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PEABODY MUSEUM OF NATURAL HISTORY
YALE UNIVERSITY

VOLUME XIV, ARTICLE 2

FISH ENDOCRINOLOGY

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Issued December 1953
New Haven, Conn., U. S. A.

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CONTENTS

A STUDY OF THE HYPOPHYSECTOMIZED MALE KILLIFISH, *FUNDULUS HETEROCLITUS* (LINN.)[1]

By

GRACE E. PICKFORD

Bingham Oceanographic Laboratory
Yale University

ABSTRACT

The following observations, made on male *Fundulus heteroclitus*, show that growth in length ceases after hypophysectomy, weight changes are irregular, no new circuli are added to the scales, and no new deposition is laid down on the otoliths. In normal wild males the liver is large in autumn and small in spring; hypophysectomy when livers are large prevents reduction in liver size whereas hypophysectomy when the liver is small results in a return to the autumnal condition. Hepatic fat and glycogen are abundant, and a ceroid-like material is present in normal minimal amounts. Testes are in complete regression, only the spermatogonial zone of the cortex being unaffected; nuptial colors are lacking. Hypophysectomized fish frequently develop renal calculi composed chiefly of carbonate apatite. After removal of the pituitary, this normally euryhaline species is unable to survive in fresh or diluted sea water. Thyroids are inactive, but no effects were noted on the pancreatic islets, Stannius corpuscles, or adrenal cortical (Giacomini) tissue. The fish are anemic.

INTRODUCTION

The literature concerning the effects of hypophysectomy on teleost fishes is comparatively small. Little is known regarding the possible physiological disturbances resulting from a deficiency of the posterior lobe hormones. After hypophysectomy, eels can survive either in fresh or salt water and can withstand abrupt transference from one medium to the other (Fontaine, et. al., 1949, 1950; Callamand, et al., 1951). The operation appears to have little or no effect on the osmoregulatory capacity of the fish. These experiments do not exclude participation of the adrenal cortical system.

Parker (1948) has reviewed the literature on the role of the intermediate lobe in the regulation of color responses. In *Fundulus*, melanophore expansion is under nervous control and innervated melanophores are unresponsive to intermedin, but denervated tail

[1] The completion of this investigation has been part of a project supported by a grant from the National Science Foundation (NSF-G50).

bands exhibit the expected darkening. The presence of the pituitary is probably necessary for melanogenesis, as it is in *Ameiurus* (Osborn, 1941) and the elver (Vilter, 1945, 1946a). The retina of the hypophysectomized eel is permanently in a state of light adaptation (Vilter, 1942, 1946b) and this problem calls for further investigation. In contrast to the melanophores, the xanthophores of *Fundulus* are responsive to intermedin, and hypophysectomy abolishes or impairs the yellow background response (Fries, 1943). It is well known that injections of crude pituitary extracts induce pallor in *Fundulus*, as in many species of fish, and Healey (1939, 1948) has shown that in *Phoxinus* this response is associated with an anterior lobe hormone. Anterior lobectomy abolishes the light background response of denervated regions.

In respect to the anterior lobe hormones, most work has been directed towards the gonadotrophins. It is known that hypophysectomy arrests sexual development in young fish and leads to a regression of the gonads in adults (*Xiphophorus:* Regnier, 1938; *Gobius:* Vivien, 1938, 1939, 1941; *Gobius* and *Ameiurus*, Buser-Lahaye, 1953). No corpora lutea were produced in the ovary of a hypophysectomized female *Rhodeus* (Bretschneider and Duyvené de Wit, 1947). There has been some uncertainty as to whether or not somatic growth can take place after the removal of the pituitary in fishes. Regnier (1938) found that growth ceased in hypophysectomized *Xiphophorus*, but according to Vivien (1938, 1939, 1941), hypophysectomized *Gobius* of either sex resume normal growth after a period of retardation. Vivien has expressed some doubts as to the validity of his results (see Pickford and Thompson, 1948), and Buser (1950) has shown that gobies fail to regenerate amputated fins after removal of the pituitary. This work is elaborated in a later study (Buser-Lahaye, 1953). The thyrotrophic hormone is undoubtedly present, and Vivien (1941) has shown that in *Gobius* hypophysectomy leads to the expected inactivation of the thyroid. This has been confirmed by Buser-Lahaye (1953). On the other hand there are no investigations concerning the effects of pituary deficiency on the adrenal cortical tissue. Laur (1950) has shown that hypophysectomy changes the response of the blood to adrenalin in a direction opposite to that which occurs in mammals; in normal fish (*Cyprinus, Anguilla*) there is a decrease and in hypophysectomized fish an increase in the numbers of red cells in circulation. One investigator (Hatey, 1951a, 1951b) has studied the effects of hypophysectomy on hepatic glycogenesis. Hypophysectomy leads to a progressive loss of liver glycogen in starving eels, but the response to injections of glucose showed that the ability of the liver to synthesize glycogen from exogenous sources is not impaired. In line with work on higher vertebrates, it is supposed that the lack of anterior lobe hormones, which directly or indirectly mediate the transformation of endogenous fat and protein to sugar, renders neoglycogenesis impossible in the hypophysectomized animal.

The present investigations were undertaken to clarify some, but not all, of the problems outlined above. In particular we were anxious to find out whether the pituitary controls somatic growth and the formation of year marks on the scales and otoliths. It had been shown previously (Pickford and Thompson, 1948; Jampolsky, 1949) that fish will respond to injections of mammalian pituitary growth hormone. Therefore, in agreement with Regnier (1938) and contrary to results reported by Vivien (1938, 1939, 1941), it was anticipated that hypophysectomized fish would not grow. Furthermore, since hypophysectomized male killifish are being used in this laboratory for the study of various aspects of fish endocrinology, it became necessary to establish basic information concerning the condition of the internal organs. Therefore the primary target organs of the anterior lobe hormones were examined and, as a result of collateral observations, special attention has been paid to the striking effects of hypophysectomy on the liver and kidney. Preliminary results have been partially reported in abstracts (Pickford, 1950, 1951, 1952a, 1953b).

I should like to express my thanks to Miss Harriet A. Chambers, who assisted in most phases of the investigation, Messrs. George M. Stone (Yale 1949), Maxwell D. Lai (Yale 1950), A. Charles Laws (Yale 1954), Mmes. Shirley M. Conover, Joan Grant, Joyce Lownsbery, and Miss C. M. Marzullo, all of whom participated at various times in the care of the fish, preparation of the tissues, or tabulation of the data. I am indebted to Mr. C. W. Coates, Curator and Aquarist, and Dr. Ross F. Nigrelli, Parasitologist, New York Zoological Society, for helpful advice on the care and treatment of the fish. A preliminary examination of the renal calculi was made with the help of Drs. John Heller and A. H. Jansen, Yale Medical School. The material was positively identified by Dr. Clifford B. Frondel of the Department of Mineralogy and Petrography, Harvard University, who made an X-ray diffraction study of the concretions. Dr. S. L. Palay, Depart-

ment of Anatomy, Yale University, has sectioned the brains of two hypophysectomized fish and has submitted a note on the results of his investigation (Palay, 1953). The photographs (Figs. 2–5) were made with the help of Prof. A. Petrunkevitch, Osborn Zoological Laboratory, Yale University. The manuscript has been read by Miss Priscilla Rasquin, Department of Fishes and Aquatic Biology of the American Museum of Natural History, and by Mr. James W. Atz, Assistant Curator of the New York Aquarium; I am indebted to both of these critics for many helpful suggestions. Facilities for collecting sea water and specimens were provided by Dr. Victor Loosanoff, director of the U. S. Fish and Wildlife Service Laboratory at Milford, Conn. Finally I should like to express my thanks to the staff of the Bungham Oceanographic Laboratory and especially to its Director, Dr. Daniel Merriman.

MATERIALS AND METHODS

The experiments reported in this article are part of a project which was initiated in 1947 and which is still in active progress. As a matter of convenience, each experiment was given a series number, in Roman numerals, at the start of the investigation. This number, which bears no relation to the subject matter of the particular experiment, is correlated only with its sequence in time. Series IV and VI, with which this article is primarily concerned, were studies of the growth of hypophysectomized male killifish as compared with that of unoperated and mock-operated controls. The tissues of 17 representative fish from Series IV were studied histologically. Series V, a study of the wild population in spring and autumn, provided basic information on the normal condition of the endocrine glands and other organs at two different seasons of the year. Series VIIA is a further study of the effects of hypophysectomy on the liver size, while Series VIIB (Pickford, 1952a) is a pilot experiment concerned with the effect of male hormone and of thyroxin on the liver size in hypophysectomized fish. Series VIII (Pickford, 1952b), which arose as an outgrowth of findings concerning liver pathology, is a study of the effects of acriflavine, used in the control of an infectious epidemic. Series IX and XI, which will not be referred to in this article, are test experiments with purified fish pituitary fractions. Series X, which arose from the attempt to prevent the development of kidney stones after removal of the pituitary, is a study of the viability of hypophysectomized killifish in fresh water. Series XII was a preliminary and unsuccessful attempt to control the development of kidney stones with injections of hyaluronidase. Series XIII, which brings the project up to date, included some experiments with injections of mammalian FSH.

Because of the enormous enlargement of the ovaries in sexually mature females, only male fish were used in these experiments in order to avoid complications in the interpretation of weight changes and indices of organ size. This procedure proved useful since the fading of the nuptial coloration in males is an external criterion of successful hypophysectomy. Nearly all fish were in their second year and were 2¾ to 3¼ inches in total length at the start of the experiment. A maximum of seven or eight fish was kept in each aquarium in 15 gallons of sea water provided with aeration and an air-lift filtration system. The tanks were illuminated by fluorescent lights for approximately eight hours each day. In Series IV and VI, the temperature, which was not regulated, fluctuated from a low of 11° C in midwinter to a high of 22° C in July. In Series VIIA and Series X the aquaria were regulated at 20° C.

Hypophysectomy was performed by a modification of the method described by Abramowitz (1937). The fish, anaesthetized with tricaine methanesulfonate (MS 222, 0.2% stock solution diluted 1:6 for use), was weighed to the nearest 0.05g and measured to the nearest 0.5 mm (total length). Sample scales were taken from the flank. The fish was then placed in a suitable groove on an operating board provided with drainage holes as well as studs for the elastic bands used to hold the fish in place. A stream of oxygenated water was allowed to flow over the gills from a small rubber tube inserted in the mouth; this served to prevent anoxia and to wash away blood and bone chips during the operation. The operculum was held open with a bent stainless steel insect pin attached to a rubber band, and the gill arches were held back by a similar device. The roof of the mouth was then visible and the pituitary could usually be seen through the bone with the aid of a bright light. An incision was made through the skin and a small circular hole was drilled through the parasphenoid bone with a No. 3 dental burr. The pituitary, thus exposed, could be removed by suction. In mock operations the gland was similarly exposed but not removed.

All fish were marked with colored beads sewn on with tantalum wire as described previously (Pickford and Thompson, 1948). However, in later experiments a heavier gauge (0.007 inch) was used, since beads were frequently lost with very thin wire.

The fish were fed daily with cooked liver-pablum (Gordon, 1943) unless otherwise indicated. In Series IV this was the only food given, but in subsequent experiments various changes and additions were introduced. Series VI received a supplement of chopped raw liver or clams about twice a week. In Series VII and all subsequent experiments the diet was supplemented with frozen raw daphnia, offered two or three times a week. Also at this time it became evident from the study of thyroid sections that the Series IV fish were suffering from a low to moderate degree of thyroid hyperplasia. Beginning with Series VII a trace of iodine[2] was added to the liver-pablum and no further trouble was experienced, with or without daphnia supplement.

In Series IV and to some extent in later experiments, trouble was experienced with an unidentified epidemic somewhat similar in its symptoms to that described by Wells and ZoBell (1934). In Series IV, sulfamerazine was added to the food for three weeks at the beginning of the experiment without beneficial results. Following this, neutral acriflavine was added to the water at infrequent intervals. Subsequently a special experiment (Series VIII) was set up to determine whether continous treatment with acriflavine would have any undesirable effects. The results of this study (Pickford, 1952b) have shown that in normal fish there is some retardation of growth and that the testes are markedly inhibited when the liver-pablum is not supplemented with raw daphnia. However, in the experiments which are the subject of the present report, the treatment was intermittent, and moreover, any retardation would presumably have affected all fish in a similar manner.

The fish were weighed and measured at approximately monthly (Series IV) or regular weekly intervals (Series VI). At autopsy they were again weighed and measured, the scales were sampled, and tissues were taken for histological study. In mock-operated and hypophysectomized fish the heads were fixed and subsequently examined by dissection to confirm the presence or absence of the pituitary.

Scales were stained with alizarin red and mounted in glycerin by

[2] Stock solution: 1% I_2 in 2% KI, aqueous. For use, dilute 1 : 99 with water and add 1cc per 100g of liver-pablum. The diluted solution does not keep.

the double coverslip method; a spring clip was used on the cover glass to keep the scales flat until the damar hardened. Otoliths, preserved in a mixture of 7 parts 95% alcohol and 3 parts glycerine, were soaked for two days in 5% KOH to remove adherent soft tissue, after which they were returned to the preserving fluid for examination.

The liver was weighed and its size recorded in terms of percentage body weight (hepatosomatic index). Except in Series IV, the color was noted against the Ridgway color chart (1886). In Series IV, liver slices were fixed in 10% formalin for fat, in 95% alcohol for glycogen, and in sea water Bouin for routine survey. This procedure was modified in Series V and later experiments, Baker's calcium formal being used for fat and 10% formaldehyde in 95% alcohol for glycogen. Fat was estimated on frozen sections cut at 15μ and stained with scarlet red and hematoxylin; in later experiments sudan black was used. Control sections were treated for one hour with scarlet red (or sudan black) in 100% alcohol. It was found that, for histological purposes, the very soluble fish fats are removed as effectively from liver sections by this procedure as by soaking for two days in ether. Glycogen was similarly estimated on paraffin sections stained with Best's carmine and counterstained with hematoxylin. In later experiments the periodic-Schiff method was used under standard conditions, which included temperature control at 25° C during the staining period, a procedure that was found necessary to obtain reproducible results. The controls were digested with buffered malt diastase in neutral saline (Lillie, 1948).

The thyroid, fixed in sea water Bouin, was studied on serial sections of the hypobranchial region. Alternate slides were stained with hematoxylin-eosin and by a modified Azan procedure in which the azocarmine G solution was used at room temperature with excellent results. Sections were studied at 200μ intervals. A single cell from each follicle was measured and the average follicle height in all cases is the mean of 100 measurements. In expressing the results for an individual fish, the standard deviation is given for 100 cells. In group averages the standard deviation is derived from the differences between the individual and group means. Another useful measure of the activity of the gland is provided by the percentage of follicles with cells over 12μ in height. This percentage, taken together with the average cell height, gives a clear picture of the state of the follicle epithelium.

The staining reactions of the thyroid colloid also provide important information on the condition of the gland. In accordance with classical concepts, and despite the scepticism of some recent investigators (De Robertis, 1949; Mayer, 1949), a clear distinction could be made between "resting" and "active" colloid by use of the Azan method. Since the fish thyroid usually contains follicles in all stages of activity, it was found convenient to divide the glands into three somewhat arbitrary groups: (1) Resting glands in which the majority of follicles contained a clear homogenous colloid stained red by Azan; (2) active glands in which the majority of follicles contained a granular colloid that stained mauve or blue with Azan; and (3) glands in which the follicles were predominantly in a transitional condition, the homogenous colloid being partially eroded and more or less completely transformed into the granular condition. Granulation can be recognized, though less strikingly, on hematoxylin-eosin preparations in which the colloid is unstained or only faintly colored. Granulation and the associated change in staining reaction is therefore not an artifact of the Azan staining procedure.

The two testes were weighed and their relative size recorded in terms of percentage body weight (gonosomatic index). They were then fixed in sea water Bouin. The principle islet of the pancreas was fixed in Helly's fluid and sections were stained with hematoxylin-eosin, Mallory or Azan. One of the Stannius bodies was similarly treated and, when possible, a second gland from the same fish was fixed in Flemming's fluid. The right head kidney, containing Giacomini (adrenal) tissue in the wall of the posterior cardinal vein, was sliced and fixed in various fluids, of which Helly, followed by hematoxylin-eosin or Azan, gave the most satisfactory results.

EFFECTS ON GROWTH

Length and Weight. The results of Series IV are given in Table I and are shown graphically in Fig. 1. It is quite evident that hypophysectomized fish do not increase in length. Small oscillations in the mean value, of the order of 1%, were undoubtedly due to the difficulty of accurate length measurement within ± 0.5 mm and are not statistically significant. The weight changes of these fish were more irregular. During the first two months there was an average increase of the same order as that in the controls, but subsequently the operated fish began to lose weight except for individual cases that developed edema. For some reason the unoperated controls did not grow as

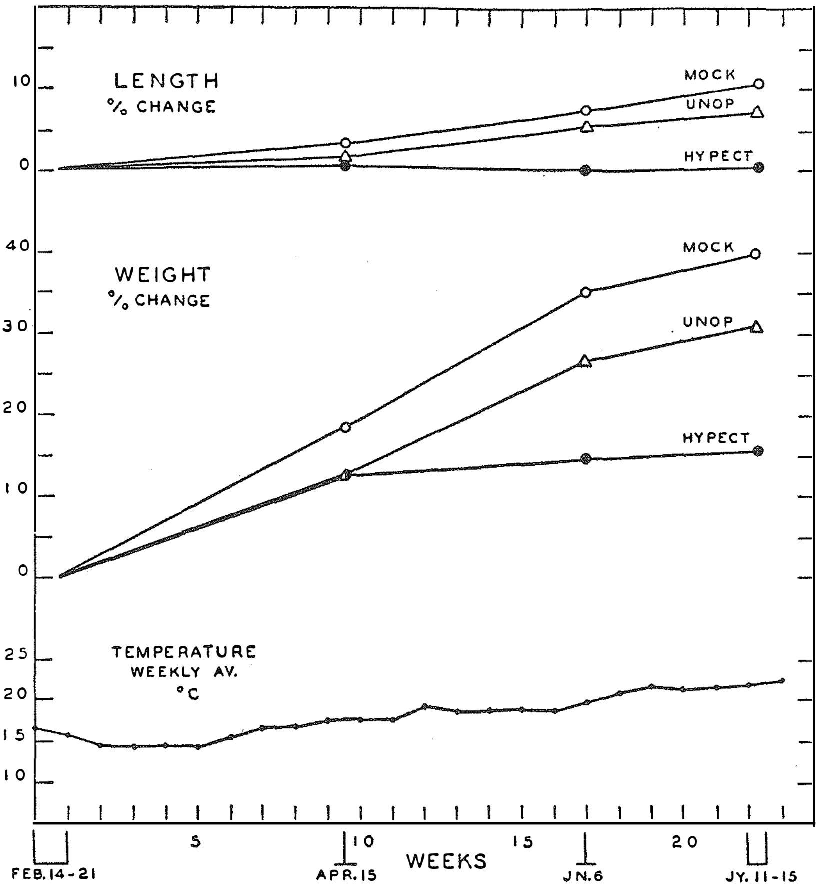

FIGURE 1. Percentage growth changes of mock-operated, unoperated, and hypophysectomized male *Fundulus*, 1949. Series IV.

rapidly as the mock-operated fish. The difference is statistically significant in respect to length but not in respect to weight. However, inspection of the graph reveals that the two control groups ran parallel after April 15, one below the other. Apparently the onset of spring growth was delayed in the unoperated fish, but once growth had commenced it progressed at approximately the same rate as in the mock-operated fish. This interpretation is substantiated by a comparison of growth changes between April 15 and June 6 (Table II). The differences between the unoperated and mock-operated fish are not statistically significant either in respect to length or weight.

TABLE I. AVERAGE PERCENTAGE WEIGHT AND LENGTH CHANGES OF EXPERIMENTAL FISH, REFERRED TO FEBRUARY 12–14, 1949 BASE LINE. SERIES IV.

Operation	*April 15*		*June 6*		*July 10–15*	
	No. of fish	*Percent increase*	*No. of fish*	*Percent increase*	*No. of fish*	*Percent increase*
Weight						
None	15	12.5±13.2†	15	26.6±17.1	6	30.7± 6.8
Mock	25	18.5±12.8	25	35.0±16.6	6	40.0± 4.8
Hypect.	15	12.5± 8.4	10	4.6±13.8	4*	5.6±11.1
Length						
None	15	1.8± 1.6	15	5.6± 3.0	6	8.1± 4.4
Mock	25	3.3± 1.9	25	7.8± 3.0	6	10.8± 3.0
Hypect.	15	0.8± 0.8	10	0.3± 1.1	5	0.6± 0.6

* By some oversight one of the five fish in this group was not weighed.
† In this and subsequent tables, ± is standard deviation, not standard error.

The delayed onset of growth in the unoperated group may have been due to chance differences in conditions between the different aquaria, but it was thought that the mock operation might have stimulated a premature release of pituitary hormones. A second experiment (Series VI, Table III, Fig. 2) did not confirm this hypothesis. Differences in the growth rates of mock-operated and unoperated fish are not statistically significant. It is concluded that the anomalous results of Series IV were due to undetermined causes irrelevent to the main purpose of the investigation. Nevertheless the experience serves as a warning in the design of future experiments.[3]

TABLE II. COMPARISON OF GROWTH CHANGES BETWEEN APRIL 15 AND JUNE 6, 1949. SERIES IV.

Operation	*Weight % change*	*Length % change*
None	12.0±11.3	3.8±2.3
Mock	14.1±12.8	4.4±2.1
Hypect.	− 5.4±11.7	−0.5±1.2

[3] It has been suggested by James W. Atz, who kindly read this manuscript, that the time of the mock operation may have affected the results of the experiments. The Series IV fish were operated early in February at a time when the nuptial colors were just beginning to develop (± or +). This indicates that the spring release of pituitary gonadotrophins, and by inference of other anterior lobe hormones, was in progress. The Series VI fish were operated in December when the nuptial colors were either wholly lacking or, in a few cases, just faintly evident (±). It is possible, therefore, that a mock operation performed in February might more easily have stimulated the premature initiation of renewed growth.

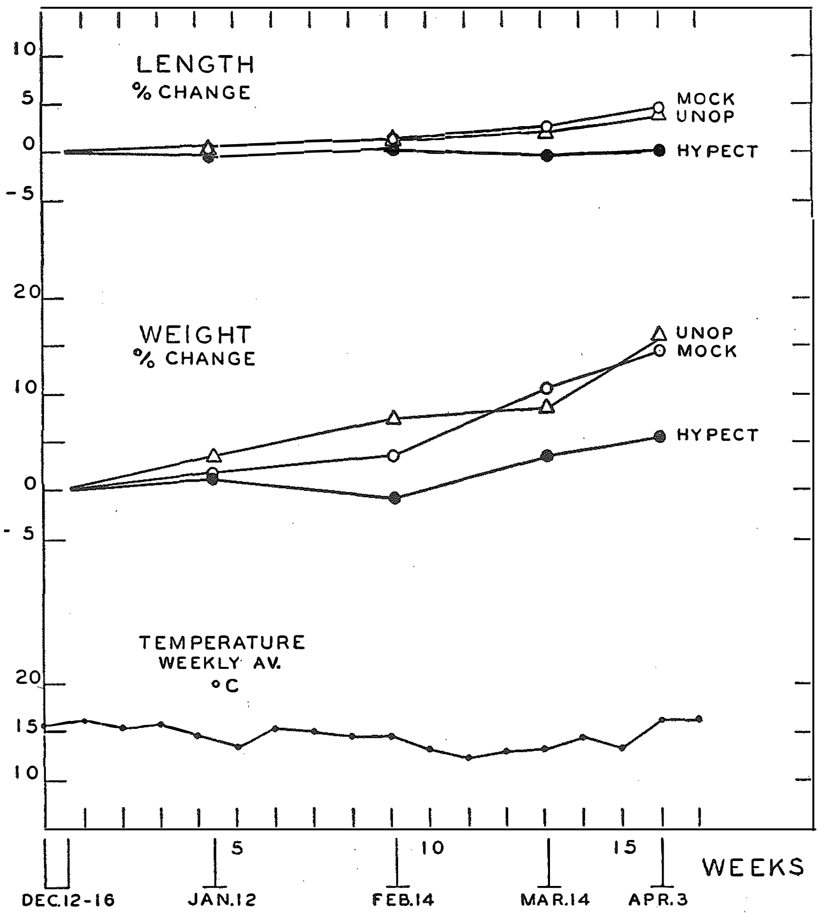

FIGURE 2. Percentage growth changes of mock-operated, unoperated, and hypophysectomized male *Fundulus*, 1949–1950. Series VI.

The fact that hypophysectomized fish do not grow in length and show only irregular changes in weight was again confirmed in a third experiment (Series VIIA, Table IV). This series received the improved diet noted previously, and the growth rate of the controls was much greater than in Series IV and VI.

Weight changes after hypophysectomy depend on a variety of factors. According to data presented below, regression of the testes would account for a 4–5% loss, whereas enlargement of the liver

TABLE III. Average Percentage Weight and Length Changes of Experimental Fish, Referred to December 12–16, 1949 Base Line. Series VI.

Operation	*No. of fish*	*Jan. 12*	*Feb. 14*	*Mar. 14*	*Apr. 3*
Weight					
None	19	−1.5±4.8	2.3±6.6	3.7±6.8	11.0± 6.7
Mock	18	−3.8±5.5	−1.3±8.2	5.5±9.8	9.5±12.0
Hypect.	20	−3.1±4.2	−5.8±7.8	−1.5±8.6	0.3± 9.6
Length					
None	19	0.2±0.6	1.2±1.1	2.1±1.3	3.8± 1.5
Mock	18	0.3±0.7	1.4±1.2	2.5±1.6	4.4± 2.1
Hypect.	20	−0.4±0.7	0.2±1.0	−0.2±1.2	0.1± 1.2

TABLE IV. Average Percentage Changes in Weight and Length Between February 6 and March 28–29, 1951. Series VIIA.

Operation	*No. of fish*	*Weight % change*	*Length % change*
None.	9	37.5± 9.9	5.7±2.0
Hypect.	11	8.4±14.2	−0.3±0.1

would offset this by about 2.4%. Edema resulting from renal calculi is responsible for a marked increase in the apparent weight of many fish, but a more common symptom of urinary lithiasis is progressive emaciation leading to death. The real criterion of the cessation of growth is the failure of the hypophysectomized fish to increase in length.

Scales. At the time of operation, in midwinter, nearly all fish showed a distinct second year mark in the form of a gap followed by one or more irregular or broken circuli close to the margin of the scale. This gap served as a convenient point of reference from which the number of new circuli added during the course of the experiment could be estimated. Fish which did not show this landmark on the scales have not been included in the calculations, since an accurate count of the new circuli was impossible. The results of the Series IV experiment are given in Table V.

In April nearly all control fish showed from 1 to 3 new circuli. By June there was an average increase of 8 to 10 new circuli per fish (compare Figs. 3, C and D). No hypophysectomized fish showed any addition of new circuli. There appeared to be some growth of the fibrous layer which projects beyond the calcified margin of the

TABLE V. AVERAGE NUMBER OF NEW CIRCULI ON SCALES OF EXPERIMENTAL FISH, REFERRED TO FEBRUARY 14–21, 1949 BASE LINE. SERIES IV.

Operation	*April 15* *No. of fish**	*April 15* *New circuli†*	*June 6* *No. of fish*	*June 6* *New circuli*	*July 10–15* *No. of fish*	*July 10–15* *New circuli*
None	10/13	0.7	13/13	4.9	6/6	8.2
Mock	15/20	1.3	20/20	5.9	6/6	10.0
Hypect.	0/14	0	0/10	0	0/5	0

* Number of fish showing new growth on scales/total number examined.

† Average number of new circuli on scales of those fish which showed new scale growth.

scale, but there was no new calcification (compare Figs. 3, A and B). Similar results were obtained in Series VI, which terminated at an earlier date; new growth was registered on the scales of all but one of the control fish whereas in hypophysectomized fish there was no change.

TABLE VI. AVERAGE NUMBER OF NEW CIRCULI ON SCALES OF EXPERIMENTAL FISH, REFERRED TO DECEMBER 12–16, 1949 BASE LINE.* SERIES VI.

Operation	*Jan. 12* *No. of fish*	*Jan. 12* *New circuli*	*Feb. 14* *No. of fish*	*Feb. 14* *New circuli*	*Mar. 14* *No. of fish*	*Mar. 14* *New circuli*	*Apr. 3* *No. of fish*	*Apr. 3* *New circuli*
None	2/19	0.5	9/19	1.0	17/19	1.5	19/19	2.5
Mock	0/18	0	4/18	1.5	11/18	1.5	17/18	2.5
Hypect.	0/20	0	0/20	0	0/20	0	0/20	0

* Explanations as for Table V.

Otoliths. Data on the growth of the otoliths is confined to 17 fish in Series IV that were killed in July. The interpretation of the observations is complicated by two factors. In the first place the sacculith of *Fundulus* shows great individual variation, although the first year mark is usually well defined. In the second place, contrary to the study of scales which may be sampled from time to time, there is no way of knowing the condition of the otolith at the start of the experiment. As in the case of the scales, incipient new growth laid down previous to the time of operation may well have been present at the start of the experiment. Thus the otoliths of four of the hypophysectomized fish showed faint opaque caps at the angles, but it is most improbable that these were formed subsequent to the time of operation. The remaining hypophysectomized fish, and also one of the controls, showed no signs of new growth. The unoperated controls and three of the mock-operated fish showed faint evidence of new deposition that may have been present at the start of the experiment. The three remaining mock-operated controls (fish nos. 2–6, 5–3, and 5–8) had well defined apical caps, more pronounced than anything observed in the other fish and clearly indicative of new growth.

EFFECTS ON THE INTERNAL ORGANS

The effects of hypophysectomy on the internal organs is largely confined to the tissues of 17 fish in Series IV that were killed in July 1949. Where necessary, the findings are interpreted in the light of a study of sample fish taken from the wild population in spring and autumn (Series V) or are supplemented by additional experiments.

Liver. In order to interpret the effects of hypophysectomy it was first necessary to consider the condition of the liver in the normal wild population (Table VII). In autumn the liver of both sexes is

TABLE VII. CONDITION OF THE LIVER IN WILD FISH SAMPLED IN AUTUMN AND IN SPRING. SERIES V.

Date	*Sex*	*No.*	*HSI**	*Usual color*	*Histological rating†* *Glycogen*	*Fat*	*Ceroid*
Nov. 1949	♂	39	6.3±1.3	Pink‡	+++	+++	±
	♀	35	6.4±1.8	Pink‡	+++	+++	±
May 1950	♂	41	2.8±0.7	Red-brown§	+	±	++
	♀	43	4.8±1.0	Red-brown§	++	±	++

* Hepatsomatic index.

† Data for five or six specimens sampled from each group. The rating is subjective, from ± (trace) to +++ (abundant).

‡ Pink: most frequently *pinkish buff, vinaceous pink,* or *vinaceous buff.*

§ Red brown: most frequently *chestnut* or *vinaceous cinnamon.*

large, its color is usually pinkish, and both fat and glycogen are present in abundance. In the spring a marked difference between the sexes becomes apparent. The livers of males show a profound reduction in size, fat reserves are almost totally depleted and, correlated with this, there is a change in color towards deeper shades of reddish brown. The livers of females are much less severely affected and, although the fat content is depleted, the hepatosomatic index remains moderately high.

It is well known that the livers of teleosts undergo a reduction in size, associated with the maturation of the gonads. This subject has

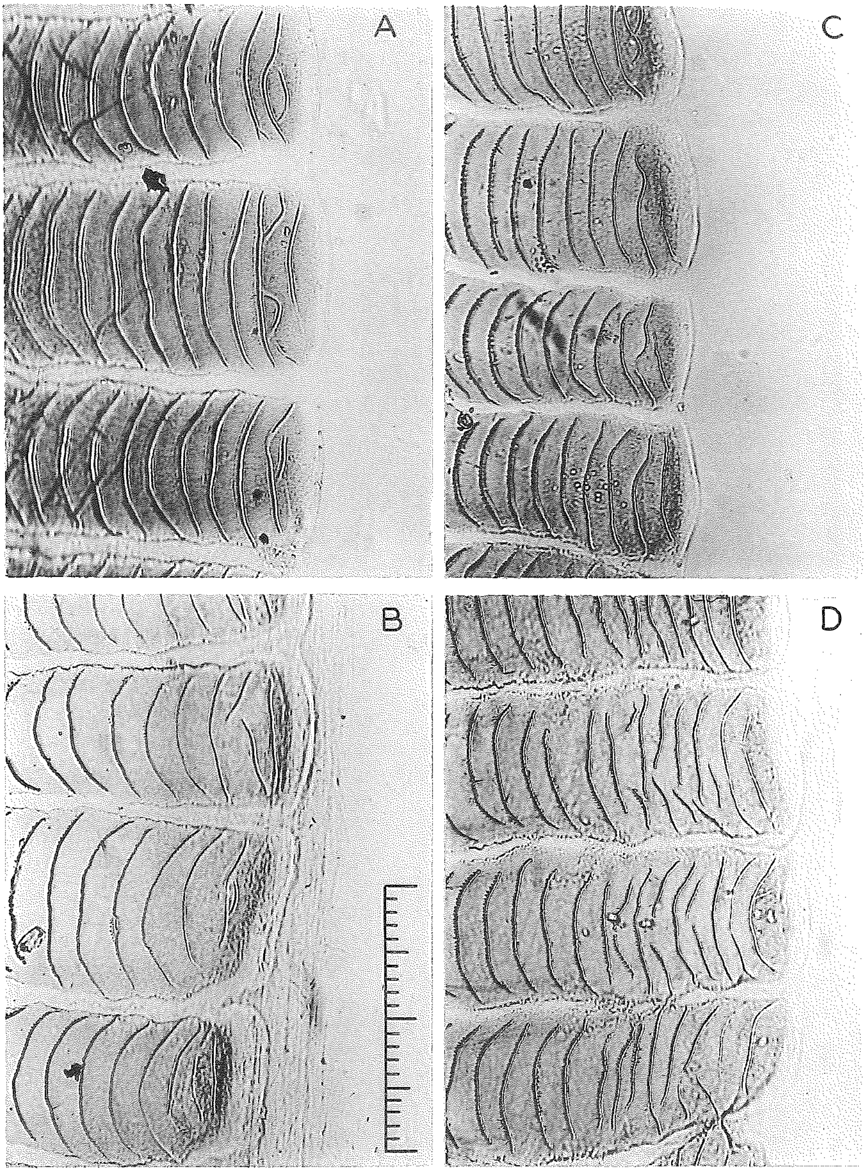

Figure 3. Microphotographs of the margin of scales sampled at the beginning and end of the experiment. Series IV. A and B, a hypophysectomized fish (no. 4-2), February 16 and July 14. C and D, an unoperated control (no. 6-6), February 17 and July 14. Micrometer scale in 10 μ intervals.

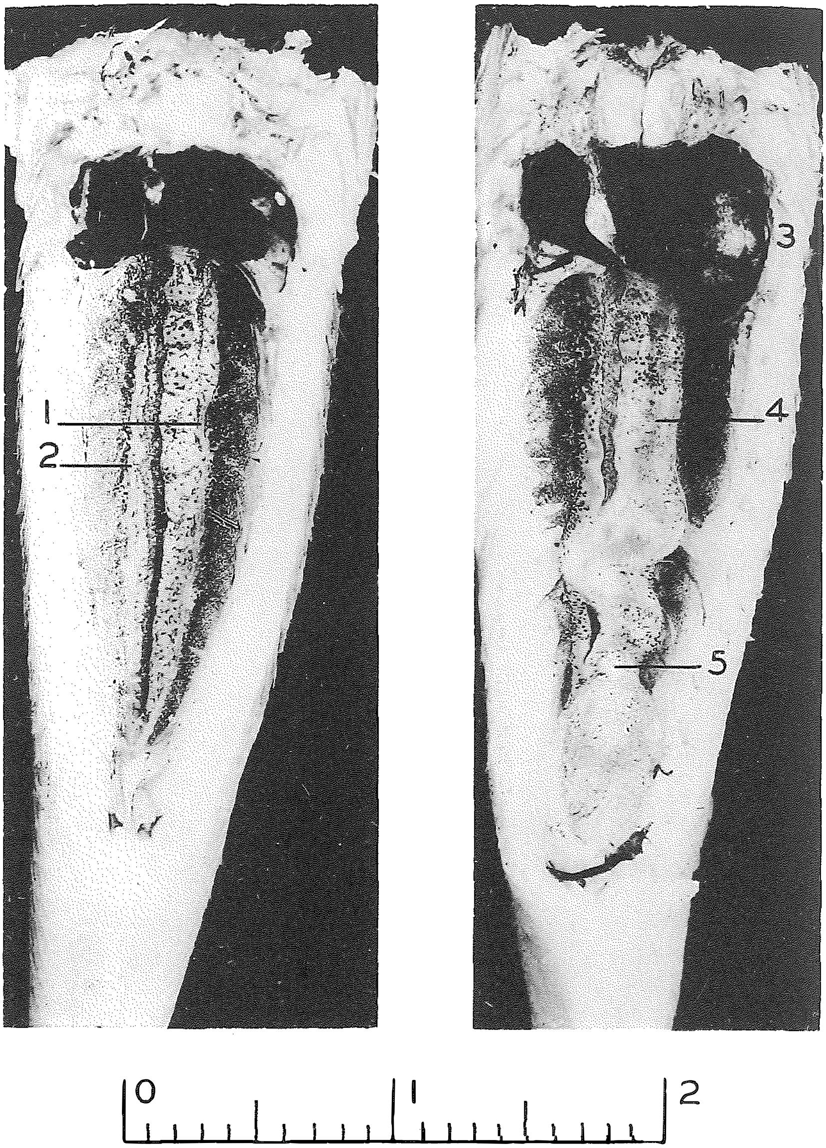

Figure 4. Dissections showing the kidneys and urinary ducts of a normal fish (left) and of an hypophysectomized fish (Series IV, fish 4-6) suffering from renal calculi (right). The abdominal viscera and swim bladder have been removed to expose a ventral view of the urinogenital system. 1 and 2, left and right urinary ducts of normal fish. 3, enlarged left head kidney. 4, greatly distended left urinary duct. 5, calculus. Scale in centimeters.

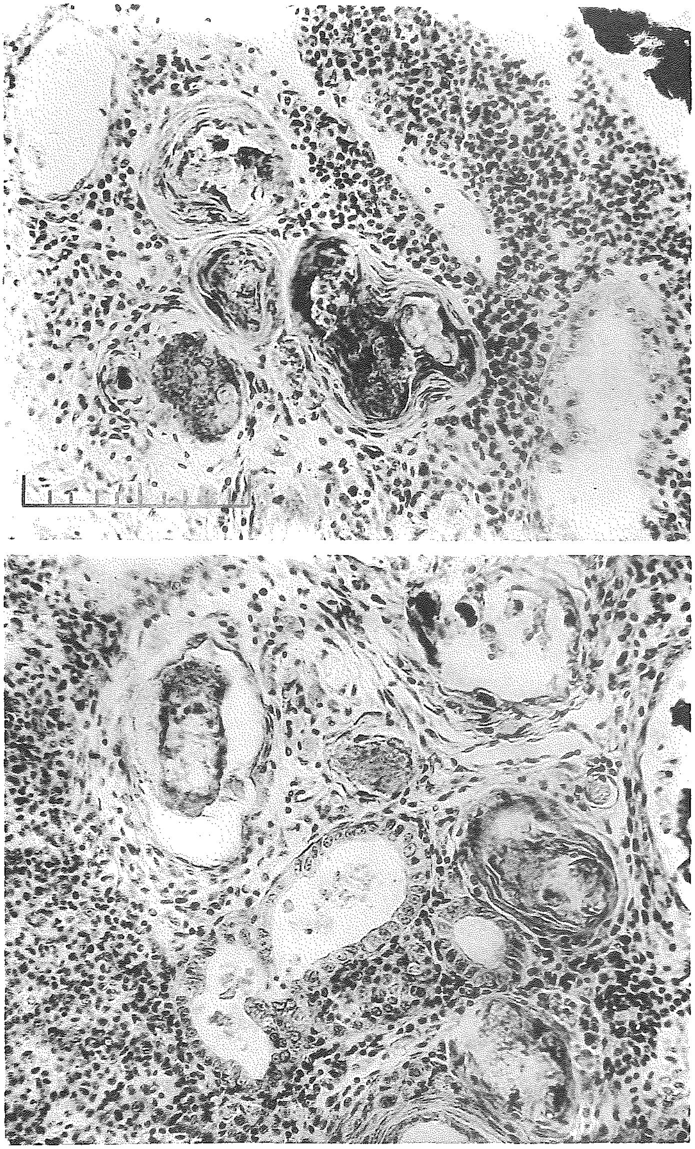

Figure 5. Sections of the head kidney of an hypophysectomized fish suffering from renal calculi (Series IV, fish 4-8). Both pictures show encapsulated concretions occluding the uriniferous tubules; some normal tubules are visible in the lower picture. Micrometer scale in 10μ intervals.

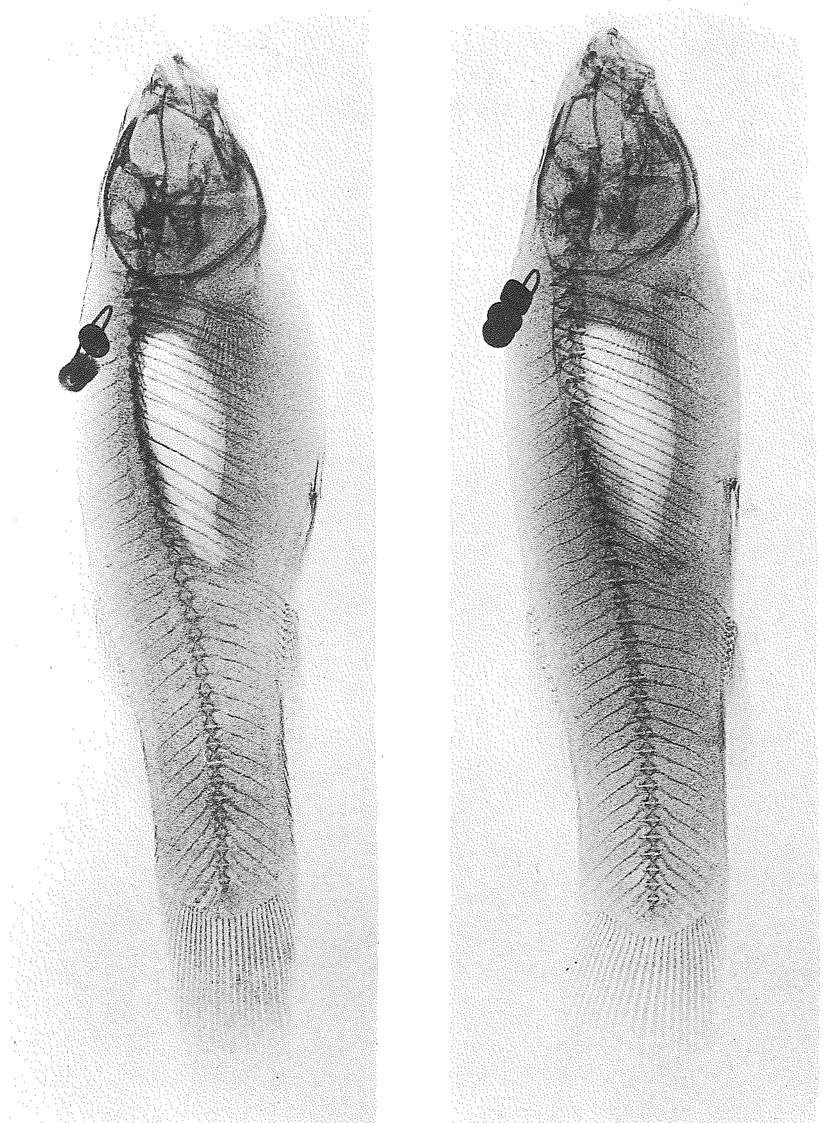

Figure 6. X-ray photographs of two hypophysectomized fish from a later experiment (Series IX). The fish on the left had macroscopically normal kidneys. The one on the right was suffering from renal calculi, and the ventral displacement of the of the swim bladder is clearly visible. (Photographs by Dr. A. H. Jansen).

been reviewed recently by Olivereau and Leloup (1950). Both sexes are usually affected, but in *Mullus barbatus* the liver of females increases *pari passu* with the reproductive organs (Bougis, 1949). The condition in the female *Fundulus* may throw some light on this situation, but the subject requires further investigation.

TABLE VIII. Hepatosomatic Index and Condition of the Liver in Experimental Fish, July 1949. Series IV.

Operation	*Fish*	*HSI*	*Histological rating**		
			Glycogen	*Fat*	*Ceroid*
None	3–5	2.4	+	+	++
	3–8	4.7	++	+++	+
	6–1	1.6	+	0	+
	6–4	2.3	±	+	+
	6–6	1.6	+	0	++
	6–7	1.3	±	0	+
	Average	2.3±1.2			
Mock	1–4	3.2	++	+++	±
	2–6	3.1	0	0	++
	4–4	2.6	++	+++	±
	5–3	2.7	++	+++	++
	5–5	3.5	++	+	+++
	5–8	2.4	0	++	++
	Average	2.9±0.4			
Hypect.	1–7	5.4	+++	+++	+
	1–9	7.4	++	+++	±
	4–2	2.6	+	+	+
	4–6	5.6	+++	++	+
	4–8†	—	+++	+++	+
	Average	5.3±2.0			

* See Table VII.

† By an oversight this fish was not weighed at autopsy, but the liver was undoubtedly large.

The results of Series IV (Table VIII) demonstrated that removal of the pituitary tends to prevent the seasonal reduction in liver size of male fish. With one exception, for which no explanation is forthcoming, the livers of the hypophysectomized fish were large, the average hepatosomatic index approaching that of wild fish in autumn. In order to amplify the data, the average hepatosomatic index was calculated for all 16 hypophysectomized fish, irrespective of date of death. The resultant figure, 5.2 ± 2.0, is close to that derived from the smaller group. This series includes one fish that had a persistent pituitary fragment but a high hepatosomatic index (5.3) and two very emaciated fish that had small livers at time of death (HSI 1.8 and 1.9 respectively). A statistical comparison of this group with the combined mean, 2.6 ± 0.9, for the 12 control fish shows that the difference is highly significant ($P = .001$).

Histological examination showed that the enlarged livers of the hypophysectomized fish that survived to autopsy were laden with fat and glycogen, just as were those of wild fish in autumn. Only the unexplained individual with a low hepatosomatic index showed some depletion of food reserves.

The livers of the mock-operated and unoperated controls were of small size, but the histological findings showed great irregularity, some livers being depleted of fat and glycogen, others not. There is a positive correlation between the abundance of fat and the abundance of glycogen. Since the autopsies were made in July, when the fish had already passed through the height of the sexual cycle, it seems probable that the variations might be associated with different stages in the onset of autumnal regression, but no definite correlations can be established between the regression of the testes and the condition of the liver (compare Tables VIII and X). Some of these fish were affected with goiter (Table XI), but again there appears to be no definite correlation between the condition of the thyroid and that of the liver.

The interpretation of these results is further complicated by the prevalence of a low to moderate degree of hepatic cirrhosis among the control fish. The disease is manifested by the accumulation of a ceroid-like material which occurs both as small inclusions within the hepatic cells and in emboli distended with ceroid-laden macrophages. The ceroid-like substance appears on frozen sections as material that is insoluble in ether or absolute alcohol but that accepts fat stains, although the color is generally less intense than that given by fat droplets. On account of its insolubility, the material remains undissolved even after paraffin embedding. It accepts the periodic-Schiff stain and is therefore conspicuous on the glycogen control sections, digested by malt diastase. No extensive histochemical study has been attempted, but the staining reactions are essentially similar to those attributed to ceroid. The literature on this subject is reviewed by Lillie (1952). The material stains blue or purple with nile blue sulfate, bright red with carbol fuchsin, and pink or red with Mallory's hemofuscin.

The problem was further investigated in a study of the livers of normal wild fish. In November, when the livers are well laden with fat and glycogen, small amounts of ceroid are always present, both within the hepatic cells and in macrophages. In the spring, when fat and glycogen reserves are depleted, it was discovered, to our great surprise, that ceroid was present in considerable amounts. As shown in Table VII, the average autumnal rating is $\pm$, whereas in the spring it rises to $++$. These observations suggest that ceroid formation may be a normal concomitant of the utilization of liver reserves.

However, the accumulation of ceroid in the livers of some of the experimental fish was more severe than anything observed in the normal wild population, amounting in some cases to an extensive cirrhosis in which large areas were replaced by ceroid-containing emboli. It was suspected that this pathological condition might be related to the use of acriflavine, and an experiment, already noted, was set up to determine this point. The results (Pickford, 1952b) demonstrated that prolonged treatment with acriflavine had no effect on the fat or glycogen reserves, or on the ceroid content, which was present in normal small amounts.

Two other possibilities suggest themselves. The pathological condition might result from a dietary deficiency or it might be an after-effect of the unidentified infection which led to the death of many fish in the early weeks of the experiment. The former explanation seems probable. In later experiments the diet was supplemented with frozen raw daphnia, and while ceroid has always been found in normal small amounts, it had not advanced to the cirrhotic condition observed in some of the Series IV fish. Infectious hepatitis is a well known cause of cirrhosis in mammals, and this interpretation cannot be excluded at the present time. The problem is still under investigation but, in any case, it is clear that the cirrhotic condition is not associated with hypophysectomy. Rather, the reverse is true, since the five completely hypophysectomized fish showed only normal amounts of ceroid.

The results of the Series IV experiments were confirmed in a further investigation under improved dietary condtions (Series VIIA, Table IX). As in the case of Series IV, the Series VIIA fish were hypophysectomized in early spring after passing the winter under aquarium conditions. In the weeks previous to the operation these fish were fed only three times a week. During the ensuing weeks of the experiment they were fed daily. A group of initial controls was autopsied on February 6 at the start of the experiment, and it was found that the livers were reduced in size, probably as a combined result of the undernourished condition of the fish and of the early onset of sexual maturation. The latter, due to the relatively warm temperature of the aquarium room, was evidenced by the slightly increased size of the testes (GSI 1.2 ± 0.2). For comparison, in autumnal regression wild males have a gonosomatic index of 0.7 ± 0.2.

TABLE IX. Comparison of the Condition of the Liver and Testes in Initial Controls (February 6, 1951) and in Final Controls and Hypophysectomized Fish (March 28–29, 1951). Series VIIA.

Group	*No. of fish*	*HSI*	*Histological rating**			*GSI*†	*Nuptial colors*‡
			Glycogen	*Fat*	*Ceroid*		
Initial controls	10	3.1 ± 0.9	+++	±	++	1.2 ± 0.2	− to ±
Final controls	9	3.4 ± 1.0	+++	+++	++	3.2 ± 1.2	± to +++
Hypect.	11	6.1 ± 2.4	+++	+++	++	0.3 ± 2.4	—

* See Table VII. Extremely uniform within each group except for one hypophysectomized fish with a low hepatosomatic index (3.0) and only traces of fat.

† Gonosomatic index.

‡ Nuptial colors: degree of development of sulfur yellow on fins and belly, rated on a subjective basis.

The small livers of the initial controls were depleted of fat but contained normal amounts of glycogen. This is probably a symptom of undernourishment. A similar condition prevailed in "pair fed" (i. e., semistarved) controls of a thiourea experiment (Chambers, 1953).

The experiment was terminated after seven weeks. The livers of the final controls remained small though well laden with fat and glycogen. The livers of the hypophysectomized group had increased to autumnal size. This experiment shows that hypophysectomized fish are capable of building up reserves of fat and glycogen and that the enlarged livers of such fish, hypophysectomized in winter, are not merely the result of a failure to utilize reserves that were already present. These results are in agreement with the work of Hatey (1951b) on glycogenesis in the liver of hypophysectomized eels.

The role of the pituitary in the spring reduction of liver size remains to be elucidated. It was thought that the effect might be mediated through the thyroid or, since it is manifested most strongly in the male sex, that it might be controlled by the male hormone. A pilot experi-

TABLE X. CHANGES IN NUPTIAL COLORATION, APRIL, JUNE AND JULY, AND GONOSOMATIC INDEX AT AUTOPSY, JULY 1949. SERIES IV.

Operation	*Fish*	*GSI*	*Nuptial coloration**		
			April 15	*June 6*	*July 10–15*
None	3–5	4.4	+	+++	+
	3–8	4.5	+	+++	+
	6–1	3.0	+++	+++	+
	6–4	4.8	+++	++	+
	6–6	3.9	++	+	+
	6–7	3.4	++	++	+
	Average	4.0±0.7			
Mock	1–4	6.8	+	+++	+
	2–6	0.7	++	+	—
	4–4	6.5	+	++	+
	5–3	5.0	+++	++	++
	5–5	6.8	+++	++	++
	5–8	3.9	+++	++	++
	Average	5.0±2.4			
Hypect.	1–7	0.6	—	—	—
	1–9	0.4	—	—	—
	4–2	0.3	—	—	—
	4–6	0.2	—	—	—
	4–8†	—	—	—	—
	Average	0.35±0.15			

* See Table IX.

† Not weighed at autopsy, but testes were in total regression.

ment in which solutions of thyroxin and of methyl testosterone were injected into hypophysectomized males has been reported in abstract (Pickford, 1952a). The results suggest that the male hormone might be the effective agent, but recent unpublished investigations indicate that the situation is more complicated.

Testes. At the time of operation, in February, all of the Series IV fish showed traces of nuptial coloration, rated on a subjective basis as ± or +. This probably resulted from the early onset of spring maturation due to the moderately warm temperature of the aquarium room. As the season progressed, the control fish advanced to their maximal coloration, rated as +++. The onset and duration of this phase varied somewhat from one fish to another (Table X), but by mid-July all fish showed a decline towards the autumnal condition. In contrast, the nuptial colors of hypophysectomized fish underwent a rapid regression, and at the end of about two weeks they had entirely vanished.

The gonosomatic indices of the unoperated and mock-operated controls were not significantly different from each other, but the testes of hypophysectomized fish were in complete regression. For comparison, the gonosomatic indices of 39 wild males taken in November 1949 averaged 0.7 ± 0.2. This value is of the same order as that found in the hypophysectomized group. On the other hand, in May 1950 the average gonosomatic index of 41 wild males was 5.8 ± 1.5. This is somewhat higher than the mid-July value recorded for the aquarium-kept controls, but the latter were already past the peak of sexual activity.

The gonosomatic index of an incompletely hypophysectomized fish, excluded from the tables, was 0.9. This fish showed a trace of nuptial coloration ($\pm$) but resembled in other respects the hypophysectomized group in that it did not grow and had a large liver. Evidently the persistent pituitary fragment, about one quarter normal size, was qualitatively or quantitatively sufficient to maintain the testes at a low level of activity.

These results are in agreement with the work of Matthews (1939) and Burger (1941) who also studied the effects of hypophysectomy on the gonads of *Fundulus*. The histological condition of the testes, studied on serial sections, is similarly in agreement with the findings of these authors. The cortex of the controls, except in the case of fish no. 2–6, which was in a state of complete autumnal regression, showed not only spermatogonia but also spermatocytes and nests of spermatids in abundance, while the tubules were filled with spermatozoa. After hypophysectomy, only the spermatogonial region of the cortex was unaffected; spermatocytes and nests of spermatids were occasionally visible, but there were no spermatozoa in the empty tubules.

Kidneys. It was discovered during the course of this investigation that the head kidneys of *Fundulus*, like those of other cyprinodonts (Krauter, 1952), are provided with numerous glomeruli. The kidneys of three of the hypophysectomized fish killed in July 1949 were highly abnormal (fishes 4–2, 4–6, and 4–8; see Tables VIII, X, and XI), and it may be significant that these are the three fish in which the thyroid colloid was totally inactive (Table XI). The head kidneys were greatly enlarged and the urinary duct, on one or both sides, was obstructed and grossly distended (Fig. 4). Sections of the head kidneys showed that many of the uriniferous tubules were also obstructed, the obstructions forming foci of encapsulation (Fig. 5). The kidneys of the other two hypophysectomized fish were both grossly and histologically normal. Nevertheless, subsequent observation and careful examination of hypophysectomized fish, dead or dying from unidentified causes, has revealed that kidney trouble of this sort is extremely prevalent. Calculi, which may develop within

TABLE XI. CONDITION OF THE THYROID IN THE EXPERIMENTAL FISH, JULY 1949. SERIES IV.

Operation	*Fish*	*Average cell height, μ*	*% cells over 12μ*	*Predominant colloid*		
				Azan-red	*Mixed*	*Azan-blue*
None	3–5	6.2±2.0	2	—	X	—
	3–8	10.4±3.6	30	—	X	—
	6–1	5.6±2.0	1	—	X	—
	6–4	7.1±2.3	4	—	X	—
	6–6	6.0±2.3	3	—	X	—
	6–7	5.6±2.1	3	—	X	—
	Average	8.2±2.4				
Mock	1–4	9.2±3.5	21	X	—	—
	2–6	11.0±4.6	41		almost lacking	
	4–4	4.8±1.8	0	—	X	—
	5–3	7.5±2.7	10	—	X	—
	5–5	11.1±3.2	44	—	X	—
	5–8	5.7±2.0	3	—	X	—
	Average	9.8±3.2				
Hypect.	1–7	5.4±2.2	0	—	—	X
	1–9	4.1±1.2	0	—	—	X
	4–2	3.8±1.4	0	X	—	—
	4–6	5.1±1.8	0	X	—	—
	4–8	3.8±1.3	0	X	—	—
	Average	4.4±0.8				

three weeks to a month after the operation, may be present as hard and well formed concretions that completely obstruct one or both of the urinary ducts, or they may be rather soft and composed of a mass of minute white granules loosely cemented together. Sometimes the uriniferous tubules are filled with white material which oozes out as a pasty mass when the kidney is cut. The affected fish often develop pronounced symptoms of edema, from which they frequently recover however. In more serious cases they become emaciated and death appears to result from asthenia. Displacement of the swim bladder by enlargement of the kidneys can be seen by x-ray photography (Fig. 6).

The calculi are composed of mineral granules compacted or more or less completely cemented together by organic material. The mineral is opaque to x-rays, soluble in dilute mineral acids, and precipitated by ammonium oxalate. The major component is therefore a calcium salt. Dr. Clifford B. Frondel of Harvard University, who kindly consented to make an x-ray diffraction study, submitted the following report:

"When the pellets are heated, they blacken rapidly at a low temperature, due to carbonization of organic material, but do not change further up to at least 900° C. At the latter temperature they have a somewhat cracked, slaggy appearance and still are black; presumably prolonged heating in air at this temperature, or lower, would burn off all the carbon but the reaction is impeded by the very dense structure of the material. X-ray powder diffraction study of the ignited pellets shows that they are composed chiefly of apatite. When dissolved in acid, the sample being watched under high magnification, there is a slight but definite effervescence and a gelatinous residue is left. The apatite type originally present thus appears to be carbonate-apatite, as is almost always the case in human concretions. On heating, the carbonate-apatite breaks down to hydroxyl-apatite which is stable at high temperatures. The gelatinous residue in acids represents the organic cementing material; human apatite (and other) calculi often show the same feature."

While these investigations were in progress, additional information has come to light from other sources. Rasquin and Rosenbloom (1953), working on *Astyanax,* discovered that kidney malformations, evidently due to obstruction of the tubules by concretions, are among the many abnormalities that develop in fish that have been kept for long periods of time in total darkness. Such fish also show skeletal malformations due to decalcification, and the ultimobranchial gland, interpreted as a parathryoid, is greatly hypertrophied. Iwai (1953) has also observed enlarged kidneys and renal calculi in Japanese killifish kept in total darkness. Pflugfelder (1953) reports abnormalities of kidneys and skeleton after epiphysectomy in newborn *Lebistes.* The skeletal malformations, due to defective calcification, resemble those described by Rasquin. The kidney abnormalities are obviously due to concretions, in fact Pflugfelder's microphotograph might have been taken from one of my hypophysectomized *Fundulus* or from Rasquin's *Astyanax.* As the problem stands at the moment it appears that the fish parathyroid, regulating calcium metabolism, is dependent on the pituitary which is in turn dependent on light stimulation mediated through the pineal. A fertile field of investigation is thus opened for future study.

Osmoregulation. It was thought that the more serious manifestations of kidney disease might be relieved if the fish were kept in fresh water or at least in a medium hypotonic to that of the body fluids. This should induce a state of diuresis that might be expected to wash away the small granules before they became cemented together to form large calculi. Matthews (1933) kept hypophysectomized *Fundulus* for "several weeks" in tap water, and since Fontaine and coworkers (Fontaine, et al., 1949, 1950; Callamand, et al., 1951) have shown that the pituitary of the eel is not necessary for the regulation of the osmotic pressure of the blood, it was presumed that the killifish would survive such treatment.

In the spring of 1952 six hypophysectomized fish were transferred to a mixture of equal parts of sea water and pond water three days after removal of the pituitary (Series X). The fish ate and appeared to be healthy. Two days later they were transferred to pond water. For three days subsequent to this they were quiescent, refused food, and became progressively more asthenic. On the fourth day one fish was near death but showed a partial recovery in a mixture of one part sea water and three parts pond water. One-tenth sea water was added to the main tank, but, since this was not sufficient, the concentration was raised to half and half (specific gravity 1.007 at room temperature). Four of the six fish came near to death even in this medium but made what appeared to be a complete recovery when they were transferred to undiluted sea water in which they resumed feeding and behaved in a normal manner. However, when taken back to the half and half dilution they again developed asthenia and all four died within a few days; two were found to have kidney stones. The two fish that survived in the half and half dilution were kept alive for one month; at autopsy one was in very poor condition and the other proved not to have been completely hypophysectomized, which no doubt accounts for its better ability to survive.

It is clear that in *Fundulus* complete hypophysectomy abolishes the ability of the fish to live in fresh or diluted sea water. However, at the time of writing there is no evidence to indicate whether it is an anterior or a posterior lobe function that is affected.

Thyroid. The condition of the thyroid in the Series IV fish is summarized in Table XI. The thyroids of hypophysectomized fish are inactive, the epithelium is low, and no follicles contain cells that are over 12μ in height. In *Fundulus*, as in *Gobius* (Vivien, 1941), the removal of the pituitary results in a permanent state of thyroid inactivity. The average epithelial height of the controls ranged from 4.8 to 11.1μ. Some fish had relatively inactive glands, but the majority showed at least some follicles with tall columnar cells, and in a few cases the percentage of such activitated follicles was extremely high.

TABLE XII. Condition of the Thyroid in Wild Fish Sampled in Autumn and in Spring. Series V.

Date	*Sex*	*No. of fish*	*Average cell height, μ*	*Colloid**
Nov. 1949	♂	6	6.5±0.9	Inactive, except for one fish
	♀	5	6.0±0.9	Inactive
May 1950	♂	5	6.9±1.1	Active
	♀	5	5.9±0.3	Active, except for two fish

* Inactive colloid: predominantly Azan-red, clear. Active colloid: predominantly Azan-blue, granular.

The condition of the colloid was very variable. Most of the glands studied were of a mixed character, some follicles containing Azan-red homogenous colloid, others particolored or blue-staining granular colloid. Granular colloid predominated in two of the hypophysectomized fish. It is not associated with increased epithelial height and presumably it represents utilization rather than secretion. Some of the control glands (fishes 3–8, 6–4, 6–6, and 2–6) showed a feature which can only be interpreted as reorganization; the epithelium of the larger follicles was disintegrated and enveloped in lymphoid sinuses while the colloid was broken up into small droplets. Fish 2–6 had a gland that was composed almost exclusively of very small active follicles with little or no colloid.

It became necessary to determine whether the changes described above, affecting the control fish, were part of a normal cycle of thyroid activity, such as that described by Buchmann (1940) in the herring, or whether the condition was one of low grade hyperplasia resulting from an iodine deficiency in the liver-pablum diet.

To answer the first question, wild fish were sampled in November 1949 and again in May 1950. The results are shown in Table XII. Three conclusions may be drawn from the data. (1) The thyroids of females are slightly less active than those of males, as measured by average epithelial height, the difference being statistically significant at the 5% level when the two sexes are compared irrespective of season (mean for 11 males, 6.7 ± 1.0; mean for 10 females, 5.8 ± 0.7). (2) There is no difference in the average epithelial height of specimens taken in spring and autumn, provided each sex is treated separately. (3) The condition of the colloid suggests liquifaction and resorption in the spring, whereas the thyroid follicles of autumn fish, with one ex-

TABLE XIII. CONDITION OF THE THYROID IN THE UNOPERATED UNTREATED MALE LABORATORY CONTROL FISH RECEIVING IODINE, WITH OR WITHOUT A DIETARY SUPPLEMENT OF RAW DAPHNIA. SERIES VIII AND XI.

Series	*Date*	*Daphnia*	*No. of fish*	*Average cell height, μ*	*Colloid**
VIII†	April 1951	No	7	5.0 ± 0.6	Active in three fish
VIII	April 1951	Yes	8	5.4 ± 0.7	Active in four fish
XI	July 1952	Yes	7	5.3 ± 0.6	Inactive

* See Table XII.

† Data for Series VIII from Pickford (1952b).

ception, are predominantly laden with resting colloid. Liquifaction of the colloid is not associated with an increase in the average epithelial height. Thus, while more complete investigations of the wild population would be desirable, it appears unlikely that there is any period of extreme activity or profound reorganization in the normal natural annual cycle.

In later experiments, after it became evident that unoperated, untreated control fish were suffering from a condition resembling goiter, a trace of iodine was added to the food. Also, in more recent experiments, frozen raw daphnia was added to the diet. The effects of these charges are shown in Table XIII. Irrespective of the use of daphnia, and irrespective of the season (spring or summer) or state of activity of the colloid, the average epithelial height was low. No hyperplasia developed in any of these fish, all of which had been in captivity for many months under conditions which had otherwise induced goiter in earlier experiments.

It may be concluded from the observations and experiments outlined above that a diet deficient in iodine readily induces thyroid hyperplasia in normal fish, as is well known, but that no such effect results after removal of the pituitary. In fish, as in higher vertebrates, hypophysectomy induces a state of extreme thyroid inactivity, and the failure of the thyroids of such fish to respond to iodine deficiency is clear proof of the absence of the thyrotrophic hormone.

Pancreatic Islets. In this species, at least in males of this age class, the principal islet has a capsule of exocrine tissue that is separated from the endocrine part of the gland by a thin connective tissue membrane. Exocrine tissue follows connective tissue trabeculae into the interior of the islet. Alpha and beta cells are present in about equal numbers, but there is a tendency for the beta cells to be more abundant around the periphery. Delta cells are of infrequent occurrence.

There are no obvious differences between the islet tissue of hypophysectomized fish and that of unoperated and mock-operated controls. Colloid transformation, described in various species of fish by Laguesse (1894), Bargmann (1937), and Pallot (1938, 1939), was not observed. According to Pallot this change is associated with hibernation and one might therefore expect that hypophysectomy would induce a similar condition. However, colloid formation was not observed in the pancreatic islet of wild fish killed in November or May.

Stannius corpuscles. According to Garrett (1942), the corpuscles of Stannius in ***Fundulus*** are usually situated in segments 13 or 14. In our experience the condition is much more variable. Normally there is a single pair in the location noted above, but at times they may be situated far forward or, quite frequently, much farther back. Sometimes the glands are opposite each other, sometimes widely separated. The pair is often of unequal size and occasionally there may be two glands on one side and none on the other. Small subsidiary glands are not infrequent.

The Stannius corpuscles of the Series IV fish, killed in July 1949, were examined histologically. Whenever possible, one gland was fixed in Flemming's fluid and the other in Helly, but frequently only one gland could be located, and in three fish they were not found at all. It is well known that the granules of the cells are extremely labile, and fixation was not good except at the periphery. So far as could be determined there were no differences between the hypophysectomized fish and the controls.

It is now generally accepted, in agreement with Baecker (1928), that the Stannius bodies are endocrine glands of unknown function, peculiar to teleost fishes. Rasquin (1951) has recently shown that they are not affected by injections of mammalian corticotrophic hormone or by implants or extracts of fish pituitary. The present findings confirm the conclusion that the Stannius corpuscles are not regulated by pituitary control.

Adrenal (*Giacomini*) *tissue.* Rasquin (1951) has shown that the so-called "anterior interrenal" or Giacomini tissue of teleosts responds to injections of mammalian corticotrophin as well as to fish pituitary implants and extracts. The controversy as to whether this tissue or the Stannius bodies should be regarded as the homologue of the adrenal cortex of higher vertebrates must therefore be regarded as settled.

In *Fundulus* the Giacomini tissue is associated with the posterior cardinal vein as it passes through the right head kidney. It forms a layer two or three cells deep in the wall of the vein and sends occasional cords into the substance of the kidney, from which it is separated by a connective tissue membrane. As in other teleost fishes, there are two main types of cell: the angular chromaffin cells with dense non-granular cytoplasm, and the adrenal cortical cells in various phases of activity, either heavily laden with granules or more or less vacuolated and degranulated.

According to Rasquin (1951) and Atz (1952) the anterior interrenal of *Astyanax* responds to injections of ACTH by hypertrophy, hyperplasia and increased vascularization; the cytoplasm is said to become slightly basophilic, but degranulation was not observed. After a single injection there is a rapid transitory increase in the number of cells which form the layer of Giacomini tissue; this reaches a maximum in four hours. Repeated injections lead to hyperplasia with frequent mitoses. In the silver eel, in which the Giacomini tissue forms a compact body on either side of the sinus venosus, Fontaine and Hatey (1953) found that hypophysectomy leads to a rapid atrophy of the gland, its weight decreasing to about one-third of its normal value. Injection of ACTH restores the weight of the atrophied glands to normal.

These investigations, made on freshwater forms, appear to offer conclusive proof that the anterior interrenal is under the control of the pituitary. It was therefore surprising to find that the Giacomini tissue of hypophysectomized killifish is not significantly different from that of unoperated or mock-operated controls. The material does not lend itself readily to accurate comparison, short of a complete reconstruction from serial sections, because the Giacomini layer is irregular, frequently interrupted, and of variable thickness even in normal fish. Any attempt to arrive at an average estimate of the number of cells forming the layer is obscured by this variation from one section to another and from one fish to another. In hypophysectomized fish the Giacomini layer usually varies from one to three cells in depth and probably averages two cells. The condition in the controls is not significantly different. The cytoplasm shows no evidence of degranulation after the operation. In agreement with Rasquin, we failed to demonstrate vitamin C granules by the silver nitrate method, although Hatey (1952) has shown by chemical analysis that ascorbic acid is concentrated in the anterior interrenal as compared with the lymphoid head kidney of the eel, and in the head kidney (plus interrenal tissue) as compared with the posterior kidney in trout.

The lack of a degenerative response of the adrenal to hypophysectomy in the killifish is as unexpected as it is difficult to interpret. It was at first supposed that the granular cells of the Giacomini tissue might be homologous with the cells of the mammalian adrenal zona glomerulosa which is believed to be independent of pituitary control and which is supposedly associated with the salt regulating function of the gland. However, this hypothesis runs contrary to the findings of Rasquin, and some preliminary tests with fish pituitary suspensions indicate that the adrenal cortical tissue of *Fundulus* also responds by hypertrophy to injections of ACTH-containing material. The adrenal tissue of wild fish (Series V) was examined to determine whether the conditions of aquarium life, in particular the hyperplastic condition of the thyroids due to iodine deficiency, might have led to adrenal cortical inactivity in the control fish. Adrenals of wild males of the same size class, captured in May, showed little if any difference from those of aquarium fish; the average number of cells in the Giacomini layer was between two and four and was therefore possibly slightly greater than the average in the aquarium fish, but the condition of the cytoplasm was the same. Only one conclusion remains possible: the adrenal cortical tissue of normal killifish living in salt water is in a state of quiescence closely similar to that which is maintained after hypophysectomy. Whether changes in the external environment might lead to different results remains to be investigated.

Blood and blood-forming tissues. The effects of hypophysectomy on the composition of the blood and on the hemopoietic tissues of fish would require a special investigation devoted to this problem alone. Hypophysectomized fish are frequently extremely anemic, as may be judged from the pallor of the gills. The blood was not examined in Series IV. In Series VIA (see Pickford, 1953) blood smears were taken from the tail at the time of autopsy. All hypophysectomized fish were somewhat anemic and a few were severely affected.

SUMMARY

1. Hypophysectomized *Fundulus* do not grow in length. Weight changes are irregular. No new circuli are added to the scales and no new growth can be detected on the otoliths.

2. In wild fish of both sexes the liver is large in autumn, but in males there is a profound decrease in size in spring. The livers of hypophysectomized fish remain large if the operation is made in winter and increase to autumnal size if the operation is made at a time when the livers are reduced.

3. Confirming the work of Matthews and of Burger on this species, the testes undergo complete regression after the removal of the pituitary. Only the spermatogonial zone of the cortex is unaffected. The nuptial colors disappear.

4. Hypophysectomized fish commonly develop renal calculi leading to an obstruction of the urinary ducts. The calculi are composed chiefly of carbonate apatite.

5. A disturbance of mineral and/or water metabolism is evidenced by the loss of osmoregulatory capacity which results in the inability of this normally euryhaline species to survive in fresh or diluted sea water.

6. The thyroid glands of hypophysectomized fish are inactive.

7. Hypophysectomy has no effect on the pancreatic islets or on the Stannius corpuscles. The adrenal (Giacomini) tissue in the wall of the posterior cardinal vein is also unaffected by the operation.

8. Hypophysectomized fish are anemic.

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A NOTE ON THE EFFECTS OF HYPOPHYSECTOMY ON THE PREOPTICO-HYPOPHYSIAL PATHWAY IN *FUNDULUS*

By

SANFORD L. PALAY

Department of Anatomy, Yale University School of Medicine

ABSTRACT

In *Fundulus heteroclitus,* as in other vertebrates, total hypophysectomy results in degeneration of the neurons in the hypothalamus (preoptic nucleus) which send their axons into the neurohypophysis.

As in all vertebrates examined thus far, the hypothalamus of fishes contains groups of peculiar nerve cells which are characterized by peripherally located Nissl substance, excentric and irregularly shaped nuclei, and rich vascularity. In the fishes these neurons comprise the nucleus preopticus, homologous with the nuclei supraopticus and paraventricularis of the mammals. In all vertebrates these neurons send axons down into the neurohypophysis where their bulbous endings lie against the capillaries. The most striking feature of these nerve cells is the presence of fine granules and droplets which stain selectively with chrome-alum hematoxylin and which appear throughout the cytoplasm and in the dendrites, axons, and terminations. These granules, first discovered in the preoptic nucleus of *Phoxinus,* a European minnow (Scharrer, 1928), have since been found in many other fishes and in all other classes of vertebrates (Scharrer and Scharrer, 1940, 1945; Bargmann and Hild, 1949; Smith, 1951). The impressive resemblance between these neurons and gland cells full of secretory material prompted Scharrer to label them "neurosecretory cells" and to term the process by which they produce their specific substance "neurosecretion."

Because the neurosecretory granules are found all along the nerve fibers extending between the nucleus and the terminals in the neurohypophysis, the suggestion has been put forth that the preoptico-hypophysial tract (or the hypothalamo-hypophysial tract in mammals) serves as a neurosecretory pathway by which the granules are conveyed from the sites of synthesis in the nerve cell bodies to the terminals on the capillaries in the neurohypophysis (Palay, 1945; Bargmann and Hild, 1949). Recent work (reviewed by Bargmann and Scharrer, 1951) has adduced evidence in favor of this suggestion and has further demonstrated that the neurosecretory material in mammals is probably identical with the hormones of the posterior lobe (Hild, 1951; Hild and Zetler, 1951; Ortmann, 1951; Smith, 1951). Current theory held by workers on this subject is, therefore, that the posterior lobe hormones are actually produced in the nerve cells of the hypothalamus, transported down the tract, and stored in the neurohypophysis to be released into the blood vessels upon an adequate stimulus. Whether this is the only function of neurosecretory cells and indeed whether their function is the same in all classes of vertebrates are questions which have not been settled. Possible functions of this system in fishes have not been examined.

The morphological manifestations of the neurosecretory process in *Fundulus heteroclitus* have been thoroughly studied both by older staining methods (Scharrer, 1941) and by the newer chrome-alum hematoxylin method (Smith, 1951; Palay, unpublished observations). It is, therefore, of some interest to examine the brains of hypophysectomized fishes in order to determine the effect of hypophysectomy on the neurosecretory cells. About 15 weeks after hypophysectomy two male specimens of approximately the same size were fixed by perfusion with Zenker-formal solution, after which the brains were removed. After dehydration and embedding in paraffin, the brains were sectioned serially 6 μ thick and stained with chrome-alum hematoxylin and phloxine (Gomori, 1941). In both of these brains the preoptic nuclei are markedly deficient in neurons. All of the large cells disappeared and only a few of the medium sized cells remained. In one specimen an estimated 10% of the cells persisted and in the other specimen about 20% remained. Many of these cells contained neurosecretory material in the form of minute granules and larger droplets. The loss of cells was correlated with a great reduction in the number of fibers in the preoptico-hypophysial tract, which all but disappeared. In the first specimen there is no trace of the tract which normally passes through the stalk except for a small contingent of chromehematoxyphil fibers in the caudal wall of the hypothalamus from which the hypophysial stalk had been avulsed. In the second specimen the proximal tip of the stalk had been left behind at the operation and in this nubbin is a cluster of nerve fibers deeply stained by the chrome-alum hematoxylin. These fibers can be traced forward along the course of the preoptico-hypophysial tract to the post-optic commissures where they begin to fray out and disappear. The larger proportions of surviving neurons in the preoptic nucleus of this specimen is directly correlated with the larger number of fibers persisting in the tract. Presumably the cells whose fibers end in the upper portion of the stalk were not fatally injured by the hypophysectomy and therefore survived. These changes are consistent with the effects observed after stalk section in mammals (Fisher, Ingram, and Ranson, 1938).

Conclusion.—As in other vertebrates, total hypophysectomy in *Fundulus* results in degeneration of the neurons in the hypothalamus (preoptic nucleus) which send their axons into the neurohypophysis.

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THE RESPONSE OF HYPOPHYSECTOMIZED MALE *FUNDULUS* TO INJECTIONS OF PURIFIED BEEF GROWTH HORMONE[1]

By

GRACE E. PICKFORD

Bingham Oceanographic Laboratory
Yale University

ABSTRACT

Hypophysectomized male *Fundulus heteroclitus* were injected thrice weekly with purified crystalline beef growth hormone at a dosage level of 10 μg per gram weight for a period of nine weeks. The response was similar to that observed in normal specimens when similarly treated (Pickford and Thompson, 1948); an initial period of weight loss was reversed at the end of the third or fourth week. At the end of nine weeks the fish had increased 14.8% in weight and 4.4% in length. An average of 3.5 new circuli was added at the margin of the scales and new deposition was formed on the otoliths. Induction of new growth on the scales leads to the formation of a zone that resembles an annulus. Three different growth hormone preparations were used in succession; No. 429 (Wilhelmi) contained sufficient gonadotrophin to induce the appearance of nuptial coloration at the end of three weeks; No. 22KR-2 (Armour and Co.) induced serious symptoms of hyperthyroidism after the fifth injection; No. 508B (Wilhelmi) was free of gonadotrophin and corticotrophin but contained a trace of thyrotrophin sufficient to maintain the thyroids at normal levels. There was a slight, but not statistically significant, reduction in liver size. Administration of growth hormone had no effect on the incidence of renal calculi or upon the anemia resulting from hypophysectomy.

INTRODUCTION

Previous work on the role of the pituitary in regulating the growth of fish is somewhat confusing. Tuchmann (1936) claimed that guppies which had been fed pituitary for two months were larger than the controls. These results were confirmed by Regnier (1938) on *Xiphophorus;* feeding with anterior lobe powder accelerated both growth and the development of sexual maturity. It is difficult to interpret these experiments, since the pituitary hormones are proteins which are presumably destroyed by the digestive juices of the fish. However, in a number of recent publications, of which only two need be quoted (Li, 1950; Kinsell, et al., 1950), it has been shown that a

[1] The completion of this investigation has been part of a project supported by a grant from the National Science Foundation (NSF-G50).

protein-free peptide mixture of low molecular weight, prepared by enzymic hydrolysis, has a high degree of adrenocorticotrophic activity. There is no such evidence in respect to the growth hormone, and therefore the results of feeding experiments must be interpreted with caution. Regnier showed further that both growth and sexual development, arrested after hypophysectomy, were restored to normal by pituitary grafts. On the other hand, Nixo-Nicoscio (1940), who injected triturated cattle pituitary into goldfish, was unable to demonstrate any effect on growth, although the color change from black to gold was said to have been accelerated. The work of Vivien (1938, 1939, 1941) also casts doubt on the supposed cessation of growth after hypophysectomy. However, removal of the pituitary prevents fin regeneration in *Gobius* and *Ameiurus* (Buser, 1950; Buser-Lahaye, 1953).

In view of the complexities outlined above, it became evident that the problem must be reinvestigated from several different angles. In the first place it was necessary to re-examine the effects of hypophysectomy. Several series of experiments have been devoted to this problem, and the results, reported in the preceding article of this issue (Pickford, 1953) provide unequivocal proof that *Fundulus* does not grow after the removal of the pituitary. There may be irregular weight changes due to various causes, but there is no increase in length and no new growth on scales or otoliths.

Secondly it was necessary to study the response of the killifish to injections of purified mammalian growth hormone, free from other anterior lobe factors. This problem was first investigated on normal unoperated fish (Pickford and Thompson, 1948). In the initial weeks of the experiment there was no growth in length and considerable loss in weight. After the fifth week, fish receiving growth hormone thrice weekly at a dosage level of 10 μg per gm weight began to show definite signs of growth and, at the end of 10 weeks, they had increased 12.7% in weight and 2.7% in length. Controls receiving saline or boiled hormone did not grow. No changes were observed on the scales or otoliths.

At about the same time, Jampolsky, working under the direction of Dr. Hoar, attempted to compare the growth promoting activity of alkaline extracts of beef and salmon pituitary by injections into rats and goldfish (Jampolsky, 1949; Jampolsky and Hoar, 1953). Rats responded to injections of the beef growth hormone, showing that the technical procedures used in the preparation of the extract were satisfactory, but they did not respond to similar preparations derived from salmon glands. Both beef and fish pituitary extracts proved toxic to goldfish, as evidenced by a very high mortality after the first week of the experiment. On the fifth to eighth day, based on mean values for several separate experiments, 19 fish receiving beef pituitary extract showed an average weight increase of 19% whereas 29 fish receiving salmon pituitary extract showed a lesser average increase of 7.2%. Approximately 50% of the fish died during the second week of the experiment and a progressive weight loss was noted in both groups. The authors are uncertain whether the initial weight increase should be attributed to osmotic disturbances or to the somatotrophic action of the growth hormone.

The present contribution, noted previously in abstract (Pickford, 1951), is devoted to an investigation of the response of hypophysectomized killifish to injections of purified crystalline beef growth hormone. It was thought that hypophysectomized fish might be more responsive than the normal fish used previously. Moreover, several improvements in procedure were introduced as the result of experience. The fish were anesthetized before injection in an attempt to minimize the shock of handling, which may have contributed to the severe loss of weight observed in the first weeks of the earlier experiment. Furthermore, to insure that the fish took food each day, they were fed regularly in the mornings; injections were made thrice weekly, in the afternoons. Finally, the injections were continued until the average percentage increase in length exceeded 4%, since a study of the spring growth of intact fish suggested that an increase of this order would be necessary to establish definite evidence of the addition of new circuli to the scales.

I should like to express my thanks to Dr. A. E. Wilhelmi, formerly at Yale University and now at the Department of Biochemistry, Emory University, who provided the preparations of purified growth hormone and who gave me the benefit of his advice during the course of this investigation. Miss Harriet A. Chambers participated in all phases of the investigation and Mr. Maxwell D. Lai (Yale 1950) helped with the preparation of the tissues and assisted in the aquarium room.

MATERIALS AND METHODS

The fish used in this experiment belonged to Series VI, reported in the preceding paper of this Bulletin (Pickford, 1953). A group of 20 male killifish had been hypophysectomized in December 1949 and their growth changes were compared with those of mock-operated and unoperated controls. By the end of March 1950 it was established that growth was at a standstill in the hypophysectomized group while the onset of spring growth was evident in all of the controls. Therefore, at the beginning of April the fish were rearranged for the new experiment. The hypophysectomized fish were divided into three groups, one to remain untreated, one to receive saline injections, and one to receive growth hormone. Some of the control fish were assigned to other experiments, but two groups of six and seven fish respectively were kept as unoperated or mock-operated untreated controls. The experiment, designated Series VIA, was begun on April 3, 1950. Injections were made thrice weekly. The fish was anesthetized with MS222 and rolled up in a piece of wet cheese cloth. A ¼ cc Tuberculin syringe was used for injection. The needle was inserted into the body cavity through the soft tissue adjacent to the anus. Care was taken to avoid puncturing the apex of the liver, the swim bladder, or other internal organs.

The growth hormone solution, prepared at approximately weekly intervals, was kept in the refrigerator. Four mg were dissolved in 2 ml of 0.6% NaCl, and 10 mm^3 of 0.1 N NaOH were added from a constriction pipette to bring the powder into solution. The dosage level was based on that found effective in normal fish (Pickford and Thompson, 1948), i. e., 10 μg per gram weight per injection, or 0.005 ml of a 0.2% solution, the volume being approximated to the nearest 0.005 ml. The fish were weighed and measured every Monday afternoon and dosages were adjusted each week in accordance with the observed weight changes. Saline controls received an equivalent volume of 0.6% NaCl.

Three different growth hormone preparations were used during the course of the experiment: No. 429 (Wilhelmi), No. 22KR-2 (Armour and Co.), and No. 508B (Wilhelmi). The growth-promoting activity of these preparations was tested on rats by Dr. Wilhelmi, who states that they were approximately equipotent; doses of 20 μg per day for 10 days induced average weight increases of 21 g (429), 14–20 g (22KR-2), and 18.3 g (508B). A gain of 16–18 g at this dosage is expected of material of unit potency. Wilhelmi states that in the electrophoretic apparatus all three preparations migrated as a single component at pH 10 but that 22KR-2 showed a second fast component at pH 4. White, Heinbecker and Rolf (1951) have published an assay of 22KR-2 in terms of Armour's standards; 1 mg contained 1.7 growth hormone units, 0.03 units of thyrotrophin, and less than 0.02 units of corticotrophic hormone; prolactin and gonadotrophins were negligible. There is no information regarding the possible activity of traces of other anterior lobe hormones in preparations 429 and 508B, but the latter is known to have been of a high order of purity.

Preparation 429 was used from April 4 through 25, a total of 10 injections during a period of three weeks. By this time it was apparent (see Table VIII) that the preparation was not free from gonadotrophin, since all fish showed the onset of nuptial coloration. The next five injections, from April 27 through May 6, were made with Armour's 22KR-2. After the fourth and fifth injections with this material it became evident that something was wrong. The protocol for May 7 reads as follows: "Hanging at surface panting, very nervous, throat red, slight exophthalmos." It was concluded that 22KR-2 contained sufficient thyrotrophin to induce serious symptoms. Fish are known to be very sensitive to thyrotrophin (Gorbman, 1940, 1946; Albert, 1945) even when it is derived from mammalian sources and, as stated above, the preparation is known to have contained small amounts of this hormone. For the remainder of the experiment, 14 injections spread over a period of four weeks, Wilhelmi's 508B was used without further trouble. The nuptial coloration slowly faded and the symptoms of hyperthyroidism rapidly vanished.

Procedures for the care and feeding of the fish, the technique of hypophysectomy, and the methods used in the study of the tissues have already been described (Pickford, 1953). In this experiment the standard liver-pablum diet was supplemented with chopped raw beef liver. Iodine was not added to the food, since the incidence of goiter in the unoperated and mock-operated controls had not been discovered. The temperature of the aquaria was not regulated and the room temperature rose slowly from about 16° C in April to 20° C at the beginning of June. To facilitate the work, the autopsies were staggered over a period of two weeks (Table I).

Near the beginning of the experiment it became necessary to treat some of the control fish for fungus infections. A combination of heat plus acriflavine proved most helpful. The fish were transferred to a "hospital" aquarium containing acriflavine (2 g/1000) and the water was slowly warmed to about 32° C. The aquarium was kept at this temperature for three days and then slowly cooled. The fish (Table I,

TABLE I. SUMMARY OF PERCENTAGE CHANGES IN WEIGHT AND LENGTH AT WEEKLY INTERVALS. SERIES VIA.

Group	*No. of fish*	*Date* IV/11/50	IV/18/50	IV/25/50	V/2/50	V/9/50	V/16/50	V/23–24/50	V/30–31/50	VI/6/50
WEIGHT										
Unop.	7	−0.7±1.4*ø	−6.7±2.1	− 2.8±3.0	4.8±4.3	9.2±4.8	14.8±6.0	15.9±6.7	——	——
Mock	6	−0.3±5.3	2.0±5.0*	0.4±4.5	7.8±5.3	9.3±5.2	12.2±7.1	10.9±7.0	12.3±8.5	——
Hypect.	6‡	−1.7±2.3	−3.4±3.0*	−7.2±4.8	−5.9±3.7	− 4.8±3.8	− 8.4±5.7	− 7.1±8.0	−10.0±7.6	——
Hypect. saline	7	−5.1±3.8	−7.9±3.6	−12.0±2.9	−13.5±3.1	−12.9±7.9	−13.7±5.7	−17.5±2.9†	−17.1±3.1§	−17.9±4.2§
Hypect. gr. horm.	7	−7.0±4.6	−7.7±4.4	−6.5±4.5	− 3.8±4.1	2.3±6.4	6.1±6.0	6.7±8.0	14.8±12.2	14.8±11.1
LENGTH										
Unop.	7	1.1±0.9	0.7±0.7	1.3±1.0	2.2±1.3	3.3±1.2	4.5±1.6	6.3±2.7	——	——
Mock	6	1.5±0.8	1.8±1.0	2.4±1.4	2.9±1.6	3.9±1.7	5.4±1.9	5.4±1.6	6.4±2.0	——
Hypect.	6‡	0.2±0.3	0.1±0.8	0.3±0.8	0.3±0.7	0.1±0.7	0.3±1.0	0.2±0.5	0.8±1.1	——
Hypect. saline	7	0.2±0.3	−0.1±0.7	− 0.1±0.8	− 0.2±0.5	− 0.6±0.8	− 0.7±0.6	− 0.5±1.0†	0.0±0.5§	0.0±0.5§
Hypect. gr. horm.	7	0.9±0.7	0.7±0.5	1.3±1.0	1.8±1.2	2.1±0.9	2.7±0.9	3.1±1.3	4.2±1.4	4.4±2.2

* Treated during interval with heat and acriflavine for fungus infections.
‡ Includes one fish which had a minute persistent pituitary fragment but which did not grow.
† Only five survivors.
§ Only four survivors.
ø In this and subsequent tables, ± is standard deviation, not standard error.

Fig. 1) showed a temporary loss in weight from which they quickly recovered. As another measure against infection, a pinch of sodium perborate was added to the water in the recovery bowl to which the fish were transferred after injection.

RESULTS

Weight and Length (Table I, Fig. 1). Since the induced growth resulting from the administration of pituitary growth hormone must be assessed against the normal growth rates of unoperated or mock-

TABLE II. Comparison of Natural and Hormonally-induced Growth Rates Expressed as Average Percentage Weekly Increments. Series VIA.

Group	*Duration (weeks)*	*No. of fish*	*Average per cent weekly increment*	
			weight	*length*
Unop.	7	7	2.3	0.9
Mock	8	8	1.6	0.8
Gr. horm.	9	7	1.7	0.5

operated controls, the growth changes of the three uninjected control groups will be discussed first. At the beginning of the experiment there was a loss in weight not only in the hypophysectomized fish but also in the mock-operated and unoperated controls. In the two last mentioned groups the onset of spring growth had already been established before the start of the experiment and therefore this temporary retardation must be attributed in part to the disturbances concomitant with the rearrangement of the fish and in part to the treatments for fungus infection. The experience merely serves to illustrate how misleading it may be to draw conclusions in respect to growth from weight changes in experimental fish. Growth in length proceeded with negligible retardation. At the beginning of the fourth week the mock-operated and unoperated groups had increased significantly both in weight and in length, whereas the uninjected hypophysectomized fish showed the expected progressive average loss in weight and no change in length. Slight differences between the weight and length increments of unoperated and mock-operated fish were not statistically significant except on April 18, when the unoperated group showed a temporary but pronounced loss in weight due to treatment.

The percentage weekly increments, averaged for the duration of the experiment, may be used as a rough measure of the growth rate of the mock-operated and unoperated control groups (Table II). Normal

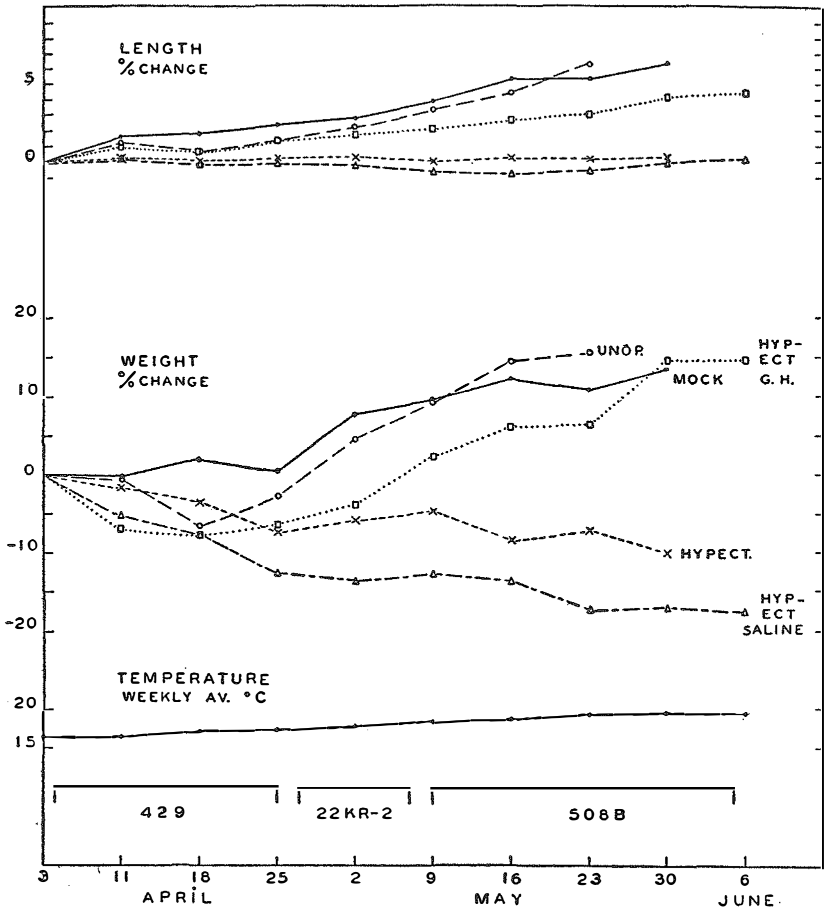

FIGURE 1. Percentage changes in weight and length of hypophysectomized fish receiving growth hormone (Hypect. G. H.) compared with unoperated (Unop.), mock-operated (Mock), untreated hypophysectomized (Hypect.) and saline-injected hypophysectomized fish (Hypect. Saline). Series VIA. Duration of treatment with each of the three different growth hormone preparations (429, 22KR-2, and 508B) is shown at the bottom.

growth increments, under the conditions of the experiments, were of the order of 2% per week for weight and 0.8–0.9% per week for length.

In a previous study (Pickford and Thompson, 1948) it was shown that even saline injections produced shock effects which led to loss of weight. It was hoped that the use of an anesthetic, together with the more careful handling of the fish, might obviate this effect. The results were discouraging. Three out of seven hypophysectomized fish receiving saline injections died during the course of the experiment. The four survivors showed a severe loss in weight which was much greater than that observed in the hypophysectomized but uninjected controls. At the end of the eighth week the uninjected fish had lost an average of 10% in weight, the saline-injected group approximately 18%.

During the first two weeks of the experiment, fish receiving growth hormone also showed a loss in weight of the same order as that observed in the saline-injected controls. At the end of the third week this trend was reversed, the differences being significant at the 5% level (Table III). At the end of the fourth week the difference was

TABLE III. Weight and Length Changes of Hypophysectomized Fish Receiving Saline Injections Compared with Those Receiving Growth Hormone.* Series VIA.

	IV/11/50	*IV/18/50*	*IV/25/50*	*V/2/50*
Weight	<0.5, >0.4	>0.9	<0.02, >0.01	<0.001
Length	<0.05, >0.02	<0.05, >0.02	<0.01, >0.001	

* Values of P in t-test, given up to and including the date on which the difference became statistically significant.

highly significant and at the end of the fifth week the fish receiving growth hormone had regained the weight lost at the beginning of the experiment. From then on the weight increased rapidly at approximately the same rate as in unoperated and mock-operated controls. At the end of the experiment the average weekly increment, i. e., the total increase divided by the nine weeks of the experiment, was 1.7% (Table II). This is probably lower than the true growth rate on account of the initial period of retardation.

In respect to length increments, which provide an even more reliable criterion of induced growth, it is probable that there was growth stimulation almost from the start of the experiment. However, because of the small weekly increments and the difficulty of making accurate measurements to within 0.5 mm, the difference between the saline-injected controls and the fish receiving growth hormone did not become statistically significant until the end of the third week (Table III). At the termination of the experiment the fish receiving growth hormone had increased on an average at the rate of 0.5% per week. This is slightly less than the rate of growth of the mock-operated and unoperated controls but clearly of the same order of magnitude.

These results may be compared with the response of normal fish, under similar but somewhat cooler experimental conditions, to the injection of corresponding doses of purified growth hormone (Pickford and Thompson, 1948). The response of the hypophysectomized fish appears to be of the same order as that of normal fish in a state of winter quiescence. At the end of 10 weeks the normal fish had increased 12.7% in weight and 2.7% in length, whereas the hypophysectomized group, injected for a period of only nine weeks, showed an average increase of 14.8% in weight and 4.4% in length. The somewhat greater response of the hypophysectomized fish is probably associated with a higher room temperature. Both groups showed a loss in weight during the initial period of the experiment; this loss was more severe under the conditions of the 1948 experiment when, during the first two weeks, the fish received daily injections. In both groups this trend was reversed after the end of the third or fourth week.

In evaluating the results of these experiments, the possible effects of temperature must be taken into consideration. The response of normal fish to injections of beef growth hormone (Pickford and Thompson, 1948) was elicited against a declining temperature which fell from 17.4° to 10.4° C during the ten weeks of the experiment. The possibility of augmentation of the response due to rising temperature is therefore excluded. In Series VIA, on the other hand, the room temperature increased slowly from 16.3° to 19.4° C, hence a possible facilitation of the response cannot be excluded. Recent experiments indicate that there is a profound increase in the response of hypophysectomized fish to growth hormone at 20° C as compared with 15° C. There is no doubt that the growth of wild killifish comes to a standstill during the cold winter months. When fish taken in the late autumn are brought into the laboratory there is little or no growth until early spring, depending on the temperature changes in the aquarium room. The data presented above appear to justify the conclusion that the onset of spring growth is stimulated by the renewed activity of the pituitary gland.

Scales. The scales from all fish were sampled at the start of the experiment and again after one month. Sample scales were also taken at autopsy. Table IV shows the number of new circuli added during the course of the experiment. As expected, no new growth was observed in untreated or saline-injected hypophysectomized fish. The unoperated and mock-operated controls added new circuli at the rate of 0.8–1.0 per week during the seven or eight weeks during which these respective groups were kept under observation.

TABLE IV. Average Number of New Circuli Laid Down at Margin of Scale, Referred to April 3, 1950 Base Line. Series VIA.

Group	*May 2*		*May 24*		*May 30–31*		*June 6*	
	*No. of fish**	*New circuli†*	*No. of fish*	*New circuli*	*No. of fish*	*New circuli*	*No. of fish*	*New circuli*
Unop.	7/7	3.5	7/7	5.5	—	—	—	—
Mock	6/6	5.0	—	—	6/6	8.0	—	—
Hypect.	0/6	0	—	—	0/6	0	—	—
Hypect. saline	0/7	0	—	—	—	—	0/4	0
Hypect. gr. horm.	5/7	1.5	—	—	—	—	7/7	3.5

* Number of fish showing new growth at scale margin per number examined.
† Average number of new circuli on scales of fish that showed new growth.

Five of the seven fish receiving injections of growth hormone showed new growth at the scale margin one month after the start of the experiment. At the end of the experiment all seven fish showed new growth, varying from one to six new circuli. However, the process does not appear to have been quite normal in that the new calcification is extremely thin and delicate. Figs. 2A and B show microphotographs of the scales of fish 4–7 taken on January 12, one month after removal of the pituitary, and on April 3 at the start of injections. It will be seen that there had been no new growth at the margin of the scale during the whole of this period. Fig. 2C shows a scale from the same fish taken on May 2, one month after the start of injections. Careful inspection reveals an extension of the fibrous layer and the deposition of one extremely delicate new circulus on its surface. Fig. 2D shows a scale taken at the time of autopsy, nine weeks after the beginning of the experiment, when about six new circuli had been laid down at the margin of the scale. The break which marks the onset of renewed growth is quite similar to the year mark that is formed on the scales of normal fish in early spring. Three things may be noticed in respect to the induced growth of the scales: (1) The new circuli are irregular, especially those first deposited; this is a normal feature of

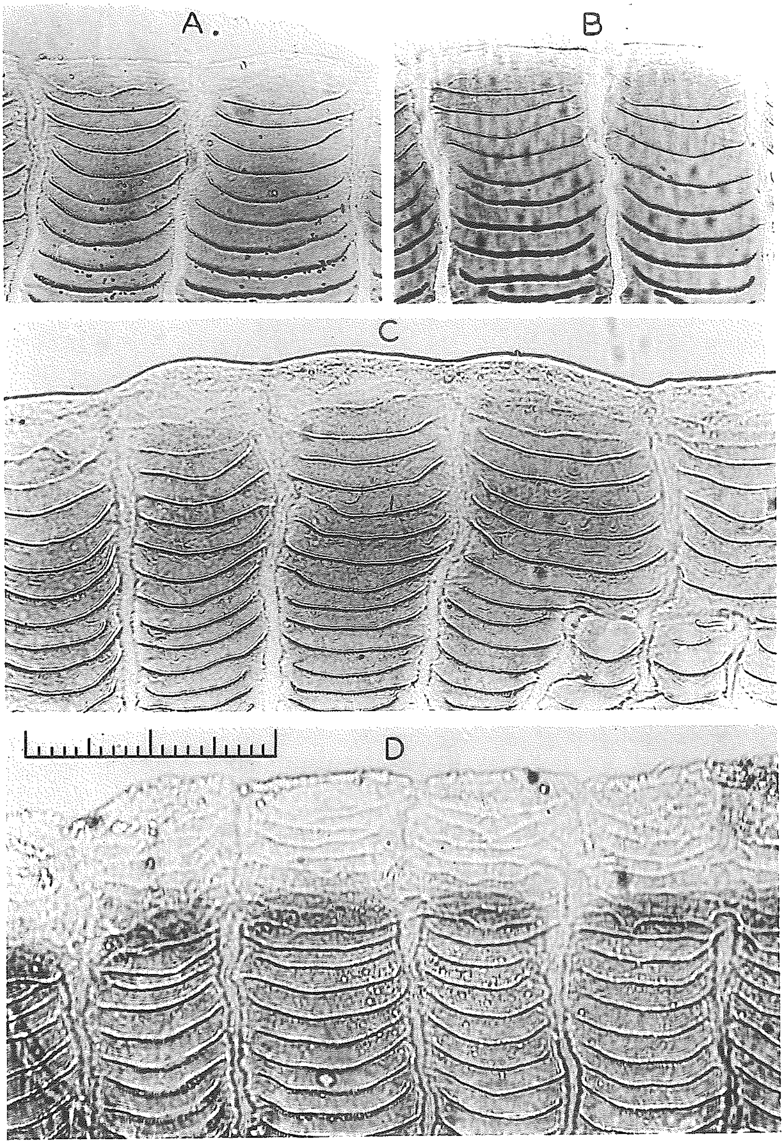

Figure 2. Microphotographs of margins of scales from fish 4-7; scales taken on January 12, 1950, one month after hypophysectomy (A); April 3, at the start of the experiment (B); May 2, after 12 injections with purified growth hormone (C); and June 6, at the end of the experiment (D). Series VIA. Micrometer scale in 10μ intervals.

annulus formation. (2) There is a tendency for new calcification to bridge the radial gap; this is an abnormal feature but one that is not infrequently seen at the annuli in scales taken from wild fish. (3) The initial zone of new calcification accepts the alizarin stain more deeply and stands out as a red band near the margin of the scale (dark grey in the photograph). New calcification, even in normal scales, tends to stain more deeply, and this phenomenon is enhanced in the experimental fish. On the other hand the most peripheral marginal zone is so lightly calcified that the staining is barely visible.

These observations appear to confirm the hypothesis that the pituitary growth hormone in fish, as in higher vertebrates, plays an essential role in the process of ossification. The somewhat abnormal features of the response may be attributed to the high degree of purity of the preparation used during the last month of the experiment. This preparation did not contribute traces of any other anterior lobe factors, except possible minute amounts of thyrotrophin, hence the fish were not in a state of normal endocrine balance. In nature, for example, the resumption of spring growth is always associated with the stimulation of the gonads, whereas in these fish the testes were in regression.

A word may be said about our past failure to observe new growth on the scales of normal fish receiving injections of purified growth hormone (Pickford and Thompson, 1948). At that time the scales were being mounted unstained. Under such conditions the deposition of faint new calcification would scarcely be visible, and furthermore, from the results of the present series, it may be concluded that perhaps only one or two new circuli could have been expected for the average length increment of 2.7%. A re-examination of the slides shows that the condition at the margin of the scale was comparable to that shown in Fig. 2C, but lack of staining makes recognition of slight new calcification very uncertain.

Otoliths. Otoliths were not recovered from one specimen, but those taken from the 11 remaining hypophysectomized fish, whether untreated or injected with saline, showed a clear marginal zone. In three cases the surface was faintly opaque and in two others there were faint caps of new deposition at the projecting angles. While the correlation is not complete, four of these five fish are the same as four of the five whose scales showed evidence of a resumption of growth in autumn before the pituitary was removed. It may be inferred that new deposition was also present on the otoliths at this time, and it is practically certain that no change occurred after hypophysectomy.

The otoliths from all of the six mock-operated controls showed new growth in the form of an opaque surface deposit with more or less well defined apical caps at the angles. The same condition obtains for four of the seven unoperated controls, but the other three showed a clear peripheral zone without evidence of new deposition; however, the scales of these three had four to six new circuli, and weight and length increments were of the same order as those in other members of the group. The amount of new growth on the otoliths was small; some of it may have been present before the start of the experiment, and it seems possible that there is a lag between the onset of bodily growth and the resumption of deposition on the otolith.

Six of the seven hypophysectomized fish receiving growth hormone showed new deposits on the otoliths, and even the otoliths of the seventh fish had a faint surface opacity. Some deposition might have been present in a few fish at the time of removal of the pituitary, but it is extremely unlikely that all seven would have been so affected. Moreover, in three of the treated fish the supposedly new apical caps were more pronounced and more clearly developed than the faint apical caps seen in some of the hypophysectomized controls. It seems safe to conclude that growth stimulation resulting from the injections was accompanied by new deposition on the otoliths.

Liver. The hepatosomatic indices are given in Table V. The livers of unoperated and mock-operated controls showed the usual reduction in size characteristic of males in the spring of the year. The difference between the mock-operated and unoperated groups is not statistically significant and the mean for the combined group of 13 fish is 3.0 ± 0.5. The livers of the untreated hypophysectomized fish are large, in accordance with the results of previous experiments (Pickford, 1953). The same is true of hypophysectomized fish that received saline; three fish in this group died during May and are excluded from the average given in Table V. The hepatosomatic indices were 3.6, 8.1, and 6.6, and the combined mean for all seven fish in this group, irrespective of date of death, is 5.5 ± 1.6. Finally, the difference between the untreated hypophysectomized fish and those receiving saline injections is not statistically significant, and the mean for the entire group of 12 hypophysectomized controls is 6.0 ± 2.1.

The hepatosomatic index of fish receiving growth hormone lies midway between the values of the hypophysectomized and unhypophysectomized controls. A statistical treatment of the data reveals that the liver size of the growth-hormone group is significantly greater than that of the pooled mean for 13 normal (mock plus unoperated) controls. On the other hand, the difference from the pooled mean for the 12 hypophysectomized controls is not statistically significant. Thus it is doubtful whether the relative size of the liver is significantly decreased by the administration of purified growth hormone.

TABLE V. Average Hepatosomatic Indices. Series VIA.

Operation	*Treatment*	*No. of fish*	*Date of sutopsy*	*HSI*
None	None	7	V/24/1950	3.2±0.6
Mock	None	6	V/30–31/1950	2.8±0.5
Hypect.	None	5	V/30–31/1950	6.6±2.8
Hypect.	Saline	4	VI/6/1950	5.1±0.9
Hypect.	Gr. horm.	6*	VI/6/1950	4.6±0.6

* Fish no. 7–8 excluded because of pathological condition of liver at time of autopsy.

The livers of the mock-operated and unoperated controls were reddish brown in color (Ridgway's *vinaceous cinnamon*, *vinaceous* or *cinnamon rufous*, or *hazel*). These shades are found normally in wild males at this season of the year. The livers of the five untreated hypophysectomized controls were also mostly reddish brown, although one was clay-colored. Among the saline-injected controls, the three that died during May had buffish colored livers (*buff*, *cream buff*, and *pinkish buff*); of the four that survived to autopsy, two livers were reddish brown (*cinnamon* and *vinaceous cinnamon*) and two were grey brown (*isabella*, *fawn*). Grey browns prevailed in four of the seven fish receiving growth hormone while two were *vinaceous cinnamon*. One (fish 7–8) which became sick shortly before the end of the experiment had a green liver; the data for this fish are not included in this part of the investigation, although its over-all growth rate was not impaired by its final sickness which came too quickly to interfere with the long term results.

There is some evidence from the work of Chambers (1953) that grey-brown livers are associated with watery vacuolization induced by the administration of thiourea. However, in the present series there is no evidence of cytological abnormality. Furthermore, no fish showed a serious development of hepatic cirrhosis, although small amounts of ceroid, of the same order as that found in the livers of wild fish, were present. Glycogen was abundant, except in the three saline-injected hypophysectomized fish which died before the tissues could be fixed. No special procedures were used for the study of fat accumulation, but its relative abundance was estimated from the number and size of the fat vacuoles in the hepatic cells, as seen on paraffin sections. Fat appears to have been present in moderate to plentiful amounts and no significant differences could be detected between the various control groups and those fish that had received growth hormone.

Blood. Blood smears were made from the cut end of the tail at the time of autopsy, except for the unoperated control group in which a study of the blood was omitted. It was at once apparent that the smears from the hypophysectomized fish were thin in comparison with those taken from the mock-operated controls. Unfortunately hemoglobin determinations were not made since our intention in taking the blood smears had been merely to make a routine check for the possible presence of trypanosomes or other parasites. However, in view of the obvious thinness of the smears, it was decided to attempt a comparative estimation of the relative abundance of red blood corpuscles by the somewhat unorthodox procedure of counting their relative frequency on selected areas of the smear. The number of red cells was counted, with the aid of a squared ocular micrometer, in a standard area of 67 square microns. Five such areas were counted for each fish. The areas were selected from the most uniform regions of the smear where the cells were evenly spread in a single uniform layer. As a further precaution, the selected areas were taken in regions which showed a uniform scattering of thrombocytes interspersed among the erythrocytes; two or three thrombocytes were present in each standard area, rarely one or four. Differences in the number of erythrocytes are thus referred to a relatively constant thrombocyte level, the possible absolute fluctuations of which are of course unknown. Although this somewhat arbitrary procedure provides only relative results of a semiquantitative nature, it is clear (Table VI) that the hypophysectomized fish are more or less anemic, with an erythrocyte count that is 30–40% below normal. This finding is in agreement with the well known effects of hypophysectomy on the blood of mammals; the "plateau level" attained in hypophysectomized rats is 30% below normal (Crafts, 1949). Furthermore,

TABLE VI. Erythrocyte Counts on Blood Smears. Series VIA.

Operation	*Treatment*	*No. of fish*	*Number of erythrocytes in standard area, 67μ²*
Mock	None	6	82.3± 7.4
Hypect.	None	5	56.0±14.2
Hypect.	Saline	4	48.0±12.6
Hypect.	Gr. horm.	7	60.5±11.5

the administration of purified growth hormone, even over a prolonged period of time, appears to have had no significant effect on the state of anemia induced by the lack of the pituitary gland. In a recent paper, Van Dyke, et al. (1952) have shown that this is also true in rats.

No abnormalities were observed in the leucocyte picture and no blood parasites were discovered in the experimental fish.

Testes and Nuptial Coloration. As anticipated from previous work, the testes of hypophysectomized fish were in a state of total regression (Table VII). The testes of the seven fish which received growth hormone were also in regression, indicating that the preparation used during the last month of the experiment, No. 508B, was free from a

TABLE VII. Gonosomatic Indices and Nuptial Coloration at Time of Autopsy. Series VIA.

Operation	*Treatment*	*No. of fish*	*Date of autopsy*	*GSI*	*Nuptial colors**
None	None	7	V/24/50	4.00±1.15	± to +++
Mock	None	6	V/30–31/50	5.60±0.81	± to +++
Hypect.	None	5	V/30–31/50	0.41±0.18	—
Hypect.	Saline	4	VI/6/50	0.23±0.14	—
Hypect.	Gr. horm.	7	VI/6/50	0.49±0.16	—†

* Degree of development of sulfur yellow rated on a subjective basis.

† One fish in this group had a persistent slight trace of yellow on the pelvic fins (see Table VIII). This was apparently nonsexual since the testes were completely regressed.

gonad-stimulating component. The nuptial colors of the hypophysectomized fish were correspondingly negative at the time of autopsy but, as indicated previously, there was a period near the beginning of the experiment when this was not true. The course of the development and subsequent regression of the nuptial coloration in these fish is shown in Table VIII. It is clear that preparation No. 429 contained sufficient gonadotrophin to elicit the first signs of nuptial coloration before the end of the third week, and therefore use of this preparation

TABLE VIII. DEVELOPMENT AND SUBSEQUENT REGRESSION OF NUPTIAL COLORATION FOLLOWING INJECTIONS OF GROWTH HORMONE PREPARATION NO. 429. SERIES VIA.

Fish	*Preparation 429*				*22KR-2*		*508B*			
	IV/3	*IV/11*	*IV/18*	*IV/25*	*V/2*	*V/9*	*V/16*	*V/23*	*V/30*	*VI/6*
1–2	—	—	—	±	±	±	tr*	—	—	—
4–7	—	—	—	±	+	±	tr	—	—	—
4–8	—	—	—	+	+	+	—	—	—	—
4–9	—	—	—	±	±	±	tr	—	—	—
7–3	tr	tr	tr	±	±	±	tr	tr	tr	tr
7–7	—	—	—	±	±	±	tr	—	—	—
7–8	—	—	—	tr	tr	—	—	—	—	—

* tr = trace.

was discontinued after April 25. Preparation No. 22KR-2 supposedly contained negligible amounts of gonadotrophin (White, et al., 1951), but the nuptial colors did not disappear until preparation No. 508B was used. However, after hypophysectomy it takes at least two weeks for the nuptial colors to fade away, and no doubt the brief period during which preparation 22KR-2 was used represents a latent period during which the testes slowly returned to a resting condition. There is no evidence that preparation 22KR-2 contributed toward the maintenance of nuptial coloration.

For comparison with this cycle of appearance and disappearance of nuptial colors, a further experiment was made in the autumn of 1952. Five hypophysectomized male fish which were totally without nuptial coloration at the start of the experiment were injected thrice weekly with pituitary gonadotrophin (Armour and Co., Lot No. 45208-R) at a dosage level of 10 μg per gram weight. Nuptial colors began to appear at the beginning of or during the second week (Table IX), but even at the end of one month they had not advanced beyond the low degree seen with growth hormone preparation 429. Testis stimula-

TABLE IX. DEVELOPMENT OF NUPTIAL COLORATION IN HYPOPHYSECTOMIZED MALE FISH RECEIVING INJECTIONS OF MAMMALIAN PITUITARY GONADOTROPHIN, NOVEMBER–DECEMBER 1952. SERIES XIIIC.

Fish	*GSI*	*XI/17*	*XI/24*	*XII/1*	*XII/8*	*XII/14*
11–1	1.87	—	—	tr	+	tr
11–2	0.58	—	—	tr	tr	tr
11–3	0.86	—	—	±	±	±
11–4	0.44	—	tr	±	±	±
11–5	0.66	—	±	+	+	+

TABLE X. CONDITION OF THE THYROID. SERIES VIA.

Group and date of autopsy	*Fish*	*Av. epith. height* (μ)	*Per cent over 12* μ	*Predominant colloid* *Azan-red*	*Mixed*	*Azan-blue*
Unop. and	3–1	5.3±1.7	0	X	—	—
uninject.	3–2	6.5±3.2	1	X	—	—
V/24/1950	3–3	13.1±6.2	54	X	—	—
	3–4	9.7±3.7	30	X	—	—
	3–5	8.4±2.7	13	—	X	—
	10–7	14.3±4.8	67	Almost lacking		
Mock oper.	2–1	10.7±4.7	39	—	X	—
uninject.	2–3	15.0±5.6	65	Almost lacking		
V/30–31/1950	2–5	14.9±4.4	72	Almost lacking		
	2–7	8.7±3.0	17	X	—	—
	5–1	11.8±4.3	42	—	X	—
	5–5	14.0±4.9	66	Almost lacking		
Hypect.	1–4	3.4±1.1	0	X	—	—
uninject.	1–5	3.3±1.3	0	X	—	—
V/30–31/1950	4–1	2.9±0.8	0	X	—	—
	7–1	3.9±1.1	0	—	—	X
	7–2	4.5±1.8	1	X	—	—
Hypect.	1–1	2.7±0.8	0	X	—	—
saline	1–9	4.4±1.5	0	X	—	—
VI/6/1950	7–5	3.7±1.2	0	X	—	—
	8–1	4.5±1.6	0	X	—	—
Hypect. gr.	1–2	5.9±2.1	2	X	—	—
horm.	4–7	5.0±1.9	0	X	—	—
VI/6/1950	4–8	5.1±2.1	1	X	—	—
	4–9	4.4±1.5	0	—	X	—
	7–3	5.2±2.1	2	X	—	—
	7–7	5.1±1.8	1	X	—	—
	7–8	6.0±2.5	3	—	X	—

tion was evident at autopsy; the mean gonosomatic index for five initial controls was 0.47 ± 0.18, the mean for five final controls (receiving saline injections during the period of the experiment) 0.32 ± 0.07; the mean for the five fish receiving mammalian pituitary gonadotrophin was 0.88 ± 0.57. The dosage level in this experiment was similar to the growth hormone dosage level (10 μg/g) and was therefore much higher than any gonadotrophin component of the latter. It merely represents the response of the fish to an upper limiting dose, hence it is not surprising that the response was more rapid. Further experiments, with a view to the possible assay of the gonad-stimulating factor, are under consideration.

Thyroid. The condition of the thyroid in treated fish and in the various control groups is shown in detail in Table X; the main results, in respect to average epithelial height, are summarized in Table XI. The glands of the hypophysectomized controls, whether untreated or injected with saline, were completely quiescent except for a single active follicle observed in one fish. The thyroids of nearly all of the unoperated and mock-operated controls were hyperactive and many of them were markedly hyperplastic.

TABLE XI. CONDITION OF THYROID, SHOWING AVERAGE EPITHELIAL HEIGHT. SERIES VIA.

Operation	*Treatment*	*No. of fish*	*Average epith. height* (μ)
None	None	6	9.5±3.6
Mock	None	6	12.5±2.5
Hypect.	None	5	3.6±0.8
Hypect.	Saline	4	3.8±0.8
Hypect.	Gr. norm.	7	5.2±0.6

Particular interest attaches to the condition of the thyroid in the fish treated with growth hormone. There were no signs of hyperplasia, but the average epithelial height is significantly greater than in the hypophysectomized controls (Table XII). The significance of the difference could not be judged from a comparison with the unoperated and mock-operated controls, since these fish were suffering from goiter. Therefore it became necessary to determine the average epithelial height in normal fish that were not suffering from an iodine deficiency so that the condition in fish receiving growth hormone might be evaluated. These data have been brought together in a previous paper (Pickford, 1953: table XIII). Normal laboratory males receiving a trace of iodine in the diet, autopsied in April or July, had an average epithelial height ranging from 5.0 to 5.4. This is of the same order as that observed in the growth hormone experimentals, and we may therefore conclude that the glands in the latter fish were in a normal state of activity.

TABLE XII. VALUES OF *P* IN COMPARISONS OF THE AVERAGE EPITHELIAL HEIGHT OF TREATED AND UNTREATED HYPOPHYSECTOMIZED FISH. SERIES VIA.

	No treatment	*Saline*
Saline	<0.7, >0.6	—
Growth hormone	<0.001	<0.01

Head Kidneys and Adrenal Cortical Tissue. The head kidneys of the treated and untreated hypophysectomized fish were compared with those of the mock-operated group. All of the hypophysectomized fish, without exception, showed a more or less pronouncedly pathological kidney condition due to the development of renal calculi (see Pickford, 1953). The kidneys of the mock-operated fish were normal.

In regard to the adrenal cortical (Giacomini) tissue, it must be stated that there were no appreciable differences between the mock-operated and hypophysectomized fish, which is in agreement with previous findings, nor were there any differences between either of these groups and the fish receiving injections of purified growth hormone. The Giacomini tissue forms a layer of from one to four cells deep (usually two to three) and the adrenal cortical cells are well laden with granules. Since there is no evidence of stimulation, it may be concluded that preparation No. 508B was free from corticotrophin.

Corpuscles of Stannius. A careful study was made of sections of the Stannius corpuscles. In the hope of obtaining better fixation, they were dissected out under a binocular and fixed in a Zenker-Formol-Osmic mixture. The preservation was no better than with Helly's fluid, and the staining was much inferior. There was no evidence that either hypophysectomy or the administration of purified growth hormone had any effect on these glands.

SUMMARY

1. Hypophysectomized male killifish were injected thrice weekly with purified beef growth hormone at a dosage level of 10 μg per gram weight for a period of nine weeks. The course of events is similar to that shown by normal fish when similarly treated: an initial period of loss in weight was reversed at the end of the third or fourth week.

2. At the end of nine weeks the fish had increased 14.8% in weight and 4.4% in length. This response is of the same order but slightly better than that induced in normal fish (Pickford and Thompson, 1948).

3. Resumption of bodily growth is accompanied by the addition of an average of 3.5 new circuli at the scale margin and by deposition of new calcification on the surface of the otoliths. Induction of new growth on the scales leads to the formation of a zone of irregular

growth resembling an annulus, but the new calcification is somewhat abnormal.

4. Three different growth hormone preparations were used. No. 429 (Wilhelmi) contained sufficient gonadotrophin to induce the appearance of nuptial coloration at the end of three weeks. No. 22KR-2 (Armour and Co.), used only five times, induced serious symptoms of hyperthyroidism. The experiment was completed with No. 508B (Wilhelmi) which was free of gonadotrophin and corticotrophin but which contained a trace of thyrotrophin sufficient to maintain the thyroids at normal level.

5. Administration of growth hormone showed a slight but not statistically significant tendency to reduce the size of the liver of hypophysectomized fish. It had no effect on the anemia resulting from hypophysectomy, and there was no disappearance of renal calculi.

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TOXIC EFFECTS OF THIOUREA ON THE LIVER OF THE ADULT MALE KILLIFISH, *FUNDULUS HETEROCLITUS* (LINN.)

By

Harriet A. Chambers

Bingham Oceanographic Laboratory
Yale University

ABSTRACT

Intraperitoneal administration of toxic doses of thiourea into adult male *Fundulus heteroclitus* (Linn.) produced the expected progressive decrease in body weight, retardation of growth in length, regression of the testes, and hyperplasia, hyperemia and hypertrophy of the thyroid gland. Also, there was an enlargement of the liver, despite depletion of glycogen and fat content. The most striking result, however, was the increase in size of the hepatic cells and their nuclei and the vacuolization of the liver cell cytoplasm.

INTRODUCTION

This investigation was undertaken to ascertain the effect of chemical thyroidectomy on the livers of adult male *Fundulus heteroclitus* (Linn.), the common killifish. The thyroid gland of *Fundulus*, like that of most teleosts (Gudernatsch, 1911), consists of a series of individual follicles distributed in a diffuse manner along the ventral aorta. Consequently, even partial surgical thyroidectomy in teleosts is extremely difficult and has not been attempted so far as is known; within the Pisces, thyroidectomy has been performed only in sharks (Vivien, 1941; Waring, et al., 1942). Inactivation of the thyroid of *Fundulus* can be achieved by hypophysectomy, which has the disadvantages of collateral effects on other organ systems, or it can be achieved by the use of an antithyroid drug, the method used in these experiments.

Many investigators have shown that, in teleosts, antithyroid agents produce hyperplasia, hyperemia and hypertrophy of the thyroid gland (Goldsmith, et al., 1943; Nigrelli, et al., 1946; Matthews and Smith, 1947; Lever, et al., 1949; Rasquin, 1949; Frieders, 1949; Hoar and Bell, 1950; Hopper, 1950; Sullivan, 1950; and Olivereau, 1952). Several of these investigators have shown also that antithyroid drugs cause an inhibition of growth (Goldsmith, et al., 1943; Nigrelli, et al., 1946; Frieders, 1949;[1] Hoar and Bell, 1950; Hopper, 1950, 1952) and a retardation of secondary sexual characteristics (Goldsmith, et al., 1943; Nigrelli, et al., 1946; Hopper, 1950, 1952). Warner (1952) reported that immersion of eggs of the German brown trout in a 0.033% solution of thiourea delayed the hatching 24 hours; also, immersion of fry up to 154 days in this solution delayed yolk sac absorption, decreased activity and viability, but did not appreciably inhibit growth in length. A decrease in oxygen consumption was demonstrated by Smith and Matthews (1949). French workers have shown an expected decrease in blood copper (Fontaine and Leloup, 1947; Leloup, 1947) and have reported the reversal of positive rheotrophism in elvers (Fontaine, 1948). Hatey (1950) is apparently the only investigator who has studied the effect of thiourea on the liver and carbohydrate metabolism in teleosts.

Concerning the use of goitrogenic substances on teleosts, the literature does not supply adequate information regarding dosage strength and volume. Most of the previous workers either put the antithyroid drug into the food or kept the fish in solutions of varying strength. In the present experiment, injections were used to obtain a more accurate record of the dosage.

METHODS AND MATERIALS

The fish used in this investigation were caught locally and were acclimatized to aquarium life for approximately two months prior to the start of the experiment. They were marked with colored beads for individual identification (Pickford and Thompson, 1948), kept in sea water of 27 °/₀₀, and received constant illumination for eight hours each day. They received daily feedings of a cooked liver-pablum mixture (Gordon, 1943) supplemented by raw beef liver about twice a week. These conditions were maintained throughout the experiment for all of the specimens except those that were injected; the latter were not offered food on days of injection, thus their feeding was reduced to four days per week. The temperature of the aquarium room rose slowly from 15.0° C on April 11, the initiation of the experiment, to 18.5° C on May 24, 1950, when the investigation was concluded. Table I gives the plan and arrangement of the experiment.

[1] In one of the four species studied, *Trichogaster trichopterus*, there was an initial retardation of growth followed by a growth rate of the phenylthiourea-treated fish which was faster than that of the controls.

The fish were anesthetized in a solution of tricaine methanesulfonate (M. S. 222)[2] prior to injection through the cloacal region into the body cavity. Injection was made thrice weekly, and the dosage employed initially was 0.005 cc of a 10% solution of thiourea, i. e., 0.5 mg/g body weight, which conforms to the dosage employed by Ratzerdorfer, et al. (1949) on lizards. However, two experimental fish died during the first four weeks, hence the concentration of the stock solution was cut to half for the duration of the investigation, thus reducing the dosage level to 0.25 mg/g body weight. Even this reduced dosage was apparently somewhat toxic, since three more thiourea-treated fish died during the next two weeks, leaving a total of seven pairs of animals plus the seven untreated controls in Tank 3.

TABLE I. ARRANGEMENT AND PLAN OF EXPERIMENT, APRIL 11, 1950

	Tank 3	*Tank 5*	*Tank 6*	*Tank 9*	*Tank 10*
Number of fish	7	6	6	6	6
Av. wgt. (g)	7.0 ± 2.1	7.2 ± 1.7	7.1 ± 1.1	8.2 ± 1.5	8.0 ± 1.1
Av. lgth. (cm)	8.1 ± 2.0	8.2 ± 0.5	8.2 ± 0.4	8.5 ± 0.5	8.6 ± 0.4
Treatment	ad-libitum fed, uninjected	Thiourea injected	"pair-fed," saline injected controls	"pair-fed," saline injected controls	Thiourea injected

After thiourea injections had begun there was an immediate decline in the amount of food consumed by the experimental fish, while saline-injected specimens in the control tanks, fed approximately the same amount of food, were voraciously hungry, thus indicating that the loss of appetite in the experimentals was an effect of the drug rather than of the handling and injection procedure. This loss of appetite on the part of the experimentals is in contrast to the report of Goldsmith, et al. (1943), who stated that the thiourea-treated fish accepted food as readily as the controls; however, their fish, immersed in a solution of thiourea, did not receive the drug directly into the body cavity. Some investigators (Leblond and Hoff, 1944; Barker, 1945; Christensen, 1945) have reported that rats take less food and that their basal metabolism is reduced after they have been injected with goitrogenic substances or have drunk water with the antithyroid drug dissolved in it.

First, the hypobranchial region containing the thyroid was removed and fixed, after which the pituitary, liver, and testis were removed

[2] Tricaine methanesulfonate obtained from Sandoz Chemical Company, New York; stock solution 1 g in 500 cc, diluted 1 to 6 for use.

and preserved, the latter two organs being weighed before preservation. The thyroid and liver were studied histologically. The gonad weights have been treated statistically, since it seemed probable that the gonosomatic index might be an adequate measure of spermatogenetic activity (Matthews, 1938). The pituitaries were not studied in the present investigation.

The hypobranchial region, fixed in sea water Bouin, was sectioned serially at 7 μ. Alternate slides were stained with a modified Azan method (Pickford, 1953) and with Delafield's hematoxylin and eosin. To avoid measuring a cell from the same follicle twice, approximately every 30th section was studied. A single cell was measured from each follicle and the results represent the mean of 100 follicles from each fish.

Before the liver was weighed, its color was obtained by comparison with the Ridgeway (1866) color charts. After being weighed, the livers were cut transversely and midslices were fixed in Baker's formaldehyde calcium (Pantin, 1948) for fat preservation and in an alcohol-formalin mixture (9 pts. 95% alcohol, 1 pt. formaldehyde) for glycogen fixation. The apices and bases of the livers were fixed in sea water Bouin.

For the study of fat, frozen sections were cut at 15 μ. Sections were stained in dilute scarlet red and counterstained in Bullard's hematoxylin. A control section was prepared by leaving the tissue for one hour in undiluted scarlet red in absolute alcohol and by counterstaining in the same manner. Sections were mounted by the double coverslip method, using buffered glycerine to prevent fading of the hematoxylin.

The alcohol-formalin fixed slices were sectioned at 10 μ. The cut surface of the block was soaked overnight in 70% alcohol to facilitate cutting since the section crumbled without this treatment. Sample sections of the alcohol-formalin fixed liver were mounted with an albumin-50% alcohol mixture, coated with a 1% celloidin covering, and stained in a water bath at a constant temperature of 25.1 ± 0.2° C by the periodic-acid-Feulgen technique of Hotchkiss (1948). Control sections, first digested for one hour in buffered malt diastase (Lillie, 1948: 141), were coated and stained simultaneously with the test section. Sample sections were also stained with Delafield's hematoxylin and eosin.

RESULTS

Length and weight. Since statistical treatment of the data for length and weight revealed no significant differences between Tanks 5 and 10 (experimentals) and Tanks 6 and 9 ("pair-fed" controls), the data for the two groups were pooled under two headings: experimentals and controls.

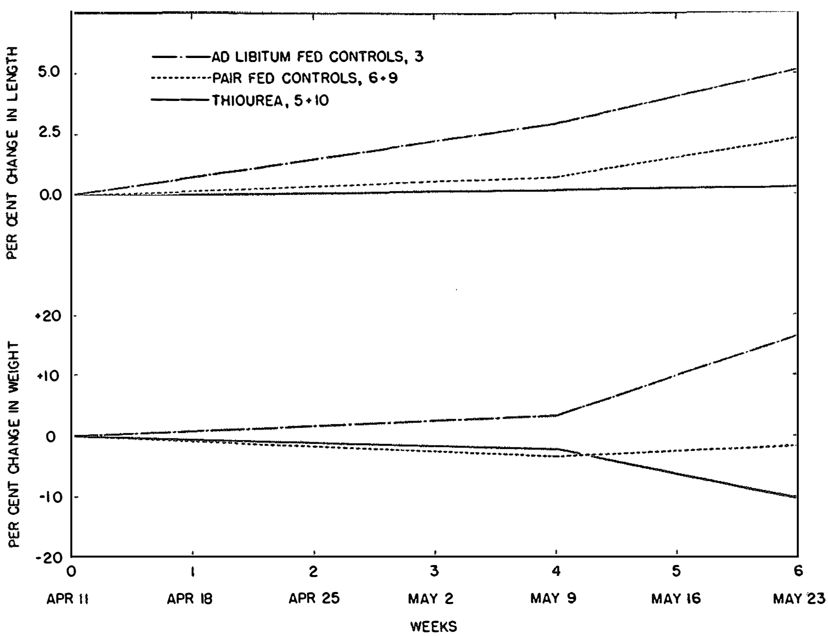

Figure 1. Length-weight changes expressed in per cent.

The data on weight and length changes (Table II, Fig. 1) show clearly that intraperitoneal administration of thiourea causes a rapid and progressive loss of weight and a retardation of growth as indicated by the failure of the experimentals to increase significantly in length (+0.25%). Undoubtedly inhibition of growth was caused by the drug, since the "pair-fed" controls, though in a state of semistarvation, increased 2.4% in length in six weeks. The "pair-fed" controls, although they lost more weight than the experimentals during the first four weeks of the investigation, showed a marked increase during the following two weeks. At the termination of the experiment, the "pair-fed" saline-injected controls had lost only 1.7% of their original weight, the experimentals 10.2%.

Testes and nuptial coloration. The gonosomatic index of each group —experimentals, "pair-fed" saline-injected controls, and ad-libitum fed controls—is given in Table III. The data were treated statistically. Also given in Table III are data for wild population samplings (Chambers, 1951).

TABLE II. LENGTH AND WEIGHT CHANGES (IN PER CENT)

	May 9	*May 24*
Tank 3. Ad-libitum fed controls		
Number of fish	7	7
% change, weight	3.10 ± 3.27	16.94 ± 6.81
% change, length	2.90 ± 1.17	5.19 ± 1.81
Tanks 6, 9. "Pair-fed" controls		
Number of fish	10	7
% change, weight	−3.41 ± 3.02	− 1.69 ± 4.48
% change, length	0.65 ± 0.78	2.36 ± 0.16
Tanks 5, 10. Thiourea		
Number of fish	10	7
% change, weight	−2.29 ± 4.00	−10.23 ± 2.42
% change, length	0.12 ± 0.91	0.25 ± 0.94

The data show, among other things, that administration of thiourea causes almost complete atrophy of the testis in male *Fundulus*. An idea of the normal size of the testis in the spring, the time of year that this investigation was carried on, may be obtained from the figures given for the various control groups and for the wild population sampling taken in May. Note that the data for the two wild population samplings confirm the work of Matthews (1938), who showed that the gonads are in a state of inactivity in the fall (0.71 ± 0.22), probably remain dormant throughout the winter, and then become greatly enlarged in the spring (5.61 ± 1.66).

The degree of maturation of the gonad is correlated with the development of nuptial coloration, displayed in this species by the appearance of sulfur yellow on the belly and anal fins. Nuptial coloration was rated on a subjective basis: +++, ++, +, ± or −. In most of the thiourea-treated fish the coloration decreased during the experiment from an average rating of + to −. The "pair-fed" saline-injected controls showed a tendency to remain at the + rating, while the ad-libitum fed controls exhibited a slight cyclical change, averaging + in April, ++ on May 9, and + again on May 24.

Thyroid. Table IV gives the mean thyroid epithelial height and the standard deviation based on 100 follicles for each fish. The data confirm the findings of all previous workers that administration of an antithyroid agent in teleosts produces hyperemia, hyperplasia and hypertrophy of the thyroid gland. All of the thiourea-treated fish had an average epithelial height of 10 μ or more, a condition which was observed in only one of the "pair-fed" controls and in two of the untreated controls.

TABLE III. GONOSOMATIC INDICES FOR TWO-YEAR-OLD MALE FUNDULUS

Group	*Treatment*	*No. of Fish*	*Gonosomatic Index**
3	Ad-libitum fed controls	7	4.01 ± 1.15
6, 9	"Pair-fed," saline-injected controls	7	5.01 ± 1.19
5, 10	Thiourea treated	7	0.64 ± 0.40
A	Wild population, Nov. 1949 (Chambers, 1951)	26	0.71 ± 0.22
B	Wild population, May 1950 (Chambers, 1951)	18	5.61 ± 1.66

* $\frac{\text{gonad weight x 100}}{\text{body weight}}$

While the thyroid epithelial height of the thiourea-treated fish is significantly greater than that of either of the control groups ("pair-fed": $t = 4.72$, P = > 0.001; ad-libitum fed: $t = 2.63$, P = 0.02), the epithelial height of the two control groups is not significantly different ($t = 1.32$, P = 0.2) although the glands of the ad-libitum fed fish were slightly more active. Recently Pickford (1953) has shown that the heightened thyroid epithelium of some of the controls is due to a moderate case of goitre resulting from an iodine deficiency in the laboratory diet. The mean thyroid epithelial height of wild male *Fundulus* sampled in May was 6.9 μ.

The thyroid follicles of the experimental fish which died before the termination of the experiment (not included in the data) were already in a hyperplastic condition on May 4, the date of the first fatality, three weeks after the beginning of the investigation.

Liver. The liver colors, recorded prior to preservation, are divided into two major groupings: red browns (vinaceous cinnamon, vinaceous rufus, hazel, brick red, chestnut and russet) and grey browns (isabella and wood brown). All of the control fish, both untreated and saline-injected, had red brown livers. However, of the original 12 thiourea-treated *Fundulus,* five died in the early stages of the investigation. Of these five, one had a cream-colored liver, two grey brown and two red brown. The liver colors of the seven surviving fish, autopsied on May 24, were grey brown (4) and red brown (3). The results are

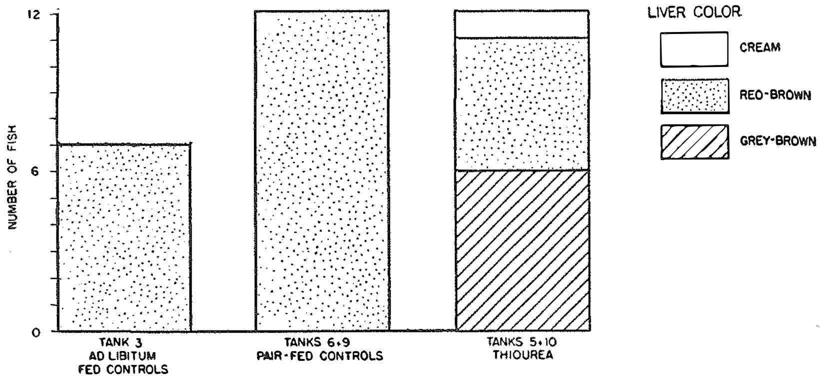

Figure 2. Graphic illustration of liver colors (all fish).

summarized graphically in Fig. 2. There appears to be no correlation of greyish liver color with reserves of fat and glycogen or with the development of ceroid, discussed in the following sections.

Of the 70 wild fish of both sexes and all ages sampled in May 1950, the liver colors of only two were grey, the rest varying from vinaceous buff to chestnut, russet or bay. Among the 74 fish sampled in November 1949, the predominant liver color was vinaceous pink or vinaceous buff, no grey being observed.

The hepatosomatic indices given in Table V show that the livers of thiourea-treated fish were significantly larger than those of both the "pair-fed" controls (t = 7.880, P = <0.001) and the ad-libitum fed controls (t = 2.914, P = > 0.01, < 0.02).

TABLE IV. Thyroid Epithelial Height (in Microns)

Tank 3		*Tanks 6, 9*		*Tanks 5, 10*	
Ad-libitum fed controls		*"Pair-fed" controls*		*Thiourea*	
Fish No.	*Epith. height*	*Fish No.*	*Epith. height*	*Fish No.*	*Epith. height*
3.1	5.3 ± 1.7	9.1	5.8 ± 1.2	9.3	13.9 ± 5.3
3.2	6.5 ± 3.2	5.3	13.7 ± 5.1	2.2	13.6 ± 4.4
3.3	13.1 ± 6.2	9.4	5.5 ± 1.7	9.2	16.6 ± 3.5
3.4	9.7 ± 3.7	5.6	5.5 ± 1.3	2.6	15.5 ± 3.4
3.5	8.4 ± 2.7	6.1	7.1 ± 1.9	10.5	11.6 ± 4.0
10.7	14.3 ± 4.8	10.3	5.6 ± 1.4	10.2	10.4 ± 4.2
6.6	*	10.1	6.6 ± 2.4	10.6	17.9 ± 5.5
Average	9.5 ± 3.6		7.1 ± 3.0		14.2 ± 2.8

Wild Population	*November*		*May*	
(2-year-old males)	*No. of fish*	*Epith. height*	*No. of fish*	*Epith. height*
	6	6.5 ± 0.9	5	6.9 ± 1.1

* Tissue lost.

The wild population sampling shows that in November the male has a large liver and that in the spring the hepatosomatic index falls to less than half of its autumn value. Only two-year-old fish corresponding to those used in the experiments have been included in the data given for the wild population samplings.

The amount of glycogen or fat, estimated on the basis of a microscopical survey of each section, is given in Tables VI and VII. The "pair-fed" controls are listed opposite their thiourea-treated partners.

TABLE V. HEPATOSOMATIC INDICES OF TWO-YEAR-OLD MALE FUNDULUS

Group	*Treatment*	*No. of fish*	*Hepatosomatic Index**
3	Ad-libitum fed controls	7	3.24 ± 0.64
6, 9	"Pair-fed," saline-injected controls	7	2.27 ± 0.12
5, 10	Thiourea-treated	7	4.00 ± 0.44
A	Wild population, Nov., 1949 (Chambers, 1951)	26	6.84 ± 1.29
B	Wild population, May, 1950 (Chambers, 1951)	18	2.85 ± 0.56

* $\frac{\text{liver weight} \times 100}{\text{body weight}}$

Fat. In the "pair-fed" saline-injected controls the fat content averaged low (c. slight trace), the cells were large, the nucleus almost completely filled the cell, and the nucleolus was conspicuous. The nucleoplasm was very finely granular and took a uniform light blue stain with Bullard's hematoxylin. The fat present in the liver appeared as bright red spherical globules, small in diameter and present in occasional cells sparsely scattered throughout the tissue. In several specimens the fat globules were concentrated at the periphery of the section, but generally there was no uniform localization of the fat deposition.

Three of the seven "pair-fed" saline-injected controls had noticeable amounts of ceroid, a term given to an insoluble lipid which stains on both the normal and control sections. The ceroid appeared either as a spherical intracellular inclusion (spherule) in the hepatic cell or as a clump of spherules in emboli throughout the tissue. In this lipid the stain was less intense, taking on a reddish-orange rather than the brilliant scarlet of the alcohol-soluble fat. On the malt-diastase digested control section for glycogen, the ceroid gave a positive reaction to the Feulgen stain after oxidation with periodic acid.

By comparison with the saline-injected controls, the ad-libitum fed controls had livers which, on the average, were laden with fat globules. The brilliantly stained scarlet fat droplets completely filled the hepatic cells, many of which had several globules of fat within a membrane.

The nuclei of these cells were not as large as those in the "pair-fed" controls and were displaced to one end of the cell by the fat inclusions. The livers of these controls contained slight amounts of ceroid.

Table VI shows that one thiourea-treated fish had a liver with an extremely high fat content (+++, fish 10.6), while the livers of the remaining fish showed negligible amounts. In the tissue from fish 9.2, the fat droplets were localized in the cells along the blood vessels and they have the appearance of cords of fat globules throughout the tissue. Six of the livers showed slight amounts of ceroid. The liver from experimental fish 10.5, noted as pathological in Tables VI and VII, contained no intracellular alcohol-soluble fat; however, it was heavily laden with ceroid, described above.

TABLE VI. FAT CONTENT OF LIVERS

Ad-libitum fed controls		*Saline-injected "Pair-fed" controls*		*Thiourea-treated*	
Fish	*Rating*	*Fish*	*Rating*	*Fish*	*Rating*
6.6	++*	9.1	0	9.3†	0*
10.7	+*	9.4	±	9.2	++*
3.5	+++	5.6	++	2.6	0*
3.2	+++	5.3	0*	2.2†	0*
3.4	+++*	10.3	+	10.2	±*
3.3	++	10.1	0*	10.6†	+++*
3.1	+++*	6.1	0	10.5§†	0*

Key 0 = none; ± = slight trace; + = trace; ++ = some; +++ = abundant.
* Ceroid
§ Pathological
† Grey-brown liver

Glycogen. The glycogen of the "pair-fed" saline-injected controls was consistently high (see Table VII), took an intense stain, and was distributed in such a uniformly diffuse manner throughout the section that no distinctly separate localizations were apparent. In many of the sections, however, there was a distinct marginal intensification of the staining, probably due to autolysis of the peripheral cells while the livers were being weighed. The nuclei were constantly central in position and glycogen granules completely filled the remainder of the cell.

The glycogen of the ad-libitum fed controls was moderately abundant (++) and the over-all staining was faint with a tendency to peripheral intensification. There was no distinct localization of glycogen; the trace amounts were generally deposited around a centrally located nucleus. The cytoplasm was reticulate and faintly stained. The hematoxylin-eosin stained slides confirmed the fact that the cells were vacuolated.

An over-all survey of the livers of the thiourea-treated fish gave a distinctly variable picture, but evidence of depletion of glycogen was apparent. The regions of glycogen deposition or depletion did not appear to be specifically localized about blood vessels, bile ducts, or in any particular region of the section. In those tissues having +++ (1) or ++ (2) ratings, the glycogen was generally diffused but was slightly more abundant toward the more peripheral regions of the section though separate from the marginal intensification that resulted

TABLE VII. GLYCOGEN CONTENT OF LIVERS

Ad-libitum fed controls		*Saline-injected "Pair-fed" controls*		*Thiourea-treated*	
Fish	*Rating*	*Fish*	*Rating*	*Fish*	*Rating*
6.6	++*‡	9.1	+++	9.3 †	0*‡
10.7	++*‡	9.4	+++	9.2	+++*‡
3.5	++ ‡	5.6	+++	2.6	0*‡
3.2	++ ‡	5.3	+++*	2.2 †	++*‡
3.4	++*‡	10.3	+++	10.2	++*‡
3.3	+++ ‡	10.1	+++*	10.6 †	+*‡
3.1	+++*‡	6.1	+++	10.5§†	±*‡

See key in Table VI.
* Ceroid
‡ Vacuolated
§ Pathological
† Grey brown liver

from autolysis. The granular glycogen in these tissues was generally deposited in one portion of the cell, sometimes occupying as much as half of it, and the nucleus occupied part of the remaining portion.

The section noted as pathological in Tables VI and VII was laden with both individual cells and groups of cells that contained spherical inclusions which gave a positive reaction to the periodic acid-Feulgen technique on both the malt-diastase control and glycogen test slide. These inclusions, described above as ceroid material and which appeared on the control sections stained for fat, were smooth in appearance and definitely limited by a membrane as opposed to the scattered granular glycogen depositions. In the hematoxylin-eosin stained slide of this tissue, these inclusions were colorless or a pale yellow. The cells themselves were highly irregular in outline. In this section there was only a very small quantity of glycogen; it was deposited around the periphery of a few cells which were sparsely scattered throughout the tissue.

Liver cell and nuclear size. Table VIII gives for each fish the mean diameter of the liver cells and nuclei together with the standard deviation. The figures are based on measurements of 50 cells and nuclei taken at random from each section. The widest and narrowest parts of each cell were measured and the average of these two measurements was used for the computation. No correction for shrinkage was attempted.

Table VIII reveals that there was an increase in size of both cell and nucleus in the liver of the thiourea-treated fish. Fig. 3 shows photomicrographs of liver sections from a thiourea-treated fish and from a "pair-fed" control to demonstrate the difference in cell and

TABLE VIII. Mean Diameter of Hepatic Cells and Nuclei (in Microns)

	Ad-libitum Fed			*"Pair-fed"*			*Thiourea-treated*	
Fish	*Cells*	*Nuclei*	*Fish*	*Cells*	*Nuclei*	*Fish*	*Cells*	*Nuclei*
3.1	16.1 ± 2.0	5.7 ± 0.9	5.6	10.1 ± 1.4	5.0 ± 0.7	2.6	12.0 ± 1.6	6.2 ± 0.7
3.2	14.5 ± 1.8	5.0 ± 0.9	9.4	10.0 ± 1.1	4.5 ± 0.5	9.2	13.4 ± 1.6	6.7 ± 1.3
3.3	11.7 ± 1.1	4.6 ± 1.0	9.1	9.0 ± 2.0	4.7 ± 0.8	9.3	11.9 ± 1.7	6.7 ± 0.7
3.4	15.1 ± 1.6	4.7 ± 0.5	5.3	10.2 ± 1.3	4.9 ± 0.6	2.2	14.7 ± 2.5	7.1 ± 0.3
3.5	13.6 ± 1.1	5.5 ± 0.4	10.3	9.8 ± 1.8	4.6 ± 0.7	10.2	13.0 ± 1.8	6.1 ± 1.0
10.7	15.6 ± 3.8	5.0 ± 0.6	10.1	10.9 ± 1.2	4.5 ± 0.5	10.6	16.6 ± 2.4	7.7 ± 1.2
6.6	14.3 ± 1.2	4.8 ± 0.5	6.1	10.5 ± 1.9	4.7 ± 0.2	10.5	18.8 ± 3.9	7.3 ± 1.2
Av.	14.4 ± 1.4	5.0 ± 0.4		10.1 ± 0.6	4.7 ± 0.1		14.3 ± 2.6	6.8 ± 0.6

nuclear size. The enlargement of the hepatic cells of the ad-libitum fed controls is explainable on the basis of the extremely high fat content; the size of their nuclei is but slightly greater than that of the "pair-fed" controls.

The difference between the thiourea-treated fish and the "pair-fed" controls is statistically significant in respect to both the size of the cell (t = 4.29, P = 0.001) and of the nucleus (t = 9.32, P = > 0.001). However, the size of the hepatic cell of the ad-libitum fed controls is similar to that of the thiourea-treated fish (t = 0.172, P = > 0.8), undoubtedly due to an accumulation of fat (see Table VII) in the ad-libitum fed fish, while the size of the hepatic nucleus of the ad-libitum fed controls is significantly less (t = 6.72, P = > 0.001) than that of the thiourea-treated fish. In the two control groups, the hepatic cell size of the ad-libitum fed group is significantly larger (t = 7.6813, P = > 0.001) than that of the "pair-fed" controls while the difference in size of nucleus is barely significant (t = 1.98, P = > 0.5, < 0.1).

The hematoxylin-eosin slides showed that the nuclear structure in the liver of both the "pair-fed" and ad-libitum fed controls consisted of a conspicuous nucleolus and coarsely granular chromatin material which stained heavily and was dispersed throughout the nucleoplasm. However, in the thiourea-treated fish the nucleus was greatly enlarged,

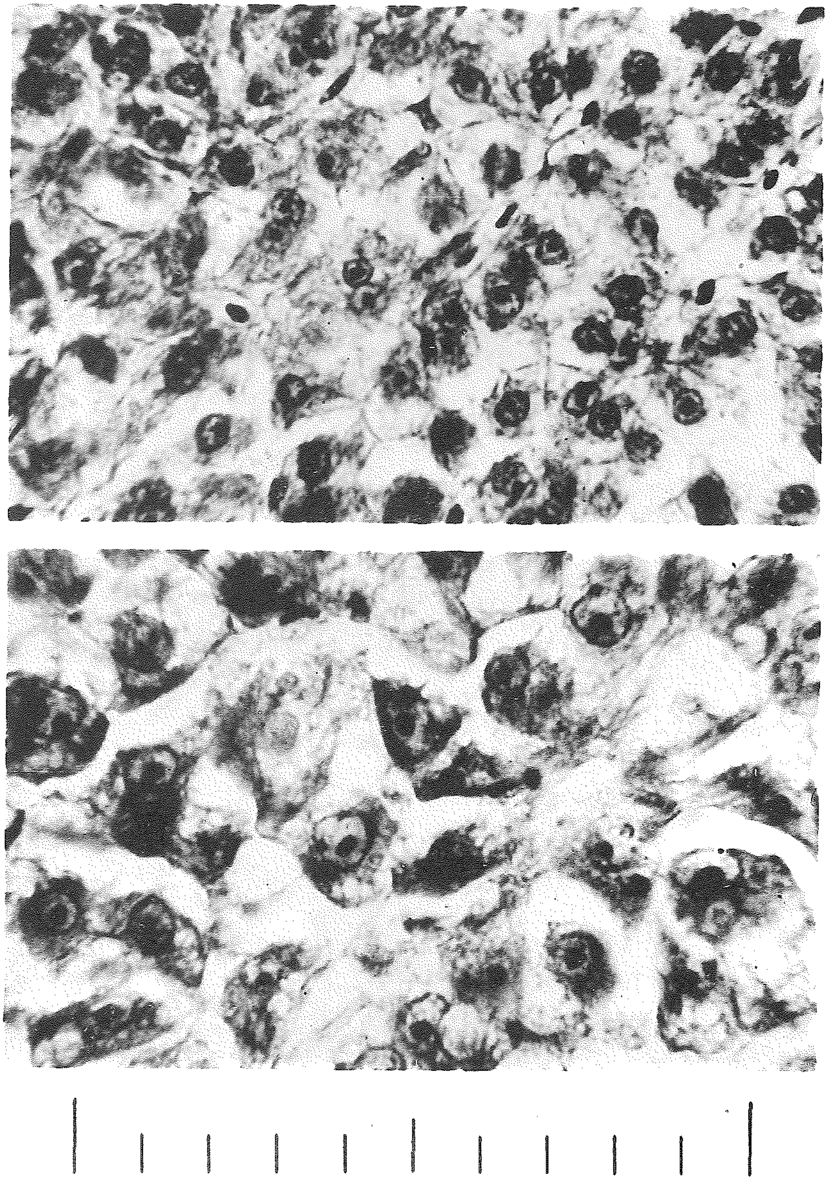

Figure 3. Microphotographs of sections of liver to show the difference in cell and nuclear size. *Upper*. From a "pair-fed" control fish (9.1). *Lower*. From a thiourea-treated fish (10.6). Scale in 10 micron intervals.

the nucleolus was conspicuous, and the chromatin material was more diffuse, less evident, and frequently peripheral in location. Also there appeared to be faintly staining fibrils radiating from the nucleolus to the nuclear membrane.

The pathological liver of fish 10.5 had very few hepatic cells that appeared to be normal. Most of the cells were intensely vacuolated with reticulate cytoplasm and enlarged nuclei. Some mitotic figures were noted and there were nests of small cells which suggested regeneration. The great variation in cell size in this liver is reflected in the high value of the standard deviation ($\pm$ 3.9).

DISCUSSION

The results reported above confirm the work of previous investigators who have studied the effects of antithyroid drugs on teleosts. In *Fundulus heteroclitus,* thiourea produced the expected hyperemia, hyperplasia and hypertrophy of the thyroid gland as well as retardation of growth and sexual development. Whereas Barrington and Matty (1952) examined the gonads histologically, other workers have used only the secondary sexual characteristics as an index of gonad development; in this investigation the testes were examined and were found to be atrophied, thus confirming the hypothesis that sexual coloration in *Fundulus* is mediated through the male gonad.

Heretofore the effect of thiourea on the *Fundulus* liver has not been reported. At first it was supposed (Chambers, 1951) that the enlarged livers of hypophysectomized fish resulted from thyroid inactivation. However, the large livers of hypophysectomized male *Fundulus* contained abundant reserves of fat and glycogen (Pickford, 1950) whereas the enlarged livers of thiourea-treated fish revealed a striking depletion of both of these reserves. Hence a new aspect of the investigation was presented, namely: the direct effect of intraperitoneal injections of thiourea on the livers of *Fundulus.* The fact that the thiourea-treated fish livers were relatively heavier than those of the control groups is the more striking in view of the 10.2% loss in total body weight of the former.

In the only reference concerning the effect of an antithyroid drug on the liver of teleosts, Hatey (1950) reported for *Cyprinus* a decrease in hepatic glycogen and a reduction in the liver size. The results of this investigation substantiate his observation of the loss of glycogen but they disagree with his results in that the thiourea-treated *Fundulus* liver was significantly larger than that of the control fish. There are several possible explanations for the difference in Hatey's results and those given here. (a) A species difference. (b) During the experiment Hatey starved his fish, consequently the livers of both controls and experimental fish decreased in size, the latter to a greater extent than the former. (c) Hatey's fish were submerged in a thiourea solution, whereas *Fundulus* received injections of the antithyroid drug into the abdominal cavity. On the assumption that the concentration of thiourea in the tissue of the fish is approximately in equilibrium with that of the external medium, it appears that Hatey's fish received twice the dosage level used in the present experiment; hence the results on *Fundulus* cannot be attributed to a higher dosage of thiourea.

As noted previously, surgical thyroidectomy in teleosts has not been performed, and therefore it is impossible to compare the effects produced by chemical inactivation of the thyroid with those resulting from surgical removal of the gland. While there is no concrete evidence to show whether or not experimental results from work on rats, guinea pigs or cats are applicable to those obtained by work on fish, such comparisons are often made.

Numerous investigators have reported the effects of antithyroid drugs on the liver of rats. Leblond and Hoff (1944), in their comparison of the effects by antithyroid drugs and surgical thyroidectomy, first noted that, although the adrenals, kidneys and heart were reduced in both groups, atrophy of the liver produced by surgical thyroidectomy was not duplicated in the drug treated group. On the contrary, the livers of the animals receiving thiouracil were significantly larger, which suggested "a unique action of thiouracil." No histological or biochemical analyses of the livers were given by these investigators. Subsequently an increase in liver weight was confirmed by May, Moseley and Forbes (1946), Leathem and Sealey (1947, 1948), Azarnoff and Leathem (1948), Leathem (1949), and Leathem and Howell (1950). Several workers have claimed that the hepatic glycogen in experimental animals receiving antithyroid drugs increases. May, Moseley and Forbes (1946) offered increased glycogen deposition as a partial explanation for the increased relative size of the experimental livers. There was a marked deposition of hepatic glycogen in spite of a reduced food consumption by the rats. On the basis of histological examination, Leathem (1949) and Leathem and Howell (1950) claimed that, in the rat, thiouracil caused an increase in liver weight, an increase in hepatic cell size, and a retention of glycogen and fat reserves which were lost in the pair-fed controls. McClosky, et al. (1947), investigating the chronic toxicity and pathology of thiouracil in cats, claimed that the most consistent hepatic alteration was "a moderate to fairly severe fine and medium fat infiltration of liver cells." Also, in the livers of six of the cats, killed at regular intervals throughout the investigation, moderate to large amounts of glycogen were histologically demonstrable, even though most of these livers showed a considerable amount of fatty degeneration.

A decrease in hepatic glycogen in rats that received thiourea was merely mentioned by Glock (1945). However, DuBois, Holm and Doyle (1946 a, b) and Dubois, et al. (1947) repeatedly reported a striking depletion of liver glycogen in rats and guinea pigs following intraperitoneal injections of lethal doses of alpha-napthylthiourea. Thus DuBois, et al. showed that the depletion of liver glycogen accompanied but did not contribute appreciably to the lethal action of the compounds. The results of the present experiment substantiate other observations on the loss of hepatic glycogen. If this loss of hepatic glycogen is a measure of toxicity, the fish used in the present experiment were receiving a toxic dose. Thus it appears that fish are more sensitive to antithyroid agents than lizards (Ratzersdorfer, et al., 1949), since the dosage employed in these experiments was based on that used by Ratzersdorfer, et al. According to Leathem and Seeley (1947), ". . . despite reduction in food intake caused by inclusion of thiourea in the diet, liver weight was found to be greater in the hypothyroid rats." In the following year these authors again reported an increase in liver weight in rats receiving antithyroid drugs (Leathem and Seeley, 1948). In the latter paper, which deals with the effect of thiouracil feeding on the liver and which compares the results on thyroidectomized and pair-fed controls, Leathem and Seeley concluded that thyroidectomy, although it induced a more rapid state of hypothyroidism than thiouracil, failed to increase the liver size or alter the percent water or protein content. Thiouracil, though not altering the percent water or protein content, significantly increased the size of the liver, thereby increasing the total liver protein per unit of body weight. It would appear, therefore, that in rats the effect of thiouracil on the liver is not caused by hypothyroidism.

Leathem and coworkers (1945, 1947, 1948, 1949 and 1950) have shown that thiouracil-induced hypothryoid rats eat less than the normal controls and should as a consequence have livers of a reduced size. However, the livers of these rats are actually greater in size with a normal water and protein content. That this should occur during a condition of semistarvation is of particular interest, since it has been claimed that liver weights in rats rapidly decrease during short periods of fasting and that part of this loss is due to the disappearance of liver protein (Harrison and Long, 1945). Kosterlitz (1944) has indicated that protein reserves are stored in the liver, not in a manner similar to glycogen or fat, but rather as part of the structural component of the hepatic cell cytoplasm. According to Kosterlitz also, the livers of rats fed on a low protein diet show an almost constant weight although there is a diminution of stainable cytoplasm and an apparent vacuolization that is probably caused by greatly increased glycogen deposition. In fasted rats the livers decrease in size and there is a subsequent chemical increase in the concentration of the cytoplasmic substance although the absolute quantity of this cytoplasmic substance decreases.

Thus it appears that investigators concerned with the effect of antithyroid drugs on rat livers are in agreement that there is an increase in the weight of this organ. However, the nature of the factors responsible for this increase is not clear. Most of the evidence favors accumulation of glycogen, and perhaps fat, as well as an increase in cell size as a result of subtoxic doses of antithyroid drugs. The present investigation also reveals that there is an increase in liver size, but it is clear that this is not due to an accumulation of fat or glycogen. This finding is in agreement with the work of DuBois, et al., who found glycogen depletion with toxic doses. The increase in cell size and the vacuolization of the cytoplasm suggest an increase in water content, probably similar to the well known watery vacuolization response elicited by cellular injury (Aterman, 1952). Tavolga (1949) has reported an increase in hepatic cell size in fish as a result of treatment with pregneninolone.

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SUMMARY

1. Twelve adult male *Fundulus heteroclitus* (Linn.) received intraperitoneal injections of thiourea thrice weekly. The seven surviving thiourea-treated animals, their pair-fed controls, and seven adlibitum fed controls, were autopsied at the end of six weeks. Thiourea produced the expected progressive decrease in body weight, retardation of growth in length, regression of the testes, and hyperemia, hyperplasia and hypertrophy of the thyroid gland.

2. The most striking result of the investigation was the direct effect of intraperitoneal injections of thiourea on the liver of *Fundulus*. Despite a 10.2% loss in total body weight by the thiourea-treated specimens, the livers were significantly heavier statistically than those of either control group. This is of all the more interest in view of the fact that there was a depletion of both hepatic glycogen and fat reserves. Histological examination of the livers of the thiourea-treated fish showed a vacuolization of the hepatic cells and an increase in size of not only the cells but of their nuclei.

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APPENDIX

Since the above manuscript was submitted for publication, a number of papers regarding the action of antithyroid agents on teleosts have appeared. Fontaine (1953) has published a comprehensive review which emphasizes the intricate inter-relationship of the action of thyroid and pituitary glands in fish. Other investigators have reported the following well known effects of antithyroid drugs: retardation of sexual differentiation (Smith, et al., 1953) and sexual development (Gaiser, 1952; Scott, 1953; Smith, et al., 1953); retardation of growth (Gaiser, 1952; Scott, 1953; Smith, et al., 1953) and hyperplasia of the thyroid (Gaiser, 1952; Scott, 1953). Scott (1953) demonstrated an increase in the number of basophil cells in the transitional lobe of the pituitary gland of *Brachydanio rerio* immersed in 0.33% thiourea. The work of Fortune (1953) has shown that thiourea-treated *Phoxinus laevis* and *Lebistes reticulatus* are able to withstand higher temperatures than untreated control fish. Osborn (1951) has reported that *Campostoma anomalum pullum*, receiving thiouracil, can withstand lower oxygen concentrations. Hoar, et al. (1952) have investigated the effects of various hormones on the behavior of salmon fry; they concluded that, although there were no changes in the basic behavior pattern, thyroxin and methyl testosterone tended to increase the general activity while thiourea intensified the grouping instinct (as distinguished from true schooling) but significantly decreased the speed of swimming after the first week's treatment.

The French school has recently published work in which radioactive iodine has been employed to study thyroid activity in teleosts. Vivien and Gaiser (1952) have shown that fish immersed in water containing radioactive iodine assimilate the iodine very slowly through the gills and intestine and that thiourea completely suppresses the uptake of iodine by the thyroid. Leloup (1952) concluded that antithyroid agents inhibit the synthesis of thyroxin in the thyroid of the marine teleosts *Mugil auratus* Linn. and *Conger vulgaris* Cuv.; and Olivereau (1952) has reported that the blocking of organic iodine synthesis in the thyroid gland of these species occurs before any histological change is evident.

The following Russian literature has also come to the attention of the author.[3] Zaks and Zamkova (1947) investigated the rôle of the thyroid gland in the embryogenesis of the vertebrata. Their experimental work on the loach, *Misgurnus fossilis*, is detailed here. Fertilized eggs were kept in conditioned tap water until they reached the 13 somite stage, at which time a control group was kept in conditioned tap water; a second group was placed in a 0.033% thiourea solution, a third group in a 1.10^{-7} concentration of thyroxin, and a fourth group in a mixture of 0.033% thiourea and 1.10^{-7} thyroxin. Up to the thirteenth day, the experimental larvae in all groups were identical with those in the control group. At the end of the 26-day experiment the thiourea-treated group showed only insignificant differences from the control group (slight retardation in resorption of the external gills and yolk sac and a slightly higher mortality rate). In the groups re-

[3] I would like to express my sincere thanks to Dr. Alexander Petrunkevitch of the Osborn Zoological Laboratory, Yale University, for translation of the Russian papers discussed here.

ceiving thyroxin and thiourea plus thyroxin, outstanding differences in external morphology were noticeable after the thirteenth day. The results observed in the group receiving only thyroxin confirm the previous work of Gerbilsky and Zaks (1947), who showed that thyroxin caused not only an increase in the number and degree of development of the scutes of *Acipenser stellatus* but also a greater degree of uniformity in the scute growth. In the groups receiving thyroxin and thyroxin plus thiourea, other observed changes (resorption of yolk sac and external gills, iris pigmentation similar to that of the adult, retraction of pigment cells, increased secretory processes in the gut, and change to an adult-shaped head) are regarded by the authors as indications of stimulation of development. The simultaneous action of thiourea greatly increased the effects of thyroxin, a fact that is little understood and for which no explanation was offered. As pointed out in the conclusions, thiourea (or exclusion of the thyroid gland) does not cause any serious disturbance in the external morphology of the larvae of *M. fossilis* up to the 26th day of development. Consequently the thyroid hormone is apparently not necessary for these stages of development. Thyroxin causes a series of changes which are regarded as stimulation of development. However, this response is elicited during the period when the morphogenic rôle of the larval thyroid is not yet manifested.

From examination of serial sections of normal larvae of sevriuga (*Acipenser stellatus*) and sturgeon (*A. guldenstaedti*) which were autopsied daily from the 1st to 9th days and on the 15th and 26th days as well, Iakovleva (1949) correlated an active thyroid with a period of active morphogenesis between the third and seventh days. Subsequent to the resorption of the yolk sac (ninth day), the thyroid entered a resting stage and remained in this condition for the duration of the investigation (26 days). In the experimental investigation, 2- and 5-day old larvae of *A. stellatus* were immersed in a solution of thiourea for 34 and 31 days respectively (i.e., until they were 36 days old) and were compared with specimens of an untreated control group. Morphologically, both groups of thiourea-treated larvae retained their larval teeth while the controls lost them, and the scutes which formed were much less numerous than those in the control fish. Histological study revealed that in the experimental fish there was a subnormal development of the thyroid: the gland was 1.5 to 2 times smaller than that of the untreated controls, no new follicles had been formed, and the total number of follicles was less than the number in the control fish.

The hypophysis, in both untreated controls and experimental specimens, was clearly not yet fully developed: no cell boundaries had become differentiated and no chromophil cells were present.

The expected hyperplasia of the thyroid gland in the thiourea-treated fish did not occur. The author attributed the lack of thyrotropic activity to the undifferentiated embryonic condition of the pituitary. According to Iakovleva, this is the first report on the independence of the thyroid gland from a thyroid stimulating hormone in early postembryonic development in fish. The work of Dormidontov (1949) regarding the effects of thiourea on the larvae of *Lebistes reticulatus*, referred to by Iakovleva (1949) and Zaks and Zamkova (1952), is in an unavailable journal and hence it cannot be discussed.

Zaks and Gerbilsky (1949) claimed that larvae of salmon and trout showed an increased resistance to lowered oxygen tension under the influence of thiourea. The matter was further investigated by Zaks and Zamkova (1952) with larvae of *Salmo salar* and *Acipenser stellatus*. Thiourea-treated larvae of *S. salar* showed a lower absolute oxygen consumption and, unlike the controls, they exhibited an ability to utilize O_2 at a lower oxygen tension. These results are in agreement with the work of Osborn (1951). Zaks and Zamkova (1952) made further experiments on the larvae of *Acipenser stellatus* and confirmed the fact that thiourea lowers oxygen consumption. The tests were made on three successive days after a period of nine days in 0.033% thiourea; the differences between the thiourea-treated and the untreated fish were 1.40, 12.7 and 18.8% respectively. Thyroxin (1 : 2,000,000) was then added to the thiourea solution and the measurements were repeated on three successive days; the differences were 4.3, 9.2 and 0.0% respectively. After three days of thyroxin treatment, the inhibitory effect of thiourea on respiration is apparently completely abolished in the larvae of *A. stellatus*.

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EXPERIMENTAL DIFFERENTIATION OF BASOPHIL CELL TYPES IN THE TRANSITIONAL LOBE OF THE PITUITARY OF A TELEOST FISH, *ASTYANAX MEXICANUS*[1]

BY

ETHEL HAFTER ATZ

The American Museum of Natural History

ABSTRACT

The demonstration of glyco- or muco-protein constituents of cells by the McManus-Hotchkiss periodic acid—Schiff reaction (PAS) has made it possible to differentiate the two functional types of basophils in the anterior lobe of the mammalian pituitary which are responsible for the production of thyrotrophin and the gonadotrophins. These hormones are considered to be glyco- or muco-proteins.

Application of the PAS stain to the pituitary gland of the characin, *Astyanax mexicanus* (Filippi), has shown that two types of transitional lobe basophils may also be differentiated in teleosts. These cells are distinguishable from each other by differences in granule size and staining reaction and by their specific response to hormonal changes. One cell type, which reacts to alterations in thyroid hormone level, is believed to be the thyrotroph; the other, which shows changes related to the physiological state of the gonad, is believed to be the gonadotroph.

Thyrotrophs show increased vacuolation and degranulation after thiourea treatment and display some regressive changes after thyroid hormone administration.

Cells suggestive of the signet ring castration cells seen in the pituitaries of certain mammals are observed in two of three *Astyanax* reared in continuous darkness. All three fish had completely undifferentiated gonads.

ACTH and cortisone administration evokes successive phases of vacuolation and degranulation in the thyrotrophs, followed by cytoplasmic reconstitution. At the same time as these changes occur in the thyrotrophs, the gonadotrophs shrink markedly in size but maintain an intense color. This loss of cytoplasmic volume is also followed by reconstitution.

INTRODUCTION

Application of the McManus-Hotchkiss periodic acid—Schiff reaction (PAS) to the mammalian pituitary by Catchpole (1949), Herlant (1949), and Purves and Griesbach (1951a) has demonstrated that this histochemical technique, which identifies the glyco- or muco-protein

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constituents of the cell, can be applied to the functional differentiation of cell types in the anterior lobe of the pituitary. Both thyrotrophin and the gonadotrophins are known to contain carbohydrate groupings (Li and Evans, 1948) and are considered to be glyco- or muco-proteins (Catchpole, 1949; Pearse, 1951). Employing this technique along with the Gomori aldehyde-fuchsin stain, Purves and Griesbach (1951a, b, c) were able to distinguish between the anterior lobe basophils or cyanophils which are responsible for the production of thyrotrophin and those responsible for gonadotrophin, the Gomori stain being specific for the thyrotrophs.

Similar study of a teleost pituitary should be of comparative endocrinological interest and might also demonstrate a method by which the results of experiments could be assessed more easily. In this laboratory, the interpretation of endocrinological data on fishes has frequently depended on the use of laborious pituitary cell counts with statistical treatment of the results and on the analysis of the general histological character of this gland. To simplify as well as amplify these procedures, the following work was undertaken.

MATERIALS AND METHODS

The pituitaries of 11 sexually mature male and female characins, *Astyanax mexicanus* (Filippi), approximately one-year old, were used in this study. To produce variations in the thyrotropic hormone content of the pituitary, four of the fish were treated with thiourea or desiccated thyroid for one month according to the methods used by Rasquin (1949).

Two fish, one male and one female, were placed in a two gallon tank containing four liters of a 0.033% solution of thiourea (thiocarbamide, c. p.). The solution, made up with "conditioned" water, was changed weekly. The fish were fed daily with only their customary food (Rasquin and Atz, 1952). Two fish, also of opposite sex, were placed in a similar small tank and were fed on alternate days a half grain of desiccated thyroid (Burroughs Wellcome tablets) along with their customary food. At the end of the experimental period (four weeks) the fish in the second tank had received a total of six grains of thyroid, and the water in that tank remained unchanged throughout this period. The four animals mentioned above, as well as two normal fish used as controls, were sacrificed by decapitation; the heads were fixed in formol-sublimate, the bodies in Bouin's fluid. After electrolytic decalcification, the tissue was embedded in paraffin and serial sections were cut at five micra through the pituitary region. Sections of the pituitary region also included the major portion of the thyroid gland. The pituitary of a male and a female of each of these groups was stained by either the Gomori or PAS techniques. The pituitaries of five untreated fish were dissected and fixed in formol-sublimate and embedded in paraffin. Sections were cut at three and five micra. There was no difference in the staining reaction between the tissues that had not been decalcified and those that had been.

In the Gomori technique and the PAS stain for glycoprotein, the procedure of Purves and Griesbach (1951a, b) was followed except for the use of a commercially prepared Schiff Reagent (Eimer & Amend Co., So-S-32). Only 10–15 minutes staining with Gomori was found necessary for these tissues rather than the half hour required by Purves and Griesbach (1951b) for rat tissue; no other modification was found necessary. A control slide of normal pituitary sections, incubated with saliva for one hour at 37° C and then stained with PAS, showed no difference in the staining reaction of the PAS-positive cells from those on undigested sections, indicating that glycogen was not present.

The effects of gonadotropic hormone variation were studied with the aid of sections of pituitary from three fish that had been reared in the dark for 23, 39 and 48 weeks respectively.[2] These fish were part of a large series maintained in total darkness from the time they were eight weeks old and sexually immature. The report of the functional disturbances resulting from this schedule has been treated in detail by Rasquin and Rosenbloom (1954). The serial sections which contained pituitary had been fixed in Zenker's or Bouin's fluid, decalcified, embedded in paraffin, and stained with Masson's trichrome. They were then restained by the PAS method with excellent results.

In addition, sections of pituitary from 25 sexually mature *Astyanax* used for another experiment (Rasquin and Atz, 1952) were restained with the PAS stain and were compared with the material used in this experiment. These fish had been given injections of either physiological fish saline, ACTH, or cortisone for ten consecutive days after which injections were discontinued. Animals had been sacrificed at various intervals during the ten-day injection period and at similar intervals after injections ceased.

[2] Sections of these fish were generously loaned to the author by Misses Priscilla Rasquin and Libby Rosenbloom.

As noted by Purves and Griesbach (1951b), it was not possible to use the Gomori stain on tissue which had been stained previously by another method. However, direct comparison of Gomori and PAS stained basophils was made by first photographing a section stained by the Gomori method and then rephotographing the same field after it had been restained by the McManus technique.

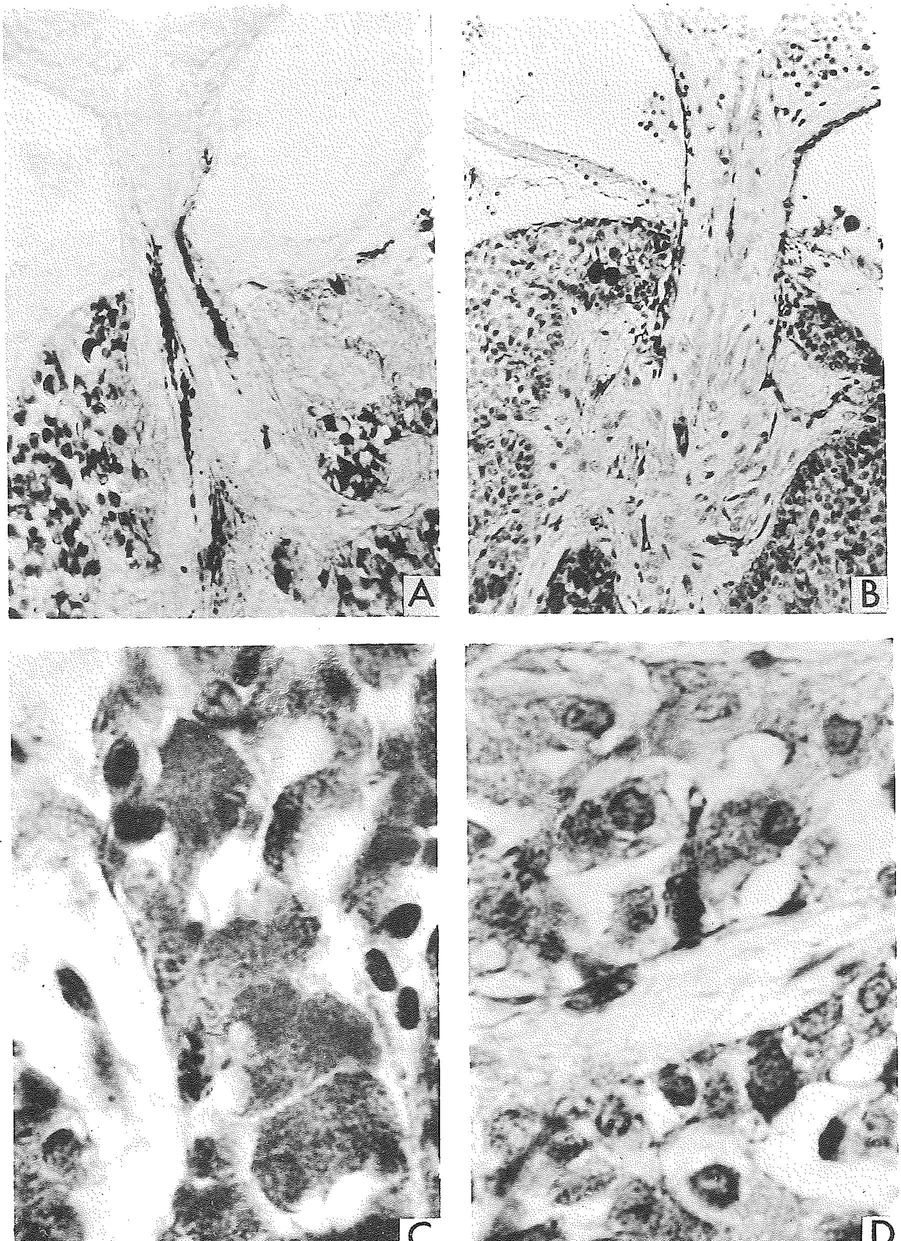
EXPERIMENTAL RESULTS

General Pituitary Histology. The anatomy and histology of the *Astyanax* pituitary has been described by Rasquin (1949). In the adult the transitional lobe is the largest part of the gland. It is this part of the fish pituitary that is homologous with the anterior lobe of mammals. It consists of acidophils and basophils that take respectively an intense red and a bright green after staining with Masson's trichrome. The term "basophil" is used in this paper, since it has been used generally in the literature and in previous descriptions of pituitary histology in *Astyanax*. It simply denotes those cells whose cytoplasmic inclusions stain with aniline blue or fast green and, as here used, includes both beta and delta cell types (Romeis, 1940; Halmi, 1950; and others). Dempsey and Wislocki (1945), among others, have pointed out the fallacy of using trichrome stains (the components of which are all acidic dyes) for the recognition of true basophilia. As an example of the confusion that has arisen from such procedure, Buchmann (1940) uses the term basophilic for cell inclusions in the pituitary of the herring which stain red with Azan, the acidophils being regarded as those that stain blue. He claimed that Delafield's hematoxylin-chromatrope stained blue those cells that had stained red with Azan. A small number of chromophobes, which are either colorless or weakly acidophilic, are also present in this lobe. Although acidophils and basophils are distributed throughout the lobe, the acidophils are more numerous next to the ramifications of the nervosa in the center of the gland while the basophils are more numerous at the periphery. The cells of the anterior lobe stain an orange-red with the ponceau of Masson's trichrome as do the cells of the intermediate lobe. The latter, however, are somewhat larger and are arranged in a cord-like fashion, separated by blood vessels and fibers of the pars nervosa. Ramifications of the infundibulum penetrate all parts of the gland as the pars nervosa, and in each lobe they are bordered by the cells characteristic of that part. The fibers of the nervosa stain weakly with ponceau. Colloid droplets which are seen throughout the nervosa stain brilliantly with the acid fuchsin, as do some of the blood elements within the gland.

Examination of all sections stained by either the Gomori or PAS method, regardless of the treatment of the specimen from which they were obtained, shows that the basophils of the transitional lobe are the only cells colored: a deep purple with Gomori and a brilliant purple-red with PAS. With both stains the acidophils are a pale greyish-pink, the acidophils of the anterior (= tuberalis) and intermediate lobes taking no positive stain. However, in the anterior lobe of Gomori-stained pituitaries, occasional cells with Gomori-positive cell membranes or a narrow peripheral band of purple cytoplasm have been observed. These occur most frequently at the border of the nervosa. Although the cells of the intermediate lobe are PAS- and Gomori-negative, they may take a pale pink color with the PAS stain; with the Gomori technique, occasional cells are seen that have very small purple granules located just at the cell membrane.

With Gomori stain, some brilliantly colored fibers are seen in the infundibulum and in the pars nervosa. Gomori-positive granules of variable size and irregular shape are also seen in the infundibulum and throughout the pars nervosa (Fig. 1A). These fibers, as well as small Gomori-positive granules, continue into the hypothalamus. In the region of the intermediate lobe, the granules are for the most part larger in size and may be so numerous that the fibrous character of the neurohypophysis is obscured. Although occasional anterior and intermediate lobe cells have small amounts of Gomori-positive material

Figure 1. A. Normal pituitary showing intensely colored Gomori-positive fibers and granules in the infundibulum. The dark cells are the Gomori-positive cells of the transitional lobe. In the upper right part of the gland, a small portion of the anterior lobe (= tuberalis) may be seen; its acidophils are Gomori-negative. Gomori aldehyde-fuchsin and eosin. Transverse section. × 260. B. Normal pituitary showing the lack of PAS-positive fibers and granules in the infundibulum. Only the reticulum of the blood vessels and the connective tissue bounding the infundibulum are positively stained. A greater portion of the anterior lobe is visible in this photomicrograph. PAS and hematoxylin. Transverse section. × 260. C. Transitional lobe of normal pituitary. At the right of the colorless tongue of nervosa, three granular thyrotrophs are seen, the lowest one of which is vacuolated. An irregularly shaped gonadotroph lies at the top of this row. Its cytoplasm is not finely granular, as is that of the thyrotrophs, but this difference is largely lost in photographic reproduction. Immediately to the right of the gonadotroph lie vacuolated and somewhat degranulated thyrotrophs. PAS and hematoxylin. × 1020. D. Transitional lobe of a thiourea-treated animal. Toward the top of the figure, note degranulated and vacuolated thyrotrophs in which the staining reaction is greatly reduced. A dark staining gonadotroph, which is smaller than normal, may be seen in the center above the tongue of nervosa; another similar gonadotroph is seen below nervosa and a little to the right of the upper one. PAS and hematoxylin. × 1020.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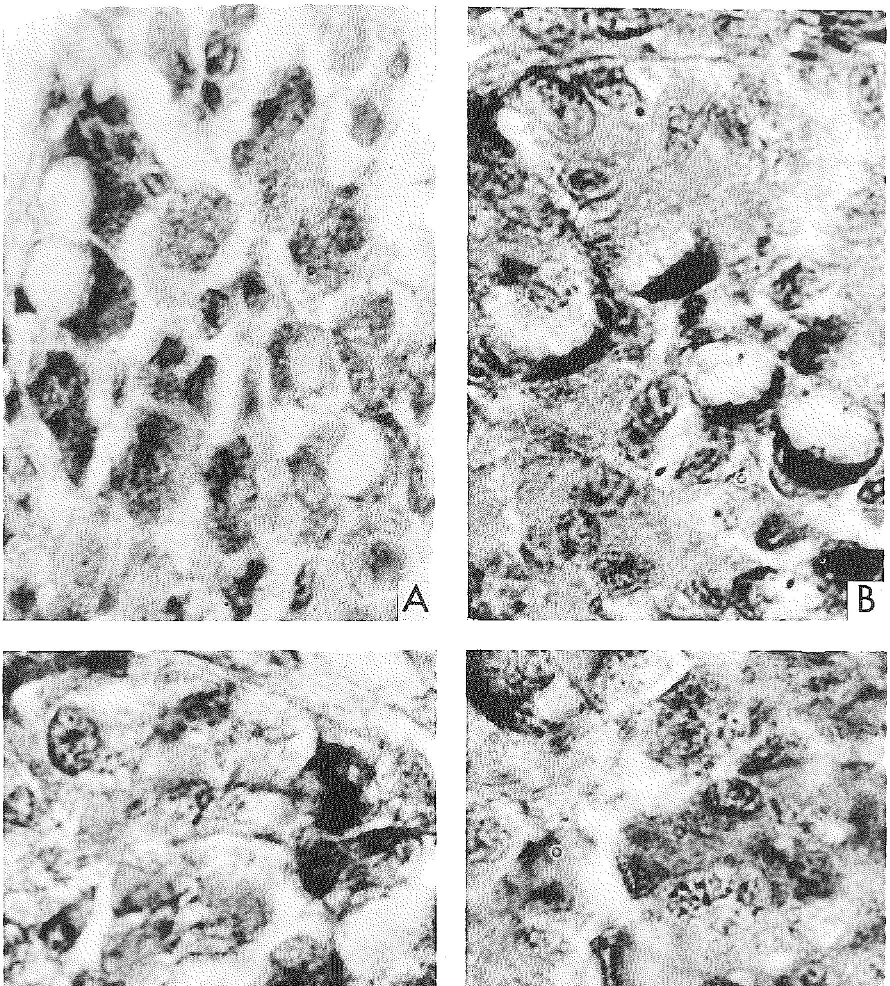
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in their cytoplasm, there is no indication that they are the source of the granules seen in the nervosa. The material seen within these cells does not resemble that observed in the nervosa. These fibers and granules are not seen with the PAS stain (Fig. 1B). Only the delicate reticulum of the hypophyseal blood vessels, the connective tissue bounding the infundibulum, and a few delicate fibers in the nervosa are realized. Occasional small granules and lightly colored granular clumps may also be seen in the nervosa.

The round regularly-outlined colloid droplets which stained brilliantly with acid fuchsin are not colored with either PAS or Gomori stains. The Gomori chromhematoxylin method was not used in this investigation, and no special attention has been paid to the problems of colloid formation and neurosecretion.

Normal PAS-positive Cells. Purves and Griesbach (1951a) found that the basophils of the rat pituitary could be separated into two groups on the basis of differences in (1) cell shape and size, (2) granule size and staining intensity, (3) prominence of the Golgi apparatus, (4) distribution and (5) function. In each group, the intensity of the glycoprotein reaction followed closely the gonadotropic and thyrotropic content of the gland as inferred by the response to experimental procedures. They concluded that one type of basophil was exclusively gonadotropic in function, the other exclusively thyrotropic. They also reported (1951b, c) that the Gomori aldehyde-fuchsin technique could be used as a specific stain for the thyrotropic basophils or thyrotrophs, the gonadotropic basophils or gonadotrophs remaining unstained.

Figure 2. A. Transitional lobe of a thyroid hormone-fed fish. At the extreme left are three gonadotrophs in a vertical row, the two upper ones being vacuolated. To the right of these cells lie thyrotrophs containing varying amounts of glycoprotein. At the lower right lies a third vacuolated gonadotroph. PAS and hematoxylin. × 1020. B. Transitional lobe of a fish reared in darkness for 48 weeks. Four gonadotrophs with intensely colored, homogeneous cytoplasm and a clear vacuole which occupies most of each cell. The nucleus has been pushed to one side, but this is apparent in only the right-most cell. The darker staining cytoplasm and greater size of vacuole in these cells as compared to the gonadotrophs of Fig. 2A are apparent. The grey colored cells are hypertrophied acidophils. PAS and hematoxylin. × 1020. C. Transitional lobe of a fish treated daily with 0.2 mg cortisone for three days. At the upper left is a thyrotroph containing few PAS-positive granules. Other similar pale cells are also thyrotrophs. At the right are four shrunken and intensely stained gonadotrophs, the lowest one being in metaphase. PAS and hematoxylin. × 1020. D. Transitional lobe of a fish five days after the cessation of ten daily injections of 0.05 mg cortisone. The cytoplasm of the thyrotrophs contains coarse, intensely PAS-positive granules. Two shrunken, dark staining gonadotrophs are seen near the bottom of the photograph. PAS and hematoxylin. × 1020.

In *Astyanax*, two types of basophils can also be demonstrated, but most of the above criteria cannot be used. Only by differences in granule size and staining intensity can the two types of PAS-positive cells be distinguished in the normal pituitary, and at best, the differences between them are not great. However, study of experimentally treated animals clearly shows a difference in response between the two cell types.

One type of basophil in *Astyanax* is of variable size and shape and ranges from a small round cell to one that is irregularly shaped and measures 20 micra across the widest diameter. The centrally or eccentrically placed nucleus is usually round or oval, although some variation in shape may occur. No differential distribution within the transitional lobe is noted with respect to cell shape, but there is a tendency for the larger cells to lie nearer the center of the lobe while the smaller ones lie toward the periphery. The cytoplasm of these cells is composed of fine granules which color brightly with both the PAS and Gomori stains. Many of these basophils show some vacuolation and degranulation, and occasionally cells are seen that have only a few PAS- or Gomori-positive granules in their cytoplasm. The smaller peripherally located cells are less frequently vacuolated, and their nuclei are somewhat hyperchromatic. They give the appearance of older less active cells. On evidence to be presented below, this group of PAS-positive cells is believed to be thyrotropic in function. They are therefore called thyrotrophs (Fig. 1C).

Other basophils, scattered singly throughout the transitional lobe and in clusters at the periphery and at the posterior border adjacent to the intermediate lobe, appear to be even more intensely PAS-positive than those described above. The cytoplasm of these cells appears to be smooth rather than finely granular, and occasionally, when the transmitted light is decreased, coarse granules, strings or clumps may be observed in it. In normal animals, these cells are seldom greatly vacuolated or degranulated. Their size and shape is variable, and the nucleus, while frequently round or oval, may also be sharply indented, bean-shaped, crescent-shaped, or twisted. There is no noticeable tendency for smaller less active-looking cells to lie toward the periphery. The appearance of this second group of PAS-positive cells can be correlated with changes in the physiological state of the gonad, and they appear to be gonadotropic in function. They are therefore called gonadotrophs (Fig. 1C).

Sections of the transitional lobe stained by the Gomori method differ from those treated by the PAS method only in the color that the basophils take. The distribution and number of these cells in the transitional lobe are similar with both stains. Direct comparison of photomicrographs of the same sections, stained first by the Gomori technique and then restained by the PAS method, showed that exactly the same cells are colored by both stains. Thus, unlike the rat, the Gomori stain does not specifically stain *Astyanax* thyrotrophs.

Response of PAS-positive Cells to Changes in Thyroid Hormone Level. Changing the thyroid hormone level in *Astyanax* by the administration of either thyroid hormone or thiourea evokes changes in the thyroid follicles and in the thyrotropic cells of the pituitary.

After four weeks immersion in 0.033% thiourea, there is an increase in the height of the thyroid follicle epithelium and a weakening of the PAS staining reaction within the thyrotrophs, indicating a reduction in the amount of glycoprotein. Both centrally and peripherally located cells are similarly affected. Many are partially degranulated, some almost completely so. There is also a great increase in the amount of vacuolation, and occasionally cells are seen that have little cytoplasm remaining (Fig. 1D). These cells give the appearance of great activity. However, the nuclei do not appear to be enlarged or more vesicular, nor are mitotic figures seen. Gomori-stained thyrotrophs are pale in color and appear active. No sex difference in response is noted. The staining intensity of the gonadotrophs is not affected by four weeks immersion in thiourea; they are intensely colored with PAS. However, they do appear to be smaller in size than are those in the control fish (Fig. 1D).

Thyroid hormone administration depresses the height of the epithelium of the thyroid follicles. Slight regressive changes also are evoked in the thyrotrophs. Pycnotic nuclei, the retraction of the cells from adjacent ones, and some loss of PAS-positive granules are noted (Fig. 2A). The amount of degranulation, however, is not as great as that seen after thiourea treatment, and little or no vacuolation is apparent. The over-all staining of this area remains bright with both the PAS and Gomori stains. No changes can be found in the staining intensity or size of the gonadotrophs. There does appear to be some increase in the number of vacuolated gonadotrophs, however, and in some more than one vacuole may be found (Fig. 2A).

Response of PAS-positive Cells to Changes in Sex Hormone Level. The normal proportion of acidophils to basophils in sexually mature *Astyanax* is approximately 40 to 60% (Rasquin, 1949). This proportion can be inverted by the injection of carp pituitary (Rasquin, 1951) and mammalian ACTH or cortisone (Rasquin and Atz, 1952). A similar reversal occurs when this species is kept in continuous darkness (Rasquin and Rosenbloom, 1954). Among fish treated in this manner may be found individuals with completely undifferentiated gonads, although they are beyond the age of normal sexual maturity; the three fish used in the present study belong to this group.

In sections of the pituitary of these fish, the PAS-positive cells are appreciably smaller than those seen after treatment with either thyroid hormone or thiourea. The majority of the PAS-positive cells of the fish kept in the dark 39 and 48 weeks contain intensely colored, homogeneous, purple-red cytoplasm surrounding a clear vacuole which occupies most of the cell (Fig. 2B). The nucleus, pushed to one side, frequently appears compressed and elongated. In some, the vacuole may be so large that only a thin rim of PAS-positive cytoplasm close to the cell membrane remains. These cells occur in clusters at the periphery of the transitional lobe and are scattered in the central areas. Not all of the PAS-positive cells have a vacuole. Most of the nonvacuolated cells have dark-staining homogeneous cytoplasm, but occasionally a lighter-staining granular cell is seen. These cells, as well as those with the vacuole, show no sign of degranulation or other indication of activity. It is not possible to differentiate thyrotrophs from gonadotrophs in these pituitaries, especially among the nonvacuolated cells.

The cells with the clear vacuole, however, resemble the description of the classical signet ring castration cells seen in the pituitaries of certain mammals after gonadectomy, and they appear similar to the PAS-stained cells of the rat pictured by Purves and Griesbach (1951a), who believed they were derived from gonadotrophs.

Although the basophils in normal animals may be vacuolated, the vacuoles can be distinguished from those in dark-reared animals (1) frequently by their smaller size, (2) by the occurrences of more than one in a single cell, (3) by the appearance of some degranulation of the cytoplasm around them and (4) by their more frequent occurrence in the centrally located thyrotrophs. In the normal pituitary, basophils are occasionally seen in which a single vacuole occupies most of the cytoplasm, thus giving the appearance of signet ring cells. However, closer examination shows that the cytoplasm surrounding the vacuole, although intensely PAS-positive, is usually granular in character. In contrast, the cytoplasm of the cells in the dark-reared fish presents a dense homogeneous appearance, and therefore the vacuole found within them has a more sharply delimited appearance. The vacuolation observed in the PAS-positive cells after treatment with thiourea differs markedly from that seen in the dark-reared fish. Here most of the cells contain numerous small vacuoles, the cytoplasm surrounding them is very degranulated, and the PAS reaction is light.

The pituitary of *Astyanax* kept in the dark for 23 weeks contained no castration-like cells despite the fact that its gonadal tissue was as undifferentiated as that of the other two fish studied. The highly variable nature of the physiological abnormalities resulting from keeping *Astyanax* in the dark has been pointed out by Rasquin and Rosenbloom (1954). The PAS-positive cells observed in the pituitary of this fish resemble those of the nonvacuolated cells of the other two dark-reared fish.

Response of PAS-positive Cells to ACTH and Adrenocortical Hormone. The normal ratio of acidophils to basophils in the transitional lobe of the pituitary was inverted only in males by ACTH but in both sexes by cortisone (Rasquin and Atz, 1952); hence male pituitaries of the ACTH series were selected for restaining by the PAS method while pituitaries of both sexes from the control and cortisone-treated series were used.

Study of PAS-stained pituitaries reveals that the thyrotrophs undergo successive phases of vacuolation and degranulation, after which there is a reconstitution of the cytoplasm. The thyrotrophs of the group given the larger dose of cortisone (0.2 mg/day) lost their PAS-positive granules three days after the injections began (Fig. 2C); after daily injections of cortisone for five and ten days they regained some of their PAS-positive granules, but the cytoplasm contained large vacuoles filled with debris. Daily injection of 0.05 mg of cortisone acetate evoked a similar response in the thyrotrophs, except that the loss of staining reaction was reached five days after the injections had begun. Unlike the degranulation noted in the above groups, the thyrotrophs of the single daily-injected ACTH fish (0.1 mg/day) still contained fine granules, but they had lost their affinity for the PAS stain three and five days after injections began. The daily double injections of 0.05 mg ACTH per injection evoked at these intervals a loss of cytoplasmic granulation. By ten days after daily single or double injections of ACTH, the thyrotrophs had regained their staining ability and there was little vacuolation. With Masson's stain, no loss of staining reaction was evident (Rasquin and Atz, 1952). Only in fish sacrificed three days after the injection of the larger dose of cortisone and in the daily double injected ACTH group are pycnotic nuclei seen. Both centrally and peripherally located thyrotrophs react similarly.

A second phase of the loss and reappearance of PAS-positive granules was observed in the postinjection period. For three or five days after cessation of injection the thyrotrophs of all experimental groups had undergone great degranulation and loss of staining reaction. By the end of the postinjection period (10 days), the cytoplasm had regained its PAS staining reaction and the cells appeared to be active. Occasional mitotic figures, seen in the thyrotrophs of both cortisone-treated groups during the entire experimental period, were more frequent during the postinjection period.

Five days after the cessation of the injection of 0.05 mg cortisone, the thyrotrophs of one of the two fish sacrificed at this interval showed numerous coarse bright PAS-positive granules of varying size (Fig. 2D). These are similar to the intensely staining material pictured by Purves and Griesbach (1951a) in the rat thyrotrophs after thyroidectomy. As in the rat, these granules were not revealed by Masson's staining. According to Purves and Griesbach (1951a), their solubility is different from that of the thyrotropic hormone, but their identity was not known.

The reaction of the gonadotrophs to ACTH and cortisone is strikingly different from that of the thyrotrophs. In the four experimental groups, these cells had shrunk greatly (Fig. 2C) and their nuclei were frequently pycnotic. With the PAS stain, their cytoplasm is an intense purple-red. The most profound alterations in the gonadotrophs occur in the groups given the larger dose of cortisone and in those injected twice daily with ACTH. Reconstitution of the gonadotrophs was observed in the injection and postinjection period. Occasional mitotic figures were seen in the gonadotrophs of the cortisone-treated series during the injection (Fig. 2C) and postinjection periods.

A similar cycle of thyrotroph degranulation and gonadotroph shrinkage followed by restoration was observed in the control fish injected with saline twice daily. No such cycle was noted in controls injected once a day. In these, an increase in thyrotroph degranulation and vacuolation was observed only at the beginning of the ten-day injection period.

There is a close correlation between the time at which the thyrotrophs are most profoundly degranulated and the time the gonadotrophs are most altered. Thus, in the groups given the larger dose of cortisone there is a loss of PAS-positive granules in the thyrotrophs and a marked shrinkage in the size of the gonadotrophs three days after injections had begun as well as three days after they had ceased. The small dark-staining gonadotrophs stand out clearly against the pale thyrotrophs, thus giving the transitional lobe a peppered appearance. All four experimental groups show this close correlation, although the time of occurrence and degree of alteration vary with the treatment.

It is not possible as yet to assign any cell type in the *Astyanax* pituitary as the source of ACTH. After ACTH or cortisone administration, changes were observed only in the thyrotrophs and gonadotrophs, and no third type of cell was distinguishable with the PAS technique. The hypertrophy and hyperplasia of acidophils described by Rasquin and Atz (1952) was also seen in the present material. They attributed these effects to unreleased growth hormone.

DISCUSSION

Although the gonadotropic and thyrotropic activity of the fish pituitary is well recognized, there are relatively few papers in which the histology of the gland has been correlated with these functions. Seasonal changes in the teleost transitional lobe have been described in the stickleback, *Gasterosteus aculeatus* (Bock, 1928), killifish, *Fundulus heteroclitus* (Matthews, 1936), herring, *Clupea harengus* (Buchmann, 1940), swordfish, *Xiphias gladius* (Lee, 1942), bitterling, *Rhodeus amarus* (Bretschneider and Duyvené de Wit, 1947), roach, *Leuciscus rutilus* (Kerr, 1948), carp, *Cyprinus carpio*, and goldfish, *Carassius auratus* (Scruggs, 1951). In all of these reports, save that of Bock (1928), an increase in basophils was associated with the spawning season. Buchmann (1940) describes what he believes to be a cycle of activity in the transitional lobe during spawning; "unripe" Azan red colloid (basophil in his terminology) becomes "ripe" Azan blue (acidophil in his terminology). In his review of the literature, Hoar (1951) concluded, "The gonadotropic production is, without doubt, localized in the middle glandular area of the teleost pituitary and is associated with the appearance of large numbers of basophils there." In this laboratory, it has been found that sexually mature *Astyanax mexicanus* show a predominance of basophils to acidophils and that the reverse obtains in the pituitaries of immature fish (Rasquin, 1949). The basophils of the transitional lobe have also been associated with changes in thyroid of the salmonid, *Salmo salar* (Fontaine and Olivereau, 1949; Leloup and Olivereau, 1950; Fontaine, et al., 1952), the characin, *Astyanax mexicanus* (Rasquin and Atz, 1952), and the cyprinid, *Brachydanio rerio* (Scott, 1953). No attempt has been made previously to localize the source of these two tropic hormones histochemically.

The use of the PAS technique on the pituitary of *Astyanax* has given results which are comparable to those found in the rat by Purves and Griesbach (1951a). Two types of basophils have been observed, and although the differences between them are not as marked in the normal fish as they are in the rat, their responses to hormonal changes are clear and specific. The differences are summarized in Table I.

The activity of the thyrotrophs after thiourea administration is greatly increased, as is evidenced by the augmented amount of vacuolation and degranulation and a resultant loss of staining reaction. Mitotic figures are not seen, however, which agrees with the observation of Rasquin (1949) who reported that there was no difference in basophil count between normal control *Astyanax* and those treated with thiourea for 12 weeks. Scott (1953), on the other hand, found that immersion of *Brachydanio rerio* in the same concentration of thiourea for 14 and 16 weeks did cause a marked increase in the percentage of basophils; he also observed mitotic figures. Mitoses were often observed in the degranulated "hypocyanophiles" in *Salmo salar* at the time of smoltification, when the thyroid appears more active histologically than that in the parr (Fontaine, et al., 1952). These authors found that the smolt thyroid and blood nevertheless contained a smaller amount of iodine. To them this condition appeared to resemble that observed after treatment with antithyroids.

The histological picture of the thyrotrophs in some of the ACTH- and cortisone-treated *Astyanax* resembles that seen in the cyanophiles of the smolt transitional lobe. In these *Astyanax*, the thyrotrophs actually appeared to be more stimulated than those of the individuals

TABLE I. Comparison of Two Types of Basophils in the Transitional Lobe of *Astyanax*

Treatment	*Thyrotrophs*	*Gonadotrophs*
Normal	Gomori and PAS+, finely granular, smaller cells peripheral	Gomori and PAS+, homogeneous or with coarse aggregations, no regional differences
Thiourea 4 weeks	Degranulation, decreased staining, increased vacuolization	No effect, or slight decrease in size
Thyroid fed 4 weeks	Slight regressive changes	No effect, or slight increase in number of vacuolated cells
Darkness (gonads in regression) 23 weeks	All basophils intensely colored, not possible to recognize thyrotrophs	No change
39 and 48 weeks	All basophils intensely colored, not possible to recognize thyrotrophs	Appearance of signet ring, castration-like cells
Cortisone 10 days	Cycle of degranulation and restoration follows both onset and cessation of treatment	Cycle of size reduction and restoration follows both onset and cessation of treatment
ACTH 10 days	Similar cycle, less striking	Similar cycle, less striking
Saline 10 days		
Double daily injection	Similar cycle, less striking	Similar cycle, less striking
Single daily injection	Increase in vacuolization and degranulation at onset of treatment followed by restoration. No changes at cessation of injections	No change

immersed in the thiourea for four weeks. However, the thyroid follicular epithelium of these fish was depressed. Halmi and Barker (1952) reported that rats treated with cortisone gave evidence of increased TSH release, but this was reflected in an increase in thyroid epithelial height. Rasquin and Atz (1952) believed that the inhibitory effect of ACTH and cortisone on the *Astyanax* thyroid was a direct one and was not mediated through the pituitary, which gave no evidence of TSH deficiency. These observations are confirmed by the present study; the PAS technique indicates rather that there is a definite transitory increase in thyrotropic activity. Three or five days after the start of the injection period, and three days after its cessation, there was a loss of PAS-positive granules, which are assumed to be the morphological sign of TSH. This was followed by a reappearance of these granules. Occasional mitotic figures were seen, especially in the cortisone-treated group during the postinjection period. It should be noted here also that, five days after the cessation of the injection of the smaller dose of cortisone, small intensely PAS-positive droplets were observed in the thyrotrophs. These granules, similar to those found in the rat thyrotrophs after thyroidectomy or antithyroid treatment (Purves and Griesbach, 1951a), may also be interpreted as a manifestation of stimulation.

The slight regressive changes observed in the *Astyanax* thyrotrophs after feeding with thyroid hormone are of the same type but of a much lesser degree than those found in thyroxin-injected rats (Purves and Griesbach, 1951a). Perhaps the amount of thyroid hormone actually ingested by the fish was insufficient to evoke any great loss of glycoprotein granules in the thyrotrophs or other signs of acute diminution of TSH production.

The PAS staining reaction of the *Astyanax* gonadotrophs is not altered by thiourea or thyroid hormone administration. These cells appear smaller after the former treatment, however, and appear to be more stimulated after the latter, as evidenced by some increase in the number of vacuolated cells.

The most striking changes in the gonadotrophs are seen in the ACTH- and cortisone-treated fish. At the same time that the profound degranulation of the thyrotrophs occurred, there was an acute shrinkage in the size of the gonadotrophs. However, they still stained an intense purple-red with PAS. This great loss of cytoplasmic volume is thought to be associated with a release of secretion. If these were regressive or inhibitory changes, presumably the gonadotrophs would have remained shrunken with pycnotic nuclei throughout the experiment. During both the injection and postinjection periods, however, they regained their normal volume, nuclear character, and staining affinity.

The gonads of these ACTH- and cortisone-treated fish are also altered. In the female, matured ova undergo resorption with a proliferation of follicular epithelium; in the male, the secretory activity of the sperm duct epithelium is so stimulated that the duct and lobules of the testis are distended with fluid (Rasquin and Atz, 1952). It was suggested that these gonadal alterations in *Astyanax* might have been caused by (1) an androgenic effect of cortical hormones in this species, (2) a nonspecific reaction to mammalian ACTH such that this hormone acted gonadotropically as well as adrenocortically and (3) a shift in the pituitary's metabolism toward the production of gonadotropic hormone which resulted from an inhibition in the production of ACTH. From the results obtained with the PAS stain, it appears that there is an activation of the gonadotropic cells, with synchronous changes in the ovary resembling luteinization, that is, the proliferation of follicular epithelium and the resorption of yolk. In this regard it is suggestive that salmon gonadotrophin has been reported extremely rich in the luteinizing hormone (Witschi, 1939). Purves and Griesbach (1951a) thought it likely that both the follicle-stimulating hormone and the luteinizing hormone were produced in the same cell, the gonadotroph. It appears that in *Astyanax* there is also only one type of PAS-positive gonadotroph.

Although there is an abundant literature to demonstrate the function of gonadotrophin in fishes, it is not known to what extent the gonadal steroids influence gonadotrophin production by the pituitary. In the absence of specific information, it may be assumed that there is a reciprocal regulation of pituitary and gonads in fishes similar to that demonstrated in mammals.

While there is a marked similarity between the mammalian castration cells and the vacuolated cells seen in two of the three dark-reared *Astyanax*, certain facts are difficult to correlate. It should be noted first that these dark-reared fish developed abnormally, not only with respect to their gonads but also with regard to the other endocrine glands. The evidence presented by Rasquin and Rosenbloom (1954) indicates that the pituitaries were hypofunctional and contained fewer basophils than those found in normal *Astyanax* of the same age reared under conditions of recurring light and darkness. Although the vacuolation of the castration cell may be considered the by-product of progressive atrophic changes, an increase in size and number of normal appearing basophils is a more consistent feature of the castrate mammalian pituitary (Severinghaus, 1939). Brolin and Theander (1945), in work on adult castrated rats, and Brolin and Löfgren (1947), in a study of infantile castrated rats, found that newly formed hypertrophied basophils appeared and that there was an increase in the degeneration of these cells (vacuolation). An increase in the number of basophils has been observed in the castrated lizard, *Takydromus tachydromoides* (Hatta, 1941). In castrated *Triturus viridescens*, Copeland (1943) found, after three and one-half months, a similar increase in the number of basophils, but six months after castration the count had returned to normal. Castration cells were observed in neither of these species. In the dark-reared *Astyanax* there is no increase in the number of basophils. Exposure of these animals to constant darkness constitutes a severe stress which results in a profound hormonal imbalance (Rasquin and Rosenbloom, 1954), and it may be that the pituitaries of such animals are no longer able to respond appropriately to a lack of sex hormones from the gonad.

With an increase in the number of basophils there also occurs in mammals an increase in the gonadotropic stimulating potency of castrated pituitaries (Severinghaus, 1939). Corresponding data for the castrated teleost are still not known. If it is assumed that such a potency increase does occur in fishes after castration, why has there been no stimulation of the undifferentiated gonads in the two dark-raised *Astyanax* which show castration-like cells? A failure of hormone release rather than production may be responsible. Severinghaus (1939) has pointed out that the increased gonadotropic potency of castrate pituitaries is referable to the newly formed large basophils of approximately normal activity in such pituitaries and not to the signet ring castration cells. In *Astyanax*, no new basophils were seen. In this regard it is also interesting to note that the ovaries of menopausal women, also physiological castrates, can no longer respond to FSH stimulation. A similar refractory situation in the target organ may exist in the physiologically castrated *Astyanax*.

Thus, while the vacuolated cells in the pituitary of two of the three dark-raised *Astyanax* are similar to the castration cells pictured by Purves and Griesbach (1951a), all of the related factors cannot be correlated. More conclusive results await successful castration of normal *Astyanax* and a series of observations on such specifically treated fish.

The question arises whether or not the thyrotrophs and gonadotrophs of the rat are homologous with the similarly designated cells of *Astyanax*. Our studies demonstrate that both types of cells in both fish and mammal react similarly to the PAS stain but that they also show some distinct differences. The latter may be best summed up by pointing out that the thyrotrophs and gonadotrophs in *Astyanax* are structurally even less distinct from each other than they are in the rat, at least in so far as they are realized by the PAS technique. Moreover, the production of most of the pituitary hormones in any animal has yet to be assigned to distinct cell types; in fact, we can only assume that differences in function are necessarily accompanied by differences in pituitary cell structure. The limits of variability of any given type of pituitary cell also remain to be determined, especially as regards the structural continuity or discontinuity between groups of cells of different endocrinological function.

Fish pituitaries present relatively difficult cytological material, principally because of the small size of their cells. Since the PAS reaction makes it possible to distinguish thyrotrophs from gonadotrophs in *Astyanax*, it gives promise of providing a useful tool for the study of teleost pituitaries and for evaluating pituitary-target gland interrelationships.

SUMMARY AND CONCLUSIONS

1. Periodic acid—Schiff staining of the pituitary gland of the characin, *Astyanax mexicanus* (Filippi), has demonstrated that two types of transitional lobe basophils can be differentiated.

2. These cells are distinguishable from each other by differences in granule size and staining reaction and by their specific response to hormonal changes. One cell type reacts to alterations in thyroid hormone level and is believed to be the thyrotroph; the other shows changes related to the physiological state of the gonad and is believed to be the gonadotroph.

3. The thyrotrophs show increased vacuolation and degranulation after thiourea treatment and display some regressive changes after thyroid hormone administration.

4. Three *Astyanax* reared in continuous darkness for 23, 39 and 48 weeks had completely undifferentiated gonads. The PAS-positive cells in the transitional lobe of these fish contain intensely colored homogeneous cytoplasm. Most of these cells in two of the three fish have a nucleus that is compressed and pushed to one side by a large clear vacuole which occupies most of the cell. The appearance of these cells is suggestive of the signet ring castration cells seen in the pituitaries of certain mammals.

5. ACTH and cortisone administration evokes successive phases of vacuolation and degranulation in the thyrotrophs, followed by cytoplasmic reconstitution. At the same time as these changes occur in the thyrotrophs, the gonadotrophs shrink markedly in size but maintain an intense color. This loss of cytoplasmic volume is also followed by reconstitution.

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