness during the entire seasonal cycle.

The testis is composed of distinct seminiferous lobules of varying shapes and sizes with definite but extremely thin fibrous lobular boundaries (Fig. 17). These lobules are distinguishable with some difficulty in very immature fish. In sexually active animals, the lobules enlarge and branch extensively, giving rise to a testicular structure composed of lobules in an amazing variety of shapes and sizes. Except for the lobules close to the posterior end of the testis, the identity of the lobules is evident even in highly active fish. Blood capillaries and elongate fibroblasts are found between adjacent and therefore adhering lobular walls (Fig. 16).

Besides these fibroblasts, elliptical lobule boundary
cells also occur close to the lobule wall. Some of these lobule boundary cells (considered homologues of the Leydig cells of higher vertebrates) lie decidedly within the lobule (Fig. 26A). They vary in shape from elliptical or oval to almost rectangular or even cuboidal and can be distinguished from the resting germ cells by the absence of any prominent nucleolus and by their less chromatic nature, their shape and disposition. The lobule boundary cells exhibit seasonal changes in abundance and histochemical reactions. Since only these testicular cells besides the blood capillaries give a positive test for cholesterol and are fairly densely lipoidal, they appear to be similar to the lobule boundary cells of *Esox lucius* (Marshall and Lofts, 1956; and Lofts and Marshall, 1957) and probably constitute the endocrine component of the testis.

The spermatogenetic components of the testis lie inside the lobule in more or less discrete cellular nests. Usually cells in one nest belong to one spermatogenetic stage, and, except for the early divisions of the primary spermatogonia, the elements in a nest undergo cell division in all-or-none fashion. Spermatogenesis *per se* has not been the main purpose of the present study. However, the various stages have been identified and delimited as far as practicable. Nuclear diameter and chromatic nature of the nucleus has been used to identify the spermatogenetic stages. Bullough (1939) has successfully distinguished the spermatogenetic stages in the cyprinid *Phoxinus laevis* by using the nuclear diameter as the sole criterion. In this study nuclear diameter as well as the affinity of the nucleus for basic dyes such as Haematoxylin has
Fig. 5. Seasonal variation in the GSI of *Couesius plumbeus* as related to changes in water temperature and daylength

- **●** GSI of the 1962-63 cycle; **0--0** GSI of the 1963 cycle (May to November)

▲——▲ Water temperature

The curved line represents the seasonal changes in daylength at 51°N
been used to distinguish the stages. The easily identifiable stages are:

I. **Primary Germ Cell**

These are found throughout the year but are comparatively rare in mature testes. A clear mass of cytoplasm can be distinguished surrounding the lightly staining nucleus. The nucleus averages 7.2 μ in diameter, and contains a single large excentric nucleolus. The nucleoplasm is otherwise clear and contains little chromatinic material.

II. **Primary Spermatogonia**

These cells have relatively less cytoplasm than the primary germ cells. The nucleus averages 5.2 μ in diameter. The nucleolus is small, central and basophilic; the nucleoplasm is more chromatic. The primary spermatogonia undergo a number of divisions to produce the secondary spermatogonia.

III. **Secondary Spermatogonia**

This stage can be distinguished from the previous by the slight decrease in nuclear diameter (average 4.5 μ) and by a marked increase in basophilia of the nucleus. It is not easy to distinguish between later generations of primary spermatogonia and the new secondary ones.

IV. **Primary Spermatocyte**

In this stage the nucleus has undergone further reduction in size and averages 3.57 μ in diameter. There is no distinct nucleolus and chromatic material is abundant.

V. **Secondary Spermatocytes**

This is a transient stage in the spermatogenesis of fishes and is rarely seen in resting phase. The average
diameter of these nuclei is 2.67\(\mu\). It is difficult to distinguish the cytoplasm in spermatogenetic stages following the primary spermatocyte.

VI. **Spermatids**

These are spherical masses consisting almost entirely of basophilic material caused by a striking condensation of nuclear chromatin. They measure 1.8\(\mu\) in average diameter.

Spermatids are the latest stage of spermatogenesis contained in nests. After spermatids are formed the nest membrane ruptures liberating the spermatids into the lumen of the lobules where they transform into sperms.

VII. **Spermatozoa**

These are the final product of spermatogenetic processes. In *C. plumbeus* transformation of spermatids into sperms takes place in the lobules so that the lobule contains mature sperms.

Primary germ cell divides to form primary spermatogonia. The stock of primary spermatogonia is derived also from certain migrating cells. These migrating cells are abundant during September and October (Stage I of the annual cycle). They arise from mitotic division of certain cell groups situated in two or three loci of the interlobular tissue. These basophilic cells appear to migrate along the interlobular path and enter the lobules to become transformed into primary spermatogonia (Fig.24A).

Both the primary and the secondary spermatogonia undergo a number of generations of mitotic division before transforming respectively into secondary spermatogonia or primary spermatocytes. Consequently nests of these three stages grow to
fairly large sizes. The division in the primary spermatogonial stage is usually slow. In nests of early generations of primary spermatogonia some dividing nuclei are frequently seen amongst non-dividing ones.

Branching efferent ducts are situated on the inner margin of each testis and the apices of mature lobules open into them. These ducts are more prominent in the middle and posterior third of the testis. In mature testes they are distended with sperms and the adjacent lobule boundary seems to break up and their contents become flush with those already in the duct. In post spawned testes these ducts collapse and their collapsed walls give the appearance of parallel cords of cells lying on the inner surface of each testis.

The cells lining these ducts and the vas deferens seem to be secretory in nature and during the spawning season show: (i) an increase in size, (ii) a displacement of the nucleus toward the basal half of the cell, and (iii) accumulation of granular substances. In spawned fish they are reduced in size and appear vacuolated (Fig. 32). Hendersen (1962) found that these cells in *Salvelinus fontinalis* possess long protoplasmic processes on their free surfaces. In *C. plumbeus* their free margins show an irregular outline reminiscent of mucus secreting cells which have discharged their mucoidal content. In post spawned testis they become flat. It is possible that the processes found in the brook trout are changes brought about in the cell after the end of its secretory activity.

In *C. plumbeus* a cortical and medullary zone cannot be distinguished. Lobules adjacent to the ducts near the
posterior end of the testis in maturing fish seem to be spermatogenetically slightly more advanced than the rest but there is no other indication of any anteroposterior seriation.

**Seasonal Changes in the Testis**

It is essential to know the normal annual cycle of the testicular changes before planning any long term investigation of the controlling mechanism. The male gonad of *Couesius plumbeus* undergoes seasonally a regular cyclical change in gross appearance, weight, and spermatogenetic activity (Fig. 7). There is little change in the lipid content of the testis; this is never present in quantities sufficient to be useful as a discriminatory histochemical test. A slight seasonal variation was observed in the cholesterol content of the lobule boundary cells (Table I).

**Spermatogenetic cycle.** Although cyclical changes in fish testes have often been studied (Turner, 1919; Craig-Bennett, 1931; Matthews, 1938; Burger, 1939; Bullough, 1939; Jones, 1940; Frederick, 1941; Weisel, 1943; James, 1946; Hendersen, 1962; Barr, 1963; Hoffman, 1963), the methods have rarely been quantitative.

The testis of *Couesius plumbeus* lends itself admirably to a discriminatory quantitative study due to the complete lack of zonation and of anteroposterior seriation, and also the fact that the organ is composed of rather discrete lobules in which various spermatogenetic nests and their state of activity can be easily charted for the estimation of relative abundance.

The annual cycle can be conveniently divided into five clearly distinguishable stages as follows:
Stage I. - Early immature testis - consisting largely of resting primary spermatogonia and primary germ cells. The beginning of spermatogenesis is evidenced by a few dividing primary spermatogonia and presence of some secondary spermatogonia but spermatocytes if present are not dividing. Resting stages constitute 69-77% of the mass of the testes. Migratory cells which appear to contribute to the new stock of spermatogonia are obvious in the earlier phase of this stage. Condition of testis in fish under natural condition during September and October conforms to this stage. The gonosomatic index (GSI) during this stage ranges from 0.712 - 0.929 on the average. Cholesterol tests are faintly positive to positive. Widespread pycnotic degeneration of later generations of secondary spermatogonia and primary spermatocyte is common. In pycnotic nests varying degrees of cellular degeneration is seen. Some cells are almost normal so that the stage of maturation can be identified; others show increased nuclear basophilia, and finally the pycnosis of the entire cell (Fig.33B).

Stage II. - Late immature testis - consists of a phase of slow sustained mitotic activity as well as a beginning of meiotic division. Spermatogonia continue to divide building up a stock of cells for transformation into spermatocytal stages. Primary spermatocytal nests increase in extent. Pycnotic degeneration slows down and finally almost ceases. Primary spermatocytes may
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divide to produce a few secondary spermatocytal nests; the cholesterol test is positive. The condition of testis in the fish in nature during November to March conform to this phase of annual cycle. The GSI averages 1.355 in November to 1.384 in March. Resting stages constitute about 75% in November to 41% in March.

Stage III. - Maturing testis - shows brisk spermatogenerative activity and the beginning of spermiogenesis. Secondary spermatogonia and spermatocytes continue to divide followed by transformation of secondary spermatocytes into spermatids and sperms. Cholesterol tests are negative. The GSI ranges from 1.505 in April to 2.069 in May. This situation is exhibited in nature by fish during April and May.

Stage IV. - Mature testis - this stage exhibits continued spermiogenetic activity while the spermatogenerative activity also continues. Resting stages now constitute only 3-4% of the total mass of the testis. Sperms fill the distended efferent ducts. Cholesterol tests are negative. The spawning testis is found in nature during June and July.

Unlike most other species (non-cyprinid) of fish, the spawning testis in Couesius plumbeus does not show any spermatogenerative arrest and slow spermatogenerative activity seems to continue. The GSI averages 2.137 in June to 1.806 in July.
Stage V. - The spent testis shows completion of spermiation. Mitotic activity is rare and resting stages constitute 94% of the total mass of the gonad. This is the only quiescent phase in the annual cycle of Couesius testes and can be called a phase of spermatogenetic recess. Primary germ-cells and spermatogonia predominate. Some residual sperms may be present.

This situation in nature occurs in fish during August. Cholesterol tests are negative. The GSI averages 0.53.

These five stages in the annual cycle are intimately related to the changes in the environment. The testicular cycle in Couesius plumbeus is continuous except for a short quiescent period in late July and August. From September up to March there is a gradual build-up of spermatogonial stages which start producing spermatocytial stages in early November (Fig. 6). The nuclear division is mitotic till the formation of primary spermatocytes up to March. The first maturation division occurs at the primary spermatocyte stage and this is held over until the beginning of spring. In late spring there is a burst of meiotic activity and the final stage in the maturation of the testis is attained. In summary the cycle is as follows: mitotic multiplication of spermatogonia → build-up of primary spermatocyte → meiotic division producing secondary spermatocytes and spermatids → completion of spermiogenesis followed by spermiation.

As evidenced from the histology of the testes and the
Fig. 6. Seasonal changes in the spermatogenetic composition of normal Couesius

- Spermatogonia, 0-0 Spermatocytes
- Sperms, X---X Pycnotic nests
Fig. 7. Cyclical changes in the activity of the testes in normal fish.

0—0 Dividing stages •—• Resting stages (sperms excluded)
appearance of early fry in the lake (Geen, 1955) spawning occurs from mid June to mid July with a possible week's extension at either end. Fish start spawning before the lobules are completely engorged with sperm, and some remnant sperms may be found in a few fish as long as one or two months after spawning. The range in gonosomatic indices in \textit{C. plumbeus} (0.5309 to 2.1028) is much less than found for most other teleost. In \textit{Fundulus heteroclitus} Pickford (1953) found a regressed fish with GSI average 0.72 which rose to 5.84 during the peak of spermatogenetic activity. Similar extremes of ranges of testicular size and weight have been reported by Harrington (1959) in \textit{Enneacanthus obesus}, Weisel (1943) in \textit{Oncorhynchus nerka}, and by Barr (1963c) in \textit{Pleuronectes platessa}. In \textit{Mylocheilus caurinum}, Scott (1952) found a five-fold increase in testes weight during the active phase. Seasonal variation in the gonosomatic indices of the male Cyprinids appear to be of a lesser order than that of other teleosts.

\textbf{Histological Features of Post-spawned Testis}

The spawned testes of \textit{C. plumbeus} have several distinctive features which will be referred to in later experimental work. Consequently some mention is made at this stage. The first distinctive characteristic of such testes is the presence of increasing numbers of migrating cells which are structurally and tinctorially distinct from all other cell types. The other feature is the beginning of pycnotic degeneration of remnant primary spermatocytes and later generation of secondary spermatogonia. In \textit{C. plumbeus} pycnotic degeneration of late secondary spermatogonial nests is a regular feature of the annual
cycle. It appears in small measure in some August fish and gradually increases to a maximum of an average of 16% of the testis mass in October. After this peak, pycnotic degeneration sharply drops to 8% in October and gradually ceases by March. Pycnotic degeneration never occurs in fish which have not yet completed their cycle. Fish which are about 57 mm fork length or less do not show pycnotic stages and consequently this is a good indication of whether the fish has reached maturity or not. Remnant sperms, collapsed seminiferous lobules and spermatid ducts are other features common to spawned fish. In C. plumbeus unspawned sperms are cast out fairly soon after spawning. A regular pycnotic degeneration of spermatogenetic nests of this magnitude is evidently of a rare occurrence in other species of fish, and has not been hitherto described. Such degeneration has been noted in frogs by Basu (personal communication), and by Robertson & Wexler (1960) in Pacific salmon.

Relation of the Annual Cycle to Environmental Changes

Temperature of the water of the lake and the amount of daylight available at the latitude of the lake were the two environmental factors investigated.

Figure 5 shows the gonosomatic indices of fish obtained from their natural environment at different parts of their yearly cycle. The lake is covered with ice from late November to early April. From late April, the temperature of the water rises rapidly and the final stages of the maturation occur during this increasing temperature period. The peak in water temperature is reached in August. This is the post-spawning period when the gonads are least active.

The final phase of maturation is also coincident with a marked increase in amount of daylength. The peak gonad weight is obtained in June when the daylength is maximum. From the present histological studies as well as the earlier field studies (Geen, 1955) it is evident that spawning commences about this time and spermiation is completed in July.

The temperature of the water declines rapidly after August and there is a simultaneous rapid decrease in daylength. The reconstitution of the testis, which is a slow process, occurs during this period of falling temperatures and decreasing daylength. The speed of reconstitution is slow at first but more rapid in October. From November to March an almost steady state is maintained as is evident in the slow build-up of both the later spermatogenetic stages and the weight of the gonad. It is obvious that water temperature remains almost constant from late November to early April (Geen, 1955) although daylength increases steadily from late December onwards.

From beginning September through March, a large store of secondary spermatogonia (SPG") and primary spermatocytes (SPC') is built up but spermatogenesis seems to stop at the primary spermatocyte level and the fish pass the entire winter in this condition. Scott (1952) thought that in a local cyprinid Mylocheilus caurinus the quiescent winter condition is passed in the secondary spermatocyte stage, i.e. in the second maturation division. It is universally agreed that the secondary spermatocyte is a transient phase in spermatogenetic cycle of fishes (Foley, 1926; Vaupel, 1929; Bennington, 1936; Weisel, 1943; Bullough, 1939) and consequently Scott's secondary
spermatocyte may have actually been the primary spermatocyte stage. Post spermatocytal development in \textit{C. plumbeus} occurs in April and May when the temperature of the water starts increasing at a rapid rate.

\textbf{Lobule Boundary Cells}

These cells also appear to exhibit a cyclical change comparable to the spermatogenetic stages with an increase in number during Stages I and II and an intensification in the cholesterol test, which later declines.

\textbf{Intralobular Lipids}

Testes from fish obtained through the various phases of the annual cycle were stained with Sudan Black B for the demonstration lipids.

In general the intralobular lipid is very low. Primary germ cells and the lobule boundary are the only cellular elements which show lipoidal nature. Unlike birds, some amphibians (Lofts, 1961) and some fish, e.g. \textit{Esox lucius} (Lofts and Marshall, 1957) \textit{Couesius} testes do not undergo any post spawning steatogenesis.

\textbf{Cholesterol Test}

The results of lipid and cholesterol staining for normal testes are given in Table I.

\textbf{EFFECT OF HYPOPHYSECTOMY}

In the present study hypophysectomised fish were maintained for periods varying from 46 to 63 days. In 14 experiments conducted from July 1962 to March 1963 and from May to October, 1963, a total of 374 hypophysectomised fish were kept successfully until autopsy. Of these 306 fish received
Fig. 8. Comparison of the GSI of hypophysectomised fish with the sham operated controls, and normals (at the time of the completion of each experiment)
replacement therapy and the rest were controls.

There is a marked decrease in the gonosomatic index (GSI) of the hypophysectomised fish in all experiments. This decrease in testicular weight is most marked in experimental groups for February 1962, March 1963, and May 1963 (Fig. 8). Differences between the GSI of hypophysectomised and sham operated controls are least in July 1962, August 1962, and July 1963 experiments, as compared to other groups.

Fish belonging to the February 1963, March 1963 and May 1963 experiments were killed between late March and end of June. This is the period of maximum testicular activity in nature and the removal of the pituitary gland at this phase has a more deleterious effect on the testes than during the relatively inactive period. Vivien (1941) also noted most acute regression of the gonads in fish operated at times of their greatest reproductive activity.

Fish in the latter set of experiments quoted above were sacrificed in late August and October. During these months the controls are most regressed as is true also under natural conditions and for this reason the GSI of the hypophysectomised and sham operated control in July and August experiments are not widely apart.

Histological Changes

At all times of the year the untreated hypophysectomised fish present a rather uniform picture of spermatogenetic arrest with testicular regression affecting both the relative gonad weight and the histological appearance.

The seminiferous lobules shrank to a rather uniform size and shape comparable to stage V or I of the annual cycle
as described on page 27. The majority of spermatogenetic stages were composed of primary germ cells and resting primary spermatogonia (Fig. 10). These nests actually consist of a greater proportion of resting nuclei with only a few showing some mitotic activity. The resting cells constitute 42% of the total mass of the testis, secondary spermatogonia are scarce constituting an average of 5.9% of the spermatogenetic stages (Table II). Mitotic figures are present but rare. The histology of the testis from an untreated hypophysectomised fish is shown in Figure 16A.

It appears that the primary spermatogonial division is suppressed although not totally stopped. Division in the secondary spermatogonial stages is rarely seen. If later stages were present at the beginning of the experiment, they are either expelled or degenerate. Some phagocytic degeneration and clumping of cells occurs during fall and winter; the stages phagocytised are later spermatogonia and spermatocytes. However pycnosis in hypophysectomised fish is rare. Remnant sperms are never found. Since clumped degenerating cells are seen in the terminal parts of the ducts in some experiments it appears that later stages are mostly expelled through the ducts.

Spermiation is not impeded by the removal of the pituitary gland. Fish operated during the prespawning and spawning phase of their annual cycle appear to spermiate normally as remnants sperms are rarely seen. Vivien (1941) found that an appreciable number of hypophysectomised Gobius paganellus are able to emit milt. Barr (1963c) has also observed that spermiation occurs normally in the hypophysectomised fish.
However, he noted the retention of remnant sperms for a considerable time.

There is an apparent increase in the number of fibroblasts in operated fish testes. An increase in the numbers of these cells sometimes gives the appearance of fibrosis in fish kept for longer periods (Fig. 16A). Such fibrotic development has also been noted by Barr (1963) in fish kept for about a year. In his fish fibrosis seems to have progressed farther, apparently due to the length of the time fish were observed. No obvious effects of hypophysectomy were seen in the lobule boundary cells histologically, but their secretory activity was completely suppressed as evidenced by negative cholesterol tests (Table III).

In the present study marked regression was evidenced within 46 days after the hypophysectomy. Both Vivien (1941) and Barr (1963c) seem to have found a much slower change. Vivien (1941) did not notice any evidence of involution in Gobius paganellus for almost three months after the operation. Tavolga (1955) however on the same fish found unmistakable signs of regression 30 days after hypophysectomy. Since Tavolga's operated fish were maintained at temperatures higher than those of Vivien's, it is possible that an acceleration in regression following pituitary removal is caused by higher water temperature. Evidence will be presented later which show that regression is speeded at higher temperatures in Couesius plumbeus.

**Cholesterol and Lipid**

There is little change in the lipid content of the testes due to hypophysectomy. The lobule boundary cells retain
their lipoidal nature although the staining seems to be less intense than in the sham operated controls. Cholesterol tests are always negative (Table III). In the normal fish, a positive cholesterol test is obtained only during the October to March part of the annual cycle. A negative test in the hypophysectomised fish during this period indicates a reduction in the activity of the lobule boundary cells due to pituitary removal. Since cholesterol tests in normal fish during the rest of their annual cycle is negative it is not possible to show any seasonal variation in the response of these cells to hypophysectomy. The remainder of the testis shows neither a marked lipoidal content nor gives any indication of the presence of cholesterol. It would appear therefore that steatogenesis or accumulation of lipid in the testes in C. plumbeus does not occur due to hypophysectomy as is the case in frogs (Lofts, 1961).

Although the hypophysectomised fish fed well during the experiment they all showed a slight decrease in body weight when compared with the sham operated controls of similar fork lengths.

REPLACEMENT THERAPY

Effect of Mammalian Follicle Stimulating Hormone (FSH)

The GSI of FSH treated fish are slightly but not statistically different from those of the hypophysectomised fish. There are slight variations in the GSI of the various experiments (Fig. 9), but these are probably due to contaminant LH. The spermatogenetic picture is more uniform than the GSI (Fig. 11).

The testicular histology of the FSH treated fish is
Fig. 9. Comparison of the GSI of FSH- and LH- treated fish with untreated hypophysectomised and sham operated controls

- untreated hypophysectomised controls
- O-----O FSH- treated fish
- o------o LH- treated fish
- O-----O sham operated controls
similar to that of the untreated controls. Primary spermatogonia are most predominant, constituting 39.9% of the spermatogenetic stage. Resting germ cells constitute 14% - 51% but are less numerous than in the untreated controls. Dividing primary spermatogonia are common. Secondary spermatogonia are present but constitute only a very small proportion (3.5% - 17.7%) of the spermatogenetic stages (Fig. 11). Division in the secondary spermatogonia are rare. FSH treated fish show testes comparable to those at Stage I of the annual cycle. There is thus an increase in the secondary spermatogonia in the FSH treated fish. This increase is definite only in experiments commenced during the months of February, March and May. The maximum effect of FSH is slight when compared with the level obtained from replacement therapy with other hormones (Fig. 19). This may be caused by a small amount of Luteinizing hormone (LH) present as an impurity in the FSH. Since the period from March to June is the most receptive phase in the reproductive cycle of this fish, the effect of this impurity would be expected to be maximum during this period.

Experiments during 1963 show a more consistent picture with the GSI of the treated fish being close to those of the untreated hypophysectomised controls (Fig. 9), in some experiments they are lower than in these controls. None of these differences are large. Of all the fish receiving replacement therapy, gonad weight was lowest among the FSH treated ones. In general, there is no clear evidence that FSH was having any effect on either the GSI or the spermatogenesis in this fish.
Cholesterol and Lipid

Cholesterol tests were always negative (Table IV). There was very little lipid material in the testes and overall picture of Sudan stained testes of FSH treated fish was similar to the untreated controls. FSH thus clearly has no stimulating action on the lobule boundary cells.

Effect of Mammalian Luteinizing Hormone (LH).

The gonosomatic indices of LH treated fish are markedly higher than those of the untreated controls (Fig. 9). In the earlier two experiments of 1962, fewer fish (three in July and two in August experiments) were injected with LH due to casualties in the operated fish caused by fungal and 'Ich' infections. The GSI of LH treated fish in these two experiments are somewhat higher than those of the following months but the differences may be due to smaller sample size. A trend towards increase in GSI of LH treated fish is apparent from January to March followed by a marked reduction in May. This trend has already been pointed out in the case of FSH treated fish. The period from February to April thus appears to be the most responsive period in the annual testicular cycle of this fish in the matter of its susceptibility to LH treatment.

In 1963 experiments the fish were similarly stimulated in each experiment except for the July experiments where the effect was less marked. The GSI of LH treated fish is always higher than those of the FSH treated or of the untreated operated controls but, except for the July and August experiments in 1962, falls somewhat short of the level of the sham operated controls (Fig. 9). The GSI of LH treated fish kept at low
Fig. 10. Cyclical changes in the spermatogenetic composition of untreated hypophysectomised fish

- Primary germ cells, □ - Primary spermatogonia
- Resting Secondary spermatogonia
- Dividing secondary spermatogonia (rare)
SPERMATOGENETIC COMPOSITION OF UNTREATED HYPOPHYSECTOMIZED FISH

EXPERIMENTS
Fig. 11. Seasonal changes in the spermatogenetic composition of FSH treated fish. (See Fig. 10 for explanations)
SPERMATOGENETIC COMPOSITION OF FSH TREATED FISH
temperature was markedly higher in June, July and August, 1963 experiments. However, as pointed out later the spermatogenetic condition of July and August 1963 experiments is not much different, whereas differences are seen in September 1963 experiment which become marked in October 1963 group.

**Histological changes.** A graphical representation of the spermatogenetic composition of LH treated testes is given in Figure 12. The proportion of resting germ cells in these fish is low (ranging from 0.6% to 14.0%) indicating a conversion of these into spermatogonial forms. Primary spermatogonia are still the most predominant form accounting for 22.8% to 63% of the various stages (Table V).

Primary spermatogonial nests in LH treated fish differ from fish belonging to FSH and hypophysectomised groups in their greater uniformity. The majority of nuclei belonging to this stage were undergoing division, that is the dividing primary spermatogonia were in active mitotic phase.

LH treated fish also show a marked difference from the FSH treated ones in an increase in the secondary spermatogonia both resting and dividing. The resting secondary spermatogonia range in numbers from a low of 17.7% to a maximum of 46.0%. The dividing forms range from 1.9% to 32.5%. Primary spermatocytes are present in all but two experiments reaching a maximum of 5.7% in January experiment. Dividing spermatocytes are rare and seen only in four experiments; occasional dividing primary spermatocytes may be present. Testicular stimulation of *Couesius* testis with mammalian LH appears to reach a climax with the development of primary spermatocytes and is comparable to
Fig. 12. Seasonal changes in the spermatogenetic composition of mammalian LH-treated fish. The right bar in June 1963 and subsequent experiments indicate condition at high temperature; the left that found at low temperature.

- - Secondary spermatogonia (dividing)
■■ - Primary spermatocytes (resting and dividing)

Other explanations as in Fig. 10.
SPERMATOGENETIC COMPOSITION OF FISH TREATED WITH MAMMALIAN LH

EXPTS
Stage III of the normal cycle.

It is obvious that LH treatment increases mitotic activity in the primary spermatogonia and probably the resting germ cells forming later generations of spermatogonia. It also stimulates division in the secondary spermatogonia leading to the formation of primary spermatocytes. Although no striking seasonal variation in the response of LH treatment can be seen, this treatment elicits a somewhat greater response during the February-April period.

Temperature effects on LH treated fish. In June experiments, a significant spermatogenetic difference is observed in the low and high temperature LH treated fish (Fig. 15). In July and August experiment although the GSI are distinctly different, spermatogenetic picture of the fish kept at low and high temperature is practically similar. In September 1963 experiment the low temperature fish possess a lower GSI as compared to high temperature, although the former shows a clear sign of spermatogenetic advance over the latter (Table V.). This is more marked in October 1963 experiment. Low temperature LH treated fish in this experiment show a degree of stimulation similar to March 1963 experiment whereas the GSI do not show this. It appears that the GSI is not always a true index of gonadal activity.

Cholesterol and lipid. Cholesterol tests results were always positive in LH experiments although the intensity of the reaction seemed to vary slightly. These variations were impractical for the purposes of the present study to quantify. The lobule boundary cells were the only areas which gave
positive results for cholesterol. These again were the only areas which showed any accumulation of sudanophilic lipid material besides the blood vessels. The lipid content of other areas of LH treated tests is meagre and therefore similar to FSH treated ones.

These results strengthen the argument that the lobule boundary cells are the physiological homologue of the Leydig cells of higher vertebrates which secrete androgens. Mammalian LH seem to constantly stimulate these cells almost irrespective of the season. It evidently possesses both the interstitial cell and the spermatogenesis stimulating property when administered to Couesius. Aside from the effect on the lobule boundary cells LH treatment has no effect on the lipid content of the testicular lobules and so unlike Esox lucius and some amphibians does not cause an artificial steatogenesis in this fish (Lofts, 1961).

**Effect of Fractionated Salmon Pituitary Gonadotropin**

The GSI of fish receiving fractionated salmon pituitary gonadotropin were higher than those of fish receiving mammalian hormones. The level of the GSI was similar in 1962 and 1963 experiments, except for marked increases in February, March and June experiments in 1963. Experiments in 1963 were commenced in May and fish were kept at room temperatures (19.5 ± 1.5°C) and at lower temperatures (10 ± 1.5°C).

**High Temperature Fish**

GSI of the injected fish remained high during May and June experiment but fell significantly during July experiment remaining the same as in the corresponding experiments of 1962,
some increase being noticed in September experiment. It is only during these periods (March-July) that the GSI of the treated fish at high temperature is greater than the sham operated controls. The level of stimulation (as judged by GSI alone) is of the same degree as that by mammalian LH except for February, March, May and June experiment, where stimulation is markedly greater in salmon gonadotropin treated fish (Fig. 13). An increase of a lesser degree is noted also in September and October experiments of 1963.

**Histological Changes**

The spermatogenetic condition is shown in Figure 14. The resting germ cells constitute a very small proportion of the testes (0.4 - 8.7%) while nests of dividing primary spermatogonia are predominant (34.6% - 64.9%). These nests show a greater uniformity of appearance and some degree of synchrony in division. The increase in the proportion of secondary spermatogonia is marked in comparison with fish treated with mammalian LH with 14.6% - 40.4% resting and 1.4% - 35.6% in dividing phase. Primary spermatocytes are present in all experiments except in August 1962, August 1963 and May 1963 experiment. In spite of the fact that salmon gonadotropin treated fish have higher GSI than the LH treated fish, primary spermatocytes are more numerous in the latter group (Tables V. & VI). Also, dividing primary spermatocytes are much less common. It appears that salmon pituitary extract causes a greater build up of the secondary spermatogonia, whereas in the LH fish some nests are stimulated beyond spermatogonial stage before a sufficient store of gonial cells is accumulated. This is obvious
from the greater extent of secondary spermatogonial nests in the salmon gonadotropin treated fish (Fig. 14).

In the present experiment there is some discrepancy between the stimulation as indicated by the GSI and the histology. In May 1963 experiment the GSI is high (0.9032) at higher temperature whereas the testes shows a greater preponderance of primary and secondary spermatogonia with few dividing secondary spermatogonial nests (Table V). The GSI at low temperature is lower (0.6497) which fits in with the histological condition of the testes in this situation. The spermatogenetic composition of both low and high temperature fish is markedly similar.

A decrease in the degree of stimulation is noted in May experiment as in all previous experiments both on the basis of the GSI and the spermatogenetic composition.

**Low Temperature Fish**

The GSI of low temperature fish is markedly lower than that kept in warmer water in May experiment. In June, July, and August, the low temperature fish show a higher GSI. In September and October experiments GSI of fish at lower temperature is again less than that at the higher, the difference being more marked in October experiment (Fig. 15).

It is obvious that maximum stimulation of the testes is obtained during the prespawning period in February and March experiments. Fish seem to remain receptive till the end of the spawning in July. A drop in July experiment may reflect the reduced receptivity of the testicular tissue to gonadotrophic stimulation immediately after spawning. It is possible that
Fig. 13. Seasonal changes in the GSI of Fractionated Salmon Gonadotropin (FSG)-treated fish, as compared to those with mammalian LH. GSI of untreated hypophysectomised and sham operated controls included for comparison.
increased weight of testes in May experiment may be due to increased hydration of the testes probably caused by infection. The low temperature GSI, therefore, reflects the correct picture of testes in May experiment. Low temperatures in the initial experiments show a slightly more stimulatory effect, but in September and October experiments high temperatures cause an increase in the GSI, which is also reflected in an increase in secondary spermatogonial nests and the primary spermatocytes (particularly in October experiment, Figure 14).

A comparison of Figure 12 and Figure 14 shows that the spermatogenetic picture in the LH treated fish and the salmon gonadatropin treated is similar except for a slight increase in the occurrence of primary spermatocytes in the former.

**Cholesterol and Lipid**

Cholesterol tests showed less uniformity in this treatment than in fish treated with LH (Table V). Positive tests for cholesterol were obtained in all experiments except August, 1962, and May, 1963, experiments. The lipid content of the testes did not change. Sudanophily was uniformly noted only in the region of the testes occupied by the lobule boundary cells.

In short, fractionated salmon pituitary extract stimulated testes of hypophysectomised testes to the level of secondary spermatogonial nests with the appearance of some primary spermatocytes. This stimulatory effect was more marked during the receptive period of the annual testicular cycle of the fish falling between February to April, which is demonstrated by the increased stimulation of the testes in January, February
and March experiments. The refractory period seems to fall in August and September; consequently stimulation is less in July and August experiments.

The fish are most susceptible to pituitary removal during May, the regression following the hypophysectomy is very complete, so much so, that replacement therapy (either with mammalian LH or fractionated salmon pituitary extract) is only partly successful in effecting a recovery. The close parallel shown by the fractionated salmon pituitary extract and the mammalian LH show that the gonadotropic factor in the former which is responsible for stimulation is physiologically similar to mammalian LH. This is borne out both by histological and histochemical characterisation of these two hormonal preparations (Tables V and VI).

Effect of Crude Salmon Pituitary Extract

Difficulties were encountered in the administration of crude pituitary extracts to operated fish. However, experiments with it were conducted from September 1962 until the end of the study. In two experiments all fish injected with crude extract died apparently due to infection. Red patches on belly and fluid in the peritoneal cavity were the only obvious pathological signs. Absorption seemed slower in this treatment which often caused prolonged distension of the belly. Survival was increased by maintaining fish during the injection at lower temperatures ($10^\circ C \pm 1^\circ C$) in May 1963 and succeeding experiments.

In spite of the mortalities, the GSI of fish treated with the crude extract was always higher than that of any other treatments, showing its most marked increase in the February and
March experiments and a low activity in July and August (Fig. 18) - the same pattern of response exhibited by the LH and fractionated salmon extract treated fish. The GSI of June experiment fish is much lower than the rest probably because the fish were autopsied after only three injections.

**Histological changes.** Fish treated with crude extract show the most active testes of all fishes receiving replacement therapy. A great increase in mitotic activity is observed in this treatment coupled with a reduction in the proportions of primary spermatogonia (16.5 - 44.8%) and primary germ cells (1.2 - 9.2%) Figure 20. Secondary spermatogonia (both dividing and resting) are predominant, except for May, July and August 1963 experiment. Most marked progress in the testes is found in the increase in the extent of primary spermatocytes both dividing (0.0% - 8.9%) and resting (0.2% - 13.3%). Some secondary spermatocytes are present in the September 1962 and February 1963 experiment (Fig. 20). The histological appearance of the testis of a typical crude salmon extract treated is given in Figure 33A.

**Cholesterol and Lipid**

Except for May experiment, tests for cholesterol were positive (Table VII). Cholesterol test was not attempted in June experiment. There was no change in the lipid content of the testes which always remained low.

The degree of stimulation attained by crude extract treated fish is higher than any other group of treated fish both in the matter of their GSI and spermatogenetic condition. Compared to mammalian LH and fractionated salmon extract treated fish, stimulation with crude extract appears to be fuller and
Fig. 14. Seasonal changes in the spermatogenetic composition of Fractionated Salmon Gonadotropin treated fish. Left bar in May 1963 and subsequent experiments indicate condition at low temperature, right denotes that at high.

Explanations same as Figure 12.
SPERMAGENETIC CONDITION OF FRACTIONATED SALMON PITUITARY GONADOTROPHIN TREATED FISH

EXPERIMENTS
Fig. 15. Effect of low and high temperatures on the Fractionated Salmon Gonadotropin and mammalian LH-treated fish in May 1963 and subsequent experiments.

- Mammalian LH-treated (high temperature)
- Mammalian LH-treated (low temperature)
- Fractionated Salmon Gonadotropin (high temperature)
- Fractionated Salmon Gonadotropin (low temperature)
Fig. 16A. Photomicrograph of a testis from hypophysectomised fish showing abundant fibroblasts simulating fibrotic degeneration. Primary germ cells are most predominant. Some primary spermatogonia are also seen (X 700).

B. Photomicrograph of an FSH treated fish testis showing mostly primary germ cells, and primary spermatogonia. A small nest of secondary spermatogonia on the left centre can be seen (X 700)
more regular, as evidenced by the great build-up of secondary spermatogonia during the non-refractory period of the annual cycle.

Crude salmon pituitary extracts effect a greater stimulation of the hypophysectomised Couesius testes as compared to replacement therapies with mammalian LH and fractionated salmon pituitary extracts. This increased stimulatory effect is exhibited both in the testes weight and in their spermatogenetic composition. The seasonal variation in response to the crude extract is similar to those obtained with the LH and the fractionated salmon gonadotropin.

Effect of Experimental Conditions on Sham Operated Untreated Controls

Sham operated controls were kept for the duration of each experiment. A slight regressive effect is noted in these controls during the summer when compared with normals from the lake. Regression is also slightly more marked during the prespawning winter months. During the post spawning period of late July (June 1963 experiment) the testes in the sham-operated control do not regress to the same degree as the fish in nature; this is probably due to the prevention of the controls from natural spawning and consequently some advanced nests and sperms are retained showing a higher GSI for such fish (Fig. 8).

Spawning in 1962 occurred earlier than in 1963, and was less extended as evidenced from the histologies of normal fish from July and August for these years. Spermiation was well on the way in July 1962 normals and in August all sperms had gone, whereas in 1963, July normals show a great proportion of active
testes full of sperms, and some remnants were present even in August. July and August normals during 1962 were more regressed (to begin with) than those of 1963. This also appears in the GSI, (Fig. 5) and explains why July and August controls in 1962 are more regressed than those of 1963 when compared to their respective normal counterpart.

The spermatogenetic condition of the sham operated control testes are given in Table II, and show a general correspondence with the GSI (Fig. 8).

**General Summary of Results of Replacement Therapy**

Positive response to replacement therapy was achieved with Crude Salmon Pituitary extract, fractionated salmon gonadotropin and purified mammalian LH. FSH and saline injections failed to produce any change. The slight stimulatory effect shown with FSH treatment might possibly be due to contaminant LH.

In *C. plumbeus* there remains little doubt that replacement therapy on hypophysectomised fish restores testicular functions. Results on the effects of mammalian gonadotropins lead to the conclusion that while LH restores and reinitiates spermatogenesis, FSH is ineffective (LH contaminants are responsible for the slight positive effect in some experiments). *Couesius* pituitary thus appears to produce a gonadotropic factor similar to mammalian LH, but subserving the functions of both LH and FSH. Among the treatments which produced positive results, maximum stimulation of the testes was obtained with crude whole salmon pituitary extract. The effectiveness of hormones in replacement therapy seemed to vary somewhat with the seasons. Such variations in response has been well known to contemporary
Fig.17A. Photomicrograph of an LH- treated testis showing the spermatogenetic composition of a typical LH- treated fish. (X 700)

B. Photomicrograph of a fractionated salmon gonadotropin treated testis showing spermatogenetic condition of a typical treated fish (X 700)
Fig. 18. Seasonal changes in the GSI of crude whole salmon pituitary extract (CSE) treated fish as compared to those treated with fractionated salmon gonadotropin (FSG). GSI of untreated hypophysectomised (HPX) and sham operated controls (SHAM) are included for comparison.
workers (Ball and Bacon, 1954, Palmer et al., 1954; Pickford and Atz, 1957). Fish in the present study were more responsive during (i) **The prespawning period when stimulation was maximal.** This period ranges from February to May of the calendar year corresponding to the January, February and March experiments of the present investigation. (ii) **Susceptible period (spawning period).** Regression was maximal during this period falling in June (corresponding to May experiment) and replacement therapies produced recoveries of lower degree. (iii) **Refractory period (early post-spawning period).** Though regression is not maximum, replacement therapies produce only a limited stimulation. This period occurs in August (July experiment). (iv) **Period of reconstitution (late post-spawning period).** Extends from September to January (corresponding to August, September, October and November experiments). Replacement therapies elicit a greater degree of stimulation during this period than during the refractory period, but lesser than the prespawning period. A summary of the replacement therapy experiments is given in Figure 30.

**ENVIRONMENTAL CONTROL OF TESTICULAR CYCLE**

**Photoperiod-temperature Experiment**

1. **Exploratory Experiment**

Fish put under long and short photoperiods were kept at low temperatures (6°C - 8°C) from January 1, 1963 to April 1, 1963. As shown in Table VIII there is almost no difference in the gonosomatic indices of the fish autopsied in February 15, 45 days later. The GSI of the long photoperiod fish is 1.12 and
Fig. 19. Seasonal changes in the GSI of fractionated salmon gonadotropin (FSG) treated and FSH- treated fish. GSI of untreated hypophysectomised (HPX) and sham operated controls (SHAM) are included for comparison.
Fig. 20. The spermatogenetic composition of crude whole salmon pituitary extract treated fish

- Pycnotic nests
- Secondary spermatocytes and spermatids
- Dividing primary spermatocytes.

Other explanations as in Figure 10.
SPERMATOGENETIC CONDITION OF CRUDE SALMON PITUITARY EXTRACT TREATED FISH
that of the short photoperiod 1.19. The testes in both groups are composed predominantly of primary spermatocytes (45%-60%); pycnotic cysts constitute 5%. The remainder of the testes are secondary spermatogonia undergoing slow mitotic activity (20%), resting secondary spermatogonia (10%) and some primary germ cell. The second autopsy 75 days after the start of the experiment did not reveal any significant difference in the testicular activity of the fish in the two photoperiod regimes.

This experiment suggested that photoperiod control initiated in January does not cause significant changes at low temperatures. It seemed possible that a combination of photoperiod and temperature might produce significant effects and lead to a clearer understanding of the controlling mechanism. Baggerman (1957) and Harrington (1957) have demonstrated some cyclic variability in the response of fishes to experimental manipulation of daylengths and temperatures. Consequently, fish were maintained under four combinations of photoperiods and temperatures with the extremes roughly those that are experienced by this species in nature.

2. **Photoperiod-temperature Experiment with Prespawning Stock**

Fish collected from the lake were placed under the following four combinations of photoperiod and temperature conditions on April 30, 1963:

1) Long photoperiod and low temperature
2) Short photoperiod and low temperature
3) Long photoperiod and high temperature
4) Short photoperiod and high temperature

Four samplings were made during the course of this experiment (Table IX).

**First Sampling** (June 14, 1963)

All experimental groups show a decline in the GSI
Fig. 21. GSI of 'prespawning fish' under four different photoperiod-temperature combinations, at the time of the indicated samplings compared with those of the normal. The point of convergence at left indicates the GSI of the normal fish at the start of the experiment.

- Short photoperiod (8 hr) and high temperature (19°C ± 1.5°C)
- Short photoperiod (8 hr) and low temperature (9°C ± 1.5°C)
- Long photoperiod (16 hr) and high temperature (19°C ± 1.5°C)
- Long photoperiod (16 hr) and low temperature (9°C ± 1.5°C)

Normal.
which is obvious within 45 days of experimental control. This decrease is most marked at the higher temperature with the fastest initial decline under high temperature and short photoperiod. Both groups of fish at low temperatures show a lesser decrease in the GSI. Under the lower temperature the GSI declines faster in long photoperiod although these photoperiod differences are not significant. It appears that a short photoperiod and a low temperature may be expected to retard testicular changes.

Second Sampling (July 6, 1963)

A further decline in GSI was found in all groups including controls at the time of the second sampling. There is almost no difference between the two photoperiod groups maintained at high temperatures where the GSI reaches the lowest point. Although the long photoperiod fish show a higher GSI at low temperatures the difference is not significant.

Third Sampling (July 30, 1963)

The GSI of the high temperature groups are similar and remain virtually unaltered from the previous sampling and are closely comparable to those found at this time in nature.

The GSI of both photoperiod groups kept at low temperature was much higher than the normal fish (Fig. 21), and again the long photoperiod group was only slightly advanced over the short photoperiod fish.

Fourth Sampling (December 16, 1963)

The final sampling on December 16, 1963 again showed a distinct separation between the two temperature situations. The GSI of the two high temperature groups remain very similar
Fig. 22. Spermatogenetic composition of 'prespawning fish' under four combinations of photoperiod-temperature at the time of the various samplings (indicated by Roman numerals)

- **N.** = Initial Normal
- **L-L°** = Long photoperiod, low temperature
- **S-L°** = Short photoperiod, low temperature
- **L-H°** = Long photoperiod, high temperature
- **S-H°** = Short photoperiod, high temperature
- **N** = Normal

- Primary germ cells; Primary spermatogonia;
- Secondary spermatogonia;
- Primary spermatocytes; Secondary spermatocytes;
- Spermatids and sperms; Pycnotic nests.

The lower block in each represents resting nests, the upper denotes those dividing.
but are slightly more advanced than at the previous autopsy. At low temperatures the GSI is much greater than at high temperature, the short photoperiod fish show slightly greater testicular growth than the long photoperiod group. Thus, the maximum GSI was found in the low temperature long photoperiod group during the period of the annual cycle when days are longest in nature; and when they start shortening the short photoperiod fish show an increase in the gonad weight (Fig. 21). The GSI of fish belonging to both the photoperiods at low temperature is higher than those of the normals. The gonads of fish at higher temperature show a slight gain in weight, the increase being identical in both photoperiod conditions. The GSI of these fish are much lower than those of the normals.

It appears that higher temperatures have a regressive effect on the testes during this period of their cycle, whereas low temperature seems to stall normal regression. It is interesting to note that a decrease in the GSI even in the low temperature groups is noted in the second sample in early July during a period when the fish spawns; but the testes instead of regressing completely as in the normal and high temperature groups, quickly recovers so that at the next sampling the GSI of these groups are the same as at the first autopsy. In short, despite an exposure to an unseasonal low temperature before spawning the fish show a fall in the GSI at a time conforming to the normal spawning period. An inherent rhythm may be responsible for this phenomenon which though suppressed by an abnormal environmental condition exerts itself in a small measure. A mild depression due to photoperiod may be noticed in the
low temperature groups with short photoperiod during the part of the annual cycle where light period is long (i.e. July). Later, from August onwards, short photoperiods seem to be slightly stimulatory with the fish attaining a much higher level than those in the nature.

In summary, it seems apparent that temperature has a far more important control of testicular maturation than photoperiod. Low temperatures accelerate testicular development and reconstitution, whereas higher temperatures speed up regression and prevent recovery of the testes during the July-December period.

**Spermatogenetic Condition**

Low temperature controlled photoperiods. The spermatogenetic picture of both the long and short photoperiods is similar at the first sampling. Unlike testes in natural condition at similar period of the annual cycle, both mitotic and meiotic activity seem to be suppressed (Fig. 25B); about 35% - 50% of the earlier stages are in resting condition (Table IX). Cell division is sometimes completely suppressed but copious sperms may fill the lobules and the ducts. In some fish there is evidence of evacuation of the lobules and ducts. The cells lining the terminal branches of the duct are partly spent although unspent columnar cells are also noticed. Sperms occupy 50.3% - 54.8% of the lobular spaces. Other stages in the order of their predominance are:

I. SPG" (17.7-20.2%), II. SPG"x (8.1-12.5%),
III. SPC'x (4.8-6.6%), IV. SPGR (3.5-4.5%), V. SPC'(7.0-1.3)

It would appear that the majority of the resting cysts
belong to early spermatogenetic stages with no marked difference save that under long photoperiods, spermatogenetic activity is almost completely stopped in two fish; no such complete cessation was seen under the short photoperiods. It would also appear that low temperatures have a limited restrictive influence on the development of later maturation stages when the testes are in a condition of normal activity. Once the secondary spermatocytes are formed they continue development undisturbed by abnormal conditions.

**High Temperature with Controlled Photoperiods**

Spermatogenetic condition in the two photoperiod groups is almost similar. In the long photoperiod fish besides the sperms and spermatids other stages represented are:

- $\text{SPG}''x(11.6-19.3\%), \ \text{SPG}''R(13.3-17.4\%), \ \text{SPG}'x(22.4-44.5\%)$
- and $\text{SPGR}(11.6-18.1\%)$

Similar stages are also seen in the short photoperiod fish although their proportions are slightly higher because of a greater degree of evacuation and regression of the testes. It is interesting to note that in higher temperature groups primary and secondary spermatocytes are rarely seen. They are either converted into sperms or degenerate and since pycnosis is not common it would appear that they do not degenerate but are rapidly transformed to sperms and expelled. Spermatocytes are quite frequent in the lower temperature groups and some continue dividing. It might be inferred from this observation that the latter stages are rather independent of environmental changes.
Second Sampling

Low temperature with controlled photoperiods. Sperms are still abundant in both photoperiod groups although there is some continued evidence of evacuation. Slow mitotic activity is evident in all fish, and a marked feature of the testes in this group is the formation of apparently fresh nests of secondary spermatogonia. Few later stages (besides sperms) are present; spermatocytes are seen in only a few of these short photoperiod fish. Pycnosis is rare; evidently the spermatocytes transform finally into sperms and are cast off. Dividing secondary spermatogonia are the most predominant stage constituting 14.9 - 19.3% and resting secondary spermatogonia are next in order (14.0 - 14.6%). The cells lining the spermatic ducts are all exhausted and rather squamose. Some migrating cells are present in a few fish belonging to both photoperiods.

High Temperature with Controlled Photoperiods

Both the photoperiod groups are spent with only a few fish showing clumped sperms in some lobules and duct. Pycnosis is rare. Dividing primary spermatogonia predominate (59.6-67.9%). Secondary spermatogonial nests are fewer (19.5-22.0%); resting germ cells account for the rest. Division in the secondary spermatogonia is rare and primary spermatocytes are not seen. Migrating cells are much in evidence in both photoperiods. Since these cells indicate the beginning of the reconstitution of the testes together with some mitotic activity in the resting germ cells and primary spermatogonia; it appears that these fish have already commenced their recovery following a complete regression. In short, no spermatogenic difference can be seen
in the two photoperiod groups at high temperature. However, regression which was slower under longer photoperiods up to the first sampling on June 14, 1963, proceeded at a faster rate thereafter as compared to the shorter photoperiods resulting in a very similar level of the GSI and spermatogenetic condition at this sampling in early July. This, however, is in marked contrast to those of the normals during this part of the year. It is obvious that higher temperature irrespective of the photoperiod causes a marked and quick regression in the testes of *Couesius plumbeus*.

**Third Sampling**

Fish were killed and autopsied on July 30, 1963, at a period when testes under natural conditions are at their minimum activity. These fish show the greatest contrast between the low and high temperature conditions apparently irrespective of the photoperiods.

**Low Temperature and Controlled Photoperiods**

The testes of both photoperiod groups present a rather similar histological picture. Sperms are present in all, although there seems to be some further evidence of evacuation, sperms occupying 42.1-46% of the lobular spaces. Mitotic activity though present is still slow. Secondary spermatogonia are the next dominant stage after the sperms, accounting for 13.0-164% of these stages. Dividing spermatogonia constitute 14.7-15.3%. Newly formed primary spermatocytal nests are present in fish of both groups. Since these stages were completely lacking in previous sampling it is obvious that they are formed anew from the recently formed secondary spermatogonia. Pycnosis
is sometimes noticed.

Since sperms are numerous in both groups it may be concluded that low temperature causes retention of sperms.

High Temperature and Controlled Photoperiods

The spermatogenetic condition in the two photoperiod groups is similar to that of the previous sampling and resembles the condition of normal testes which is extremely regressed at this time of the year. Slight differences from the previous samplings are noted in a general increase of secondary spermatogonia; these account for 28.1-29.5% of all the stages and also include some dividing nests (1.1-7.9%). Primary spermatogonia still predominate (58.2-58.5%). Some migrating cells are also present. It has been suggested earlier that low temperatures favour retention of sperm, it is now obvious that high temperatures accelerate spermiation. All the high temperature fish are almost completely spent, in nature also, spermiation peak coincides with the peak in water temperature. However, spermiation may also depend on other factors, notably the behavioural, the presence of mates, and suitable environmental condition. Sperm retention at low temperatures should be viewed with these factors in mind. Nevertheless, the acceleration of spermiation at higher temperature shows some temperature dependence of this phenomenon.

Final Sampling

The fourth and final sampling of the prespawning stock of fish was on December 16, 1963. These fish also showed the same temperature based variations noted in previous samplings.
Low Temperature and Controlled Photoperiods

Spermiation is now complete in both photoperiod groups with only a few sperm remnants seen particularly in the long photoperiod groups. Mitotic activity has increased slightly. Secondary spermatogonia are the most important cell type in the long photoperiod contributing up to 49.7% of the total of the various stages while primary spermatocytes (59.4%) distinctly outnumber the secondary spermatogonia (34.0%) under short photoperiod regimes (Table IX). The increase in the extent of primary spermatocytes and in pycnosis is also a distinctive feature of the short photoperiod fish at low temperature. Short photoperiod fish, in spite of a slower mitotic activity, are spermatogenetically slightly more advanced than the long group as evidenced by larger proportion of primary spermatocytes. Although these differences are slight it is obvious that testicular maturation during this part of the annual cycle is retarded under long photoperiod; under short photoperiods, the testes reach their maximum seasonal limit faster and therefore the pace of development is slackened earlier. The histological picture of both these groups is somewhat more advanced than the normal fish during the same period of the year.

High Temperature and Controlled Photoperiods

The two photoperiod groups at high temperatures show no definite difference and are both much retarded as compared to the normals (Table IX). However, they do show a slight increase in spermatogenetic activity over the previous sample with increased appearance of the primary spermatocytes (3.8-4.6%) and also in the secondary spermatogonia (Fig. 22). Migrating
cells are not seen. Fish at higher temperature under either light conditions thus show a very slow increase in the spermatogenetic activity over the previous sample. Figure 21 shows that there is a gradual build up of the normal testes after complete regression in early August. The same trend is shown to a limited extent in these two experimental groups.

In summary, by the end of the experiment both the GSI and the testicular development of the high temperature fish is much below that of the normal fish. On the contrary, at low temperatures, a testicular regression never occurs; there is a sustained mitotic activity over the entire period of the experiment ending in a histological condition more advanced than those obtaining in fish in nature. It is interesting to note that spermatogenetic progress even in the low temperature groups is blocked at the level of primary spermatocyte production during the pre-freeze up period of the year, and the testes are prevented from passing into the meiotic phase of development.

13. Photoperiod - Temperature Experiments with the Post-Spawning Stock

This experiment commenced on August 9, 1963, and fish were sampled on three occasions with the final sampling on February 2, 1963 (Table X).

At the first sampling (September 10, 1963), changes in the low temperature photoperiod groups appear to be similar to those found in nature; the GSI of the long photoperiod fish is significantly higher than that of the normals which closely approximated to the short photoperiod GSI. Testicular development does not seem to advance significantly in the high temperature groups. In fact, GSI of both groups is lower than the normals;
Fig. 23. GSI of 'post spawning' fish under four different photoperiod-temperature combination at the times of the indicated samplings compared with those of the normals. For explanations see Figure 21.
Fig. 24A. Photomicrograph of a Stage V or early Stage I testis showing lobules consisting mostly of primary germ cells. Migrating cells are shown in the interlobular space, some of which have entered the lobules which lack a lumen. (X 700)

B. Photomicrograph of a section of testis from photoperiod-temperature fish showing premature formation of primary spermatocytes and the stopping of any further spermatogenetic progress at lower temperatures (X 700)
Fig. 25A. Photomicrograph of a portion of an LS of a normal spawning testis showing dividing nests of spermatogonia and spermatocytes lining the lobule filled with sperms (X 700)

B. Photomicrograph of a portion of an LS of a testis of similar fish under low temperatures (either photoperiod) showing suppression of spermatogenetic activity. (X 700)
Fig. 26A. Photomicrograph of a portion of testis showing lobule boundary cells (X 1260)

B. Photomicrograph of a testis of *C. plumbeus* stained with Sudan Black B showing lipoidal lobule boundary cell areas, and absence of any intralobular lipid accumulation (X 135).
the long photoperiod fish show slightly less regression.

In the second sampling the low temperature groups show a considerably higher GSI than the normals with the long photoperiod fish still slightly higher in GSI than the short photoperiod animals. Fish in the high temperature groups also show an increase in their GSI although the change is more gradual; the shorter photoperiod GSI is higher than the long at these temperatures (Fig. 23).

At the final sampling the GSI of the low temperature groups remain higher than the normals although the GSI of the short photoperiod group now exceeds that of the long photoperiod fish. This change is similar to the situation seen in the previous experiment (Fig. 21). Thus, in both experiments testicular development was at first faster under long photoperiod and low temperature but later it proceeded at more rapid rate under the short photoperiods (low temperature).

Fish in the two photoperiod groups at high temperature have similar GSI which indicates that the long photoperiod group has continued to develop at a slightly faster rate than the short photoperiod ones ending up at levels with them. Actually, the high temperature groups have changed only slightly from their initial condition. It is concluded that in the post-spawning stock testicular regression at high temperatures occurs irrespective of the photoperiod. On the other hand, maturation at low temperature is faster and considerably greater than the normals with little difference in the GSI of the two photoperiod groups. The small photoperiod effect seems to be confined to the stimulatory nature of the long photoperiod in the initial
Fig. 27. Spermatogenetic composition of 'post spawning fish' under four combinations of photoperiod-temperature at the time of the various samplings indicated by Roman numerals.

Other explanations as in Figure 22.
stages of the experiment.

Spermatogenetic Condition of Post-spawning Fish under Experimental Conditions

First Sampling

Low temperature with controlled photoperiods. At the first sampling, some mitotic activity is noticed in fish under both photoperiod regimes at low temperature. Dividing nests constitute 43.9-44.0% of all stages, and dividing secondary spermatogonia are the most predominant stage in the long photoperiod fish (30.8%), followed closely by resting secondary spermatogonia (29.4%). Dividing primary spermatogonia constitute 12.6% of the total and the rest is made up by primary spermatocytes 6.7% (Table X). A few secondary spermatocytes are also seen. Fish under the short photoperiod show a similar picture except that they show a greater proportion of spermatocytal stages (Fig. 27). Pycnosis is not common.

High Temperature with Controlled Photoperiods

The two photoperiods seem to produce some difference in fish at high temperature (Fig. 27). In particular, the long photoperiod fish show very little mitotic activity (dividing stages constituting only 1.0%) as compared to those under short photoperiod (dividing stages 10.8%) leaving aside the primary spermatogonia whose division in these fish is restricted to a few nuclei here and there. This activity difference is also reflected in the spermatogenetic composition. In long photoperiod fish, slowly and sparsely dividing primary spermatogonia predominate (61.7%); there are fewer secondary spermatogonia (25.3%); the remainder are primary germ cells (12.0%). Contrasted
to this, secondary spermatogonia are the most common stage (37.0%) in the short photoperiod group. There are also some dividing nests of this stage (2.0%). Dividing primary spermatogonia constitute only 31.7% of the spermatogenetic stages. Pycnotic nests are noticed only in the short photoperiod group. It appears that these represent the degenerating later generations of secondary spermatogonia and primary spermatocytes.

Second Sampling

Low temperature with controlled photoperiods. The spermatogenetic condition of fish under the two photoperiod situations are much more similar. Mitotic activity is of the same order as in the first sample with primary spermatocytes the predominant form in both groups (29.6-32.5%). Pycnosis is present (1.9% in the long photoperiod and 1.6% in the short). Secondary spermatogonia are only slightly less common than the primary spermatocytes.

High Temperature with Controlled Photoperiods

The two photoperiod groups show some spermatogenetic difference. Mitotic activity is low (2.8-11.5%) in both, but the short photoperiod groups is spermatogenetically rather more advanced than the long photoperiod group. Although dividing primary spermatogonia are the major cell type in both groups (Table X), there is a higher proportion of secondary spermatogonia in the short photoperiod fish. Also, in these fish, some primary spermatocytes are seen (3.6%); these are less (1.5%) in the longer group. Pycnotic nests are not seen in fish in these groups.

The normal fish during a similar period of the year show a higher proportion of primary spermatocytes (35.4%) and
secondary spermatogonia (34.3%). These are thus much more advanced than the high temperature fish, but compared to the low temperature fish they are less advanced and less active.

**Final Sampling**

*Low temperature with controlled photoperiods.* At the final sampling the spermatogenetic condition in the two photoperiod groups at low temperature is rather similar, although the short photoperiod fish are slightly advanced over the long photoperiod group. In the latter, most of the testes is composed of primary spermatocytes (52.2%). Next are the secondary spermatogonia (21.3%) and their dividing stage (17.4%). Some secondary spermatocytes appear for the first time in this sample in small numbers (0.5%). Pycnosis is common, with the long photoperiod group showing more pycnotic nests (3.8%). In only one fish under long photoperiod a few nests of spermatids and some sperms were noticed.

The short photoperiod fish have slightly more active testes. In 50% of them sperms are present in small quantity. The bulk of the testes is composed of primary spermatocytes (63.2%) with about half of these nests undergoing division. Dividing secondary spermatogonia constitute 16.2% of the stages. Some resting secondary spermatogonial nests are present. Sperms and a few resting germ cells complete the spermatogenetic picture. In those fish which do not show sperms, primary spermatocytes are the most predominant stage. The short photoperiod fish thus show a more advanced testicular development than the longer photoperiod. The spermatogenetic situation even in the long photoperiod group is in advance over that of the normals from
same period of the year.

High Temperature with Controlled Photoperiods

Fish belonging to the two photoperiods show similar spermatogenetic appearance. Mitotic activity in both is low. Secondary spermatogonia predominate (42.0-42.6%), followed by dividing primary spermatogonial nests (24.5-27.2%). Dividing secondary spermatogonia are fewer (11.6-20.9%). In some fish, primary spermatocytes are also seen. Pycnotic nests are much fewer in these fish than the low temperature ones.

Fish kept under normally changing daylengths at low temperature have GSI lower than those of the experimental fish at similar temperatures, and the spermatogenetic condition is also less advanced. These testes are composed of primary spermatocytes and secondary spermatogonia, and later stages are absent. Mitotic activity is less than the short photoperiod low temperature fish. Pycnotic nests in the normals are absent.

Summary of Effect of Photoperiod-temperature Combination on Post-spawning Fish

Low temperatures seem to be stimulatory for the testicular maturation in *Couesius* during the post-spawning period of the annual cycle. Long photoperiod is slightly more activating than short photoperiod in the earlier part of this period but in the winter months the short photoperiod appears to be more efficacious. Continued high temperatures have a regressive effect on the testes.

4. Photoperiod-temperature Experiments with the 'Pre-freeze Up' Stock

In this experiment, started on November 9, 1963, the fish were sampled twice, with the final sampling on April 1, 1964.
At the first sampling (December 6, 1963) the GSI of the long photoperiod group was higher than the initial values or the normals at this sampling. Short photoperiod fish showed only a slight increase in the GSI (Fig. 28). At the high temperatures, both photoperiod groups exhibited a marked but similar decrease in the GSI.

At the final sampling, the short photoperiod group at low temperature showed a much higher GSI than those under the long photoperiod (again similar to earlier experiments), while the GSI of both groups were significantly higher than the normals. The high temperature photoperiod groups showed no change in their GSI, the GSI of the long photoperiod fish is slightly higher than the short photoperiod animals but there is relatively little change from the first sampling. Thus, high temperatures seem to completely block testicular growth during the 'freeze up' months of the annual cycle in Couesius.

**Spermatogenetic Condition**

**First Sampling**

*Low temperatures with controlled photoperiods*. Histological appearances of the two contrasting photoperiod groups are similar with a slightly increased cell division in the longer photoperiod fish (Fig. 29). The testes in the short photoperiod group have similar amounts of dividing secondary spermatogonia (22.6%) and primary spermatocytes (25.2%). Dividing nests are rather fewer in the short photoperiod than in the long. Pycnosis is slightly greater in the long photoperiod which also has a greater proportion of primary spermatocytes (31.0%). Some dividing primary spermatocytes are seen only in the long photoperiod fish (Table XI).

As pycnosis occurs in later generations of secondary
Fig. 28. GSI of 'pre-freeze up' fish under four different photoperiod-temperature combinations at the time of the indicated samplings. The converging point at left indicates the GSI of the normals at the start of the experiment. ⬤-----⬤ Normals. Other explanations same as in Figure 21.
Fig. 29. Spermatogenetic composition of 'pre-freeze up' fish under four combinations of photoperiod and temperature at the time of the various samplings indicated by Roman numerals.

Explanations same as in Figure 22.
spermatogonia and primary spermatocytes, an increase in the extent of pycnosis in the long photoperiod group which also shows greater activity may indicate that superfluous or excess nests of these stages are degenerated by an inherent regulatory mechanism.

High Temperatures with Controlled Photoperiods

Spermatogenetic condition in the two photoperiod groups is very similar. Testes are mostly composed of secondary spermatogonia (35.6-42.2%) and dividing primary spermatogonia (35.1-36.8%). There are some dividing secondary spermatogonia and a few spermatocytal nests (Fig. 29). No later stages are seen. Fairly extensive pycnotic nests are observed in both groups, and mitotic activity is low. These groups thus show unmistakable signs of regression.

Final Sampling (April 1, 1964)

Low temperature with controlled photoperiods. Both photoperiod groups are more advanced than the normal animals killed at the same period. The short photoperiod fish are the more advanced of the two experimental groups. A small number of mature sperms are seen in fish at this autopsy, with 40% of the long photoperiod fish and 80% of the short photoperiod fish showing few lobules containing sperms. The ducts are still collapsed and empty.

Spermatocytes are the dominant form in both groups, with the short photoperiod fish showing a marked increase in the secondary spermatocytes (27.7%), Figure 28. An interesting feature of these two groups of fish at this autopsy is the complete absence of pycnotic nests. Short photoperiod fish are
similar in activity (72.1% dividing stages) to the long group (71.6% dividing stages) but include a slightly greater proportion of advanced stages. There is no change in the cells lining the spermatic ducts; they remain squamose.

Normal fish at this autopsy show a uniform picture. Sperms are seen in only one fish out of eight. Lobules are composed of actively dividing nests, with a preponderance of the primary spermatocytes (53.3%) over other stages. Dividing secondary spermatogonia make up for 20.3% of the various stages. Secondary spermatocytes constitute 10.6% of the total stages. These fish are thus less active than any of the two low temperature groups, but much more advanced than any of those at high temperature.

High Temperature with Controlled Photoperiods

Fish belonging to both photoperiod groups show regressed testes. Mitotic activity is rare. Lobules are narrow and the nests are small. Secondary spermatogonia (52.1-52.9%) and dividing primary spermatogonial nests (35.1-36.8%) are the commoner cell types. Primary spermatocytes are seen in some fish. No later stages are found, and the cells lining the ducts remain squamose.

Summary of Effects of Photoperiod-temperature Combinations on "Pre-freeze Up" Fish

When Couesius plumbeus is subjected to temperature-photoperiod manipulation, normal maturation occurs only at low temperature. The speed of maturation is faster (in both long and short photoperiod group) than in the normals kept under normally changing daylength at low temperatures. Short photoperiod is slightly more stimulatory in the later part of the winter; whereas
in the early winter long photoperiod seems to be more efficacious. Photoperiod differences are not apparent at high temperatures where both groups regressed to the same level.

**General Summary of Photoperiod-temperature Experiments**

The results of the photoperiod-temperature experiments indicate that temperature is a far more important environmental factor for the spermatogenetic process of *C. plumbeus* than light. Low temperature has a retarding effect on the spermiogenesis and spermiation in the prespawning fish; in the post spawning and pre-freeze up fish low temperatures stimulate earlier spermatogenetic development, and fish under these experimental conditions outstrip the spermatogenetic progress of the fish under normal environmental conditions.

Higher temperatures speed spermiation and induce quicker regression in prespawning fish. They restrict the earlier development of testes in fish during all the three phases of the cycle studied. A diagrammatic summary of the photoperiod-temperature experiment is given in Figure 31.

A feeble photoperiodic effect is also noticed. In the initial phase of each experiment at low temperatures the long photoperiod is slightly stimulatory, but later short photoperiod seems more activating. At the final sampling of the pre-freeze up fish, post spermatocytal changes (at low temperatures only) are noticed under both photoperiods; the testes of the short photoperiod fish is slightly more advanced than those of the long photoperiod fish. This trend though consistent in all the three stocks of fish is not statistically significant (prespawning fish -- value of $t = 1.468$, post-spawning fish $t = 0.818$ and pre-freeze up fish $t = 1.036$).
Fig. 30. Summary of the replacement therapy experiments showing seasonal effect. The smooth curve indicates the seasonal change in the activity of the normal testes. Roman numerals indicate the five stages of the annual testicular cycle. Signs at the top of the figure indicate the timing of the various experiments with reference to the annual cycle, and their effects. A (-) sign denotes regression; and a (+) sign stimulation. Increases in stimulatory and regressive effect is indicated by increase in number of these signs.
Fig. 31. Summary of the photoperiod-temperature experiments. Symbols above the curve indicate the condition of fish at later samplings at higher temperature, the ones below the curve denote those at low temperature. The spots on the curve where these symbols are placed indicate the level of stimulation or regression exhibited by the fish at the final sampling.

Other explanations same as in Figure 30.
POST SPAWNING FISH

PRE FREEZE UP FISH

PRE SPAWNING FISH

POST SPAWNING FISH
Fig. 32. Photomicrograph showing cells lining the distal spermatic ducts of spawning fish. Note three cells in the centre with elongated nuclei in their basal half and granule filled lumen-half; adjacent cells on left are spent (X 1260)
Fig. 33A. Photomicrograph of a portion of an LS of testis of fish treated with crude whole salmon pituitary extract. Note the lobule on left upper corner almost full of primary spermatocytes. Some secondary spermatocytes are seen dividing at right upper corner (X 700).

B. Photomicrograph of a portion of an LS of a normal Stage II testis showing pycnotic nests among healthy spermatogenetic stages (X 700)
DISCUSSION

Seasonal Testicular Cycle

The testes of *Couesius plumbeus* undergo regular cyclical changes. The changes are similar to those described in detail by Bullough (1939) in a closely related fish, *Phoxinus laevis* and Harrington (1957) in *Notropis bifrenatus* another allied species. The spermatogenetic cycle in *C. plumbeus* can be divided into two distinct phases on the basis of the kind of the multiplicative processes:

(i) The **mitotic phase** - this is very slow and commences in September, progressing slowly till April.

(ii) The **meiotic phase** - this is rapid and shorter in duration. During this period, which begins in April, secondary spermatocytes are formed which proceed towards final maturation and formation of sperm.

There is no time lapse between these two phases, and a similar scheme can be found in the testicular cycle of all other cyprinid fishes studied (Bullough, 1939; Scott, 1952; Harrington, 1957). In *Couesius* spawning commences before the lobules become fully packed with sperms; spermiogenesis continues during the spawning period. There is no indication of any spermatogenetic arrest during spawning, and since spermiation and spermiogenesis occur simultaneously repeated sperm discharges are possible. It is interesting that a low ratio of males to females was found in all catches from the lakes; repeated spermiation might consequently be of a decided advantage in this species. After spermiation reconstitution of the testes commences subsequent to a brief
spermatogenetic inactivity in August.

The annual testicular cycle in this fish is closely correlated with the changes in its environment. When the ice goes from the lake and the water temperature and daylength start increasing there is a burst of spermatogenetic activity leading to the formation of sperms and ending with spawning. Spawning occurs in June and early July (as evidenced from the testicular histology) when water temperature is still rising but the daylength is maximum. In August when temperature is maximum spermiation is completed, and during this period very little mitotic activity is present. Reconstitution commences in late August and during the period of decreasing light and temperature slow mitotic activity continues. Fish immediately before the "freeze-up" have developed a stock of primary spermatocytes and considerable spermatogonial reserve. During the period of ice cover little change occurs, although slow mitotic activity continues. Later development occurs only when the temperature of water rises in mid-April; daylength has been continually increasing since late December. In short, the first phase of testicular changes occur during the fall and "freeze over" months and the final phase commences immediately on the warming of the lake in the springtime.

Seasonal Factors affecting Testicular Cycle

It is natural to expect that like many other animals gonadal maturation in *Cousius plumbeus* is regulated and controlled by the environmental variables notably light and temperature. Testicular cycles in *C. plumbeus* have been closely correlated with changes in the environmental factors in the present study. It is
supposed that these correlations have a cause-effect relationship. This belief is supported by similar earlier studies on seasonal changes in teleost testicular cycle. Out of various possible factors, photoperiod and temperature are the most likely candidates for the regulation of the testicular cycle. Studies in this field are numerous. The literature has been summarized by Pickford and Atz (1957) and in reviews edited by Winthrow (1959). Some of these works favour changing photoperiods as the major regulator, whereas others deny it.

A few have shown temperature as the sole regulating factor, while others have compromised by including both light and temperature as important in the control of testicular changes. Some workers have reported discordant results in the same species. It is possible that some of the discrepancy in the experimental work is due to differences in the response to manipulations of photoperiod and temperature at the various phases of the life cycle; also possibly to short-time of the experimental controls. To test the relative significance of these two factors in Couesius, fish at three distinct periods of the annual cycle were subjected to four combinations of photoperiods and temperatures and the experiments conducted for an extended period.

Role of Temperature and Light

When prespawning fish showing advanced spermiogenesis were subjected to different photoperiod-temperature conditions, normal testicular activity was interrupted at low temperatures irrespective of the photoperiods. At higher temperatures, on the contrary, spermiogenetic changes were accelerated and the fish showed spent testes at least 20 days earlier than the fish in
Difference between the two higher temperature groups on the basis of photoperiod was almost absent.

At low temperature spermiation was retarded for considerable time and only mild activity was retained in the fish, a greater proportion of spermatogonial and spermatocytal nests stopped dividing. This temperature effect was again emphasized in the later part of the experiments with the prespawning fish.

Similar changes were noted with the post-spawning stock, and the "pre-freeze up" stock of fish. In both cases temperature and not photoperiod is the prominent regulator. However, two pertinent facts were apparent:

(i) Although there was little difference in the long and short photoperiod fish at higher temperature, fish at lower temperature held under short photoperiod always showed a slightly advanced testicular composition at the final sampling.

(ii) Some post-spermatocytal changes occurred in the low temperature fish under both conditions of photoperiods when experiments were extended until early April, the normal prespawning period.

These two factors suggest a mild effect of photoperiod and an endogenous rhythm which cannot be completely suppressed by either the low temperature or the photoperiod.

The probability of light as a trigger in the control of testicular cycle is further reduced by the irregularity of illumination reaching the fish during the critical prespawning phase. In midwinter the thickness of ice cover in the lakes is about 18 inches which is often covered by a layer of snow. The
period of snow cover is irregular. During the time when dry snow covers the lake very little or no light penetrates below the frozen layer. There are periods when there is no snow cover, or, if present, it is wet. Under such conditions there is some penetration of light; the amount varying with the several parameters concerning the ice and snow. Fish during the period of ice and snow cover are subjected to a fluctuating action of light which is extremely irregular and not predictable. Had the snow cover been uniformly present and also dry, light penetration would have been prevented. At the time of thawing, then, there would have been a sudden onset of illumination. If it were so, an abrupt increase in light would then be a logical candidate for a triggering mechanism. As the fish experience irregular periods of intermittent light and near darkness, a trigger role cannot be attributed to light. Even if it were possible that light could trigger off changes in testicular activity, the amount of light would fall below the threshold level demanded for stimulation.

In summary, it is clear that temperature is the more important regulator of the testicular cycle of *Coriesius plumbeus*. When temperatures are low spermatocytal stages lose most of their activity, and spermiation is hindered; while high temperature accelerates these changes and facilitates spermiation. High temperatures, moreover, impede normal gonial proliferation as a result of which high temperature fish never reach the spermatocytal stages. The inhibition imposed by temperature (low in the case of spermiation and spermiogenesis; and high for gonial proliferation) is not overridden by either photoperiod.
Another significant fact is that at low temperatures both pre- and post-spawning stocks reach the prefreeze up spermatogenetic condition much earlier, but development stops at the primary spermatocyte level for a considerably long time. It is only in the instance of aforesaid sampling with pre-freeze up stock that this barrier is somewhat broken.

The environmental regulation of the annual testicular cycle may now be explained. The first phase of spermatogenetic process commences and continues when water temperature is low, and as late as March spermatogenesis proceeds only up to primary spermatocyte stage (Fig. 6). The second phase of spermatogenesis occurs when the lake thaws and water temperatures start rising rapidly and final stages of maturation and spermiation occur at higher temperature. Gonial proliferation (= the first phase) commences only when the temperatures are lower and progresses steadily till the freeze up.

**Endogenous rhythm**

Theoretically if the temperature was the only regulator no post-spermatocytal development should have occurred at low temperature and no spermatocytes formed at the higher. Since these changes do occur in a mild way there is some endogenous rhythm to the annual testicular cycle in this species. This phenomenon has been encountered by some other workers. Hendersen (1963) in male *Salvelinus fontinalis* has suggested that the male germ cells are more stable than the female insofar as modification by experimental photoperiod and temperature conditions are concerned. Bullough (1941) found some maturation in *Phoxinus laevis* maintained in dark.
It is proposed that in *Couesius plumbeus*, temperature is the major environmental factor responsible for the triggering of the second phase of the annual testicular cycle; it is also important in the normal progression of the first phase. The testes possess an inherent rhythm which is more effectively suppressed by higher temperature (hence no normal gonial proliferation in fish under such situation) than by the lower (hence a limited post spermatocytal development is possible in fish at lower temperature but approaching normal spawning season). The nature of this endogenous regulatory rhythm in teleosts and even in other vertebrates is unknown, but it is reasonable to suppose that it is genetic and peculiar to each species. Photoperiod does not play any significant role in the regulation of the testicular changes in this fish.

**Mechanism of Control of Testicular Cycle**

The relation of pituitary to the gonads is well established in the entire vertebrate series, and it is logical to assume that in *Couesius* also the regulatory mechanism of testicular activity operates through this gland.

**Evidence from Hypophysectomy**

Out of a number of teleosts successfully hypophysectomised the effects of pituitary removal on the gonads has been studied in only nine species, and among these only in a few forms have the histological changes been followed particularly in the females (Vivien, 1941, and Barr, 1963). Seasonal variations in response to this operation have been taken into account only by Matthews (1939, 1941), Vivien (1941), Burger (1941), and Barr (1963,a,b,c).

From these earlier works and the present study the
following conclusions may now be made:

(i) Removal of pituitary gland affects the mitotic activity of the spermatogonial cells with complete suppression of spermatogonial activity. Thus, conversion of spermatogonia into spermatocytes is completely stopped. Mitoses may occur but are rare.

(ii) If, however, spermatogenesis is well under way hypophysectomy does not seem to halt abruptly the process.

(iii) Spermiation occurs normally.

Seasonal variation in the response to pituitary removal is evident. In this study, it was maximum during the peak of the spermatogenetic activity closely followed by the effect on the fish immediately before spawning. Hypophysectomy produces its least effect immediately after spawning. It should be noted that these seasonal effects of hypophysectomy affect only the speed of regression; the end result is always the same and in all cases the testes assume a resting condition.

The effects of hypophysectomy on spermiation is so far unsettled. In the present study all fish spermiated completely and the spermatic duct and their branches were collapsed and almost devoid of sperm. Spermiation therefore, appears to be independent of pituitary control. Generalisations should, however, be made with caution as retention of sperms and completion of sperm formation has been shown to be a species peculiarity. In a wide variety of non-cyprinid fishes sperm formation is completed before actual spawning (Barr, 1963; Harrington, 1956) and some
fish may show sperms throughout the year (Weisel, 1949). In *Couesius*, photoperiod and temperature studies have shown that high temperatures accelerate sperm discharge whereas low temperatures cause sperm retention for a longer time. It may be possible that higher temperatures *per se* affect the motility of the sperms causing the discharge and lower temperatures reduce it in this fish. If this is true, it would not be surprising that spermiation occurred in some fish normally in the absence of the pituitary.

The present study is in full agreement with those of Barr (1963c) on a marine teleost *Pleuronectes platessa* and Dodd *et al* (1960) on the elasmobranch *Scyliorhinus canicula*, on the question of the precise stage of spermatogenesis most affected by hypophysectomy. Withdrawal of pituitary completely suppresses the vital transformation of secondary spermatogonia into primary spermatocytes. It is well known that meiotic divisions are much more elaborate phenomenon than the mitotic. They involve far reaching biochemical and genetic processes. In *Couesius*, first meiotic division occurs at the primary spermatocyte stage which is the climactic stage of the first phase of the testicular cycle. Little post-spermatocytal activity occurs in fish at low temperature, whereas pituitary removal blocks development at a stage earlier. It is presumed that the change from secondary spermatogonia into primary spermatocytes requires some exceptionally specific triggering so that the spermatocytes that are formed can embark on meiotic divisions.

Lobule boundary cells are recognised in *C. plumbeus* both histologically and histochemically as homologue of vertebrate
Leydig cells. They undergo a cyclical change in activity, and their normal functioning also is suppressed by pituitary withdrawal. In short, the pituitary gland in C. plumbeus is not only essential for the maintenance of the first phase of spermatogenesis and successful completion of the second, it is also necessary for the maintenance of the normal secretory activity of the lobule boundary cells. The question now arises as to which hormone or hormones of the pituitary are responsible for this control? To answer this question known extracts from this gland were utilised in replacement therapy on hypophysectomised Couesius.

Role of Gonadotropin(s) in the Control of the Testicular Cycle

Extracts from pituitary glands of various animals have been used in fishes to accelerate gametogenesis and inducing spawning. Almost all of these works involved homo- or heteroplastic injections of pituitary suspension into intact fish usually approaching gonadal maturity. Varying degrees of success were obtained in these studies (Pickford and Atz, 1957). These tests are instances of augmentation therapy since no effort was made to eliminate endogenous gonadotropins and are not critical since the identity of hormones used were not known.

Reinitiation and restoration of spermatogenesis was obtained in this study with purified salmon gonadotropin, crude whole salmon pituitary extract and pure mammalian LH. Mammalian FSH failed to produce any stimulation. Stimulation with mammalian LH was almost as great as that with purified salmon gonadotropin; during the receptive prespawning period salmon gonadotropin was slightly more efficacious. The differences
might have been due to contaminants or dosage and do not provide evidence for any large differences between them. It is suggested that the salmon pituitary possesses a factor similar to mammalian LH which is effective in the restoration of spermatogenesis in *Couesius plumbeus*.

There is no satisfactory evidence, so far, in any fish of an action of mammalian FSH in testicular maturation of fishes. Pickford (1953) concluded that mammalian FSH was 1/5th to 1/10th as efficacious as LH. She suspected that the contained 3% LH impurity might have caused the stimulation. Witschi (1953) and Kirshenblat (1949) have found evidence for an FSH-like factor in fishes using standard assay methods on mammals and birds. Otsuka (1956) claims to have separated two gonadotropins from salmon pituitary exhibiting FSH and LH properties when tested by mice bioassay. Barr (1963b) demonstrated vitellogenesis in primary oocytes of hypophysectomised plaice with FSH-like preparations. He, however, did not study the effect of purified mammalian hormones on his fish; nor did he study the effect of replacement therapy in the male. Ball (1960) cautions the reliance on mammalian bioassay for determining the nature of fish gonadotropins. The positive assays might have been obtained by the effect of increased endogenous secretion of gonadotropins in the mammals caused by the action of the releasing substances possibly present in the fish pituitary. These releasing substances according to Martini et al. (1959) are probably the posterior lobe polypeptides. Although Olivereau (1962) differentiated two kinds of gonadotrophs in several species of fish, the nature of their secretions remains yet to be
established. Ramaswami (1962) did not use any test specifically for FSH but found specific evidence for LH in the pituitary of *Heteropnuestes fossilis*. Ahsan and Hoar (1963) tested for FSH on pituitary suppressed *Gasterosteus aculeatus* and found negative indications. It is possible that fish pituitaries produce a substance with an FSH-like action in higher vertebrates, but there is presently no evidence for any gonadotropin effect in fishes which cannot be initiated by the LH-like factor. Testicular activity in *Couesius* is physiologically controlled by gonadotropin(s) similar to mammalian LH.

Complete restoration of spermatogenesis was not obtained by replacement therapy during the present investigation. Although the hurdle of the spermatocytal transformation was crossed, the later stages were not numerous. Testicular stimulation reached only up to secondary spermatocytal stage at best. There are several possible explanations. Incomplete activation might either be due to specific differences in the quality of *Couesius* gonadotropin and the exogenous gonadotropins used, or to an unphysiological dosage, or to the duration of the treatment.

Although the fish gonadotropin dose might be less (there is little chance of this if the dose is considered in terms of wt/wt and not in numbers of equivalent whole pituitaries involved), there is no doubt that a dose of 0.35 mgm mammalian LH and FSH for an average of 2.5 gm fish is sufficient when compared to the dosages successfully used by Ahsan and Hoar (1963) and Pickford (1953). Gonadotropins are either glycoprotein (FSH) or mucoprotein (LH) in mammals (Barrington, 1963). The
The chemistry of fish gonadotropins has not been studied but there are indications that they are similar (Pickford and Atz, 1957). Most animals usually react more strongly to pituitary hormones from their own or closely related species. However, varying degrees of response have been noted in the gonads in the case of phylogenetically remote donors and recipient (Pickford and Atz, 1957; Greaser and Gorbman, 1939; Gorbman and Bern, 1963). Limited response to replacement therapy is possibly due to species specificity of the hormones.

It is assumed that the gonadotropins in the various vertebrate groups possess certain common physiologically active molecular sections which are the active core, and species differences may be related to the remainder of the molecule. Some molecular differences have been found in the LH (ICSH) obtained from various mammalian sources (Li, 1963). It may be supposed that the degree of specificity may be related to the degree of dissimilarity between their gonadotropic molecules. On the basis of this hypothesis it is not surprising that most instances of specificity are of relative nature and absolute specificity is rare.

It is possible that the greater stimulation obtained with crude whole salmon pituitary extract is due to the synergestic action of other pituitary hormones. Fontaine (1961) in the discussion after Ramaswami's paper reported that maturation of gonads in Anguilla was obtained with TSH which had a little LH as impurity. Ramaswami (1961) himself found TSH containing small LH impurities effective in completion of the maturation of gonads in intact Heteropnuestes fossilis. Ahsan and Hoar (1963) showed that testicular and ovarian
maturation was enhanced with TSH containing LH impurities. Pickford (1954) has also reported similar synergistic action of gonadotropins with TSH and STH in *Fundulus*. In mammalian work also, addition of STH to gonadotropins and testosterone increased the response by the gonads to replacement therapy (Woods and Simpson, 1961; Boccabella, 1963). It is suggested that spermatogenetic processes respond specifically to gonadotropins while other factors like TSH and STH, with a general stimulatory action on metabolic processes, accentuate the effect of the gonadotropin on the testes of *Couesius plumbeus*.

A thesis regarding the regulatory mechanisms of the annual testicular cycle of *Couesius plumbeus* can now be presented on the basis of these experimental findings.

The controlling mechanism of the regulation of the testicular cycle in this fish is complex. Temperature variations act as the major factor which affect the secretory activity of the pituitary gland; light plays no significant role. This temperature effect is in the nature of a trigger rather than the general increase in metabolic activity commonly associated with temperature changes. The pituitary gland is solely responsible for the maintenance and continuation of testicular cycle. Importance of this gland in the testicular regulation is underlined by the fact that it is not only related to the gonads through gonadotropins and steroids, but also acts as an intermediary between its target organs and the animal's external environment through its close associations with the nervous system. Temperature changes act on the central nervous system and affect the neurosecretory activity in the hypothalamus.
which in turn regulate the production of the gonadotropin; this in the final analysis controls the testicular changes. Increasing water temperatures in spring cause enhanced gonadotropin secretion which result in a sudden burst of meiotic activity in the testes leading to the completion of testicular maturation. Sperms are shed as the water temperature rises and it is possible that higher temperature may act directly upon sperms and accelerate spermiation by increasing their motility. Reconstitution of testes after spawning is slow in August as gonadotropin secretion is stopped after a certain limit. When water temperature falls again, small amounts of gonadotropins are secreted maintaining the slow activity during the first phase of the testicular cycle in this fish.

The gonadotropin responsible for testicular maturation is similar to mammalian LH, and a factor like mammalian FSH is probably not physiologically active in this fish.

Although gonadotropin is primarily responsible for testicular maturation, evidence is present that other factors (possibly thyrotropin and somatotropin) may have some stimulatory effect by acting synergistically with the gonadotropin. Testes of *Couesius* possess an unknown but feeble endogenous rhythm which is almost completely suppressed by higher temperatures (19 ± 1.5°C). It is possible that this rhythm is genetic and peculiar to the species possessing it.
SUMMARY

1. The annual testicular cycle in *Couesius plumbeus* is in harmony with the cyclical changes in its environment, and the spermatogenetic processes are controlled mostly by temperature changes.

2. Hypophysectomy causes marked regression in the testis suppressing spermatogonial multiplication and preventing transformation of spermatogonia into spermatocytes. There is some seasonal variation in the effect of hypophysectomy, it is maximum in prespawning and spawning period.

3. Lobule boundary cells (- the homologue of Leydig cells of tetrapod) also show cyclical changes of activity as evidenced by Schultz's cholesterol test, and are markedly depressed by pituitary removal.

4. Spermatogenesis is largely restored by injections of fractionated salmon pituitary gonadotropin, pure mammalian LH, and crude whole salmon pituitary extract. It is possible that other factors such as STH and TSH contained therein, act synergistically and enhance the effect of the gonadotropin.

5. Fractionated salmon pituitary gonadotropin, mammalian LH and whole salmon extract restore the activity of the lobule boundary cells of hypophysectomised fish.

6. Temperature is a far more important factor than light in the regulation of the testicular cycle and the variations in this environmental parameter has different actions at different phases of the spermatogenetic process.

7. Spermiation is speeded by rise in temperature and is probably autonomous of pituitary control.

8. Pycnotic degeneration of excess and precocious later generation of secondary spermatogonia and of primary
spermatocytes is a regular feature of the annual cycle of this fish. This characteristic can be used in finding whether a fish has spawned or not.

CONCLUSIONS

Integration of the present findings suggest that the testicular cycle in *Couesius plumbeus* has a dual basis reflected in its controlling mechanism which is partly endogenous (gonadotropin, similar in character to mammalian LH) and in part exogenous (variations in the water temperature). An interplay of both these factors is essential for spermatogonial multiplication, spermatocyte formation and completion of the maturation stages. The annual testicular cycle of *Couesius*, is closely related to changes in the environmental conditions, and mediated by the pituitary gland whose own secretory activity is regulated by these changes (of which temperature is the most important). Stimulation of testes of hypophysectomised fish is brought about by fish gonadotropin and mammalian LH, but mammalian FSH is ineffective. Response to replacement therapy is maximum during the prespawning period and least immediately after the spawning; and maximum testicular regression is noted when pituitary is removed at the peak of gonadal activity. Temperature is the most important exogenous regulator of testicular cycle. Low temperatures facilitate spermatogonial multiplication and high temperatures are essential for spermiogenesis and spermiation. Temperature effects are rarely overridden by photoperiod. An internal rhythm, completely suppressed at high temperatures, may be a contributory factor in the regulation of the testicular cycle in this species.
LITERATURE CITED


------, 1940. Some further experiments on the relation of the external environment to spermatogenetic cycle of Fundulus heteroclitus (L.) Bull. Mt. Desert Island Biol. Lab, 20: 2-1


________, 1954. The response of hypophysectomised male killifish to purified growth hormone as compared with the response to purified beef growth hormone. Endoc. 55: 274-287.


- 121 -


- 121 -


Table I. Summary of lipid stain and cholesterol test results on normal Couesius testis

<table>
<thead>
<tr>
<th>Month 1963</th>
<th>Lipids Sudan Black B Stain</th>
<th>Cholesterol test</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>Diffuse lipoidal granules</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lobule boundary cell lipoidal</td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>March</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>May</td>
<td>Little evidence of lipoidal substance</td>
<td>-</td>
</tr>
<tr>
<td>June</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>July</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>August</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>September</td>
<td>Diffuse lipoidal granules</td>
<td>-/+</td>
</tr>
<tr>
<td></td>
<td>Lobule boundary cells lipoidal</td>
<td></td>
</tr>
<tr>
<td>October</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>November</td>
<td>&quot;</td>
<td>+</td>
</tr>
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</table>
Table II. Effect of hypophysectomy on the testes in *C. plumbeus*.
Figures under spermatogenetic condition are the percentage composition of various spermatogenetic stages from planimetry.

<table>
<thead>
<tr>
<th>Date hypophysectomised</th>
<th>Date of Expt.</th>
<th>Spermatogenetic condition in hypophysectomised fish</th>
<th>Spermatogenetic condition of control fish</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>SPGR</td>
<td>SPG'x</td>
</tr>
<tr>
<td>1962</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>July 14-19</td>
<td>Aug. 31</td>
<td>46</td>
<td>56.3</td>
</tr>
<tr>
<td>Aug. 15-19</td>
<td>Oct. 2</td>
<td>46</td>
<td>49.6</td>
</tr>
<tr>
<td>Sept. 3-7</td>
<td>Nov. 1</td>
<td>56</td>
<td>47.5</td>
</tr>
<tr>
<td>Oct. 23-26</td>
<td>Dec. 19</td>
<td>56</td>
<td>66.5</td>
</tr>
<tr>
<td>Nov. 23-26</td>
<td>Jan. 26</td>
<td>63</td>
<td>52.0</td>
</tr>
<tr>
<td>1963</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jan. 3-5</td>
<td>Feb. 25</td>
<td>52</td>
<td>31.3</td>
</tr>
<tr>
<td>Feb. 3-6</td>
<td>Mar. 23</td>
<td>47</td>
<td>31.7</td>
</tr>
<tr>
<td>Mar. 5-7</td>
<td>May 2</td>
<td>58</td>
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<td>May 2-4</td>
<td>June 30</td>
<td>57</td>
<td>40.7</td>
</tr>
<tr>
<td>June 6-8</td>
<td>July 31</td>
<td>54</td>
<td>24.8</td>
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<td>July 3-5</td>
<td>Aug. 27</td>
<td>53</td>
<td>32.0</td>
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<td>Aug. 12-14</td>
<td>Oct. 8</td>
<td>55</td>
<td>42.0</td>
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<td>Sept. 5-7</td>
<td>Oct. 28</td>
<td>52</td>
<td>30.3</td>
</tr>
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<td>Oct. 11-13</td>
<td>Dec. 5</td>
<td>53</td>
<td>25.0</td>
</tr>
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Table III. Effect of hypophysectomy on the testes in *C. plumbeus* as measured by gonosomatic indices (GSI) and cholesterol test.

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<tr>
<th>Expts.</th>
<th>GSI Hx</th>
<th>Control</th>
<th>Cholesterol Test</th>
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<tr>
<td>July</td>
<td>0.4701</td>
<td>0.5423</td>
<td>-</td>
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<tr>
<td>August</td>
<td>0.5043</td>
<td>0.5385</td>
<td>-</td>
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<tr>
<td>Sept.</td>
<td>0.4934</td>
<td>0.8121</td>
<td>-</td>
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<tr>
<td>Oct.</td>
<td>0.4689</td>
<td>0.7898</td>
<td>+</td>
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<tr>
<td>Nov.</td>
<td>0.4916</td>
<td>0.7525</td>
<td>+</td>
</tr>
<tr>
<td>1963</td>
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<tr>
<td>Jan.</td>
<td>0.4694</td>
<td>0.9154</td>
<td>+</td>
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<tr>
<td>Feb.</td>
<td>0.3856</td>
<td>0.8317</td>
<td>+</td>
</tr>
<tr>
<td>March</td>
<td>0.3641</td>
<td>0.8720</td>
<td>+</td>
</tr>
<tr>
<td>May</td>
<td>0.2104</td>
<td>0.6903</td>
<td>-</td>
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<tr>
<td>June</td>
<td>0.411</td>
<td>0.7460</td>
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<tr>
<td>July</td>
<td>0.525</td>
<td>0.8568</td>
<td>-</td>
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<tr>
<td>August</td>
<td>0.404</td>
<td>0.8521</td>
<td>-</td>
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<tr>
<td>Sept.</td>
<td>0.460</td>
<td>0.899</td>
<td>+</td>
</tr>
<tr>
<td>Oct.</td>
<td>0.4226</td>
<td>0.8573</td>
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Table IV. Summary of effect of replacement therapy with mammalian purified follicle stimulating hormone (FSH). Symbols as in previous table.

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<td></td>
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<tr>
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<td>Nov. 1963</td>
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<td>48.6</td>
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<tr>
<td>1963</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>47</td>
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<td>0.8317</td>
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<td>16.4</td>
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<td>May</td>
<td>58</td>
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<td>July 31</td>
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</table>
## Table V. Summary of the effect of replacement therapy with mammalian purified luteinizing hormone (LH).

Symbols as in Table II. The upper row denoted by H indicates condition of treated fish at high temperature, the lower row L that at low temperature.

<table>
<thead>
<tr>
<th>Month of Expt.</th>
<th>Month of Autopsy</th>
<th>Expt. Days</th>
<th>Hx</th>
<th>Control</th>
<th>Treated</th>
<th>SPGR</th>
<th>SPG'X</th>
<th>SPG&quot;</th>
<th>SPG&quot;x</th>
<th>SPC'</th>
<th>SPC'x</th>
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</thead>
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Spermatogenetic condition in treated fish.

Cholesterol in treated fish.
Table VI. Summary of the effect of replacement therapy with fractionated salmon pituitary gonadotropin. Symbols as in Table II. In May 1963 and subsequent experiments the upper row denoted by H indicates condition of treated fish at high temperature, the lower row L that at low temperature.

<table>
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<tr>
<th>Month of Expt.</th>
<th>Month of Autopsy</th>
<th>Expt. Days</th>
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<th>Control</th>
<th>Treated</th>
<th>Spermatogenetic condition in treated fish</th>
<th>Cholesterol in treated fish</th>
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<td>SPG'x</td>
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Table VIII. Exploratory Photoperiod Experiment
Fish in Experimental Condition - January 1, 1963.

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<th>Gonosomatic Indices</th>
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<td></td>
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<td>I. 1963</td>
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<tr>
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<td></td>
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<td>II.</td>
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Table IX. Photoperiod-temperature Experiment: Effect of Photoperiod-temperature Combination on Prespawning Stock - Fish in Experimental Condition - April 30, 1963.

L-L\textcircled{O}, Long photoperiod, S-L\textcircled{O}, Short photoperiod, Low temperature; L-H\textcircled{O}, Long photoperiod, S-H\textcircled{O}, Short photoperiod, High temperature; Other Symbols as in Table II.

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Table X. Summary of the Photoperiod-temperature Experiment: Effect on Postspawned Stock.
Fish in Experimental Condition from August 9, 1963.
Symbols as in Table II & IX.

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Table XI. Summary of the Photoperiod-temperature Experiment: Effect on Pre-freeze up Stock.
Fish in Experimental Condition from November 1, 1963.
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