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Effects of Estradiol Hormone on the Maturation of the Gonads and Associated Duct System of the Fish *Lebistes reticulatus*[1]

MICHAEL THOMAS STORY[2]

Abstract. It was the purpose of this study to describe the secondary sex characters and the histological appearance of the gonads, including the associated duct system, of the fish *Lebistes reticulatus* from birth through differentiation and maturation when subjected to the hormone estradiol. Sixty-four *Lebistes reticulatus* were fed 0.125 mg of estradiol, beginning at birth, at intervals of 60 hours. An equal number of fish were used as the control. Fish were sacrificed at ten-day intervals following birth, for a period of 80 days, and examined for the presence of secondary sex characters. Serial cross sections of the gonads were prepared from 32 experimental fish and 16 control fish. Estradiol hormone, when fed to male *Lebistes reticulatus* suppressed the appearance of male secondary sex characters, but did not stimulate the appearance of female sex characters. Estradiol had no apparent effect on the secondary sex characters or gonads of females. The effects produced by estradiol feeding on the testes were: a stunted testicular size, a suppression of spermatogenesis, an accumulation of connective tissue stroma within the medulla of the organ, a suppression of secretory activity of cells lining the sperm ducts.

[1] Revised thesis in partial fulfillment of the requirements for the degree Master of Arts, Department of Biology, Drake University, Des Moines, Iowa.

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Hormones as agents in gonadal sex differentiation have been the object of study in a number of cold-blooded vertebrates. The existence of considerable degree of embryonic bisexuality in most species proved a definite morphologic basis for experimental transformation of sex. The fact that the secondary sex characters in these vertebrates are modified by sex hormones is well known. A number of investigators have also reported that sex hormones have an effect on the gonads of certain fish and amphibians. However, on the basis of evidence available from these studies, a considerable variation appears to exist in the histological and physiological patterns in various groups and species of these vertebrates.

The embryological development of the gonads of *Lebistes reticulatus* has been described by Goodrich, et al. (1934) and Dildine (1936). These authors reported that gonadal differentiation occurs in both sexes just prior to birth.

Winge (1930) reported the occurrence of a few XX males in normal breeding of *L. reticulatus*. Winge (1934) also obtained some females of the male genotype XY in certain varieties of this fish. It was suggested that in this species sex might be determined by the sum of the hereditary units (sex chromosomes and autosomal factors) acting in the male or female direction. These findings seem to indicate that sex differentiation in certain species of *L. reticulatus* is very weak. This has also been shown by embryological studies on the differentiation of the gonads by Dildine (1936) who reported juvenile hermaphroditism in this fish.

The appearance of secondary sex characteristics in the male *L. reticulatus* has been correlated with the maturation of the testes (Blacher, 1926; Goodrich, et al., 1934). Winge (1922, 1927) has shown that male color dimorphism depends on the genes associated with the Y chromosome. Berkowitz (1938, 1941) and Eversole (1939, 1941) have, however, demonstrated that secondary sex characters in *L. reticulatus* are modified by antagonistic sex hormones. In addition, Berkowitz (1938) reported that estrone and estriol, when fed to *L. reticulatus*, from the time of birth, inhibited spermatogenesis and resulted in involution of the testes. Upon further investigation, with various dosages of these hormones, Berkowitz (1941) observed the appearance of an ovotestis in nearly all genetic males. It was also observed that both the intertubular stroma and the duct system were markedly hypertrophied.

Eversole (1941) and Mohsen (1958) were also able to demonstrate various degrees of masculization of the ovaries in *L. reticulatus* with androgens.

The above studies indicate that the gonads of *L. reticulatus* are in a state that can be influenced by antagonistic sex hormones. However, a complete and functional sex reversal has not been obtained in this organism.

Yamamoto (1953) has reported complete sex reversal from male to female in the fish, *Oryzias latipes,* by the influence of estrogens. By the use of androgens, Yamamoto (1958), induced functional sex reversal in genetic females. Yamamoto (1961) stated that so far *O. latipes* is the only animal in which induction of sex reversal, in both directions, has been accomplished. This induction was shown to be functional and permanent as verified by the offspring producted by sex reversed females with sex reversed males in the 1:1 expected sex ratio for two consecutive years.

Experiments involving heterotypic sex hormones in fish indicate that some species are able to undergo complete sex reversal, whereas, in others this appears not to be the case. Further studies with special attention to the histological detail during hormonal treatment would be of considerable value to a knowledge of the mechanisms involved in sex reversal and differentiation. The purpose of this investigation has been to describe the secondary sex characters and the histological appearance of the gonads, including the associated duct systems, of *L. reticulatus,* from birth through differentiation and maturation when subjected to the hormone estradiol.

Materials and Methods

A parent stock of *Lebistes reticulatus* was obtained from a supply maintained at Drake University. From this stock a total of 128 offspring were obtained. Newborn fry were used exclusively in this study.

Within 24 hours after birth, newborn *L. reticulatus* were divided into groups consisting of 16 litter mates, and were placed in 5-gallon aquariums. A total of eight such groups were required for this study. Four groups were used as experimental material, while the remaining groups constituted the control.

All aquariums were equipped with a bottom filter (Wil-Nes). The water temperature was maintained between 76-80° F by means of thermostatically controlled heaters (Wil-Nes). Approximately 12 hours of light was maintained daily.

The normal diet for both experimental and control groups consisted of brine shrimp. Periodically this diet was supplemented with liver paste (Gordon, 1955). This diet was fed in small amounts at approximately 12-hour intervals.

The source of estradiol hormone used in this study was Progynon buccal tablets (Schering Corporation). Each tablet, containing 0.25 mg of estradiol, was finely ground in a mortar and pestle. One-half of the tablet weight, containing approximately 0.125 mg of estradiol, constituted a single dosage. This dosage was added to the aquarium water of each experimental group, beginning on the third day following birth, and was continued at intervals of 60 hours replacing the normal diet. The entire duration of the hormone feeding was 80 days.

Two fish were selected from each of the experimental and control groups at intervals of ten days for morphological study. The fish were observed for the presence of the following secondary sex characters: color, gonopod, gravid spot, and length. The length was measured from the tip of the snout to the end of the tail. On the basis of secondary sex characters, fish were considered to be males if the color factor was discernible, and to be females if the gravid spot was present. Fish lacking both of these factors were considered sexually immature. The fish were then sacrificed by cutting off the head just posterior to the operculum, and the tail was removed just posterior to the anus.

One control group and two experimental groups were used for histological study of the reproductive system. The portion of the body containing the gonads was fixed in Zenker's fixative and washed in running tap water. The tissue was dehydrated in ethyl alcohol, cleared in toluene, and embedded in Tissuemat paraffin (Fisher Scientific Company). The tissue was serially sectioned at 6 microns with a rotary microtome (International Equipment Company), and stained with Mallory's triple stain. The sections were studied microscopically to determine the effects of estradiol on the histological appearance of the gonad and associated duct systems during maturation. Graphic reconstruction of the gonads at representative stages of maturation was made from serial cross sections. All measurements were made with an ocular micrometer (Bausch and Lomb Incorporated). Photomicrographs of various sections were taken to illustrate significant structures.

Results and Interpretation of Data

Sex distinction, on the basis of secondary sex characters, was evident in all fish of the control group by 40 days of age. No sexually immature fish were found in the control group between 40-80 days of age.

Females, which had received estradiol, showed no apparent effect on the secondary sex characters. Histological study of

the gonads and associated duct systems revealed ovarian development similar to that of the control group. These findings agree with those reported when females *L. reticulatus* were subjected to other estrogens by Berkowitz (1938, 1941).

Males of the experimental group failed to exhibit secondary sex characters from birth to 60 days of age. The suppression of these characters was not, however, complete. Two members of the experimental group began to show some male coloration when sacrificed at 60 days of age. One fish sacrificed at 70 days and a second at 80 days of age also showed some male coloration as well as the beginning of gonopod development. There was no evidence of female secondary sex characters appearing in males of the experimental group.

On the basis of the histological appearance of the gonads of experimental fish considered to be sexually immature, it would appear that inhibition of the male secondary sex characters by estradiol might be due to an inhibition of the testes by this hormone. However, this inhibition does not appear to be as great as that described for estrone and estriol by Berkowitz (1938, 1941).

Studies of the reproductive system of males of the control group, from birth to 80 days of age, revealed maturation similar to that reported by Goodrich et al (1934), Vaupel (1929), and Evans (1961).

Examination of the testes and associated duct systems of the experimental fish, which were sacrificed from 10-30 days after birth, revealed no significant variation from that observed in the control group at the same stages of maturation.

The appearance of the testes of the 40-day experimental fish showed minor variation from that of the control group at this age. Measurements indicated no significant difference in size between the testes of males in the experimental and control groups. The length of the testis in the control fish was 498 microns as compared to 528 microns for the experimental fish. This is reconstructed graphically in figure 1.

Figure 2 is photomicrograph of a cross section of the central portion of the testis of a 40-day control fish. Fusion of the bilobed testis was incomplete. Spermatogonia were primarily confined to the periphery of the organ. Activity in the anterior region of the gonad suggested the initiation of spermatogenesis. The duct system was confined to the medulla and appeared as narrow slits. Lumina of the ducts were devoid of secretory products. The major ducts had fused within the posterior end of the testis, and a single duct then entered the urogenital sinus.

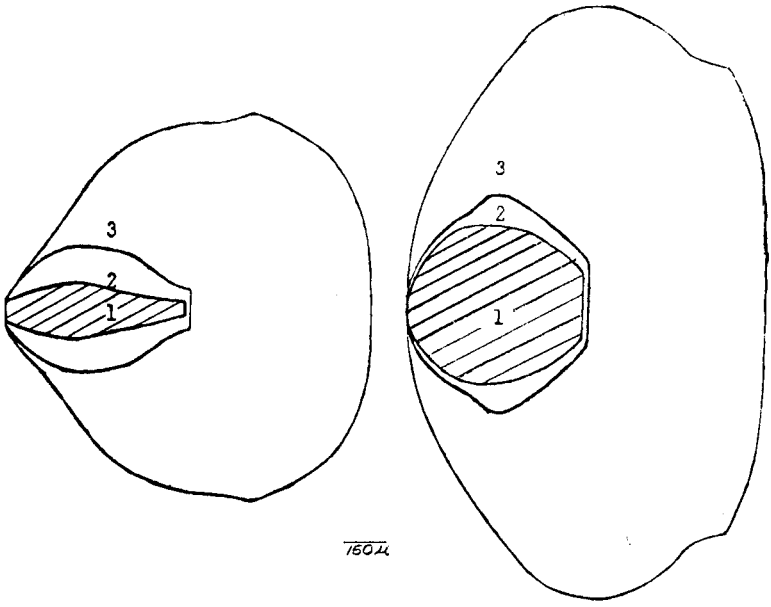

Figure 1. A (on left). A representative graphic reconstruction of the height and length of the testes of *Lebistes reticulatus* fed estradiol hormone. 1. experimental and control fish at 40 days of age, 2. experimental fish at 60 days of age, 3. control fish at 60 days of age.

B (on right). A representative graphic reconstruction of the width and length of the testes of *Lebistes reticulatus* fed estradiol hormone. 1. experimental and control fish at 40 days of age, 2. experimental fish at 60 days of age, 3. control fish at 60 days of age.

Figure 3 is a photomicrograph of a cross section of the central portion of the testis of a 40-day experimental male. The fusion of the embryonic bilobed gonad appeared not to be as complete as that exhibited by the control group at this period of development. More connective tissue stroma was evident within the medullary region of the testis. Spermatogonia were confined to the cortex and no stages of spermatogenesis were evident. The duct system appeared similar to that of the control fish, with fusion of the major ducts occurring posterior to the testis.

Sections of the testes in the 60-day control males were markedly larger than those observed in the 40-day control males. Length of the testis in the 40-day male of the control group was 498 microns; at 60-days of age, 1098 microns. This increase in size is shown graphically in Figure 1.

Figure 4 is a photomicrograph of a cross section of the anterior portion of the testis of a 60-day control fish. The major ducts within this portion of the organ were the only remaining evidence of the embryonic bilobed origin of the gonad. The stroma was scattered between the numerous cysts. All stages

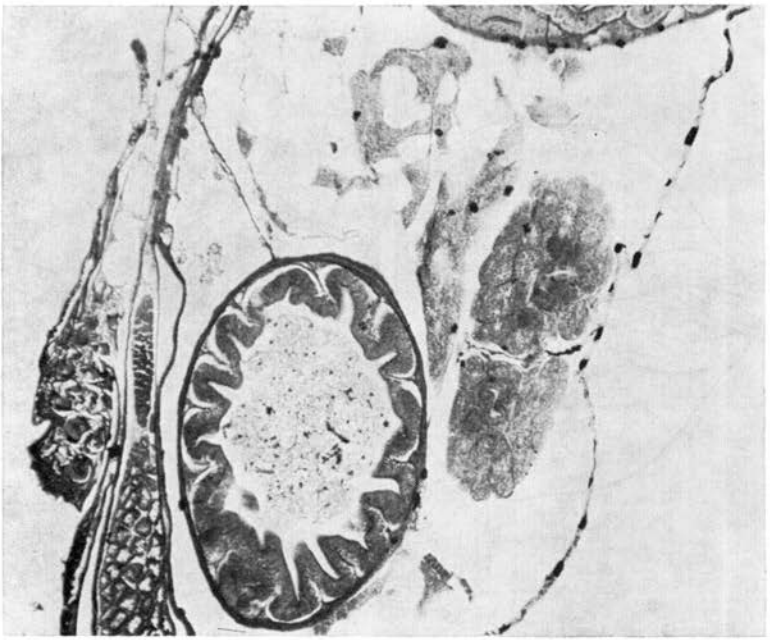

Figure 2. Cross section of the central portion of the testis of a 40-day control fish. 325X.

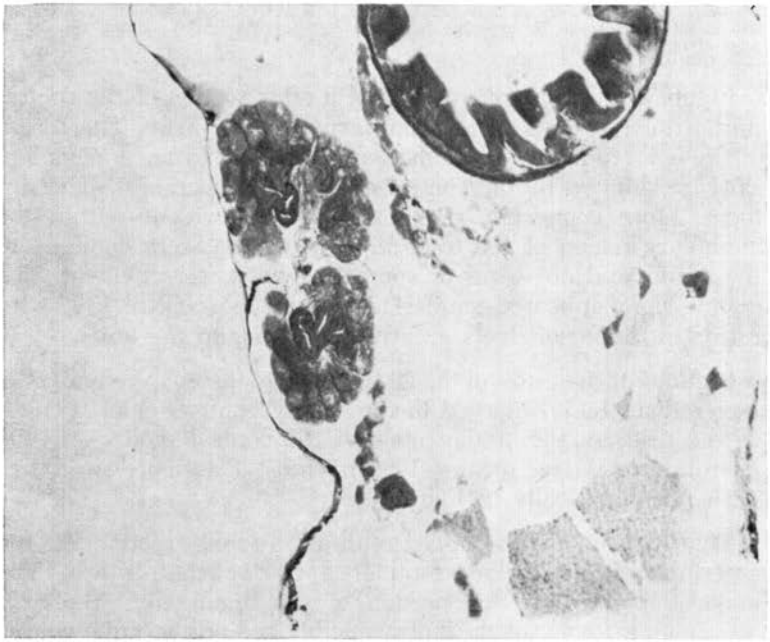

Figure 3. Cross section of the central portion of the testis of a 40-day experimental fish. 325X.

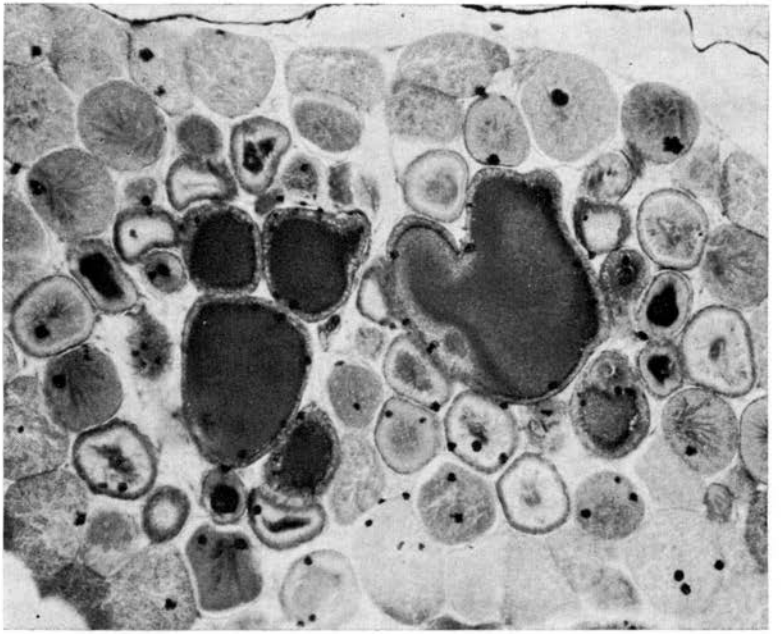

Figure 4. Cross section of the anterior portion of the testis of a 60-day control fish. 325X.

of spermatogenesis as described by Vaupel (1929) were represented. The duct system showed a significant development over that of the 40-day control males. The major ducts fused in the posterior portion of the testis to form a seminal vesicle as shown in Figure 5. A single duct then left the gonad as described for 40-day males of the control group. The cells lining the lumina of the major ducts were low columnar to cuboidal epithelium as evident in Figure 6. These cells were highly granular and appeared to have a secretory function. The Lumina of the major ducts contained a homogenous colloidal material ranging in staining qualities from red to blue with Mallory's triple stain. The colloidal material appeared to be a product of the cells lining the tubules.

The testes of the males among the experimental group between the ages of 40 and 60 days did not show the degree of maturation characteristic of the control group. Length of the testes among the experimental group increased from 528-534 microns from 40-60 days of age, respectively; however, the length of the testes in males of the control group increased from 498-1098 microns during the same period of maturation. These results are shown graphically in Figure 1.

Figure 7 is a photomicrograph of a cross section of the central

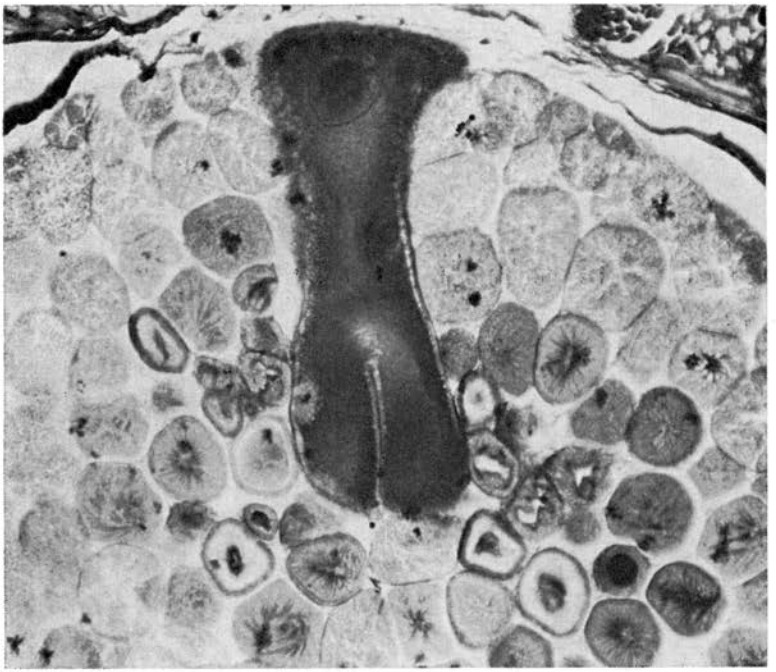

Figure 5. Cross section of the posterior portion of the testis of a 60-day control fish displaying the seminal vesicle. 325X.

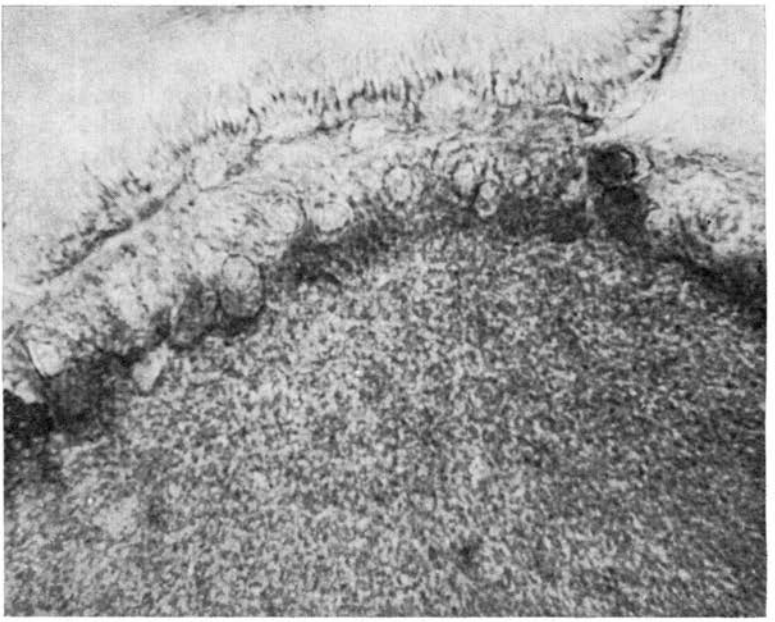

Figure 6. A major duct of the testis with secretory cells in a 60-day control fish. 3000X.

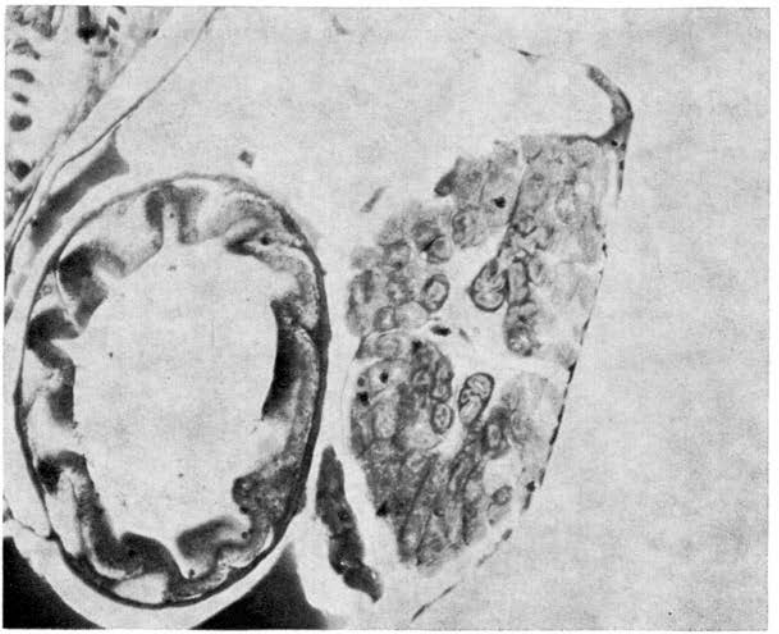

Figure 7. Cross section of the central portion of the testis of a 60-day experimental fish. 325X.

portion of the testis of a 60 day experimental fish. The sperm ducts were surrounded by a large amount of connective tissue stroma, and were located in the center of the organ. Fusion of the embryonic bilobed testis appeared less advanced than that shown in the control fish. The testes of the 60-day experimental males were highly compact structures. There were no spermatogenic elements with the exception of spermatogonia confined to the cortex of the organ. No female germ cells were evident in the testes of the experimental or control fish at any stage of maturation. The major sperm ducts appeared similar to those described for the 40-day control males. The major ducts at the posterior end of the testis had not fused. Fusion of the sperm ducts was evident some distance posterior to the gonad as indicated in Figure 8. There was no evidence of secretory activity in the epithelial lining of the ducts.

Involution of the testes and inhibition of spermatogenesis has also been reported in *L. reticulatus* by Berkowitz (1938, 1941). With a dosage of 30 R. U. of estrone and estriol Berkowitz (1941) reported female ova in the testes of 90% of the males. However, with a dosage of 600 R. U. the testes were described as being involuted and lacking in oögonia.

The estradiol dosage used in this study was 0.125 mg (700

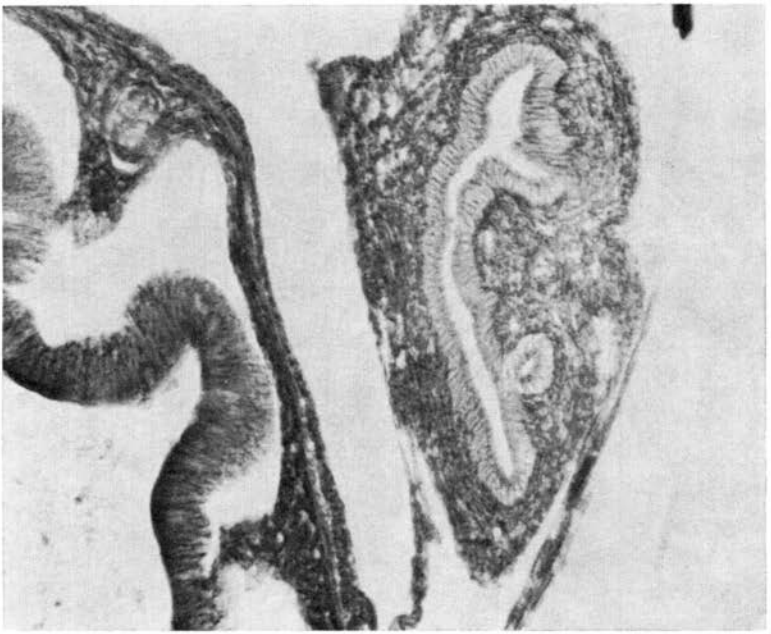

Figure 8. Cross section of the fused major ducts, posterior to the testis, of a 60-day experimental fish. 1375X.

R. U.). It would appear that suppression of spermatogenesis and inhibitory effects on the maturation of the testes were similar to results reported for 600 R. U. of estrone and estriol by Berkowitz (1941).

The influence of estradiol on the duct system of the testes, as described in this study, was in sharp contrast to that reported by Berkowitz (1938, 1941), who reported an increase in the number and size of the sperm ducts, with an abnormal accumulation of colloidal material within the lumen as a result of estrogen feeding. These results were not restricted to males receiving low dosages of the estrogen, but were also shown in the group receiving high dosages of the hormones.

Apparently estradiol had a different physiological effect on the duct system of the testes of *L. reticulatus* than that reported for estrone and estriol. This study, however, does not permit a comparative study of the cellular activities of the estrogens.

Sex differentiation in *L. reticulatus* appears to be established at birth. Attempts at sex reversal by antagonistic sex hormones might, therefore, be expected to be less successful than in cases of indifferent sex distinction at birth.

Functional sex reversal in *O. latipes* (Yamamoto 1953, 1958,

1961) might be attributed to sexual indifference present at hatching and to a comparatively long duration of gonadal indifference prior to differentiation.

Future studies of sex reversal in *L. reticulatus* might be directed toward hormonal administration to pregnant females in an attempt to influence embryos from the beginning of the differentiation process.

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