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THE REPRODUCTIVE SYSTEMS
OF THE ZEBRAFISH,
BRACHYDANIO RERIO (HAMILTON-BUCHANAN)

by

Helen Jenny Jervis

Submitted to the
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of The American University
in Partial Fulfillment of
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of
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Signatures of Committee:

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The assistance of the members of the Thesis Committee is here gratefully acknowledged. Thanks are due to Dr. S. O. Burhoe for his patience in reading and correcting the manuscript, for his unfailing advice when needed, and his tactful non-interference at other times. Thanks are due also to Dr. A. B. Chaet for his help in interpreting histological details and to Dr. H. Seversmith for his interest and encouragement. Last but not least, my husband and son deserve my gratitude, since without their understanding and patience this work could not have been accomplished.

TABLE OF CONTENTS

LISTING OF FIGURES

LISTING OF FIGURES (continued)

INTRODUCTION

The developmental stages of fishes, in recent years, have engaged the attention of biologists searching for more varieties of animals suitable for embryological study and experimentation.

In many aspects, fishes are particularly suitable for these studies because of the fact that most of them are oviparous, with external fertilization and with semitransparent eggs. The developmental stages and any attempted experimental alterations are very easy to observe, much more so than in the old stand-bys, frog and chicken.

For instance, the developmental stages of *Fundulus heteroclitus* have been described and extensive transplantations in the embryos have been performed.

Recently, experimental studies on another fish, the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan), have made their appearance in the literature. Hisaoka and Battle (1958) described its developmental stages, and they and others have used it successfully in experimental work.

The zebrafish has the advantage of being a freshwater fish which is inexpensive, very easily obtainable in practically all tropical fish outlets, readily maintained in the laboratory and, with a few precautions, easy to breed at any time of the year. The eggs, completely transparent, are fertilized in the water, demersal, and, at 26° C, develop from deposition to hatching in about 4 days. The first few cleavages succeed each other in a matter of minutes (Hisaoka and Battle, 1958; Hisaoka, 1960). Except for the experimental work mentioned above, the zebrafish is practically unknown in the literature. There exist some taxonomic descriptions and cultural directions in aquaria magazines (mentioned by Dean 1923) and in handbooks, but no anatomical description. This lack was strongly felt during a course in Experimental Embryology given at The American University in the spring of 1960 by Dr. Seversmith, instructor in the course. The zebrafish was one of the animals chosen as a subject of study, and it was the opinion of the instructor and of the students that the experimental work would have been easier if a description of the normal morphology of the fish had been available. The present work is meant as a beginning toward providing such description. It is limited, at least for the moment, to a study of the anatomy of the male and female reproductive systems of this species, since these are the most useful for the embryological studies for which this fish is particularly well adapted. In addition, the reproductive system seems to be the least known part of fish anatomy. It is sharply divergent from that of most other vertebrates, especially as far as gonoducts are concerned. Homologies with other vertebrates and among fishes are vague, and opinions about them contradictory. Even fairly closely related groups show important differences in the disposition of the gonads and gonoducts. It is hoped that this work will make a small contribution toward the more comprehensive picture that eventually will emerge from the meager and confused reports now available. To this end the reproductive systems of the zebrafish will be compared to those in other fishes already described, and the similarities and differences will be noted. Also the development of the gonads and the accessory sex organs will be traced back to as early a condition as can be followed, in an effort to determine the mechanics of gonads and gonoduct formation.

Brachydanio rerio comes originally from India. It is found in Bengal and in the rivers of Coromandel as far south as Masulipatapam (Day, 1878). It was first described by Francis Hamilton (formerly known as Buchanan, 1822), who discovered it in the Kosi River and named it Cyprinus rerio. It was one of the many Cyprini that he had grouped in the Danio division, Danio being derived from the name Dhani given by the Bengalese to these fishes.

Later the Danios were subdivided in several genera. F. Day (1878) classified the zebrafish as Danio rerio of the subfamily Cyprininae, family Cyprinidae. By that time it had been described by five more authors under 5 different names (quoted by Day, 1878).

Under the name of Danio rerio it was introduced in Europe early in the twentieth century; it became very popular, judging from the number of articles dealing with its care, and breeding, which appeared in aquaria magazines. The Bibliography of Fishes (Dean 1923) gives 11 titles on Danio rerio alone between 1905 and 1913. Weber and Beaufort (1916) proposed a subgenus Brachydanio to include those danios having 6-7 branched rays in the dorsal fin and an incomplete or missing lateral line. Simple Danios were to be those with 12 or more dorsal fin rays and a complete lateral line. Myers (1924) gave to Brachydanio full generical rank not only on the basis of the number of branched rays in the dorsal fin, but also because of the fact that the Brachydanios have demersal eggs while the known eggs of the Danios are adhesive. Hora in 1937 also came to agree with this view (cited by Myers, 1953). Smith (1945) did not approve of this breakdown of the genus Danio, since the criteria for the separation were based mostly on gradual variations of one character, the number of branched rays in the dorsal fin. Myers (1953), however, contended that there are no intermediate genera to fill the gap between Danios and Brachydanios. One such, described by Hora (1937, cited by Myers, 1953) on the strength of one specimen, Myers considered to be a natural hybrid (not uncommon among Cyprinidae); while another, described by Smith (1945), he considered to be a completely different genus.

The modern consensus of opinion seems to support Myers and Hora, and the appellation Brachydanio rerio is generally accepted in the scientific literature and in popular works for fish fanciers (Innes, 1947; Brittan, no date; Axelrod & al., 1960).

MATERIALS AND METHODS

Description of Subject of Study

The fish used in this study is the zebrafish, Brachydanio rerio, (Hamilton-Buchanan, 1822). This is a small fish, described originally as being about two inches long. The specimens studied, however, were never more than 38-40 mm long (32-34 mm standard length).

It has an oblong body with a fairly straight back and a more rounded belly. It is silvery, striped longitudinally with blue with one stripe middorsal and 4 more on each side. The caudal fin has 7 stripes, the 3 central ones continuous with those of the body. The anal fin has 4 horizontal stripes, while the others have no definite color pattern.

The back has an olive-brown coloration. The color in the stripes is due to subcutaneous chromatophores, while the coloration of the back is due to smaller chromatophores set right under the epidermus of the scales. The distribution of the pigment in the chromatophores is influenced by ambiental conditions, fishes becoming darker when placed in a tank with a dark bottom. According to Brown (1952) this should be due to a reflex response to reflected illumination from the bottom.

The head is small, without scales, the mouth is oblique with the lower jaw extending beyond the snout.

There are two pairs of maxillary barbels: the first two, placed forward near the nostrils, are short and thin; the second, starting at the angle of the jaws, longer and thicker.

There are three rows of pharyngeal teeth.

There is no lateral line, or at least none was observed.

The scales are cycloid, of moderate size, in regular rows.

The fins are all soft rayed, and most rays are branched. The dorsal fin starts behind the ventral fin; it has 7 branched rays. In the few specimens observed (since this is not a taxonomic study), 2 single rays, not spines, have been observed anterior to the branched rays. The candal fin is homocercal; the anal fin is larger than the dorsal and begins close behind the excretory opening. The ventral fins are abdominal, set close to the midline, anteriorly to the anal papilla. The pectoral fins are set low. There is no adipose fin. Brachydanio rerio is classified as belonging to the class of Pisces or Osteichthyes, Super-order Teleostei (Jordan 1923), order of Eventognathi or Cyprinifarmes, family of Cyprinidae, subfamily of Rasborinae.

The zebrafish are Teleosts as befits their being bony fishes. They may be recognized as Eventognathi because of their cycloid scales, branched soft rays in the fins, abdominal ventral fins, and toothless jaws;

the other taxonomic characters, less easily checked, have not been verified during this study.

Their naked heads and well developed pharyngeal bones, with a limited number of teeth, place them in the Cyprinidae. Finally H. Smith (1945) placed them in the subfamily of Rasborinae because of the number of rays in the dorsal fin, without any spines, and because the dorsal fin arises behind the origin of the ventrals. They lack a lateral line, and have 2 pairs of barbels.

It is difficult to distinguish the sexes, except when the females are mature. Then the contours of their belly are much more rounded than in the male, and they bulge out ventrally and laterally.

Females are reported by Brittan (no date) to be more silvery and to have lighter blue stripes, while the males are darker and their light bands have a golden cast. Males also have golden and blue bands in the anal fin (Axelrod, 1960). Some breeders (Brittan, no date) have observed that the anal fin of the male has a more rounded tip. All these characters, however, depend on graduated differences, and thus are difficult to recognize.

In this study an attempt was made to establish a relationship between sex and the shape of the anal-excretory papilla. The papilla in many females examined seemed to be more prominent than in the male, but then an occasional female would have a smooth anal contour, and an occasional male a prominent papilla, possibly due to physiological conditions.

In some specimens the ventral fins were found to extend beyond the caudal edge of the anal-excretory region, while in others they did not reach quite as far as the edge. The gonads of these fishes, when examined macro- or microscopically, showed that the longer ventral fins belong to males (Fig. 1), and the shorter to females (Fig. 2).

Fish culture and breeding.

The adult fish for this study were obtained from various commercial sources. Very few full grown specimens were found, dealers stocking mostly young fish.

Male and female fish were kept in separate aquaria, filled with tap water, and provided with a sandy bottom, vegetation and a small catfish as a scavenger. The catfish was recommended by the fish dealer as a substitute for the more commonly used snails as it is less injurious to the vegetation. The water was aerated with an electric pump and thermostatically maintained at 26° C with a Metaframe heater. However, often it reached higher temperatures, due to the high ambient temperature of the laboratory. Gradual changes in temperature do not seem to affect adult fishes, which are able to withstand temperatures as low as 14.5° C and as high as 35° C.

Explanation of Figures

Fig. 1 - Male zebrafish; anal region.

Fig. 2 - Female zebrafish; anal region.

Key to figures:

A, anus
AF, anal fin
P, pit
VF, ventral fins

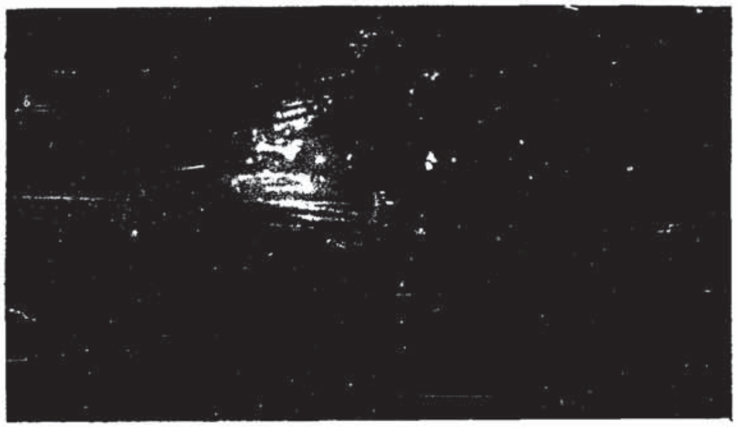

Fig. 1

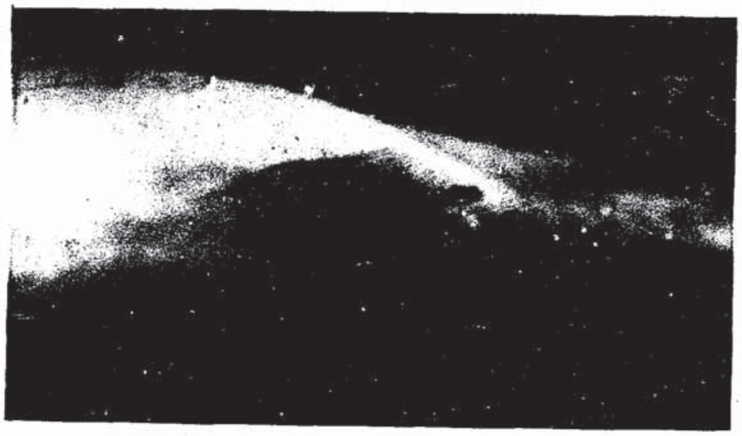

Fig.2

Larvae, on the other hand, are much more sensitive and won't live if the temperature varies more than a few degrees from the optimum of 26° C.

The water which evaporated was originally replaced with tap water kept at least overnight in open vessels to lose the chlorine. Later, according to suggestions of Hisaoka (1960), it was replaced with distilled water, so as not to increase unduly the ionic concentration.

The fish were fed mostly frozen brine shrimps and frozen daphnia; occasionally, live daphnia, and dried commercial fish food. They were fed once a day except when eggs were desired, in which case they were fed several times a day, with at least one meal of live food. To obtain the fish larvae required for the study of the development of the gonads, it is necessary to use a device to prevent the parent fish from eating the eggs as soon as deposed and fertilized. Several methods have been described in the literature to achieve this end. They all take advantage of the fact that the eggs are demersal and settle on the bottom. Rugh (1948) and Corms (1953) recommended putting layers of glass marbles on the bottom of the breeding tank; the eggs would fall among the marbles and so be protected. Legault (1958) also recommended marbles in a two-level fish tank. Marbles were tried but without success: if any eggs were produced it was impossible to find them between the marbles. A commercial breeding trap was also used; this consist of a slatted plastic cage hung in a 5-gallon aquarium. Breeding fishes were put in the cage and the eggs fell through the slats to the bottom. Since the bottom was kept clean, it was easy to see the eggs and collect them. It was felt, however, that the cage was too small (a surface of 10 x 20 cm), since the breeding activities consists of fast swimming to and fro with the males chasing the females and vice versa.

The best results were obtained with a nylon net cage made in the following way: a rectangular frame of glass rods (17 x 26 cm) is placed in the center of a rectangle of nylon netting, (45 x 60 cm) with a mesh large enough to let the eggs fall through. The frame was fastened to the net with long stitches to form the flat bottom of the cage; the free edges were folded vertically to form the sides and were kept in place with a few stitches. The cage was immersed in water for about 5 cm. and kept at that depth by clipping it to the edges of the tank. Enough space was left at one end for heater, aerator, and thermometer. To collect the eggs, the cage was lifted on one side and the bottom of the tank was made readily accessible without taking the fish completely out of water.

One or two females were bred with several males. It has been found that under the conditions present in this laboratory a female would depose eggs over a period of 2-3 days, and not a great number of eggs at any one time; nowhere near the number of eggs obtained by Hisaoka (1960). It is possible that the fish used had not reached full maturity or that not enough live food was provided.

The eggs were pipetted or syphoned out into finger bowls filled with water from the breeding tank. The bowls were kept almost completely immersed in water at 26° C. Either water grasses were added to the water, to develop oxygen, or an aerator with a slow flow of air was provided. The larvae fed in part on the infusoria developed in the water and in part on paramecia, cultured separately and added several times a day, a pipetteful at a time.

It had been planned to obtain larvae in all stages, from hatching to at least the sixth month, at regular intervals of two days in the first month and at longer intervals as needed later. However, a series of unexplained incidents, mostly believed due to lethal increases in temperature, prevented the regular development of several batches of eggs and the collection of the necessary intermediate stages.

Some of the early stages have been secured, as well as the later ones, when the gonads are already well differentiated. The stages whose age after hatching is known will be so designated, the others will be identified by overall size.

Gross examination methods.

All gross observations were made under a dissecting binocular microscope.

A paraffin dissecting pan was prepared in a Petri dish with a grove to hold the fish belly up. Watchmaker's forceps, iridectomy scissors and fine needles were used. All the dissections were made under water.

The first observations were made on freshly killed fish, but they were not satisfactory, due to the gelatinous consistency of most organs. Later, the fish were fixed in toto in 10% formalin or Bouin for at least 24 hours and then transferred to water for dissection.

Histological procedures.

For adult tissues, three fixatives were used: Bouin, Tellyesniczki, and 10% formalin. Of the three, Bouin seems to have given the best results as far as distortion of tissue and staining properties are concerned, and was used more than the others.

The fish were dropped in the fixative alive. Several methods were tried to insure complete penetration through scales, mucus covering and all the way into the swim bladder. Since it was thought desirable to process whole fishes, after death the fixative was injected in the abdominal cavity with a syringe and fine needle. However, it was difficult to gauge exactly the necessary pressure and several fishes ruptured. Even the ones that did not show external damage had their abdominal organs badly displaced. The swim bladder, on the other side, due to its toughness and resiliency, was seldom punctured. For all these reasons, on later specimens the heads and tails were severed right after death to afford rapid penetration through the cut surfaces. After fixation, fishes of 12 mm. and up were decalcified in a sodium citrate and formic acid solution (Armed Forces Institute of Pathology, 1957), until the organic salts were completely removed. Then they were washed for 24 hours in running water, dehydrated, cleared and embedded in paraffin according to standard techniques. However, standard embedding methods did not prove satisfactory. The swim bladder and often the intestine were never impregnated properly and rendered practically impossible the cutting of good sections.

It was found necessary to open the swim bladder with a sagittal dorsal incision, for specimens to be sectioned transversally, or by slicing off the back of the fish for specimens to be sectioned frontally or by slicing off one side for specimens to be sectioned sagittally. Moreover, the impregnation in the third paraffin was made in a vacuum oven.1/ By these means well impregnated

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1/ Thanks are due to the Dental Department of the Armed Forces Institute of Pathology and to Miss E. Buddo for the use of their vacuum oven.

paraffin blocks and readable sections have been obtained.

The larvae were all fixed in Bouin and processed according to Rugh methods (1948) for amphibian eggs and fish larvae. Two-hour fixation gave better results than one hour with embedding in 53-55° C Tissuemat.

Sections were cut at 10 microns, except a few cut at 6 microns to obtain finer histological details. Sections were cut on 3 planes: approximately transversal, frontal, and sagittal. Complete serial sections were cut on all material. In the first blocks cut on adult material, only every fifth section was mounted on slides, about 5 sections to each slide; the remaining sections were preserved in order, on folded paper towels and kept in the refrigerator until needed.

In later blocks complete ribbons were mounted on slides, since it had been found that cutting the dry ribbons tended to tear the sections further.

In general it has been found that adult material is very difficult to cut and requires unusual care and precautions.

Larvae were all cut transversally in continuous ribbons and offered no technical problem.

Most sections have been stained with H&E; some with Weigert's iron hematoxylin and eosine or PAS; others with Gomori aldeheide-fuchsin or van Gieson stains to emphasize particular structures.

Photographic techniques.

The photographs were taken with an Exacta camera equipped with a bellows attachment for close-ups.

The microphotographs were taken with the same camera mounted on a Baush and Lomb microscope equipped with a 10x ocular and with different objectives: 3x, 10x, 43x, and oil immersion 97x.

All photographs were taken on 35mm Kodak Panatomic-X film.1/ They were further enlarged when printed.

1/ The help of E. R. Jervis in taking the photographs, and of R. Musick in developing and printing them is gratefully acknowledged.

RESULTS

Adult Male Reproductive System

a) Gross description

In the male zebrafish the anal and excretory papillae protrude from the bottom of a deeply indented pit (Fig. 1). This cavity in a fish about 31 mm long is about 1 mm long and half that wide. It is situated at the very base of the most anterior ray of the anal fin. Two scales on each side partially cover it.

The anal papilla projects in a ventro-caudal direction from the anterior end of the pit. The anus is very clearly visible; it has a gaping, roughly circular opening, surrounded by deep radial folds whose axis is parallel to the axis of the lumen. These are visible also in fresh material. If given the proper illumination, the folds are seen to continue longitudinally inward.

Right behind the anal papilla there is the uro-genital papilla with the uro-genital pore. This, however, is in the pit under the anal papilla, so that it is impossible to see it even under a dissecting microscope.

To study the internal anatomy, the abdominal wall was removed in its entirety, from pectoral girdle to ventral fins.

In the center, under one of the lobes of the liver, there is a large doubly looped intestine. The first descending branch is the most ventral and is displaced slightly to the right. Then it bends to the left anterad, and finally bends back toward the anus. After removing the alimentary tract and its connected glands, it is possible to see the reproductive system.

The testes appear as 2 thin long irregular bodies extending from the posterior end of the abdominal cavity to the anterior lobe of the swim bladder, between the swim bladder and the body wall (Fig. 3).

In a 31 mm fish (overall length) they are 6 mm long.

At their caudal end they form a loose single mass, from which they diverge in an antero-dorsal direction. Following the ventral contour of the swim bladder, which is very well developed and bilobed, they bend ventrally and forward for the last third of their length. In cross-section they are roughly triangular, with one side convex against the body wall and two sides concave where they adjoin the intestine ventrally and the swim bladder medio-dorsally.

They are connected to each other by membranes (mesorchia) that continue laterally to connect them with the lateral wall of the body cavity. These membranes are very well developed, especially at the posterior end, and may be easily dissected out together with the two testes for more detailed study.

When examined against a dark background, the testes appear as a fairly compact, irregularly granular mass. The granules appear held in a semitransparent stroma,

Explanation of Figures

Fig. 3 - Testes; dorsal view.

AF, anal fin held by a pin

D, tubules suspended in median membrane

Fig. 4 - Kidney of male zebrafish; ventral view. Black spots are melanophores.

AD, archinephric ducts

US, urinary sinus

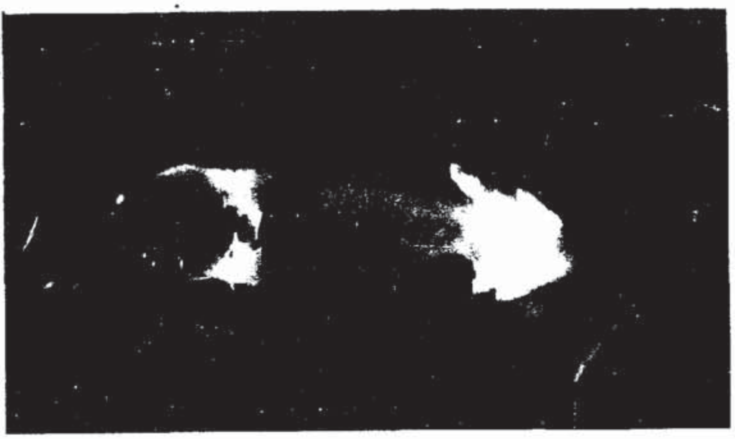

Fig. 3

Fig. 4

surrounded by a thin membrane closely adherent to the organ and its ramifications. On the edges of the testes the granules resolve themselves in convolute tubules. More mature fishes have larger and better defined tubules than the smaller specimens. A distinct longitudinal tubule may be seen on the dorso-lateral edge of the anterior third of the testes but cannot be followed caudad. Many more tubules are visible at the posterior end. At this point, the 2 organs seem to resolve themselves in a number of tubules anastomosing and converging in a more or less antero-posterior direction. From them, many tubules appear to branch anterad in the membrane connecting the 2 testes (Fig. 3).

After excising the testes and the swim bladder it is possible to observe the kidney. This is a single organ closely adhering to the dorsal surface of the body cavity; it ends in 2 archinephric ducts (Weichert, 1958), running very close together and opening in a long urinary sinus. This follows the contour of the body cavity caudad, bending ventrally in an open S curve and ending just dorsally of the genital tubules (Fig. 4 ~~& 5~~).

Actually it has not been possible to follow any of the abdominal ducts -- alimentary, genital and urinary -- to their external openings, due to the small size of the fish and to the fact that all ducts converge together, surrounded by dense connective tissue and muscles.

Their respective relationships at this point have been determined through the study of histological preparations.

b) Microscopic description.

Gonads - Microscopic study of serial sections confirms the findings of the macroscopic examination. Cross sections have been particularly useful in the study of the testes.

There are two testes. They are composed of a very tight mass of tubules, (Fig. 5 & 6) extremely convolute so that it is rare to find any cut along a longitudinal axis. The tubules have a central irregular lumen and a "seminiferous" layer, surrounded by an external layer of connective tissue, containing collagen and reticular fibers. This layer is very thin and, in closely adjacent tubules, practically invisible. The stroma is very scarce and is composed of the same elements as the external layer of the tubules. There are few cellular elements, fibrocytes and free cells. Outstanding among these are some large oval cells, with an oval nucleus displaced to one pole against the cellular membrane, and cytoplasm that stains brick red with eosine and is intensely PAS positive. No attempt has been made to interpret these cells. The testes have no capsule, the external layer of the tubules being the only covering of the organs. However, they are not free in the celomic cavity, but are suspended in loose connective tissue: the "membrane" observed macroscopically.

The "seminiferous" layer has a very characteristic structure. It is formed by closely adjoining cysts, to use a common term, filled with gametocytes in different stages of development, (Fig. 5 & 6). They are firmly attached to a basement membrane and present a convex surface to the lumen. A very thin layer of squamous cells surrounds them, (Fig. 7).

The size of the cysts varies and does not depend on the stage of development of the gametocytes. These are in the same stage of development in each cyst, and adjacent cysts may be in the same stage or in widely different stages (Fig. 6).

Some cysts have a very limited number of large cells, with large nuclei, with very little chromatin in fine threads and a prominent nucleolus. Similar cells, either singly or in groups of 2 or 3, may be seen between cysts close to the basement membrane, (Fig. 6).

Other cysts are composed of cells in later stages. Figures are seen that could be interpreted as tetrads and diads. Finally there are cysts filled with small cells with practically no cytoplasm. The total volume of these cells appears to have shrunk since they are the only ones not to fill their cysts completely (Fig. 5-7). Some of these cysts are ruptured, and the cells are spilling in the lumen (Fig. 7). When they are not too

Explanation of Figures

Fig. 5 - Longitudinal section of "seminiferous" tubule. H&E; 970X, oil immersion.

Fig. 6 - Cross section of "seminiferous" tubules. H&E; 970X, oil immersion.

Fig. 7 - Cyst discharging spermatozoa. H&E; 970X, oil immersion.

Fig. 8 - "Seminiferous" tubule changing into an efferent duct. H&E; 430X.

Key to figures:

C, cysts

Cd, cysts with spermatids

Cr, ruptured cyst

CW, cyst wall

ED, efferent duct

L, lumen with spermatozoa

SP, spermatogonia

St, stroma

Fig. 5

Fig. 6

Fig. 7

Fig. 8

close together, it is possible to see that they have tails and are therefore fully developed spermatozoa. These fill the lumina of the tubules. (Fig. 6 & 7). The spermatozoa have perfectly spherical heads and the tails are exceedingly thin and short (not more than 2-3 times the diameter of the sperms). Many of the tails have fallen off, and it has not been possible to photograph them.

The percentage of mature cysts increases slightly from the medio-ventral portion of each testis toward the lateral portion, with an increase of the relative diameter of the lumen as compared to the size of the cysts (Fig. 8).

Gonoducts - At the latero-dorsal edge, the tubules take the function of efferent ducts. The cysts become smaller and appear only at intervals along the connective walls (Fig. 8). The ducts have thin walls, similar to those surrounding the tubules, and are lined by a squamous, simple epithelium. There are many of them, running along the lateral face of the testis. At the posterior end of the organs they converge and form almost a mat of ducts extending from side to side in that membrane already mentioned (Fig. 9-12). Their epithelial lining thickens and is intermediate between squamous and cuboidal. Due to the number and intricacy of their convolution, it has not been possible to follow them in detail, and to determine if any of them could be interpreted as one of the

Explanation of Figures

Fig. 9-11 - Serial cross sections of male zebrafish 34 mm long in craniocaudal succession. H&E; 100X.

Key to figures:

BW, body wall

Coe, coelom

ED, efferent ducts

G, gut

LC, loose connective tissue in which the testes are suspended

MM, medial membrane

SB, swim bladder

T, testis

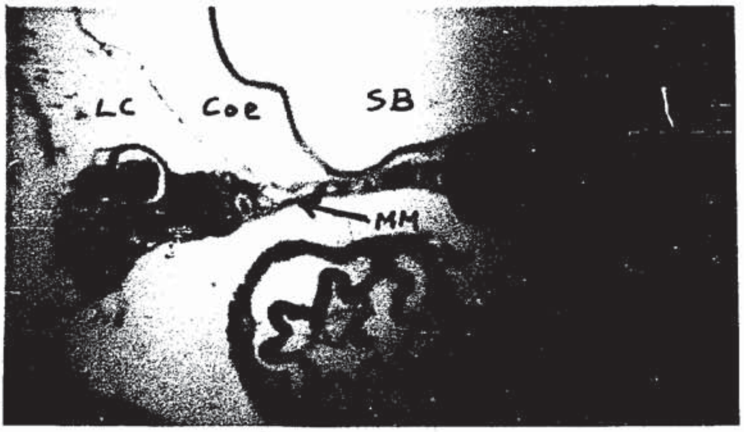

Fig. 9

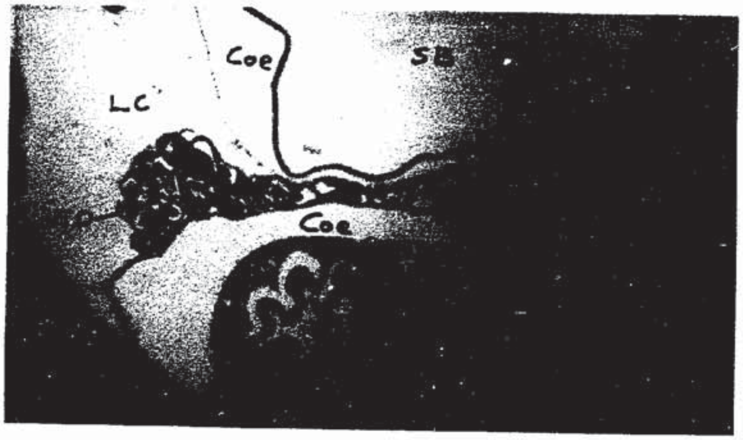

Fig. 10

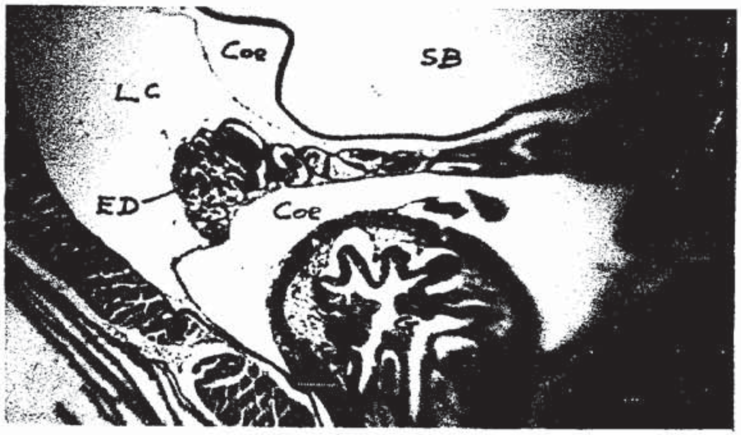

Fig. 11

Explanation of Figures

Fig. 12,13 - Serial cross sections of male zebrafish 34 mm long in cranio-caudal succession. They follow Fig. 11. H&E; 35X.

Fig. 14 - Sagittal section of male zebrafish 32 mm long. H&E; 35X.

Key to figures:

A,	anus
BW,	body wall
Coe,	coelom
ED,	efferent ducts
G,	gut
GU,	genito urinary duct
P,	pit
R,	rectum
SB,	swim bladder
SV,	seminal vesicle
US,	urinary sinus

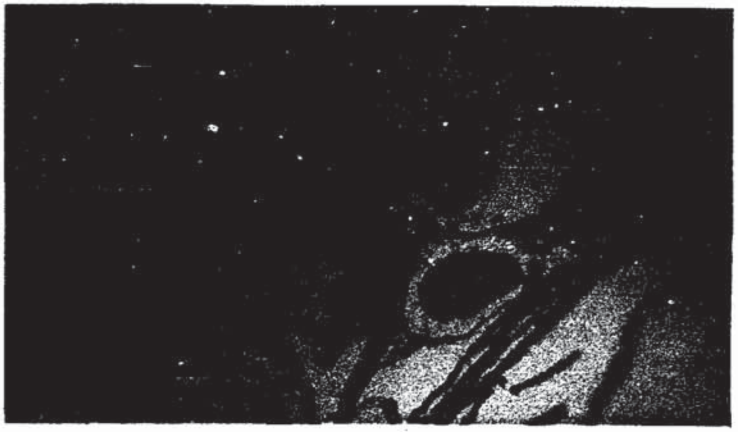

Fig. 12

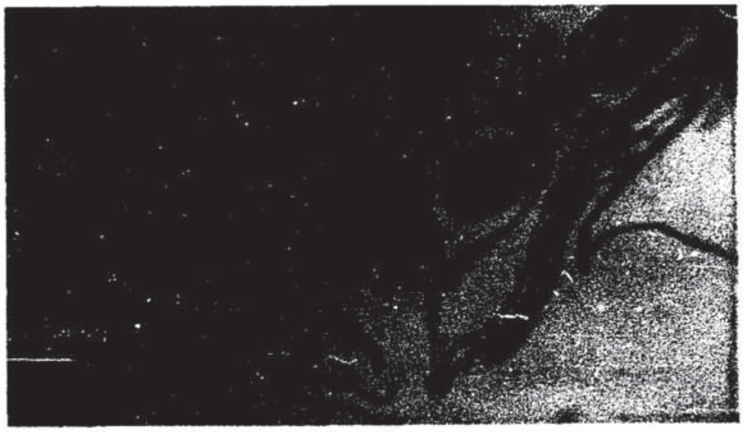

Fig. 13

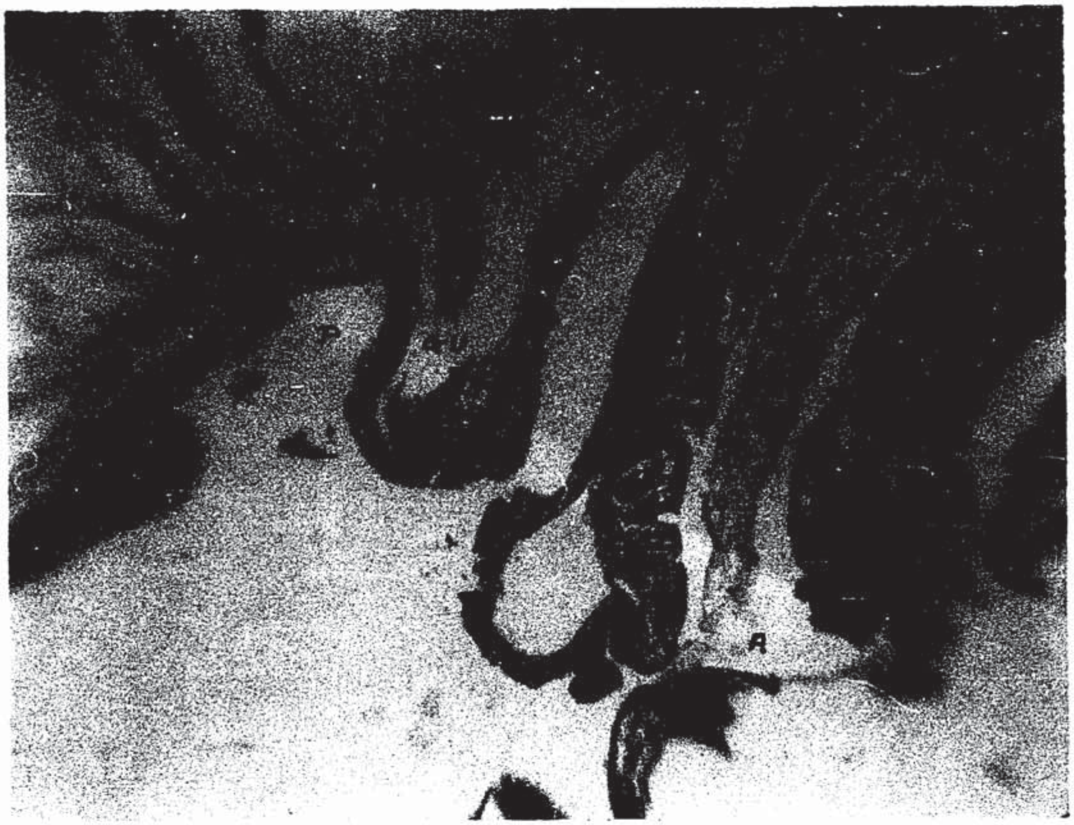

Fig. 14

forward branching formations visible in the dissected testes.

Some ducts finally combine to form an elongated cavity that functions as a seminal vesicle. The rest of the ducts open into it individually along its ventral and lateral sides (Fig. 13).

It is flattened in a dorso-ventral direction, but this might be due to processing. Its walls form irregular folds and are lined with a simple cuboidal epithelium; in older specimens, however, this appears stretched out.

As may be remembered from the macroscopic description, the two archinephric ducts join in a long S-shaped urinary sinus. The seminal vesicle opens onto the ventral side of this sinus by way of a narrow vertical slit, just below the second curve of the S, (Fig. 14).

The urinary sinus at this point narrows, acquires a columnar epithelium (it was cuboidal before) and becomes the common uro-genital duct. This ends in a single, well defined pore, the uro-genital pore in a papilla located behind the anal papilla and separated from it by a deep furrow (Fig. 14).

The uro-genital papilla is formed by fairly dense connective tissue interspersed with smooth muscle fibers; some of these run roughly longitudinally in the papilla, (others transversally); there does not seem to be a distinct sphyncter.

Adult Female Reproductive System

a) Gross description

In the adult female the anal papilla is also located at the base of the first ray of the anal fin, but there is no pit. The ventral fins do not extend beyond it and are more rounded, (Fig. 2).

The papilla is rather prominent and is directed toward the tail, so that the distal portion of the rectum is almost horizontal. This may possibly be due to the pressure of the egg mass in the abdomen. The anus is circular, surrounded by folds like the anus in the male.

The excretory pore is not visible, being tucked under the anal papilla.

To study the anatomy of their reproductive system, the females were prepared like the males, their body cavity exposed and the digestive system removed. The digestive systems are similar in the two sexes.

There are two ovaries; they are tightly packed between the intestine medio-ventrally, the body wall laterally, and the swim bladder medio-dorsally. Their adult shape is conditioned by the surrounding organs. They are deeply indented medially, on the ventral side against the intestine, and on the dorsal side, (Fig. 15) against the two lobes of the swim bladder. Their size varies greatly according to the maturity of the fish and to the spawning time.

The two ovaries converge caudally. In immature fish they are easily distinguished as separate entities, but in mature fish they are so closely associated that only an irregular line of demarcation is still visible (Fig. 15). In a large mature female, 33 mm long, the two ovaries together form a mass 8 mm long, 5 mm. wide and 4.5 mm. deep (after fixation).

The ovaries appear as closely packed masses of eggs of different sizes. The larger eggs, full of deutoplasm, are most easily noticed but among them are visible smaller semitransparent eggs. The ovaries are covered by a transparent membrane or capsule continuous with the mesovarium and the peritoneum. This membrane envelops the individual ovaries anterad. Posteriorly, where the ovaries come together, the membrane disappears on the surfaces in contact, and both ovaries are covered by a continuous membrane. This membrane seems to be closely applied to the lateral surface of the ovaries, but loose on the medial surfaces and all around their posterior common end.

The membrane continues in a funnel-shaped ovarian sac that becomes progressively narrower and ends in a duct parallel and dorsal to the rectum. The caudal end of this sac is continuous with the connective tissue that forms the body walls and, in mature individuals, cannot be excised without tearing.

Explanation of Figures

Fig. 15 - Mature ovaries; dorsal view. The line of demarcation between them is clearly visible, as well as the ova.

Fig. 16 - Kidney of female zebrafish; ventral view. Black spots are melanophores.

Key to figures:

AD, archinephric ducts

US, urinary sinus

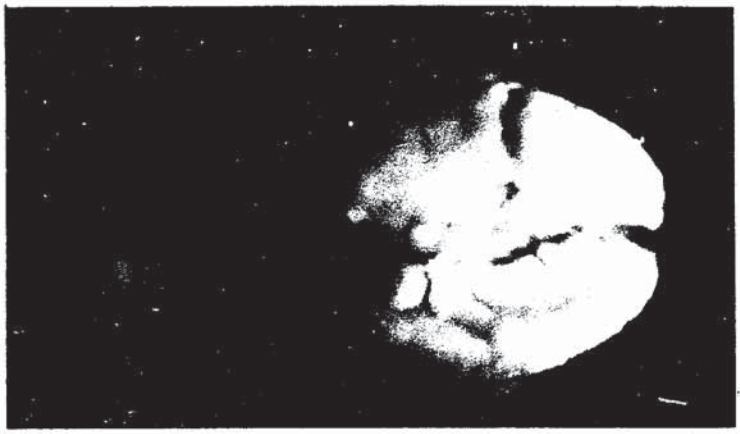

Fig. 15

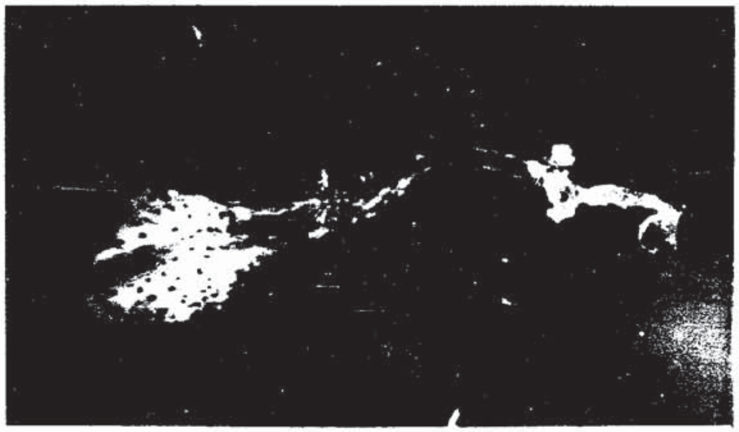

Fig. 16

The excretory system in the female is similar to that of the male; there is a single central kidney with two archinephric ducts, this time quite separate, entering a long sinus equally S-shaped, (Fig. 16). This last, at its caudal end, is very close to the dorsal surface of the oviduct. It has not been possible to dissect the three ducts separately because, as is the case of the male, they are very closely surrounded by dense connective tissue and muscles. Their relationship has been determined through the study of histological preparations.

b) Microscopic description

Gonads - Microscopic study of sections of female zebrafish also confirm the findings of the macroscopic study.

Frontal and transversal sections have been found particularly useful in the study of the ovaries, and sagittal sections in the study of the anal region.

The ovaries occupy most of the abdominal cavity, molding themselves on the abdominal organs. In histological sections it is difficult to distinguish a line of demarcation between the two ovaries.

The capsule is missing on the surfaces in contact, and the stroma is so scarce as to render it difficult to follow; also, one ovary may be more developed than the other, thus pushing it to one side and displacing the demarcation line. This, however, has been observed in enough sections of different specimens to confirm that the ovaries are indeed two.

The ovaries consist of masses of eggs in many stages of development, (Fig. 17). These are distributed apparently at random, with mature or almost mature eggs interspersed with eggs that are just beginning to grow and with cells that resemble primordial germ cells.

The stroma is extremely scarce and in many places practically invisible. In some sections it is possible to distinguish a kind of lobation of the ovary, possibly starting from the lateral surface. However it has not been possible to establish how these lobes, if such they are, are distributed.

The different stages of development of the eggs may be recognized first by their size and then by the different staining properties of the protoplasm.

Germ cells are visible in all ovaries examined; they appear singly or in small clusters practically anywhere in the ovaries; their nuclei have diffuse chromatin and one or few nucleoli. Later stages are represented by growing oogonia. They still have a clear cytoplasm, large nuclei with well-defined membranes, filamentous chromatin and indistinguishable nucleoli. They are surrounded by a thin layer of cells, flattened and barely visible. The next stage is characterized by a further increase of the nucleus and the cytoplasm, that becomes strongly basophilic, while the nucleus

Explanation of Figures

Fig. 17 - Cross section of mature ovary after ovulation. PAS and Weigert Hem.; 35X

Key to figure:

BW, body wall

C, capsule of ovary

FE, empty follicles; the cavity of one is already obliterated

GC, germ cells

L, interlobal spaces

N, nucleus

Nn, nucleus with nucleoli

O, fully developed egg

Oa, oogonium with basophilic cytoplasm

Ob, oogonium with strongly PAS-positive peripheral globules

Oc, oogonium with weakly PAS-positive globules

St, ovarian stroma

Fig. 18 - Frontal section of mature zebrafish. H&E; 100X

Key to figure:

O, ovulated ovum

OS, ovarian sac

T, tail

US, urinary sinus

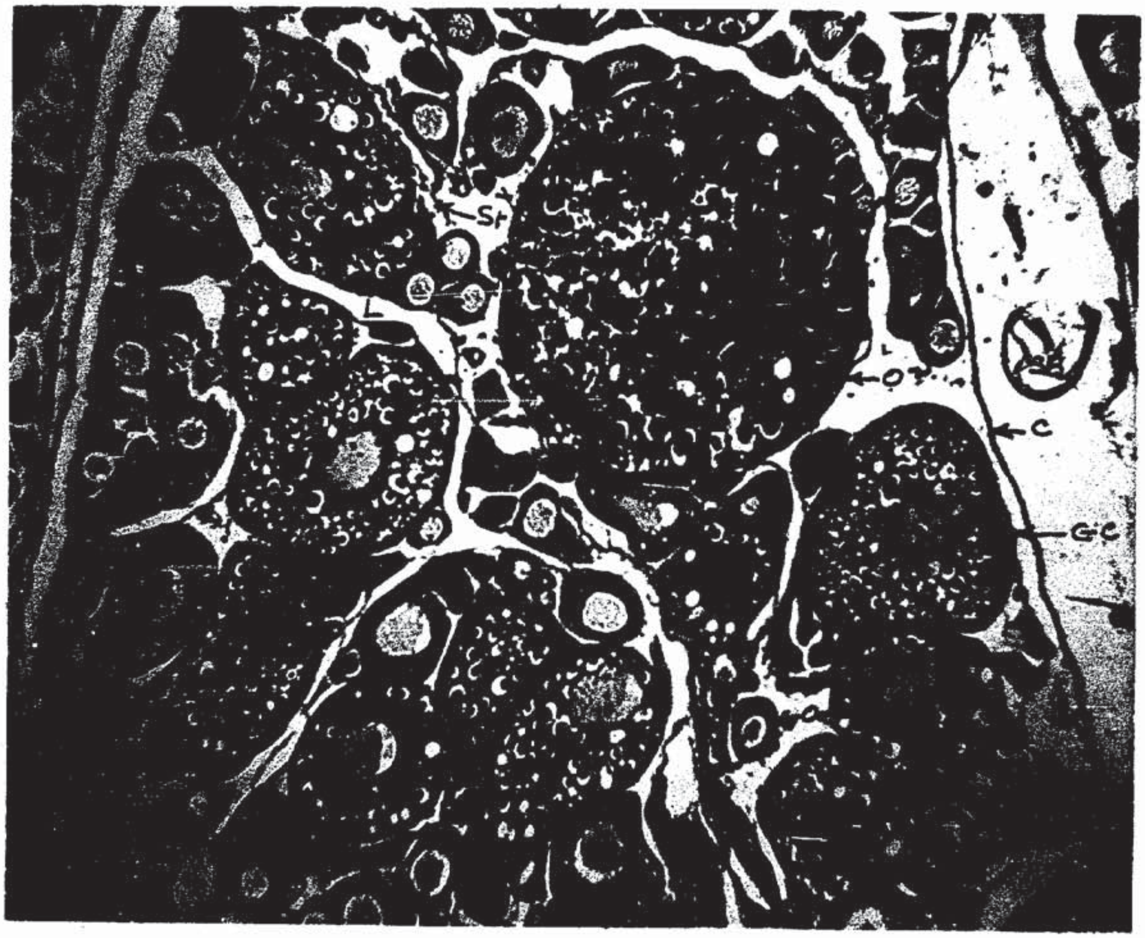

Fig. 17

Fig. 18

becomes more acidophilic and acquires many conspicuous nucleoli. The cytoplasm stained with Weighert's ferric hematoxylin appears finely granular and very dark. The eggs are surrounded by a distinct layer of follicular cells and a layer of connective cells, mixed with elastic fibers, the theca.

In larger eggs some vacuoles appear in the peripheral cytoplasm; these are filled with an eosinophilic substance, strongly PAS positive. At this time also begins the secretion of the zona pellucida between the follicular layer and the cell membrane. The follicular cells at this stage have become more numerous and have assumed their final cuboidal shape.

Later the vacuoles become more numerous and move toward the center of the cell. The outlines of the nuclei become blurred, due to the many fine ramifications of the nuclear membrane, many nucleoli are still present. In the zona pellucida can be distinguished a lighter internal layer, which stains pink with eosine and light purple with PAS, and outside it a thin layer of darker-staining material.

Next, a granular substance makes its appearance in the cytoplasm; the granules are less acidophilic than the content of the vacuoles and with PAS do not give quite so positive a reaction. They appear first around the nuclei and from there move toward the periphery, coalescing and becoming quite large globules. The basophilic fine granules are still present, scattered between the newly acquired substances that will constitute the deutoplasm of the egg. The zona pellucida meanwhile, has become still thicker and shows fine striations, perpendicular to its surfaces, the zona radiata of many authors.

The largest eggs present in the ovaries show a sharp change in staining properties. The yolk consists of a large mass of large, lighter eosinophilic globules, often coalescing. The basophilic granules are still present but very scattered. The PAS positive material has been pushed to the periphery, only a few small globules are present in the mass of the deutoplasm. The nucleus is still more or less central, very faintly basophilic, with few and small nucleoli. (Most of the details described may be seen on Fig. 17.)

The one free egg which has been found in the gonoduct, a badly damaged one, (Fig. 18) has a clearly visible chorion, stretched quite thin and limited on the outside by a very thin basophilic layer. To this are attached a few basophilic granules, possibly remnants of follicular cell nuclei. As far as it is possible to ascertain the cytoplasm of the cell occupies the periphery of the egg while most of the deutoplasm, in the center, due to the preparation, was lost. The nucleus could not be recognized as such, but could also have been lost.

In the mature ovaries of females that have recently laid eggs are visible a number of collapsed follicles, (Fig. 17). Their walls are variously folded; the internal layer, probably formed by the follicular cells, has clear cells with rounded pale nuclei. Presumably, they later proliferate and invade the lumen which is completely obliterated.

Some eggs, atretic, seem to disintegrate right in the ovary. The zona pellucida disappears, and the follicular cells no longer form a regular layer around it. In later stages they look like late ovulated follicles. Other small atretic eggs have been reduced to a mass of debris surrounded by clear epithelial-like cells and connective layers.

Gonoducts - In a sagittal section of a mature female, reproduced in Fig. 19 and 20, it is possible to see the three ducts -- intestinal, genital, and urinary -- leading independently to the exterior. They all open on a single large papilla. The anus is the most anterior; next is the genital pore whose connection with the ovary is visible to the right (the dorsal wall of the oviduct is missing); finally, the urinary duct, which opens on the tip of a small sub-papilla of its own. They may be seen in cross section in Fig. 21.

The opening of the oviduct is practically obliterated by the longitudinal folds of its epithelium; those near the pore become so deep that they meet and overlap, making it difficult to find a section demonstrating a clear opening at the caudal end, (Fig. 20). From Fig. 19, however, it is evident that the oviduct is separate from the urinary tract.

The oviduct, in its course through the papilla, is lined with one layer of columnar cells without cilia. It leads into a more or less funnel-shaped sac. It actually assumes different shapes in different specimens, according to the size of the ovary and possibly to fixation. In less mature specimens the sac is elongated; in more mature ones it is wide and bulges ventrad below the opening in the oviduct.

The walls of the ovarian sac are very thin. They are formed by one layer of cells, backed by very loose connective tissue continuous with the body wall. In some individuals these cells are columnar, while in others they are so low as to appear cuboidal. At its anterior end the wall of the sac separates from the body wall; it consists then of a thin mesothelium on the outside, and a layer of connective tissue with collagen and elastic fibers; it is lined by a ciliated epithelium.

The ovarian sac is continuous with the capsule that envelops the ovary. This seems to be closely adherent to the lateral surfaces of the ovaries, being continuous with the ovarian stroma, while on the medial surfaces and on the anterior and posterior ends it is detached, even if very close to it, (Fig. 22). At the anterior

Explanation of Figures

Fig. 19 - Approximately sagittal section of female zebrafish, 32 mm long. H&E; 35X.

Fig. 20 - Detail of Fig. 19 showing the oviduct. H&E; 970X, oil immersion

Fig. 21 - Frontal section of female zebrafish, 27.5 mm long. H&E; 100X

Key to figures:

A,	anus
Coe,	coelom
O,	ovary
OS,	ovarian sac
Ov,	oviduct
R,	rectum
SB,	swim bladder
UP,	urinary sub-papilla
UT,	urinary tract

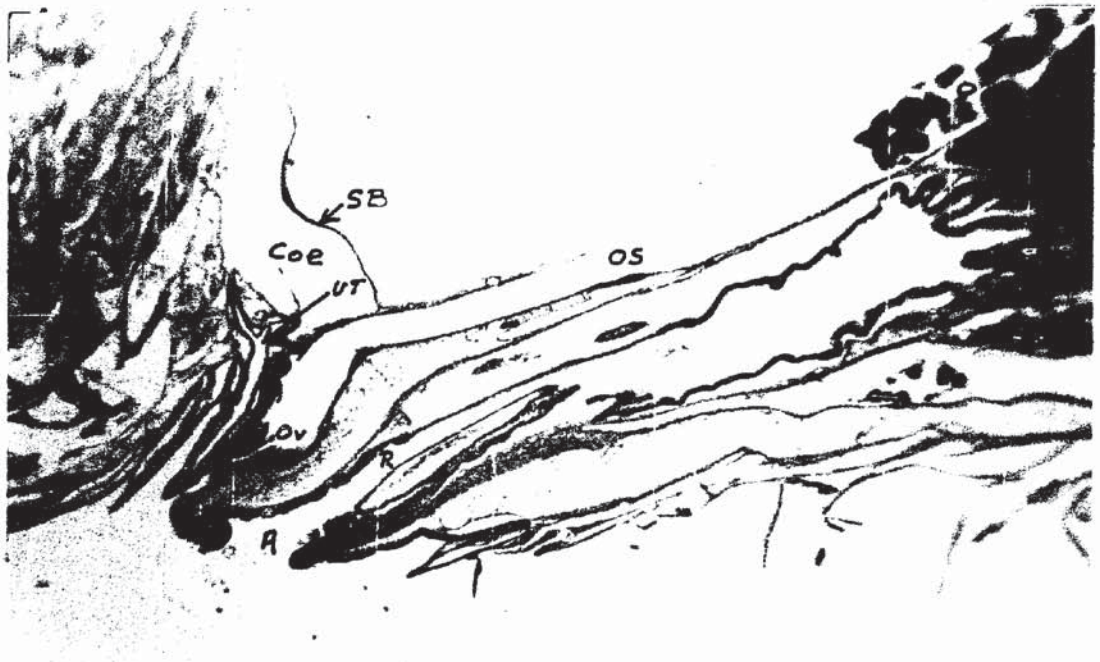

Fig. 19

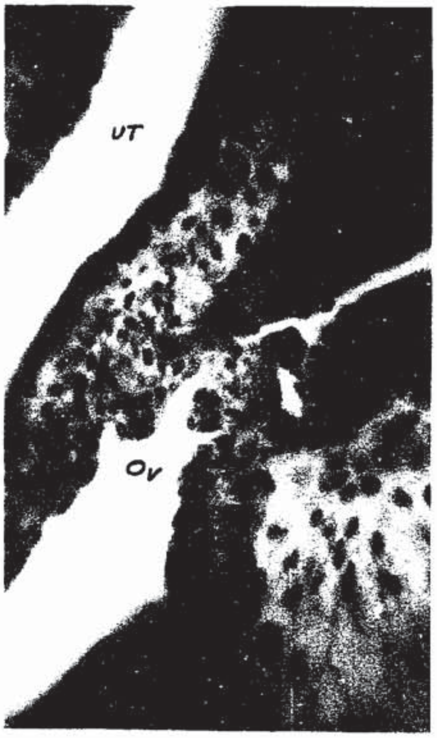

Fig. 20

Fig. 21

end, where the ovary is in contact with the liver, the capsule of the ovary seems to be continuous with that of the liver, (Fig. 23). No explanation has been found for this fact. There is no cavity within the body of the ovaries to receive the ovulating eggs. The only way out seems to be the space between ovary and capsule where this last does not adhere to the ovary.

Development

Not enough stages were secured to make possible a description of the mechanics of development of the reproductive system. Large gaps exist, too wide and too critical to be bridged by extrapolation. Here are recorded only the few observations made on the material available.

Very early development - The youngest larvae studied were three days old (after hatching). They have a straight intestine, single swim bladder, 2 clearly visible archinephric ducts meeting at the distal end in a common sinus, and easily recognizable primordia of liver and pancreas. There are no signs of gonads, ducts or even a genital ridge. However, the larvae still have a substantial quantity of yolk in the yolk sac. On the surface of this yolk there are a number of large cells that might be interpreted as primordial germ cells, (Fig. 24). Their nuclei are large, with dispersed chromatin and one or more nucleoli. The cytoplasm is more basophilic than the yolk,

Explanation of Figures

Fig. 22 - Frontal section of female zebrafish, 33 mm long. Posterior end of ovaries. H&E; 35X.

Fig. 23 - Frontal section of female zebrafish, 33 mm long, showing the continuity of the ovarian capsule with the liver capsule. H&E; 430X.

Key to figures:

BW, body wall

Cl, capsule continuous with ovarian stroma on lateral surface of the ovary

Cm, capsule detached on medial surface

DL, demarcation line between the two ovaries

SB, swim bladder

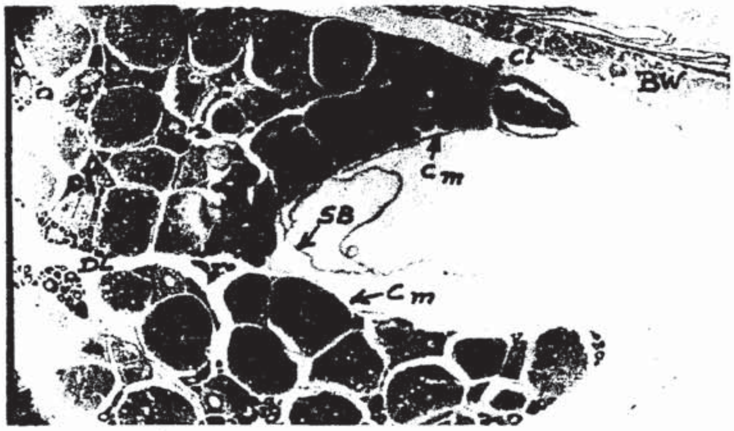

Fig. 22

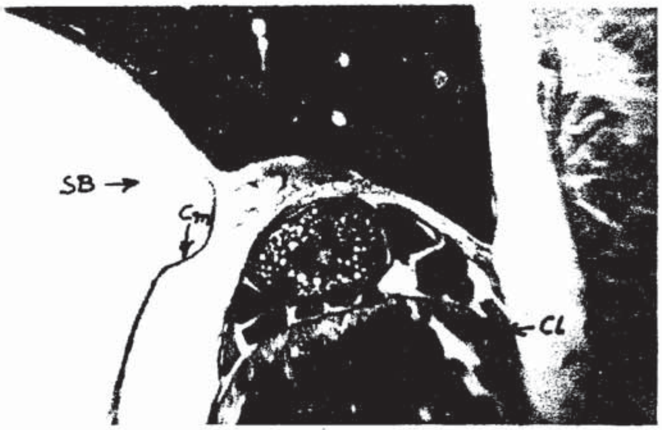

Fig. 23

is finely granular, and forms an almost continuous layer along the ventral surface of the yolk sac. The cellular membranes are indistinct. The cells are especially numerous at the lateral edges of the yolk sac.

The 4-day-old larvae are similar (Fig. 25), except that the quantity of yolk is greatly reduced. The primordial germ cells appear more numerous, possibly because more concentrated.

In the 6-day-old larvae it is possible for the first time to see germ cells in the genital ridge region on the ventral surface of the swim bladder, lateral to the intestine. They are located caudal to the primordia of liver and pancreas. In each section only a few cells (2-3) are visible, surrounded by a few very flattened cells, and can be considered primordia of the gonads.

In 10-day-old larvae the picture is substantially the same, except that the groups of primordial germ cells are a little larger in cross section and longer in extent. Also they are in a more lateral position, (Fig. 26).

At 12 days it is possible to see some differentiation in the gonads. In one specimen the gonad is divided in two lobes surrounded by flat cells with small dark nuclei. The appearance of the germ cells themselves has not changed. In later larvae this picture is not substantially changed, except for an increase of size and the appearance of more subdivisions.

Explanation of Figures

Fig. 24 - Cross section of 3-day-old larva. H&E; 970X, oil immersion

Fig. 25 - Cross section of 4-day-old larva. H&E; 970X, oil immersion

Fig. 26 - Cross section of 10-day-old larvae. The gonad shows two germ cells in a mesothelial capsule. H&E; 970X, oil immersion

Key to figures:

BW,	body wall
Coe,	ceolom
G,	gut
GC,	germ cells
SB,	swim bladder
Y,	yolk-sac

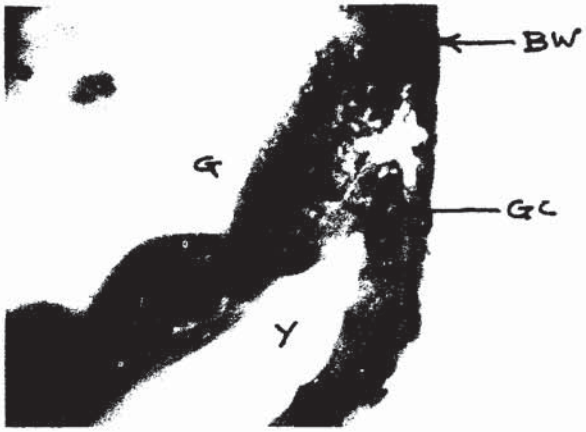

Fig. 24

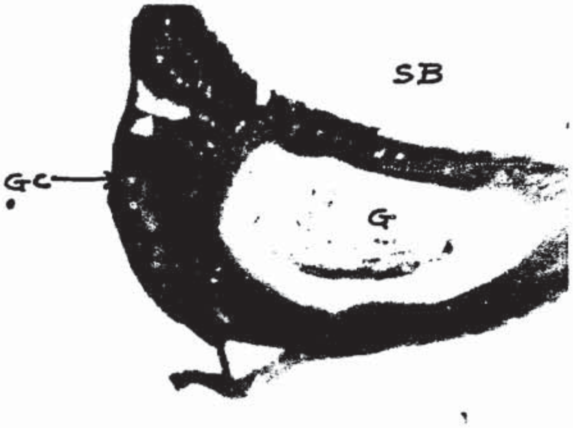

Fig. 25

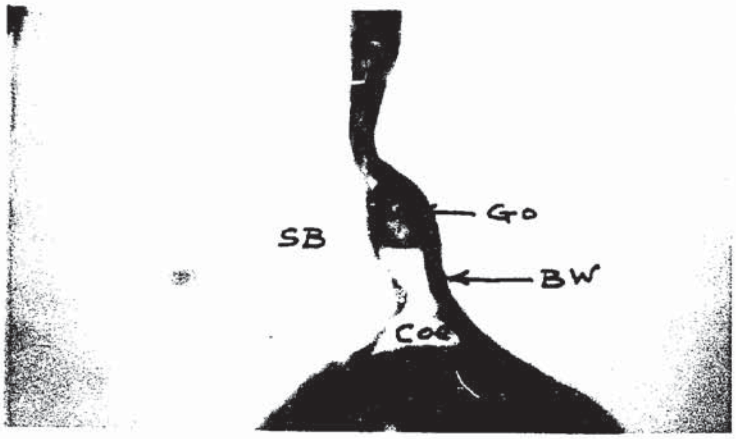

Fig. 26

In all these stages nothing has been noticed which may be considered a primordium of the gonoducts. The urinary tract alone is present.

Sexual differentiation - Sexual differentiation has been first noticed in young fishes of about 10 mm. The gonads then have still the same elongated oval shape, but gametogenesis has progressed far enough to make possible a distinction between ovaries and testes. In one specimen (10 mm overall), the gonads seem to be formed by lobes or cross sections of cords, some of them showing syncronous divisions, a situation that makes one think of the testis.

The next specimen unmistakably male is a fish of 14 mm. The testes are proportionally as long as in the adult, and spermatogenesis is quite advanced. There are many cord-like formations composed of cells similar to the germ cells, but a number of cysts are also present, with spermatocytes in different stages. Some have reached the spermatid stage, and there are tubules already filled with spermatozoa. These are found toward the caudal end of the testis in a fashion reminiscent of the adult. The sections in this particular specimen, however, are too shattered to make possible a positive identification of the gonoducts.

Developmental stages for the female are better represented. In a fish of 8 mm the ovaries consist of lobes of germ cells, surrounded by flattened mesenchymal cells. Some of the germ cells have begun to differentiate in oogonia, and are in an advanced stage of development, (Fig. 27-28).

In a 12 mm. female the ovaries are quite well developed; they hang freely in the coelom. There is no trace of the capsule so evident in the adult. On the dorsal side of the organ, however, there is a cavity, (Fig. 29) that looks as if the ovary had pulled away from the coelom wall.

In a slightly larger female (12.5 mm), the two ovaries have come together caudally. At their caudal end they are surrounded by loose connective tissue and there is no sign of the oviductal sac. Only a very flattened lumen is visible, lateral to the lower edge of the ovaries, (Fig. 30). The next stages examined have adult characteristics.

Explanation of Figures

Fig. 27 - Cross section of female zebrafish, 8 mm long, taken at about mid-body. H&E; 100X.

Key to figure:

G, gut

K, kidney

L, liver

O, ovaries

P, pancreas

SB, swim bladder

Fig. 28 - Cross section of ovary from same specimen of Fig. 27. H&E; 970X oil immersion.

Key to figure:

Coe, coelom

O, oogonia in a cluster or lobe

Oa, oogonium dividing

Ob, oogonium with basophilic cytoplasm

Fig. 27

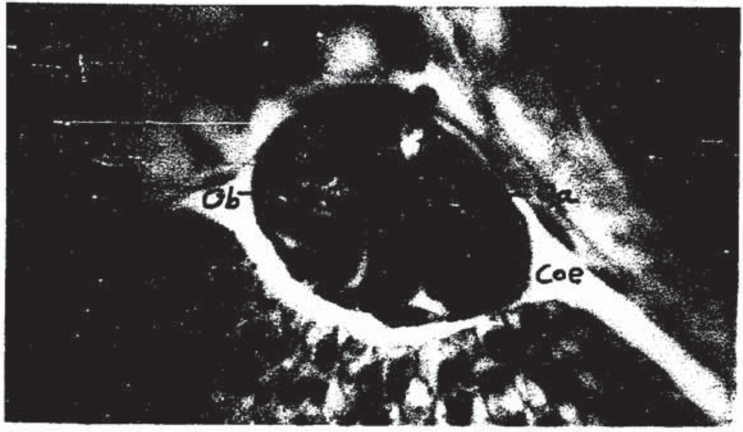

Fig. 28

Explanation of Figures

Fig. 29 - Cross section of an ovary of a zebrafish, 12 mm long. H&E; 430X.

Key to figure:

BW, body wall

Coe, coelom

PC, parovarian cavity (?)

Fig. 30 - Cross section of the ventro-lateral edge of an ovary of a zebrafish 12.5 mm long. H&E; 970X oil immersion.

Key to figure:

BW, body wall; loose connective tissue separates the ovary from the muscular wall

Coe, coelom

PC, parovarian cavity (?)

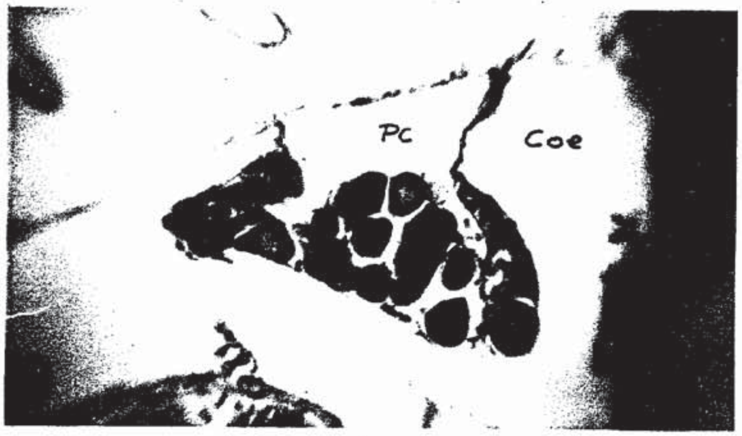

Fig. 29

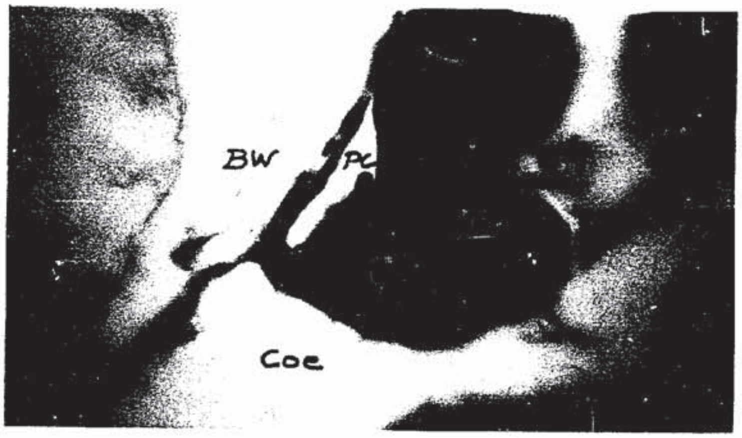

Fig. 30

DISCUSSION

Male Reproductive System

The male reproductive system of the zebrafish, Brachydanio rerio, is represented by two testes, molded in a rather elaborate shape by the surrounding organs, and composed of a highly convoluted mass of tubules. These tubules converge to form an undetermined number of efferent ducts, which join posterad in a seminal vesicle opening in the distal part of the urinary tract. Except for this last connection, they have no relations with the excretory system similar to those observed in the amphibians, where the ductuli efferentes open in the mesonephros and the sperm is carried out in the Wolffian ducts.

Two separate testes must represent the primitive condition, since the primordia are separate.

Separate testes have been found also in Fundulus heteroclitus (Matthews, 1938), Umbra limi (Foley, 1927) and Spirynchus lanceolatus (Ito, 1958).

In other fishes, as the large-mouthed bass and the bluegill (James, 1946), the perch (Turner, 1919; Parker, 1942), the testes are fused posteriorly, while in still others as in the Gambusia holbrooki (Dulzetto, 1931) and in the two phallostedid fishes studied by Te Winkel (1939) there is but one single testis; Dulzetto (1931) however adds that the testis is derived from the fusion of two primordia.

As regards the structure of the testes, (Brock 1878, cit. by Hann, 1927), who studied the reproductive organs of 30 different species of teleosts recognized two types:

I - Acanthopterygian type, with tubules or lobes radiating from a more or less central sperm duct to the periphery of the organ; the sperm duct continuing with the deferent ducts.

II - Cyprinoid or acinous type, with tubules branching and anastomosing to form a network, so that the actual structure of the testes could not be determined.

This last description could fit quite well the testes of the zebrafish; anastomoses have not been observed, but given the branching and the complexity of the structure they cannot be excluded. Being a cyprinoid and having testes without a well defined lumen, the zebrafish may be assumed to have testes of the cyprinoid type.

Most testes mentioned in the literature belong to the acanthopterygian type, therefore any comparison of their gross anatomy with that of the zebrafish would be irrelevant. They will be considered, however, because even to a superficial observer, the spermatogenetic sequence seems to be the same no matter what the disposition of the tubules or lobes. A detailed spermatogenetic study is outside the scope of this study.

All authors agree that in the testes the gametes are first represented by primordial germ cells that transform themselves in primary spermatogonia and encyst in connective tissue. After successive spermatogonial divisions, followed by maturation divisions, they give origin to spermatozoa. Turner (1919) compares the cysts to those found in the testes of arthropoda, and they do look like them. Bennington (1936), who has studied the spermatogenesis in Betta splendens (another fish with cyprinoid testes), also reports that the size of the mature cysts is not constant, and attributes it to the fact that the number of spermatogonial divisions varies.

There are some differences in the timing of the gametes release from the cysts. Bennington (1936, in Betta) reports that they are released in the spermatid stage and finish their transformation in the tubules.

James (1946) mentions seeing bundles of spermatozoa in the cysts themselves, with their tails together and heads fanning out. Dulzetto (1931) describes intact cysts filled with spermatozoa, leaving the testis to proceed to the seminal vesicle, where they break open. The spermatozoa, however, do remain clumped together in spermatozeugmata, similar to those of coleoptera.

In the zebrafish, as may be remembered, the cysts release mature gametes.

The timing of the spermatogenetic cycle is also different, but this is to be expected, since most fishes described are seasonal breeders. The zebrafish is a year-round breeder, so that spermatozoa are always present in the tubules, and cysts are found in all phases of spermatogenesis. The adult zebrafish studied were killed between May and November, and no seasonal differences were observed.

The appearance of the testes of seasonal breeders, instead, vary considerably with the seasons. In the perch (Turner, 1919), shortly after spawning, the lobules consist of germ cells which eventually develop into cysts, etc., so that before spawning the lobules are filled solidly with spermatozoa. Hann (1927, in Cottus) also describes the testes at the beginning of the spermatogenetic cycle as being formed by cords of germ cells; eventually these cords develop a central lumen surrounded by cysts, a condition similar to that of the immature testis of the zebrafish.

The sperm ducts of the zebrafish are rather unique. Most fishes studied have the well defined sperm ducts associated with acanthopterygian type testes. The lumen may be central or more or less displaced to one side, but each testes has a single duct.

Stromsten (1931) in his work on the development of the gonads in Carassius, mentions that the collecting tubules are in the mesorchia and in the dorsal coelom, without elaborating further. Bennington (1936), who also studied a cyprinoid type of testis, having worked on excised testes, does not even mention the ducts.

In the zebrafish no single efferent duct has been found. Even the longitudinal formation, observed in the macroscopic examination, has not been confirmed in the study of cross sections. Only a number of superficial and more or less longitudinal ducts are present in that region. These same efferent ducts combine with others from the caudal end to form the seminal vesicle; they open into it individually.

Unless there has been a gross misinterpretation of the slides, it may be stated that there is no single sperm duct carrying the sperm from the testis to the seminal vesicle. Stromsten's description (see above) might apply to the efferent ducts of the zebrafish.

Goodrich (1958) states that, generally, in the teleosts of both sexes there is a separate median genital pore between anus and urinary pores; he continues: "occasionally, however, the sperm ducts open into the basis of the mesonephric ducts, or together with the anus, or with both kidney and anus."

The male zebrafish obviously belongs to the second group, as do many other fish studied. This condition was reported by Goodrich (1958) in the Anguilliformes and others, in the perch by Turner (1919) and Parker (1942), and in the large-mouthed bass and bluegills by James (1946).

In Gambusia (Dulzetto, 1931) there is a small independent genital pore, and in Cottus (Hann, 1927) the two sperm ducts unite and lead to a genital papilla.

Umbra limi (Foley, 1927) and the crappies (Cooper, 1952) have been described as having a cloaca.

From a review of the literature it would seem that, in the male at least, a single opening for both urinary and genital tracts is the most common condition. The number of fishes studied, however, is much too small to make any valid generalization.

Female Reproductive System

The female reproductivw system of the zebrafish, Brachydanio rerio, is represented by two compact ovaries, very closely associated at their caudal end, but not quite fused.

The ovaries are completely separate in a few fishes, like the black bass (Johnston, 1951), but in most of the other fishes mentioned in the literature they are either partially fused and bilobed or single. It could very well be that some of the bilobed ovaries are similar to those of the zebrafish; Fundulus heteroclitus, for instance, has a ventral groove like the one present in the zebrafish ovaries.

Numerous single ovaries have been recognized: in the guppy (Anteunis, 1959), Gambusia holbrooki (Dulzetto, 1937), Neoteca bilineata (Mendoza, 1943), the yellow perch (Parker, 1942), Oryzias latipes (Robinson and Rugh, 1943) and the two phallostedid fishes described by Te Winkel (1939). Most of them belong to viviparous fishes, a condition always connected with a single ovary with a central cavity (Mendoza, 1943). One might wonder if it is not their viviparity that has made them a subject of study.

The ovaries are formed by large masses of eggs in different stages of development, within a scarce framework of connective tissue. There are a number of straight descriptions of oogenesis like the one by Beach (1959) for the goldfish, Yamamoto K. (1956) for the flounder and Yamamoto T. (1955) for the Japanese medaka. Different authors may subdivide the process in different ways, from the two stages considered by Vakaet (1955, cit. by Anteunis, 1959) to the eleven stages described by Yamamoto K. (1956). No fundamental differences have been noticed; Yamamoto K. (1956) describes, on the basis of differential staining, 3 kinds of yolk material; the first, peripherical, contains carbohydrates. This yolk might correspond to the PAS positive globules first observed in the cytoplasm of the zebrafish eggs. Yamamoto, T. (1955) reports the presence of oil droplets in the medaka egg. No free lipids are believed to exist in the zebrafish eggs. No differential staining for fats has been attempted; however, after regular paraffin processing most vacuoles in the cytoplasm do show some kind of content, and if they are empty, there are signs that the content has been lost for mechanical reasons. The adult eggs at any rate have no vacuoles. Mature ovaries of most fishes studied have shown eggs in different stages of development, and atretic follicles of different sizes. This is true also for ovaries of seasonally breeding fishes, if not to the extent found in the zebrafish. After seasonal spawning, however, only empty follicles, many atretic ones and the small cocytes of the following year crop, are left in the ovaries (James, 1946; Matthews, 1938; etc.).

No large spawnings comparable to those described by Hisaoka and Battle (1958) and Hisaoka (1960) have been obtained in this laboratory. The ovary in Fig. 17, taken from a female that had shed up to 100 eggs in the two days prior to fixation, shows numerous collapsed follicles, but is not otherwise different from other mature ovaries.

The description of ovulated and atretic follicles corresponds exactly with the observations made in the zebrafish. Matthews (1938), in the killifish, speaks of them as "corpora lutea," without mentioning any hormonal function. So do Hisaw and Hisaw (in Acanthias, 1959), who think that their main function is to reabsorb the yolk and debris. Beach instead (1959) suggests some hormonal function at least on the part of atretic follicles.

The egg membrane, zona pellucida (oolemma of Beach, 1959), zona radiata, or chorion as it is called by most authors, is present in all eggs studied. Dulzetto (1936) noticed its absence in the Gambusia and attributed it to the fact that Gambusia is viviparous. Neoteca (Mendoza, 1943), also viviparous, does not have a zona radiata either, but has a thickened vitelline membrane.

The follicular cells are present around all oocytes and may be more or less high. They are surrounded by the theca. Some authors (Beach, 1959) speak of a theca interna and a theca externa; in the zebrafish it is at times difficult to distinguish one theca, let alone two, as may be seen in Fig. 17.

Some of the authors mentioned above have studied the ovogenesis as a function of seasonal changes; (Beach 1959, Matthews 1938) others have emphasized the histochemistry of the developing oocytes (Marza, et al, 1937; Yamamoto, K, 1956; Yamamoto, T., 1955); still others have studied the physiology of the formation of the deutoplasm (Chaudhary, 1951; Narain Dharan, 1956).

Ovogenesis in all its aspects, however, is outside the scope of this work and will not be further considered.

Teleost ovaries may be either free, as in the trout (Parker, 1943), discharging eggs in the coelom; or saccular, when they are enveloped in a capsule continuous with the so-called oviduct, which closes off portions of the coelom (Weichert, 1958). The saccular ovaries may have a central canal, entovarian, or a lateral one, parovarian to receive the ovulated eggs. In the first case the stromal folds supporting the oocytes, the ovigerous lamellae of the literature, radiate from the periphery toward the center of the ovarian cavity, while in the second the ovigerous lamellae line only one side of the ovarian cavity.

The zebrafish ovary, with its capsule and oviduct continuous with it, is certainly of a saccular type. The ovarian cavity is more difficult to recognize. There is no entovarial cavity; therefore, there must be a parovarial cavity, probably that thin space between the ovary and the capsule which surrounds the ovary on all sides except on part of its lateral surfaces.

In the ovary of Histrio (Rasquin, 1958), the parovarian cavity is self evident. The ovary is shaped like a coiled flattened tube; the medial wall supports the ovigerous lamellae (in this case more in the shape of clubs than folds) while the lateral wall is detached, leaving a cavity in which the eggs are liberated; this cavity is continuous with the oviduct. It is possible that the lobes shown in Fig. 17 correspond to the lamellae ovigerae; the stroma of the ovary is continuous with the capsule on the lateral surfaces, but it has not been possible to distinguish any pattern in the structure of the lobes. Nor is it possible to explain the fact that in Histrio the lateral wall is detached, while in the zebrafish it is the medial.

Aronson and Tucker (1949) speaking of Tilapia and "many other teleosts" state that no ovarian lumen may be recognized macro- or microscopically. In Tilapia monocephala during ovulation the follicle springs back toward the midline of the ovary, pulling with it immature ova and interstitial tissue and leaving a cavity under the ovarian wall continuous with the oviduct. From the above description, however, one must postulate a virtual cavity, which becomes visible only during ovulation, and that would correspond to the one described in the zebrafish.

Weichert (1958) states that the oviduct in most teleosts has a separate opening from the intestinal and urinary tracts. The zebrafish conforms to this pattern as do most fish which have been studied.

One exception is Lebistes reticulatus whose oviduct opens in the urinary tract near its opening (Anteunis, 1959). Another is Fundulus heteroclitus (Braumgart, 1951) who has a tubelike formation that envelops the distal ends of both the urinary and genital tracts and may be considered a urogenital sinus, homologous to the genital pouch of Hubbs (1924 cit. by Braumgart, 1951).

Development

A number of papers, some quite old have been found which bear on the origin of the germ cells in fishes. In this class also, the germ cells do not originate in the gonadal anlage, but they are segregated elsewhere and by devious routes migrate to their final destination.

Primitive germ cells may be recognized because they are very large, compared to somatic cells, have clear nuclei with distinct nuclear and cellular membranes.

The appearance of the cytoplasm may vary according to different species and possibly different techniques (Everett, 1945).

Robertson (1953) reports that in the bass they have been traced back to the midpoint of the dorsal lip of the blastopore.

Woods (1902) in selachians, recognized germ cells in the first differentiating endoderm before the formation of a mesoderm. Later they could be found at the edge of the blastoderm where the 3 layers come into contact.

Eigenmann (1891), studying Micrometrus viviparous, recognized as primitive germ cells, some large cells with clear nuclei, observed at the time of the closing of the blastopore. At the 2 somites stage he found them scattered in the endoderm, the mesoderm, some in the ectoderm from the head region down. In older larvae they had disappeared from the head region but could be found in the mesoblast and the mesentery, from the anus forward.

In a 4 mm. Carassius auratus (Stromsten, 1931) germ cells were found in the dorsal peritoneum, at the root of the dorsal mesentery, and in the mesentery itself, only later in the gonadal region.

Woods (1902) seems to think that the migration of the germ cells is compatible only with ameboid movement. Stolk (1958) on the other side, since in many fishes he found ectopic oocytes in strange places (the mesencephalon for instance), is of the opinion that they travel by way of the vascular system. If one is to accept Eigenmann's data, however, oocytes in the head should not be so surprising.

The large cells found in the yolk sac of the 3-4 day old zebrafish larvae do fit the description of the germ cells. The cellular membrane only is not too distinct, but the other characteristics are very similar, and therefore they are going to be so identified. It has not been possible to find them in the mesentery or in the parietal peritoneum, but then the immediately following stages are missing. The fact that they are crowded at the lateral edges of the yolk sac seems significant. Also significant is the fact that when the germ cells make their appearance, rather scattered on the dorsal surface of the coelom, the cells in the yolk sac have disappeared from this location.

For convenience sake the dorsal region of the coelomic wall where the germ cells congregate has been called the genital ridge; however in the case of the zebrafish the term is rather misleading. The genital ridge in the higher vertebrates has a mesenchymal core which is invested later with the germinal epithelium. The primitive germ cells of the zebrafish may be found isolated against the coelomic wall, later they come together in groups and then in a cord, to form the primitive gonads. There is no sign of a proper mesenchymal ridge; it is only as the number of the cells increases, bulging into the coelom, that a ridge is formed.

At this point the gonads are represented by a core of germ cells, possibly already divided in lobes, surrounded by a multiple layer of mesenchymal cells and mesothelium.

Anteunis speaking of the early gonadal stages in Lebistes mentions a continuous layer of germ cells covering the gonads, some of them later changing into endothelial-like cells to form the external capsule of the ovary. At the same time some mesothelial element of the parovarial cavity would acquire the characteristics of germ cells. If this is so it would mean that germ cells may change into mesothelial cells and vice versa.

Since Anteunis micrographs of the very early gonads look like the early gonads of the zebrafish, it is to be wondered if her description of a germinal epithelium is not an attempt to fit the gonads of the fishes in the conventional vertebrate pattern.

While the early gonadal formation is fairly well documented, later development stages have been largely neglected.

Stromsten (1931) has studied a series of _Carassius auratus_ larvae from an early gonadal stage to the formation of the primordia of the gonoduct.

In the male goldfish the testes may be distinguished from the ovaries because they have less interstitial tissue. Later the cells segregate into nests (cross sections of cords) and then arrange themselves around a central lumen to form seminiferous tubules. Stromsten does not describe later stages, except to mention the location of the collecting tubules; he does not say where they open.

The description of Stromsten (1931) corresponds to the conditions found in the 10 and 14 mm. zebrafish, so that it may be suggested that the organogenesis of the testis is similar in both species as far as it has been followed.

The early ovary of the goldfish (Stromsten, 1931) hangs anteroposteriorly in the coelomic cavity as a long thin band eliptical in cross section. In the 18 mm embryos the distal edge of this ridge becomes attached to the peritoneal wall, thus forming a parovarial cavity. This is the process of parovarial sac formation which has been described by many embryologists from MacCleod (1881, cit. by Goodrich, 1958) on. It might apply to the ovary of the zebrafish as it is shown in Fig. 29, which is very similar to one of the micrographs of Stromsten. Of course this is only an educated guess, since the intermediate stages have not been examined.

The oviducts of the goldfish (Stromsten, 1931) originate from an extension caudad of the gonadal folds, minus the gonocytes. Their free edge, also does attach itself to the peritoneal wall, continuing the parovarial cavity in two oviducts, that end blindly near the anus. This does not seem to happen in the zebrafish.

At this point it is useless to speculate on the processes that will transform conditions like the ones observed into those found in the adult zebrafish.

Gerard (1958), in commenting on the different theories proposed to explain the formation of the gonoducts, recognizes that most authors reject the idea that there are any homologies or relationships with Wolffian ducts or Muller canals.

On the basis of this study, it may be stated that in the zebrafish no embryological relations have been found between the excretory system and the reproductive one. Only a minor secondary one, represented by a common genito-urinary pore is present in the adult male. If the mullerian ducts had contributed to the formation of the oviduct they should have been evident in the very early stages, when, however only the archinephric ducts are present.

CONCLUSIONS AND SUMMARY

This is a study of the male and female reproductive systems of the zebrafish, Brachydanio rerio (Hamilton-Buchanan).

1. The taxonomic position of this fish has been defined on the basis of the existent literature.

2. An attempt has been made to find an external character to distinguish the sexes. The ventral fins of the male are longer than in the female and extend beyond the ano-excretory region.

3. Methods of breeding the fish are given and evaluated.

4. Methods of dissecting the fish and of collecting and processing histological materials are described and evaluated.

5. The anatomy of the reproductive systems is described through dissections and the study of serial sections. In the male adult zebrafish the reproductive system consists of:

a. Two testes composed of a highly complicated mass of branching tubules. These tubules have an irregular lumen filled with spermatozoa and are lined with cysts containing developing sperm. The cysts seem to originate from germ cells which are found scattered along the basal connective tissue of the tubules.

b. A number of convoluted efferent ducts which are a continuation of the tubules of the testis.

c. A seminal vesicle, formed by the confluence of the efferent ducts which open in it individually. The vesicle discharges in the caudal end of the urinary tract so that there is a single uro-genital pore.

In the female adult zebrafish the reproductive system consists of:

a. Two ovaries closely associated at their caudal end. They are composed of numerous eggs in different stages of development, also held in a connective tissue stroma. Together they are enveloped by a capsule which is connected with the ovarian stroma only on the lateral surfaces of the organs.

b. One oviduct continuous with the above capsule. It is sac-like cranially where it receives the common posterior end of the ovaries. It opens independently on a large papilla, between the anus and the urinary tract.

6. The development of the gonads has been studied through serial sections of larvae and young fishes.

The gonads first appear on the dorsal wall of the coelom, on both sides of the mesentery in the 6-day old larvae. They form by the coming together of primordial germ cells that have been observed in the three day old larvae, along the walls of the yolk sac.

Later stages have also been described. It has not been possible to trace the complete development to maturity due to insufficient data.

7. All descriptions have been supplemented by photographs and microphotographs.

8. The results of this study have been discussed in the light of published work on the same subject and any similarities and divergences have been noted.

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