ORAL INCUBATION IN *TILAPIA MACROCEPHALA*

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1. *EMBRYOLOGICAL STUDIES*

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INTRODUCTION

"This same fish [*Geophagus*] has a most extraordinary mode of reproduction. The eggs pass, I know not how, into the mouth, the bottom of which is lined by them, between the inner appendages of the branchial arches, and especially into a pouch, formed by the upper pharyngials, which they completely fill. There they are hatched and the little ones, freed from the egg case, are developed until they are in a condition to provide for their own existence."

Thus wrote Louis Agassiz in 1868 to his colleague, Professor Milne-Edwards, of the Jardin des Plantes. Since the time of Agassiz, mouthbreeding has been a subject of particular interest to aquarists and biologists. This unusual mode of parental behavior has been discovered in several unrelated teleost families. Among these is the family Cichlidae in which many species of several genera have developed various modifications of oral incubation. Several detailed studies have appeared on various aspects of oral incubation in cichlid fishes. In these, as well as in the voluminous popular aquarists' literature, there is a tacit assumption that the mouthbreeding habit is primarily an adaptation for protection of the embryos and young fry from predators. No one, as far as could be ascertained, has ever investigated the possibility that mouthbreeding may be otherwise necessary to the survival of the embryos.

During the course of extensive studies on reproduction in the mouthbreeding cichlid fish *Tilapia macrocephala* (Aronson, 1949; Aronson and Holz-Tucker, 1950), it was found that the embryos, when removed from the mouth and placed into aquarium water taken from the tank in which the spawning had occurred, did not survive for more than two or three days. These observations reported by Shaw (1950, 1951) strongly suggested that the mouth serves an additional function, possibly a physiological one, necessary for the survival of the embryos. Casual observations were made by Baerends and Baerends-Van Roon (1950) who reported that the eggs of *Tilapia natalensis* and *Haplochromis multicolor*, when removed from the female's mouth within a week after spawning, died and soon became moldy. The failure of eggs to develop outside the mouth of *Astatotilapia strigigena* was noted by Seitz (1940).

Cichlid fishes are fresh-water forms found in South America and Central America, north through Mexico to Texas, also in Africa and in southern Asia. Several of the species are known to have a rather high salt tolerance. For this reason, Norman (1931) and Myers (1938) classify the cichlids as members of the secondary division of fresh-water fishes which means that they may behave like true fresh-water fishes but are less sharply restricted to fresh water. One species, *Tilapia nilotica*, is known to live in the estuaries of the Dead Sea (Steinitz, 1951) and in other places of high salt concentration. *Tilapia macrocephala* is found in West Africa from the Gold Coast to Nigeria. It is often taken in the river estuaries and coastal lagoons (Boulenger, 1915; F. O. Otorubio, personal communication) in which the salinity at times approaches that of sea water (Irvine, 1947). However, we were able to maintain and breed this *Tilapia* species in fresh-water aquaria in the laboratory.

Virtually nothing is known about the reproductive habits of *Tilapia macrocephala* in their natural habitat, but much information is available concerning reproduction in this species in laboratory aquaria (Aronson, 1949). Aronson describes a rather involved and lengthy courtship during which an excavation or nest is built in the substratum. The female deposits her eggs in this nest, and they are fertilized as the male slowly rubs his genital papilla over the eggs. Within a minute, the male draws the eggs into his mouth (pl. 35, fig. 1). Here they are incubated from six to 22 days (mean, 13.8 days) during which time they are almost continuously churned by the respiratory movements of the adult. Hatching occurs in the mouth on the fifth or sixth day of development. When the brood is released at the end of incubation, they are young fry. The adults breed readily in captivity and reproduce frequently over the entire year.

On the basis of the considerations discussed in this section, a series of experiments were planned to investigate the role of oral incubation in embryonic survival. The approach

chosen was to find methods of raising the embryos extra-orally and to relate the factors of extra-oral survival with factors in the milieu of the oral cavity.

It was apparent, early in this study, that a knowledge of the early embryology of the species was necessary for an adequate investigation of the survival value of oral incubation. The most useful method of describing embryological development, when needed as a prerequisite for experimental research, is the designation of stages which show gross morphological changes. Oppenheimer (1937) emphasized the value of this technique as superior to older methods where the number of somites or the time after fertilization was used as a criterion for describing the degree of development. Recently, a great number of experimental studies have been based on stages, and there are now available among the more widely used experimental animals reference stages for the killifish, *Fundulus heteroclitus* (Oppenheimer, 1937), the platyfish, *Xiphophorus maculatus* (Tavolga, 1949), the leopard frog, *Rana pipiens* (Shumway, 1940), the salamander, *Ambystoma punctatum* (Harrison, published by Rugh, 1948), and the chick, *Gallus domesticus* (Hamburger and Hamilton, 1951).

The first part of this report is devoted to a description of stages in the development of *Tilapia macrocephala*. Since the studies on cichlid embryology are relatively brief (Hoepfel, 1913; Konnertz, 1913; Jones, 1937a, 1937b; McEwen, 1930, 1940) it is hoped that the present description will contribute further to the knowledge of cichlid ontogeny. The second part of the report deals with experimental studies on oral incubation.

ACKNOWLEDGMENTS

MATERIALS AND METHODS

Adults 6 to 10 cm. long were selected from laboratory-bred stock. The males were chosen for the brightness of their golden opercula, a secondary sex characteristic. Females have a complementary sex characteristic, namely, a transparent area in the operculum through which the red gills can be seen (Aronson, 1951). Females that exhibited a deep red color through the transparent area were selected.

The fish were paired at random and were placed in 54-liter aquaria, 60 cm. by 30 cm. by 30 cm., each containing roughly 36 liters of water. The aquaria were located in a greenhouse, in which the temperature was maintained at 26° C. throughout the year, with a fluctuation of about 3° to 4° C. The fish were fed a dehydrated preparation consisting of dried shrimp, Pablum, beef liver, lettuce, and spinach (Gordon, 1950). To insure a regular supply of fertilized eggs, between 100 and 125 pairs were maintained at all times. These were replaced whenever their spawning frequency dropped markedly or whenever death occurred to one member of a pair. In such cases the surviving member was removed from the aquarium, and a new pair was placed in the tank. This large number of adult pairs produced at least 10 spawnings per week. At least half of these yielded viable embryos; thus about 300 to 400 embryos per week were made available for the embryological and experimental studies.

Observations were made daily to detect the presence of incubating embryos. If a male (or occasionally a female) was found to be carrying a brood of embryos, the fish was caught in a net. Gentle squeezing caused him (or her) to eject the embryos into the net. The fish was then returned to the aquarium, and the embryos were placed in a finger bowl.

Embryos were examined in the living state and were allocated to various developmental

stages which were arbitrarily designated by the appearance of certain gross morphological characteristics. Photomicrographs were made from living embryos in each of the stages. A number of embryos were fixed in Bouin's picroformal and dehydrated in dioxan. They were sectioned serially at 10 μ, and stained with Harris' hematoxylin and eosin or a modification of the Masson trichrome stain. Sectioning of the embryos was done with the yolk attached. Photomicrographs were made of representative sections.

Approximately 5000 eggs which had been fertilized and were developing normally, ranging from stages 1 to 11,[1] were treated in various aqueous media or were subjected to certain mechanical tests, and in most cases the following routine procedure was used in handling the embryos. Ten embryos were placed in bowls containing 50 cc. of the test solution. Control embryos in all experiments were reared in a similar manner in 40 per

cent sea water (with 60% aquarium water).

In most cases, the solutions were not changed during the period of treatment, since preliminary experiments indicated no deleterious effects to the embryos if they remained throughout the experiment in the same solution. Since the primary interest of the investigation centered on the effects of the experimental media or on the effects of mechanical manipulations on embryonic survival, experiments were terminated at hatching.[2] The conditions of the embryos were observed at least once every two days, and the stages of development were recorded. If dead embryos were found, the stage at which death occurred was noted, and they were removed from the solution.

Further details regarding techniques and procedures are presented in the descriptions of the individual experiments.

[1] For definition of stages, see pages 384 to 390.

[2] Experiments were terminated at hatching (stage 24), since preliminary experiments showed that there was rarely any change in embryonic mortality after this time.

1. EMBRYOLOGICAL STUDIES

DESCRIPTION OF GAMETES

OVA

Plate 35, figure 2

THE UNFERTILIZED, MATURE OVUM is asymmetrical (Breder, 1943), and measures between 2.0 and 3.5 mm. in length and between 1.5 and 2.5 mm. across the widest part of the yolk. There is considerable variability in mean egg size among different batches of spawn, but this is not correlated with the size of the adults. Within each batch the eggs are uniform in size. The yolk varies in color from light yellow to deep orange-yellow. The animal pole or apex of each ovum is capped by a thin, white, lenticular, protoplasmic disc, which continues as a membranous layer over the entire yolk. Lipoid globules are peripherally arranged around the blastodisc. A central micropyle, visible as a small opening, can be seen above the blastodisc. The chorion closely adheres to the yolk, and the entire ovum is soft and pliable.

SPERM

The sperm, when examined under a phase microscope, have rounded heads, which appear biconcave when viewed from their edge. At the anterior end of the head, there is a small pointed projection, probably an acrosome. The middle piece is U-shaped, approximately one-fifth of the length of the head. The tail is rather short, only about eight times the length of the head. The entire sperm measures about 190 μ in length. Spermatogenesis in *Tilapia macrocephala* has been described by Jakowska (1950).

FERTILIZATION

In order to obtain mature ovulated eggs for the study of fertilization, it was necessary to select females that were exhibiting the last phases of courtship, namely, "passing-nest" and "spawning-quivers." These behavior patterns indicate that ovulation has occurred (Aronson, 1949). Only ovulated females can be stripped, although milt is obtainable from males at any time. It was not possible to fertilize unovulated eggs.

Eggs were successfully stripped from seven females and were dropped into 40 per cent sea water (with 60% aquarium water). Each batch of eggs was fertilized with milt stripped from several males. Immediately after introduction of the sperm, no obvious morphological changes in the eggs were observed when studied under a magnification of 20 times. (It is assumed, but was not verified, that the gametes united almost instantaneously, as in other oviparous fishes.)

Gradually the diffuse, thin, protoplasmic disc became more compact. About one-half an hour after introduction of the sperm, the diffuse appearance of the blastodiscs had disappeared, and a clear-cut demarcation was noted between the blastodisc and the yolk. The eggs swelled, and the chorions "water-hardened" and became rubbery. They then lifted away from the yolk, and a perivitelline space filled with fluid was formed.

STAGES IN EMBRYONIC DEVELOPMENT

The stages described in the following pages are based on relatively clear distinctions in the gross morphology of embryos raised in 40 per cent sea water (with 60% aquarium water). Most of the descriptions are supplemented by brief observations on internal microscopic changes in the embryo. The length measurement in each stage represents a mean derived from 10 embryos, each from a different female. Table 1 represents the time-temperature relationships relative to the growth of embryos raised in 40 per cent sea water. Twenty-five embryos obtained from five different females were raised at each temperature. The majority of embyros in each group attained the indicated stage at the end of each 24-hour period. A small number, approximately 10 per cent, were in the previous stage of development. These embryos did not always survive to hatching.

STAGE 1: FERTILIZED OVUM

Plate 35, figure 3

The fertilized ovum exhibits a raised blastodisc at its apex, which is surrounded by small lipoid globules. The blastodisc continues from its periphery as a thin proto-

TABLE 1

RELATION OF TEMPERATURE TO STAGES OF DEVELOPMENT

Tempera-ture	No. of Days after Fertilization									
	1	2	3	4	5	6	7	8	9	10
25° C.	9	14	16	18	19	20	21	22	23	24
29°	11	15	17	19	20	23	24			
33°	13	17	19	21	23	24				

plasmic layer over the yolk. The chorion is clearly separated from the ovum by a peri-vitelline space.

STAGE 2: TWO-CELLED OVUM

Plate 35, figure 4

Two cells are formed by cleavage along a meridional plane. Just prior to cleavage, protoplasmic blebs appear in the blastodisc at the poles opposite the cleavage plane, and the entire disc gives evidence of much activity. This is seen in subsequent cleavages, which occur at intervals of three-quarters of an hour to one hour at laboratory temperatures (25° C.).

Longitudinal serial sections show that the cleavage furrow does not completely divide the two cells which are connected ventrally by a thin strand of protoplasm. Wilson (1889) terms this the central periblast. Nuclei were not discerned in these sections.

STAGE 3: FOUR-CELLED OVUM

Plate 36, figure 1

The second cleavage plane, which appears at right angles to the first plane, divides the blastodisc into four cells. In a few ova the micropyle is still visible in the chorion. In serial longitudinal section, each pair of cells is connected ventrally by a thin protoplasmic strand.

STAGE 4: EIGHT-CELLED OVUM

Plate 36, figure 2

The third cleavage is double and parallel to the first, resulting in the formation of eight cells. The two medial cells are almost square and are smaller than the lateral cells. Until now, the cells had been of equal size. Nuclei are not discernible in any longitudinal sections.

STAGE 5: SIXTEEN-CELLED OVUM

Plate 36, figure 3

The fourth cleavage, double and parallel to the second plane, forms 16 cells, which are of unequal size. Cleavages do not occur simultaneously, leading occasionally to irregularities in the typical pattern of four rows of cells containing four cells each.

STAGE 6: THIRTY-TWO-CELLED OVUM

Plate 36, figure 4

Beyond the 16-cell stage divisions are less synchronous, and it is difficult to determine cleavage planes. Thirty-two cells are generally selected as the number present in this stage, but actually very few ova exhibited precisely that number of cells. Therefore, for convenience, 32 ± 4 cells are included in stage 6.

In longitudinal sections, a marginal periblast, without nuclei, was noted.

STAGE 7: EARLY BLASTULA

Plate 37, figure 1

The cells rapidly divide horizontally and vertically, forming the early blastula. Macroscopically, individual surface cells are distinguishable.

In transverse section (pl. 42, fig. 1) the blastula is about seven cells deep. The outermost, superficial layer of cells becomes flattened, representing the initial stage in the formation of the epidermic stratum. It is contiguous with the underlying marginal periblast. Small scattered spaces, apparent between the blastomeres, will eventually become confluent and will give rise to the segmentation cavity.

STAGE 8: BLASTULA

Plate 37, figure 2

The blastula is flattened, forming a lenticu-

lar cap over the yolk. In sectioned material, a large segmentation cavity is present (pl. 42, fig. 2). The thinner central periblast covers the underlying yolk, and a syncytium of nuclei is just appearing in the thicker marginal periblast. A few cells are proliferating medially into the segmentation cavity from the periphery of the blastula.

STAGE 9: EARLY GASTRULA

Plate 37, figure 3

A small, transparent, crescent-shaped area is visible on one side of the blastoderm, lateral to the apex. This is the first appearance of the extra-embryonic membrane, which will eventually give rise to the yolk sac epithelium. The periphery of the balstodisc is thickened, forming the germ ring. The embryonic shield can be seen as a thickened mass extending anteriorly from a sector of the germ ring on the side opposite the crescent. In longitudinal section (pl. 42, fig. 3), the cells in the shield, although compact, are not yet oriented into two layers.

STAGE 10: GASTRULA

Plate 37, figure 4

Epiboly begins with the migration of the peripheral germ ring over one-sixth of the yolk. The embryonic shield is now a clearly defined, triangular area, with a broad, thick base, resulting from invagination, proliferation, and migration of cells into the area. The antero-posterior axis of the embryo is indicated. Lipoid globules are visible through the transparent yolk sac epithelium.

In longitudinal serial section two distinct layers of cells, a dorsal and a ventral, can be seen in the shield (pl. 42, fig. 4). At the posterior end of the shield, an undifferentiated mass of cells, the caudal knob, is present, which is homologous to the amphibian dorsal lip of the blastopore. From the knob, a median ventral layer of cells extends cephalad. This layer of cells, the chorda-endoderm, will eventually give rise to the notochord and the medial section of the endoderm. Lateral to the chorda-endoderm, aggregations of cells will form the mesoderm and the lateral sections of the endoderm (pl. 43, fig. 1).

STAGE 11: NEURULA

Plate 38, figure 1

The germ ring has migrated over one-fourth of the yolk, and the embryo has become more elongate. The anterior end of the embryonic shield lies in much the same position as in the previous stage. Elongation occurs principally in the posterior end of the embryo.

Sections show that basic embryonic differentiation has taken place at this stage. The solid neural keel has invaginated. It is broad at the cephalic end. Caudad, the chorda-endoderm has separated into two distinct regions, consisting of the notochord and medial endoderm. Laterally, the mesoderm has become compact, and the endoderm is a broad layer of cells in juxtaposition with the underlying periblast (pl. 43, fig. 2).

STAGE 12: HEAD FOLD

Plate 38, figure 2

The germ ring at this stage is slightly above the equator. The embryo extends anteriorly from the periphery of the germ ring as a narrow, elongate streak. A head fold lifts the cephalic end of the embryo from the yolk by the formation of a subcephalic pocket.

In cross section, the nerve cord has pushed deeply into the underlying yolk (pl. 43, fig. 3). The cord extends for the entire length of the embryo but is not found in the undifferentiated caudal knob. Lateral to the nerve cord, mesenchyme cells are abundant. Midbody, the mesoderm has divided into two regions, the somite (epimere) and the lateral plate mesoderm (hypomere). (See pl. 43, fig. 4.) At this stage the mesomere is not distinguishable from the hypomere. Three to five pairs of somites are present. The notochord is an ovoid aggregate of cells, running along two-thirds of the embryo from the caudal knob. Kupffer's vesicle appears just anterior to the caudal knob (pl. 44, fig. 1). It is bounded ventrally by the periblast and dorsally by the endoderm, which sends up dorsolateral projections.

STAGE 13: OPTIC BUDS

Plate 38, figure 3

This stage is characterized by the appearance of optic buds as large, lateral outgrowths

of the prosencephalon. The germ ring is at the equator. Nine or 10 pairs of somites are present. Kupffer's vesicle, which looks like a lipoid globule, is visible through the posterior region of the embryo.

In cross section, the optic primordia are solid (pl. 44, fig. 2). The nerve cord cells are horizontally flattened, and they are oriented perpendicular to a median vertical line, where a split will occur and form the neurocoele. The auditory placode is present lateral to the rhombencephalon (pl. 44, fig. 3). On the mesial border of the hypomere, bilaterally symmetrical aggregates of cells which give rise to the heart are barely distinguishable. The more posterior notochord cells are becoming vacuolated. The hyomandibular pouch and the first branchial pouch are seen as dorsolateral evaginations of the pharynx. The floor of the pharynx is incomplete and caudad; the gut is a ventral cord of cells, without a lumen.

STAGE 14: BRAIN CONSTRICTIONS

Plate 38, figure 4

The outstanding characteristic of this stage is the appearance of constrictions which divide the brain into three primary regions, the prosencephalon, the mesencephalon, and the rhombencephalon. Increase in depth of the brain causes marked elevation of the cephalic end of the embryo above the yolk. The mesencephalon possesses slightly thickened walls. Melanophores are visible for the first time on the yolk sac epithelium lateral to the embryo and just posterior to the brain. They are very light brown in color and are separate. Fifteen to 16 pairs of somites are present.

In section, various lumina have appeared. A lumen is seen in the anterior portion of the gut, corresponding in position to the future esophagus. A lumen can also be seen in the optic vesicle and as a thin vertical slit in the nerve cord. Three regions are present in the mesoderm, namely, the somites, mesomere, and hypomere. A sheet of splanchnic mesoderm is folded medially and surrounds paired pericardial cavities. Cells of the notochord are horizontally flattened, and the pharynx does not appear to have a floor of endoderm.

STAGE 15: HEART BEAT

Plate 39, figure 1

The heart, which is located anterior to the head, contracts rhythmically on the yolk, but no blood corpuscles are seen. The germ ring has migrated over seven-eighths of the yolk. The brain has increased in size. The lens placode is thickened and cradled in the optic cup. A ventral choroid fissure is visible. The auditory vesicle is elliptical and has sunk beneath the surface. Between 20 and 23 pairs of somites are present. The melanophores have darkened and anastomosed. A small tail bud is formed.

In cross section, the optic vesicles are folded to form a two-layered cup. Columnar cells line the cup and form the retinal layer. They are separated mesially from the peripheral layer of cells, the pigment layer, by a narrow cavity which is confluent with the third ventricle (pl. 45, fig. 1). The rhombencephalon roof is becoming thin, and the mesencephalon and prosencephalon walls continue to thicken. The prosencephalon is growing ventrad and caudad. Olfactory placodes have appeared. Rapid development of the circulatory system commences at this stage, and the first major vessel to appear is the dorsal aorta. A single endocardial mass, the heart, is visible ventrolateral to the pharynx. The somites are becoming myofibrillar and >-shaped. Vacuoles appear in the notochord. The stomach anlage is a dorsolateral diverticulum from the gut. Dorsolateral evaginations of the hyomandibular pouch have deepened. The first branchial cleft is formed by the junction of the pouch with the ectodermal invagination (pl. 45, fig. 2). A second branchial pouch is evaginating. The pharynx has a floor, and a lumen has appeared in the gut. The posterior portion of the gut is lined with cilia, and Kupffer's vesicle is still present.

STAGE 16: CLOSURE OF BLASTOPORE
(3.1 MM.)

Plate 39, figure 2

Between the present and the previous stages, the lips of the germ ring meet as a longitudinal raphe just posterior to the tail bud. The actual time of closure is proportional to the quantity of yolk. The ventral

and lateral lips of the germ ring migrate dorsally to meet the dorsal lip of the germ ring. The posterior part of the embryo is elevated above the yolk. In the heart, very light pink blood corpuscles are visible, and melanophores are orienting along the ducts of Cuvier.

The prosencephalon is narrow, and the lens is completely invaginated. The mesencephalon has laterally widened, forming the optic lobes. Two regions can be distinguished in the laterally widened rhombencephalon, the metencephalon and the myelencephalon. The metencephalon is a pair of small, transverse, posterolateral bands. The myelencephalon, much broader than deep, possesses the characteristic neuromeres and a prominent posterior tela choroidea. Twenty-three to 25 pairs of somites are present, showing that the rate of somite formation has decreased. Muscular contractions were not observed when a mechanical stimulus was applied to the embryo. In side view the first two branchial clefts and the third branchial furrow can be seen.

In section, the prosencephalon is clearly divided into telencephalon and diencephalon. The infundibulum is seen ventral to the mesencephalon, and the hypophysial anlage is present. The lateral and ventral walls of the myelencephalon have thickened, and the fourth ventricle is conspicuous. The auditory vesicle has thick lateral and ventral walls. A number of thickened ectodermal placodes lie dorsolateral to the brain. They are presumed to be the anlage of the lateral line system. The fifth, seventh, eighth, ninth, and tenth cranial nerves arise from the myelencephalon. A large sinusoid blood vessel, the vitello-caudal vessel, can be seen postanally. Many blood vessels are visible in the head and trunk of the embryo, including the main cardinal veins and internal carotid artery. A sheath is present around the notochord. The pectoral fin anlage is seen as thickenings in the somatopleure. Differentiation of somites is proceeding with the formation of myofibrils in the anterior somites. A myoseptum divides the somites into dorsal and ventral bundles. The mesonephric tubules are seen at the level of the second to fourth somites. The lateral portion continues posteriorly as the mesonephric duct. Liver cells, containing

hepatic sinuses, can be seen surrounding the gut.

STAGE 17: OTOLITHS IN EAR VESICLES (3.5 MM.)

Plate 39, figure 3

The mesencephalon is expanding across the head, and the embryo is considerably broadened. The eyes are pushed forward by the enlarging midbrain. The neuromeres are still visible in the laterally expanding myelencephalon. Two otoliths are present in each auditory vesicle. The vitelline circulation commences with the formation of large blood sinuses on the yolk. Many pink blood corpuscles are massed in each blood sinus, and they do not appear to partake in the yolk circulation. The blood leaves the embryo through the ducts of Cuvier, drains into the vitelline system, and collects at the sinus venosus. The prominent vitello-caudal vessel drains the caudal vein. The heart beat is regular, about 130 beats per minute. The thinner-walled atrium is to the left of the thicker-walled ventricle. The somites are closely packed and less distinct, and the tail is elongate and free swinging when the chorion is removed. Four branchial arches are visible in side view.

In section, the epiphysis anterior to the tela choroidea can be seen as a dorsal evagination of the diencephalon (pl. 46, fig. 1). The optic lobes of the mesencephalon are larger and rounder. The otocyst has become slightly irregular, preparatory to the formation of the semicircular canals. The ventricular walls are thickening, and blood cells are visible in the embryonic circulatory system. The mesonephric ducts merge and then enter the anus, forming a cloaca (pl. 45, fig. 3). Blunt ventrolateral projections from the dorsal aorta into the mesonephric tubules are the anlage of the glomeruli. Swim bladder and pancreas anlagen can be seen surrounding the gut.

STAGE 18: RETINAL PIGMENT (3.7 MM.)

Plate 39, figure 4

The diagnostic feature of this stage is the first appearance of retinal pigment. A few melanophores have migrated onto the trunk of the embryo. Blood sinuses have become

abundant on the yolk, giving it a soft, spongy appearance. The tail is more elongate.

STAGE 19: PECTORAL FIN BUDS (4.0 MM.)

Plate 40, figure 1

The pectoral fin buds are small and rounded. The somites have clearly become myotomes. The trunk and tail regions are laterally compressed. The tail is slightly upturned, giving a heterocercal appearance. Aortic arches are seen in the first three branchial arches. The circulation of the deep pink blood cells is visible in the body. Blood vessels are ramified throughout the entire embryo. The first three branchial clefts are open at this stage.

The downward and backward growth of the forebrain continues. The optic lobes steadily enlarge and overhang the diencephalon. The metencephalon is a narrow transverse band, while posteriorly the myelencephalon has become broadened, containing the expanded fourth ventricle. Pigment is now clearly visible in the eye.

In section the olfactory bulb has become cup-shaped. The diencephalon has deepened ventrally; the optic lobes have thickened anterior walls, and the layers of the eye are differentiating. A vascular plexus is present dorsal to the cerebellum. The cell bodies of the neurons in the brain are oriented medially and the fiber tracts peripherally, thus separating the brain into gray and white matter. Many blood cells can be seen in the vessels of the body. The ventricle is muscular, and the heart tube is bending on itself. The operculum is just beginning its posterior growth from the hyoid arch (pl. 46, fig. 2), and the gill bars, in sagittal view, are narrow, double rows of cells. Strips of cartilage, the trabeculae, are seen lateral to the infundibulum on the floor of the brain.

STAGE 20: OPERCULA PRESENT (4.2 MM.)

Plate 40, figure 2

The optic lobes are enlarging continuously, growing upward, backward, and outward. The eye is more prominent, occasionally containing a few iridiophores. The auditory vesicle is becoming irregular, with three blunt projections extending anteriorly, posteriorly, and ventrally, initiating the formation of the semicircular canals. Lateral and posterior extensions of the hyoid arch form short, blunt opercula, which cover the first two branchial arches. The pectoral fin is triangular and enlarged. Stellate melanophores are more abundant on the trunk region of the embryo. In sectioned material, the neurocoele is becoming obliterated because of the growth of the walls in the fore and midbrain. The swim bladder arises as a lateral outgrowth to the right of the gut. It is connected to the gut by a pneumatic duct. Liver tissue is now highly vascular.

STAGE 21: IRIDIOPHORES IN EYE (4.7 MM.)

Plate 40, figure 3

Iridiophores are more abundant in the eye, and a few are found along the ventrolateral edge of the trunk. Stellate melanophores are found on the dorsal part of the rhombencephalon. The mesencephalon is becoming the dominant part of the brain, and the roof of the optic lobes is almost solid. The entire brain has deepened considerably and is demarcated from the rest of the spinal cord by a slight elevation of the rhombencephalon above the spinal cord. Many vessels, including the prominent ducts of Cuvier, are distinguishable on the vascular plexus of the yolk sac epithelium. Short, blunt gill filaments are apparent on the first two branchial arches.

In section, the olfactory bulbs connect with the telencephalon by the olfactory nerves. The optic nerve is completely fibrous, and eye muscles are forming near the eye. The infundibulum now lies beneath the myelencephalon. The mandibular arch possesses the palatoquadrate cartilage in the upper jaw and Meckel's cartilage in the lower jaw. Between the oral epithelium and the base of the brain lies a sheet of cartilage, which forms the ventral part of the chondrocranium. The otocyst is surrounded ventrally and laterally by the otic capsule, and the pectoral fins also contain cartilage in their bases. The pharynx extends far forward, but the stomodeal plate has not yet broken through to form the mouth. Thyroid follicles can barely be distinguished around the ventral aorta. Pigment cells are lining the body cavity and surrounding the

digestive organs. The gill filament anlagen make their first appearance. Gonadal ridges can be seen attached to the dorsal body wall at the level of the stomach.

STAGE 22: 4.9 MM.

Plate 40, figure 4

The quantity of iridiophores has markedly increased in the eye and along the trunk. Melanophores are still heavily concentrated on the yolk. A migration of melanophores onto the body of the embryo apparently occurs, because, in the next few stages, they are more abundant on the embryo and less abundant on the yolk.

In section the main change seen at this stage is the reorientation of the brain in relation to the body. The brain is assuming its adult characteristics by the anterior growth of the snout. The diencephalon has shifted to a more dorsal and anterior position, and the infundibulum is contiguous with the myelencephalon.

STAGE 23: MOUTH OPEN (5.1 MM.)

Plate 41, figure 1

Melanophores are spreading on the myelencephalon and are concentrating in the mid-gut region. Many iridiophores are seen in the eyes and along the ventral part of the trunk. The metencephalon is a transverse plate, which has grown laterally to an extent equal to the lateral growth of the optic lobes. The auditory vesicle or inner ear has become more irregular in shape, and the semicircular canals, sacculus, and utriculus are formed. Circulation is clearly visible in the embryo. An aortic arch extends through each branchial arch. Many blood vessels are seen in the head, and the intersegmental arteries arising from the dorsal aorta can be seen in the trunk and tail. In the bucco-pharyngeal region, the mouth has broken through terminally (pl. 47, fig. 1).

STAGE 24: HATCHING (5.3 MM.)

Plate 41, figure 2

Heterogonic growth of the embryo results in an enlarged brain, which has become elongate and has broadened laterally. The telencephalon extends anteriorly between the eyes, and small lateral telencephalic vesicles are visible. The mesencephalon possesses a solid roof. Many melanophores are present on the mesencephalon and the myelencephalon. Smaller dot-like melanophores can be seen on the caudal fin. The eyes are mobile, and the mouth irregularly opens and closes. The operculum is functional, covering the four gill arches which bear blunt gill filaments. The caudal and pectoral fins contain fin rays.

Shortly before hatching, the chorion becomes ridged. Within the chorion, vigorous movements of the tail are noted. These may contribute to the rupture of the chorion. The first part of the embryo to emerge is the tail. The thrashing movements of the newly, partially hatched larva eventually are effective, and the head of the larva emerges from the chorion. As soon as hatching is completed, the larvae actively swim on their sides along the bottom of the dish for a few seconds and then come to rest. The large yolk sac and the swim bladder, which is not fully developed, may contribute to the inability of the larvae to swim upward in the medium.

SUMMARY OF EMBRYONIC DEVELOPMENT

The foregoing study of embryonic development in *Tilapia macrocephala* revealed no fundamental differences when compared with developmental studies in other cichlids (McEwen, 1930, 1940; Jones, 1937a). Typical teleostean cleavage patterns and modes of embryonic formation were observed. The most interesting difference is the manner in which the germ ring closes. Generally, in teleosts, the closure occurs at the level of the tail bud. In *T. macrocephala*, the lips of the germ ring continue migrating posteriorly far beyond the tail bud, so that closure is delayed and the point of closure is extra-embryonic. The large quantity of yolk may be the determining factor in the method and time of blastopore closure. From the figures of Jones (1937a) it is apparent that the same is true in the cichlid fish *Etroplus maculatus*.

2. EXPERIMENTAL STUDIES

EXPERIMENTAL ORGANIZATION

THE BASIS for virtually all of the experiments stemmed from an initial discovery that sea-water dilutions maintained the embryos extra-orally, up to and beyond hatching. This raised several questions. Was there any relationship between extra-oral embryonic survival in sea-water dilutions, and embryonic survival in aquarium water in the adult's oral cavity? Was there a common factor or factors between these two apparently unrelated conditions?

The following three factors or combinations of factors could conceivably enhance survival in sea water: an optimum concentration and assortment of ions; a higher and possibly more favorable osmotic pressure; and fungicidal and bactericidal properties.

In addition, examination of the adult's oral cavity suggested that embryonic survival in the mouth might be associated with mechanical agitation or churning of the embryos caused by the respiratory flow of water in the adult, and the presence of unusual glands in the oral cavity possibly related to oral incubation.

With these possibilities in mind a series of experiments was designed to determine the relation of extra-oral survival in sea water to the special features of the adult's oral cavity. The organization of these experiments is charted in figure 1.

SURVIVAL IN ADULT'S ORAL CAVITY

Females 6 to 10 cm. in length deposit between 65 and 120 eggs at spawning. However, the number of embryos surviving is considerably smaller. For example, when 48 pairs of *Tilapia* of comparable size were permitted to incubate their offspring for 10 days after spawning, the mean brood size was 36.4. This figure includes eight pairs from which no offspring at all were obtained. Twenty-two other pairs were allowed to incubate their young for five days after fertilization, at which time the embryos had reached stage 24 (hatching). The mean brood size was 44.6. This included two pairs from which no offspring were obtained. Hence there is a discrepancy between the number of eggs laid,

the number of embryos at hatching, and the number of embryos 10 days after spawning. Aronson (1949) discusses several factors that undoubtedly contribute to the loss of embryos during incubation. Broods contain eggs in large numbers which had never been fertilized. Embryos die during development as males are frequently found incubating dead embryos. Embryos are sometimes found in the gut, indicating that the parent may swallow them.

EXTRA-ORAL EMBRYONIC SURVIVAL IN AQUARIUM WATER

When embryos were removed from the oral cavity prior to the third day of development (which is about stage 19) and placed in finger bowls containing aquarium water, generally none survived longer than three days (tables 9–13). Embryos removed from the mouth and placed in aquarium water from stages 1 through 11 did not develop beyond stage 19. When the embryos were permitted to develop in the mouth until stages 12 to 15 and were then immersed in aquarium water, they generally survived to about stage 21. Embryos removed from the mouth between stages 16 and 19 occasionally survived through hatching. If removed after stage 19, extra-oral survival rates in aquarium water were high. Observations on embryos in aquarium water frequently showed masses of fungus growing on the chorions. In a number of cases, where a fungus mycelium was noted at stage 14, survival until stage 19 was recorded. Some exhibited muscular twitches while a fungus growth was abundant on their chorions. The chorion usually split prematurely, and through the split round yolk blebs were extruded. The appearance of yolk blebs generally indicated that the death of the embryo was imminent. There were indications that the fungus growth may contribute to the splitting of the chorion.

SURVIVAL IN SEA-WATER DILUTIONS

Non-polluted sea water was collected at Jones Beach, Long Island (salinity of 3.3%), and solutions were prepared containing 100, 80, 60, 40, 20, 16, 12, 10, 8, 6, 4, and 0 per cent sea water. All dilutions were made with
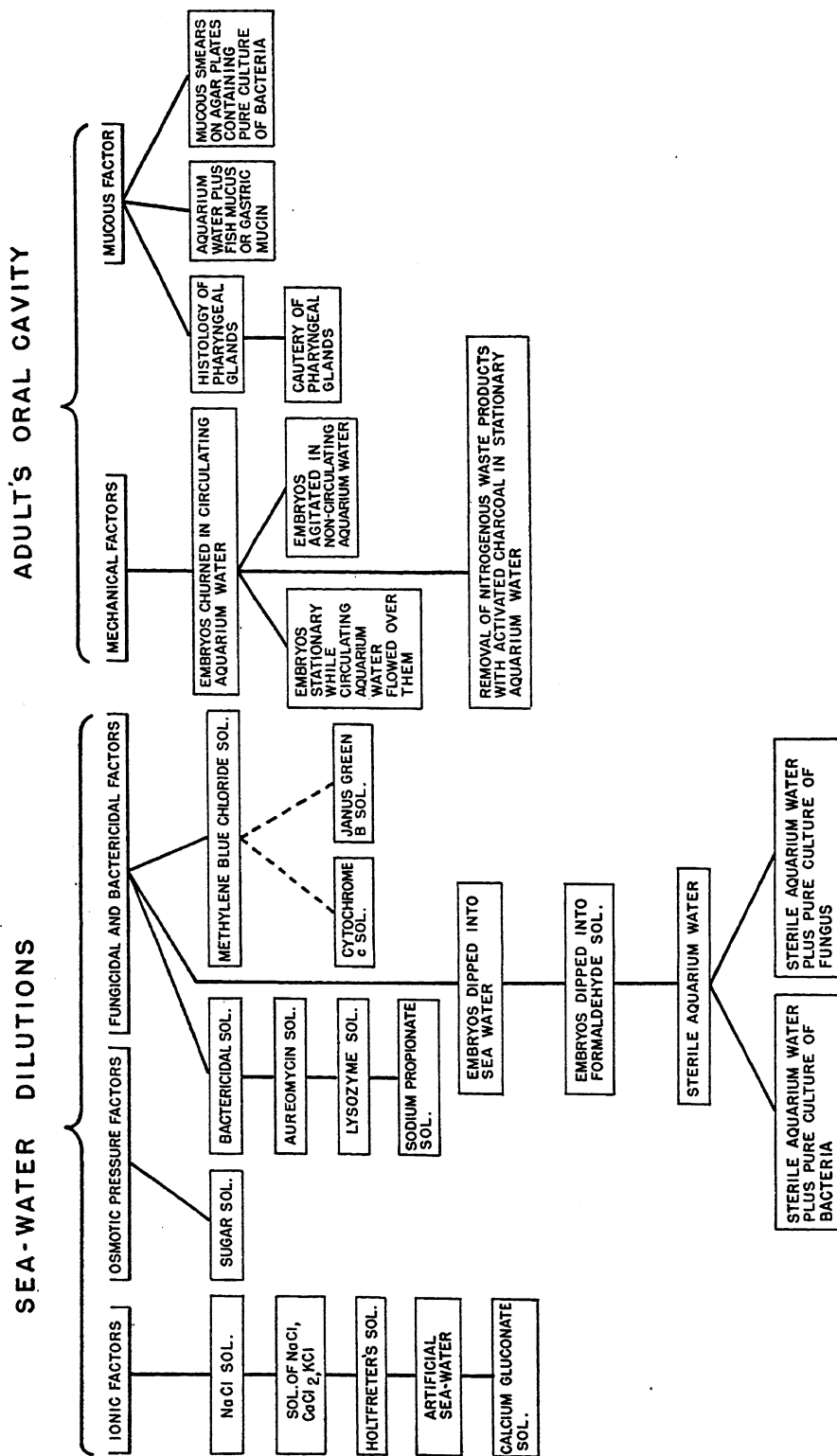
FIG. 1. Experimental organization.

aquarium water. Large numbers of embryos were reared in each of the above concentrations (table 2). In contrast to survival in aquarium water, virtually all of the groups of embryos immersed in the various sea-water dilutions showed some degree of survival. A critical concentration of sea water was noted to be about 20 per cent. Above this concentration, embryonic survival was greatly enhanced (fig. 2). Survival in dilutions containing 16 per cent or less of sea water was irregular. Before death many embryos had yolk blebs extruding through broken chorions. Two embryos in 10 per cent sea water exhibited a decreased amount of yolk pigmentation, although they did survive beyond hatching. The embryonic yolk sacs in the 10, 8, 6, and 4 per cent solutions were softer than the yolk sacs of embryos in 40 per cent sea water. This soft yolk sac created some difficulty in handling, and there was a tendency for the yolk to ooze out readily if the embryos were slightly mishandled. In 100 per cent sea water embryonic development was markedly slow. Loeb (1894) also found that *Fundulus* embryos developed more slowly in sea water than in brackish water.

Optimum survival occurred in 40 per cent sea water. No matter at what stage the embryos were introduced into this medium, survival was consistently high (table 3). The embryos were normal, healthy-looking, and quickly responsive to mechanical stimuli. No fungus growth was ever noted in this medium. For these reasons 40 per cent sea water was used regularly as a control in all experiments which followed.

INVESTIGATION OF IONIC FACTORS

As stated above, factors likely to enhance survival in sea water were separated into three major categories. Concerning the first, a number of tests were performed, with the use of various salt solutions in order to determine whether any specific ions might be necessary to the embryo's survival. Since sea water contains mainly sodium chloride, four groups of embryos were immersed in a 1.1 per cent solution of NaCl in aquarium water. This is approximately equivalent to the quantity found in 40 per cent sea water. No embryos survived beyond stage 19. Several salts (0.8% NaCl, 0.2% $CaCl_2$, and 0.1%

TABLE 2

SURVIVAL OF EMBRYOS IN VARYING DILUTIONS OF SEA WATER

Sea-Water Dilutions[a] (Per Cent Sea Water)	No. of Groups in Each Dilution	Mean Survival to Stage 24[b]
100	5	4.6
80	5	7.0
60	5	7.0
40	46	8.4
20	11	6.5
16	5	2.8
12	6	1.0
10	8	2.2
8	6	0.8
6	9	2.3
4	6	1.3
0	46	0

[a] Sea water diluted with aquarium water.
[b] Each group contained 10 embryos.

KCl) were then combined, and 12 groups of embryos were immersed in the aquarium water solution containing these salts. The results are shown in table 4. In five out of 12 groups some embryos survived to hatching. The mean survival at hatching was 2.3. The greatest mortality occurred between stages 15 and 19. Many embryos that survived to hatching were abnormal. Hence the medium was not satisfactory. One of the most predominant anomalies was a persistent pericardial edema which remained until hatching. Many small white masses aggregated on the pericardium. In a few embryos large vesicles formed which were filled with static blood at the point of entrance of the ducts of Cuvier into the sinus venosus.

Holtfreter's solution was also unsuccessful in promoting embryonic survival. Anomalies, similar to the ones previously described, were present. Table 5 shows the results of seven groups of embryos tested in this medium. The mean survival was 1.1 embryos.

Artificial sea water, prepared according to the formula of McClendon *et al.* (1917) was tested.[1] This artificial sea water was diluted 50 per cent with aquarium water, and five

[1] Because of unavailability, Na_2SiO_3 and $Na_4Si_4O_9$ were omitted in the formula.
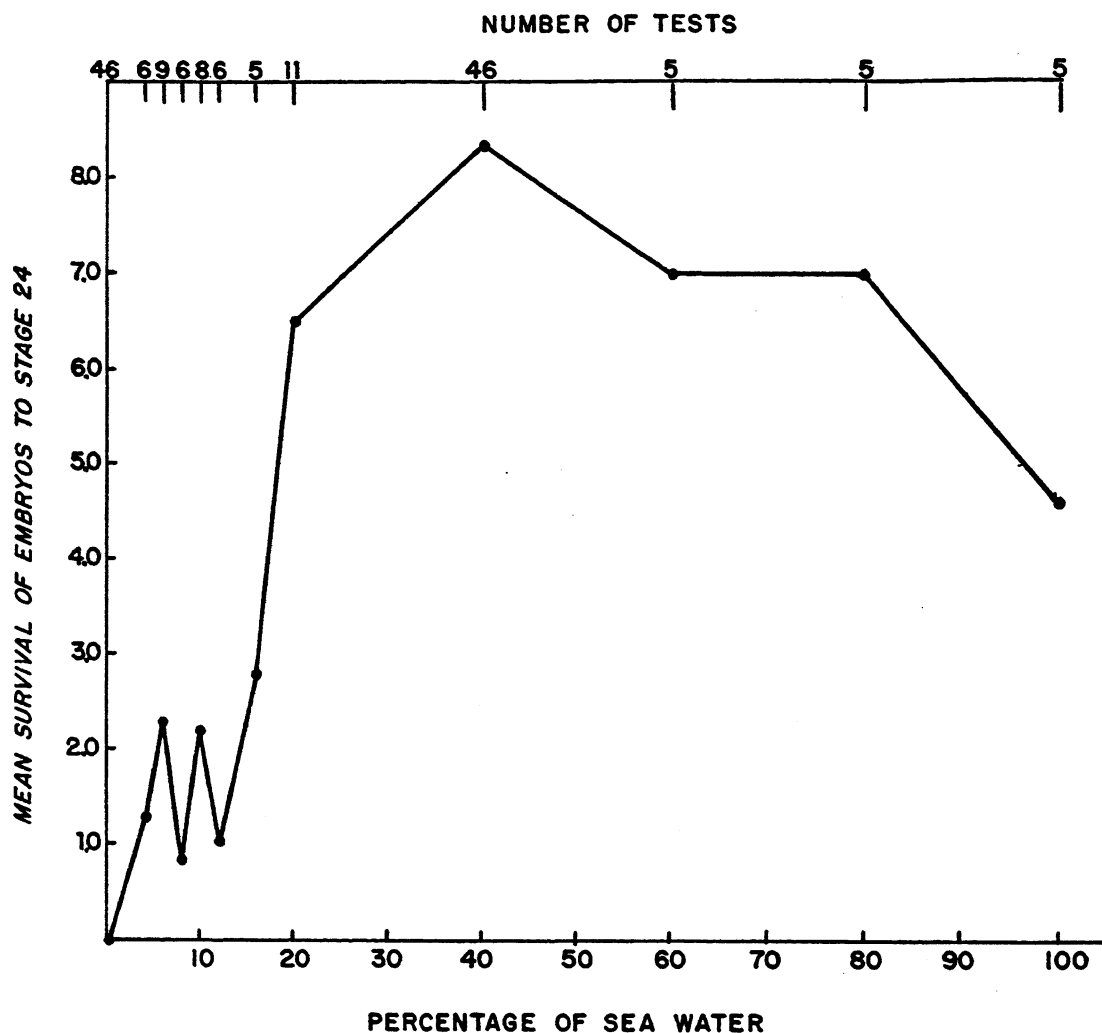
NUMBER OF TESTS

FIG. 2. Graph of embryonic survival in various dilutions of sea water.

groups of embryos were immersed in this medium. None survived, and abnormal embryos were observed, many exhibiting laterally twisted bodies and pericardial edema.

Calcium gluconate[1] was used primarily to test the effects of the calcium ion. Five groups of embryos were immersed in a concentration of 0.1 per cent of calcium gluconate in aquarium water, while four groups were immersed in a 0.5 per cent calcium gluconate solution of aquarium water. Six embryos in one group survived to stage 24 in 0.1 per cent calcium gluconate. These embryos were ab-

[1] Calglucon, 195001, courtesy of Sandoz Chemical Works, Inc.

normal, exhibiting the typical pericardial edema with a stretching and narrowing of the heart. They died soon after hatching. All of the remaining groups died during the early stages. The solutions were usually cloudy, and much debris accumulated on the chorions, which fragmented easily. The yolk seemed to be more swollen than usual.

INVESTIGATION OF OSMOTIC FACTORS

Since sea water has a high osmotic pressure, sugar solutions were tested in order to discriminate between the osmotic effects and the ion effects of sea water. The osmotic pres-

TABLE 3

SURVIVAL OF EMBRYOS IN 40 PER CENT SEA WATER WHEN
INTRODUCED AT DIFFERENT STAGES

Stage Introduced	No. of Groups in Each Stage	Mean Survival to Various Stages				
		15	17	19	21	24
1	3[a]	10	9	8	[b]	8
2	8	10				10
3	3	10				10
4	3	10	9	8		8
5	5	9				9
6	6	10	9			9
7	10	10	9			9
10	5	9				9
11	1	10	7	6		6
Means		9.8	9.0	8.7	8.7	8.7

[a] Each group started with 10 embryos.
[b] Blank spaces indicate no change in number of survivors.

sure of sea water according to the formula in Sverdrup *et al.* (1942) was calculated to be about 22 atmospheres at 25° C. Two solutions of cane sugar were prepared, a 10 per cent solution, which exerts a pressure of about 6.5 atmospheres, and a 20 per cent cane sugar solution which exerts a pressure of approximately 10 atmospheres, the latter having a pressure which is close to the osmotic pressure of the 40 per cent sea water used in this experiment.

A total of five groups were immersed in these solutions, three in the 20 per cent solution and two in the 10 per cent solution. No embryos survived beyond stage 17 in either solution, and the solutions became contami-

TABLE 4

SURVIVAL OF EMBRYOS IN A SOLUTION CONTAINING NA, CA, AND K IONS

Stage Introduced	No. Surviving to Various Stages in NaCl, CaCl₂, and KCl Solution[a]					No. Surviving to Various Stages in 40 Per Cent Sea Water (Control)				
	15	17	19	21	24	15	17	19	21	24
2	0[b]				0	2	[c]			2
6	7	0			0	6			5	4
6	5	0			0	9		8	6	6
7	0				0	8				8
7	0				0	10				10
7	10	8			8	8				8
7	6	4	3		3	10				10
7	7	6	1		1	10				10
7	10	8	7		7	8				8
7	8				8	6		5		5
10	5	5	0		0	5	3			3
10	8	8	2	0	0	9		8		8
Means	5.5	3.9	2.3	2.3	2.3	7.6	7.4	7.1	6.9	6.8

[a] 0.8% NaCl; 0.2% CaCl₂; 0.1% KCl.
[b] All experimental and control tests started with 10 embryos.
[c] Blank spaces indicate no change in number of survivors.

TABLE 5

SURVIVAL OF EMBRYOS IN HOLTFRETER'S SOLUTION

Stage Introduced	No. Surviving to Various Stages in Holtfreter's Solution					No. Surviving to Various Stages in 40 Per Cent Sea Water (Control)				
	15	17	19	21	24	15	17	19	21	24
2	10[a]	9	8	[b]	8	10				10
6	7	7	3	0	0	9		8	7	6
6	5	—[c]	2	1	0	6	—	—	5	4
7	9		6	2	0	10			9	8
7	7	0			0	10				10
7	3	0			0	10				10
10	10	6	5		0	6	4			4
Means	7.3	5.1	3.4	2.3	1.1	8.7	8.4	8.3	7.9	7.4

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in number of survivors.
[c] Dashes indicate no observation at this stage.

nated with fungus growths. The embryos were dwarfed and developed more slowly than the control embryos. Heart beats were never observed. Stockard (1907) reported similar results in *Fundulus* embryos reared in sea water containing various concentrations of cane sugar. He never reported any fungus growths in sea water plus cane sugar, but he did note fungus growths in solutions of cane sugar and distilled water. Piiper (1933) found shortened bodies, no tails, and hydrocephaly and other abnormalities in *Rana temporaria* when it was grown in a 25 per cent cane sugar solution.

INVESTIGATION OF BACTERICIDAL AND FUNGICIDAL FACTORS

PRELIMINARY TESTS

Embryos were immersed in a bactericidal solution containing a number of known salts. This solution is believed to inhibit the growth of molds and bacteria and was also used as a synthetic growth medium for *Oryzias latipes* (Rugh, 1948). It consists of 1.0 gram of NaCl, 0.03 gram of KCl, 0.03 gram of $CaCl_2$, 0.08 gram of $MgSO_4$, and distilled water to make one liter. Table 6 summarizes the results in 15 groups of embryos. A number of embryos survived to hatching in nine groups giving a mean survival of 3.7 embryos. In some instances fungus growths were noted on the embryos. It was difficult, however, to determine whether the beneficial effects of this solution were due to the ion content of the solution or to its bactericidal properties. Therefore, other bactericides, such as aureomycin, lysozyme, and methylene blue, which do not contain large quantities of inorganic salts, were tested.

Aureomycin,[1] which is widely advertised as a general bactericide for tropical fishes, was ineffective. Five groups were immersed in solutions of aquarium water containing 0.00006 per cent and 0.00003 per cent aureomycin. Each day the solutions were freshly prepared and changed. The embryos were abnormal, and in some instances only clumps of protoplasm were seen on the yolk, suggesting that development was disrupted. Lysozyme[2] was also ineffective in concentrations ranging from 1/50000 to 1/1000000. Nine groups of embryos were treated in various concentrations. The embryonic chorions always appeared opaque, and many fungus spores were noted in the medium. In table 7 the survival of embryos in lysozyme is tabulated. In one case six embryos hatched prematurely but died shortly afterward.
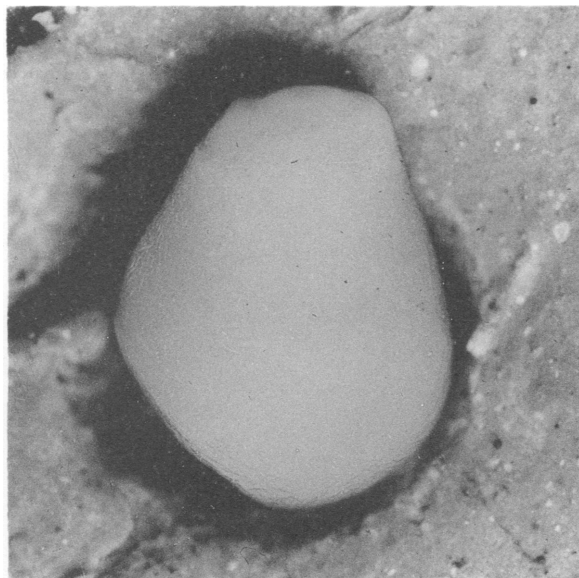
Methylene blue, which is not thought to be as effectively bactericidal as aureomycin, was tested. Concentrations of methylene

[1] Aureomycin hydrochloride, Lederle No. 7-5374, courtesy of Lederle Laboratories Division, American Cyanamid Company.
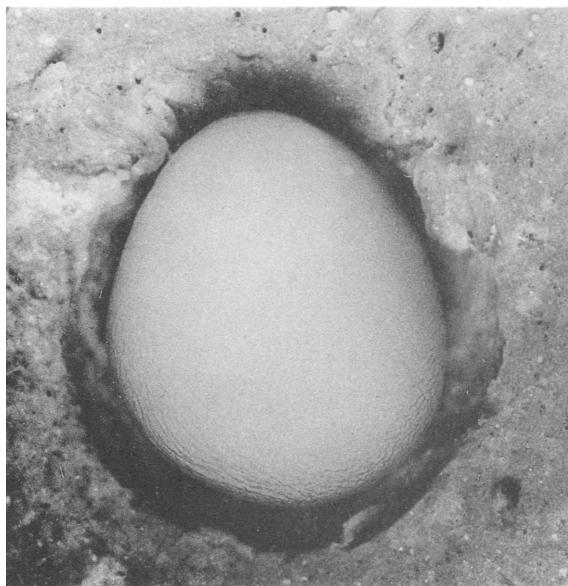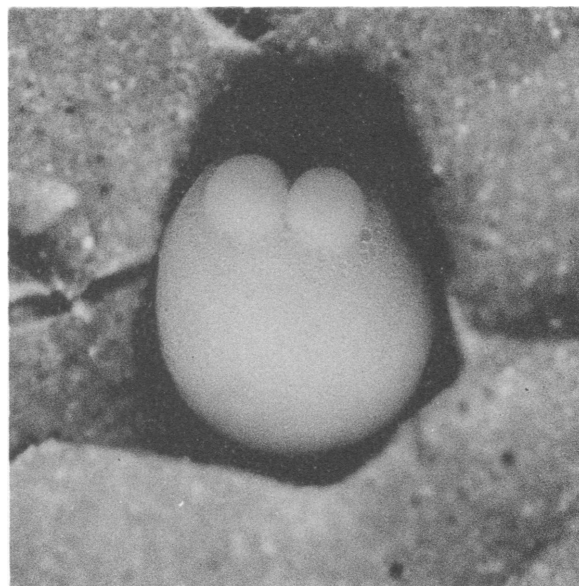[2] Lysozyme, Armour Batch No. 99321.
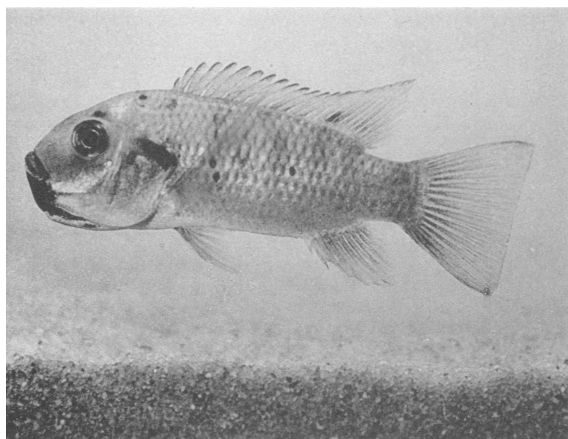
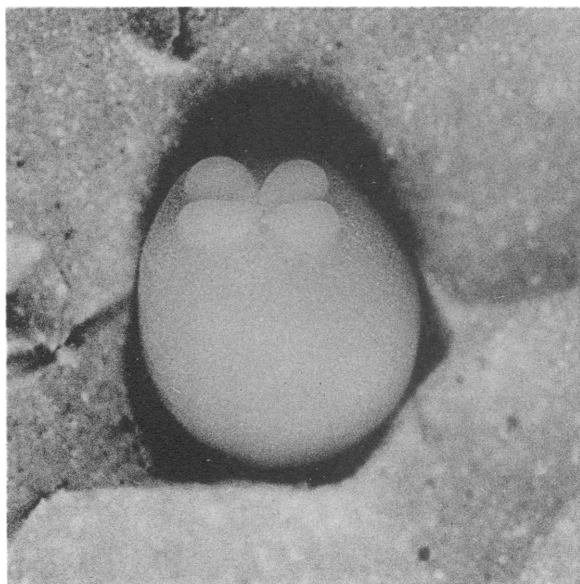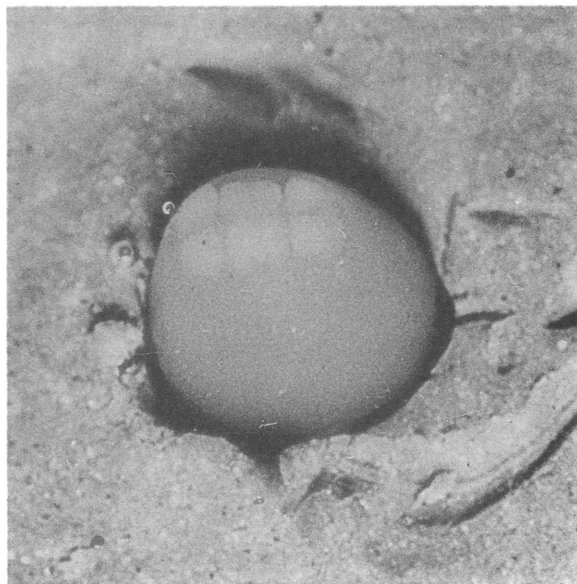1. Male incubating eggs. × 1.5. Photograph courtesy of New York Zoological Society. 2. Unfertilized ovum.
: 18.   3. Stage 1, fertilized ovum. × 18.   4. Stage 2, two-celled ovum. × 18

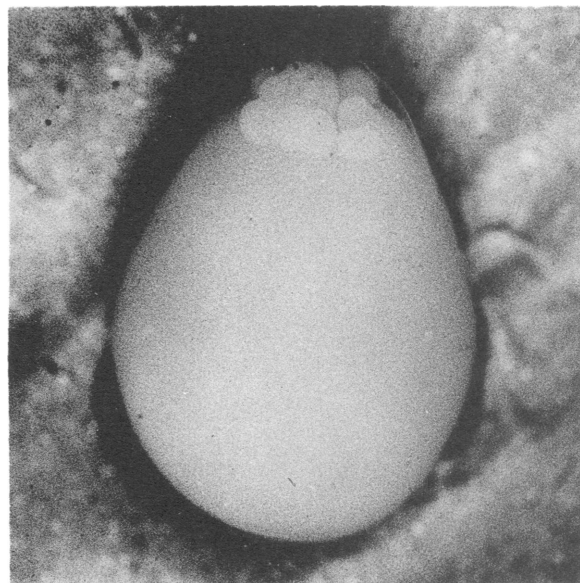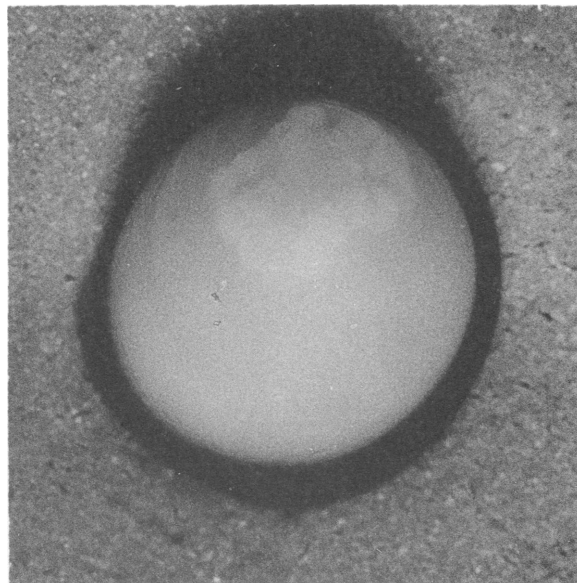1. Stage 3, four-celled ovum. × 18.   2. Stage 4, eight-celled ovum. × 18.   3. Stage 5, 16-celled ovum. × 18.   4. Stage 6, 32-celled ovum. × 18
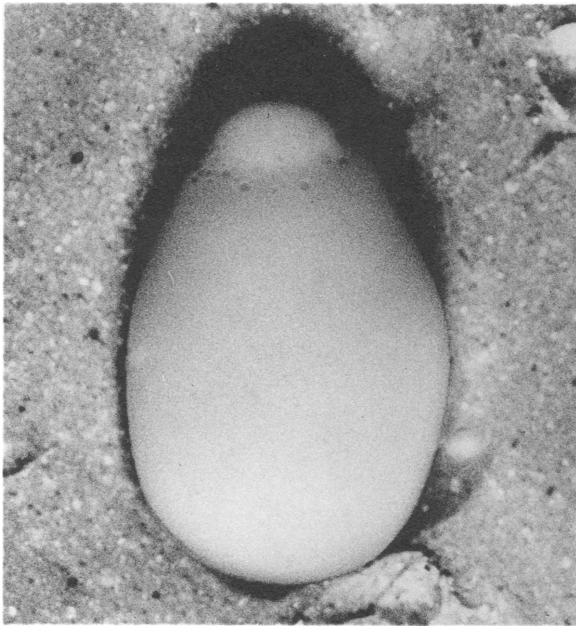
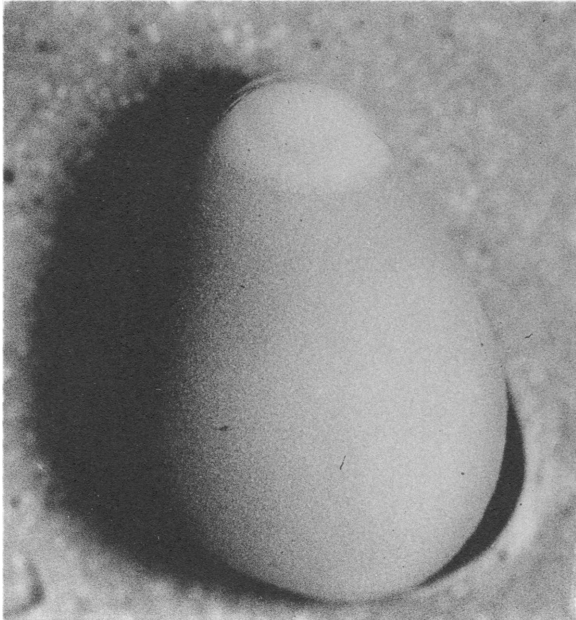1. Stage 7, early blastula. × 18.   2. Stage 8, blastula. × 18.   3. Stage 9, early gastrula. × 18.   4. Stage 10, gastrula. × 18
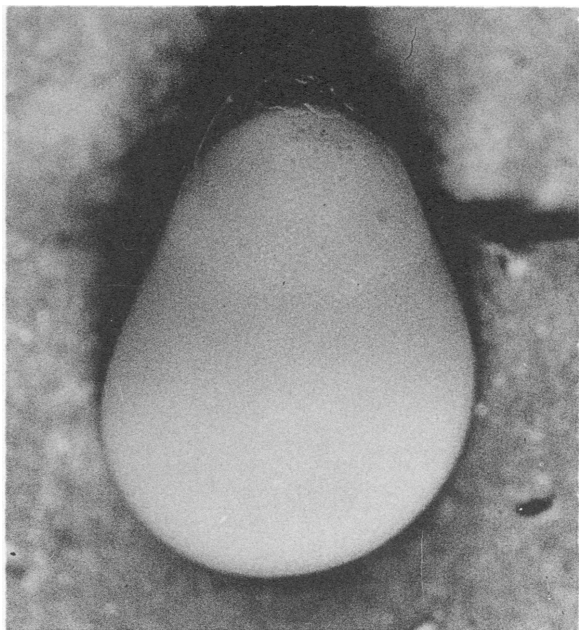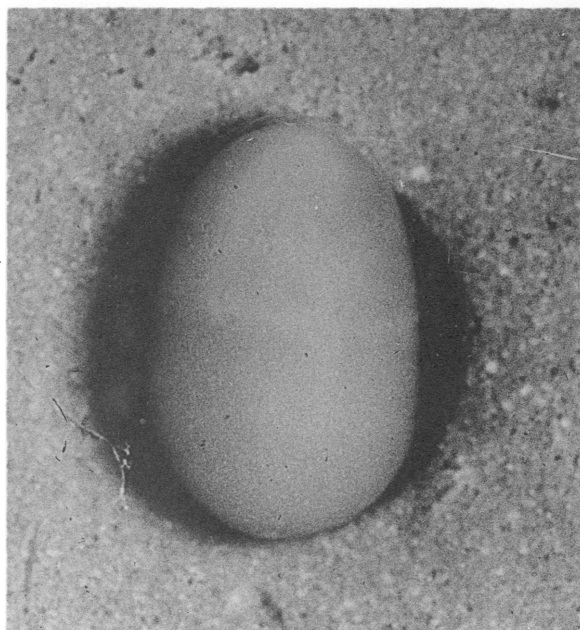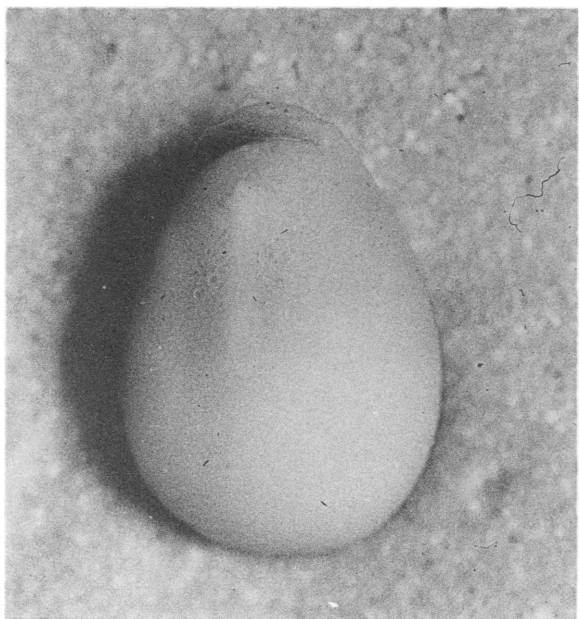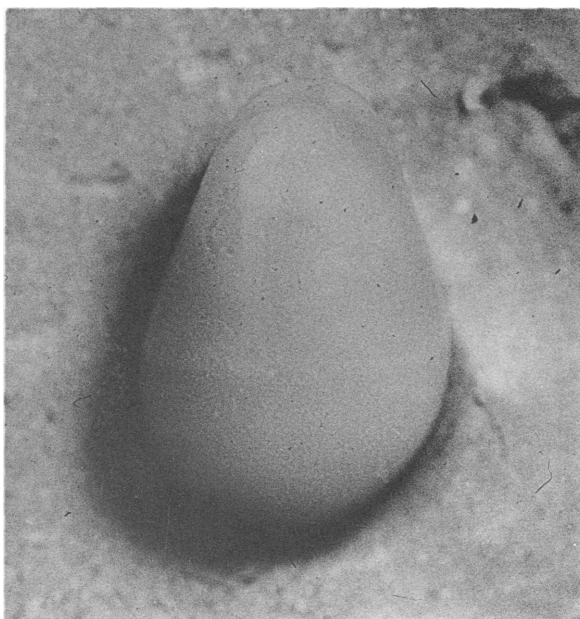
1. Stage 11, neurula. × 18.   2. Stage 12, head fold. × 18.   3. Stage 13, optic buds. × 18.   4. Stage 14, brain constrictions. × 18

1

2

3

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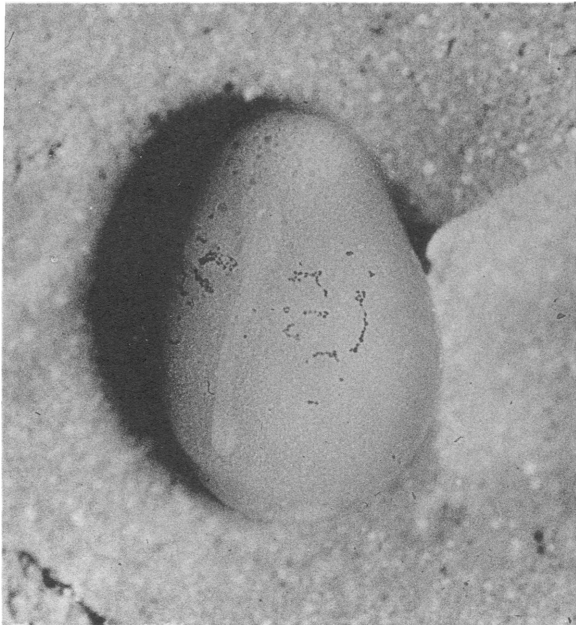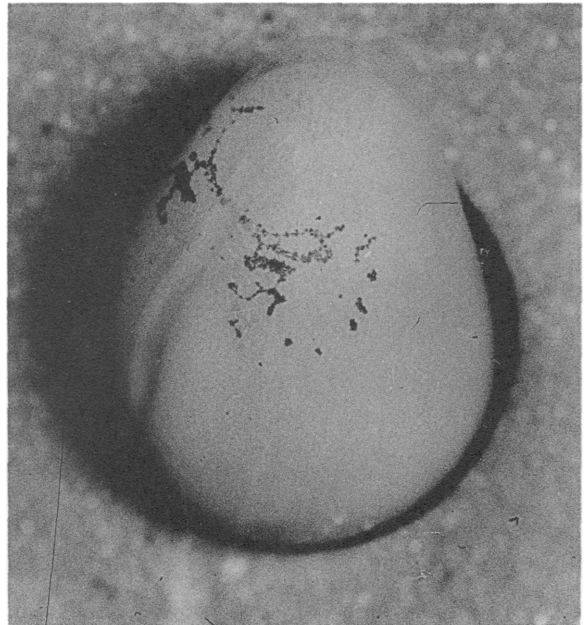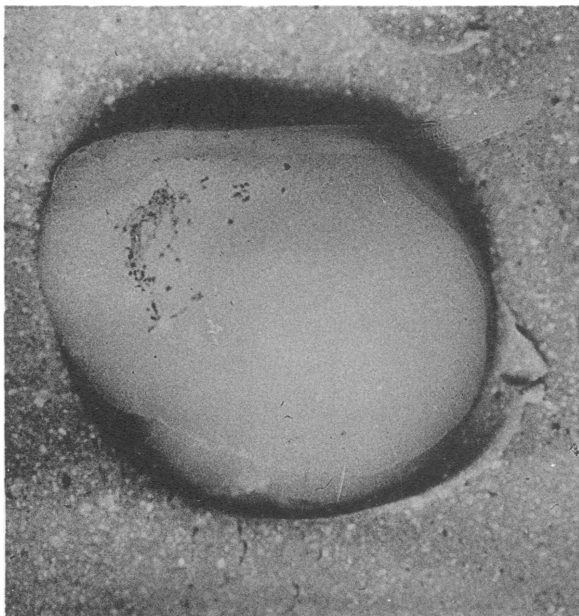
1. Stage 15, heart beat. × 18.　2. Stage 16, closure of blastopore; 3.1 mm. × 18.　3. Stage 17, otoliths in ear vesicles; mm. × 18.　4. Stage 18, retinal pigment; 3.7 mm. × 18
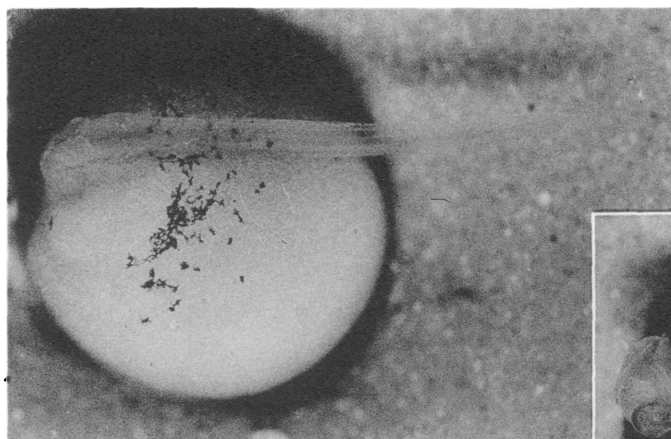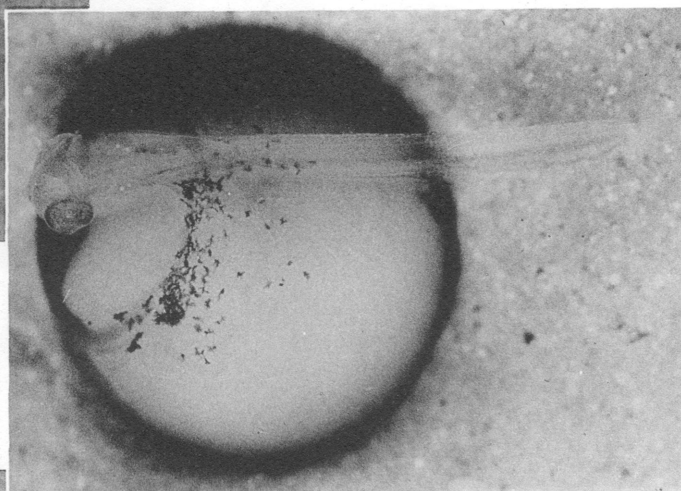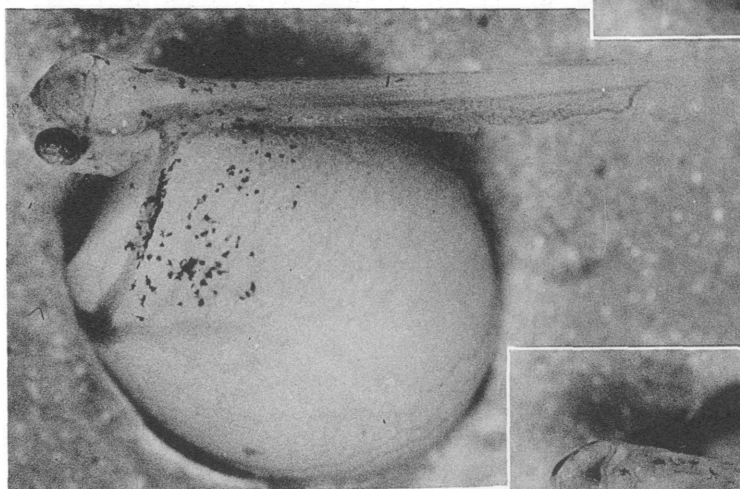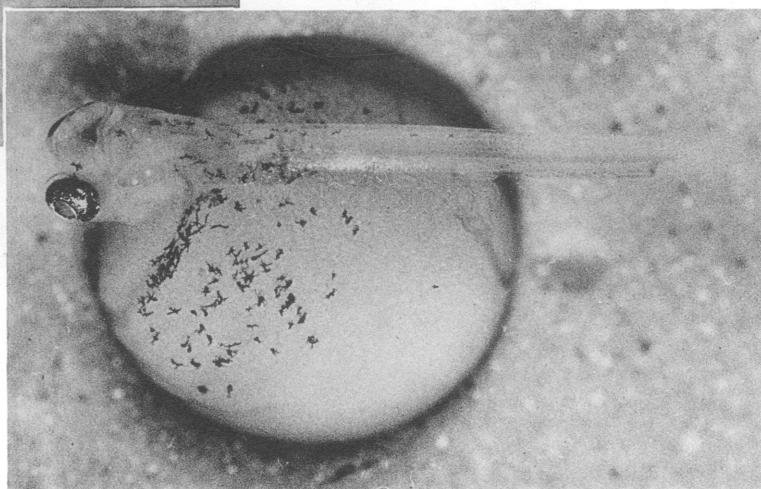
1. Stage 19, pectoral fin buds; 4.0 mm. × 18.   2. Stage 20, opercula present; 4.2 mm. × 18.   3. Stage 21, iridiophores in eye; 4.7 mm. × 18.   4. Stage 22, 4.9 mm. × 18

1

2
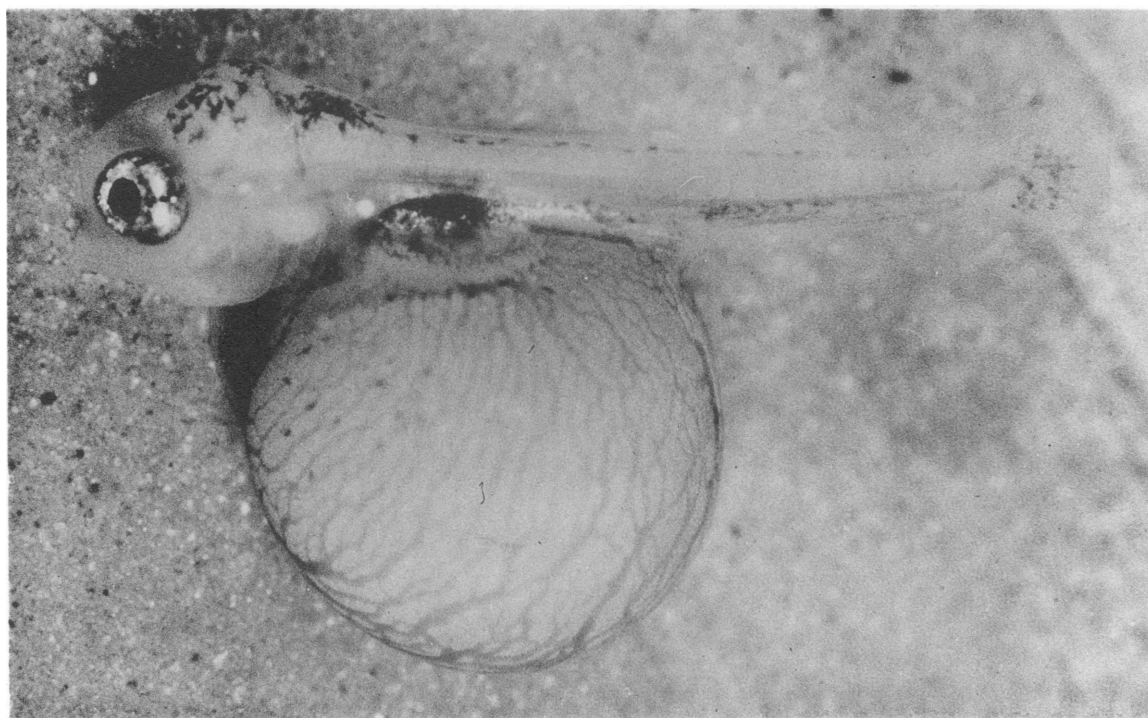
1. Stage 23, mouth open; 5.1 mm. $\times$ 25.   2. Stage 24, hatching; 5.3 mm. $\times$ 25
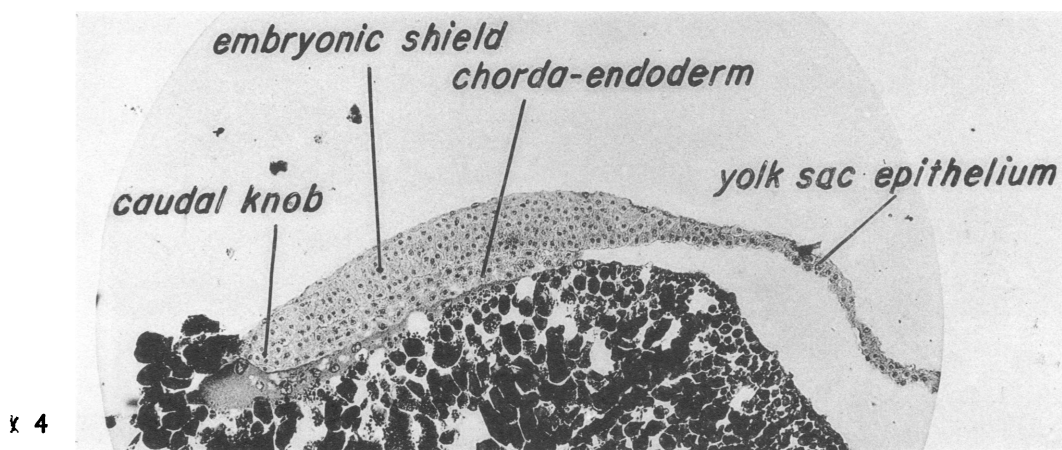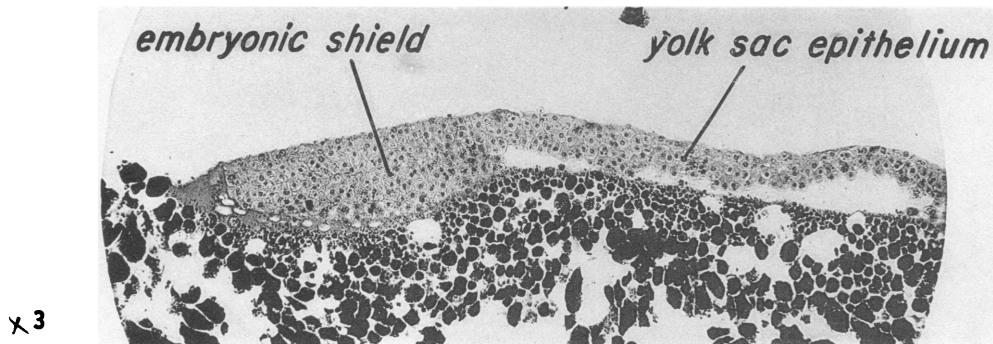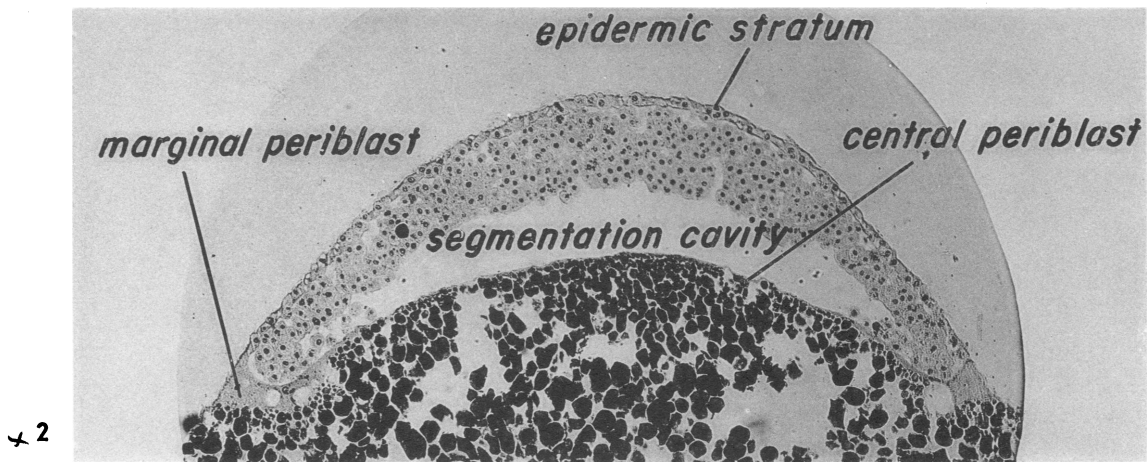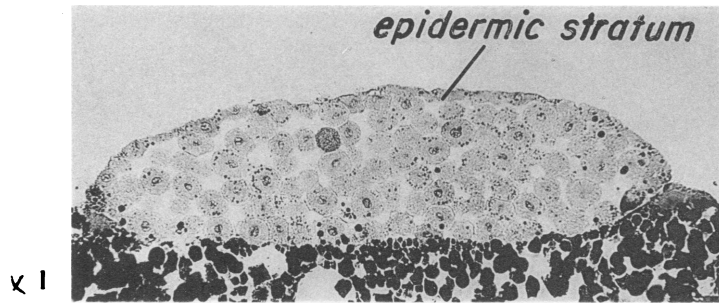
1. Stage 7, early blastula, cross section. × 100.   2. Stage 8, blastula, cross section. × 100.
3. Stage 9, early gastrula, longitudinal section. × 100.   4. Stage 10, gastrula, longitudinal
section. × 100
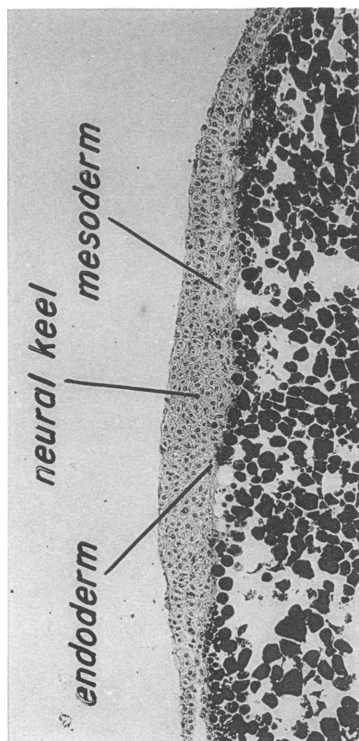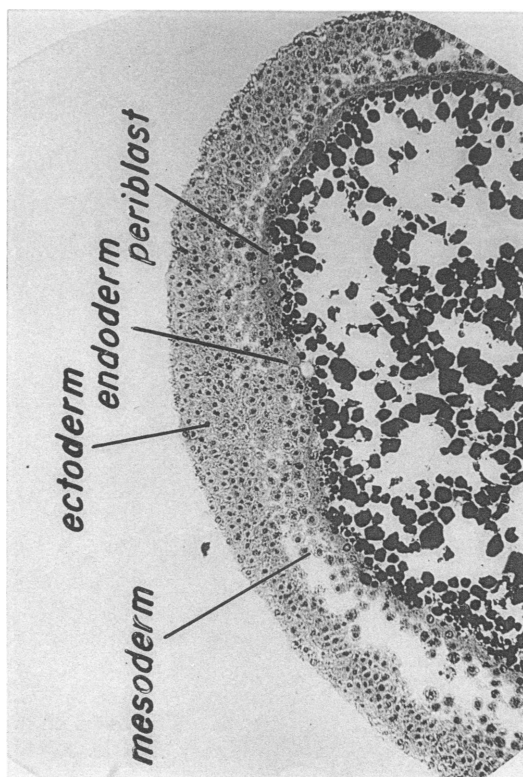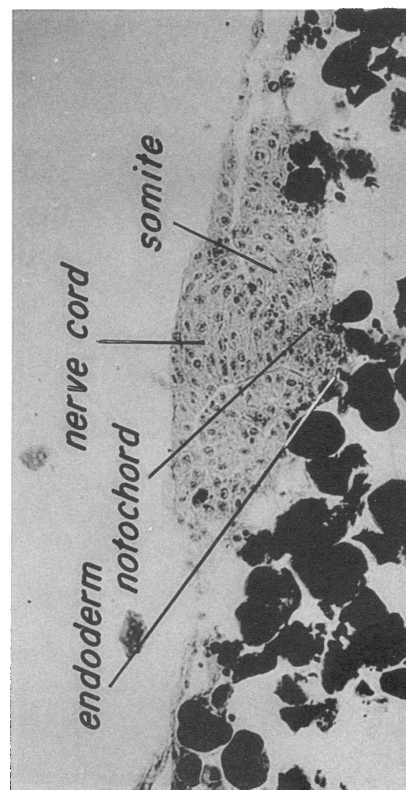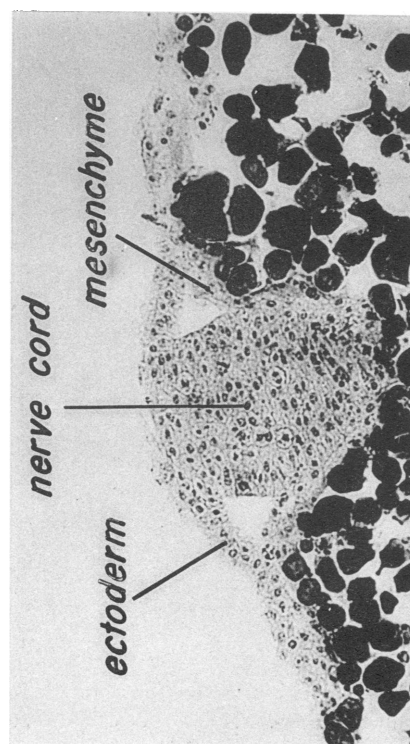
1. Stage 10, gastrula, cross section. × 100.   2. Stage 11, neurula, cross section. × 100.   3. Stage 12, head fold, cross section through cephalic end. × 440.   4. Stage 12, head fold, cross section through midbody. × 440

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✕ 2
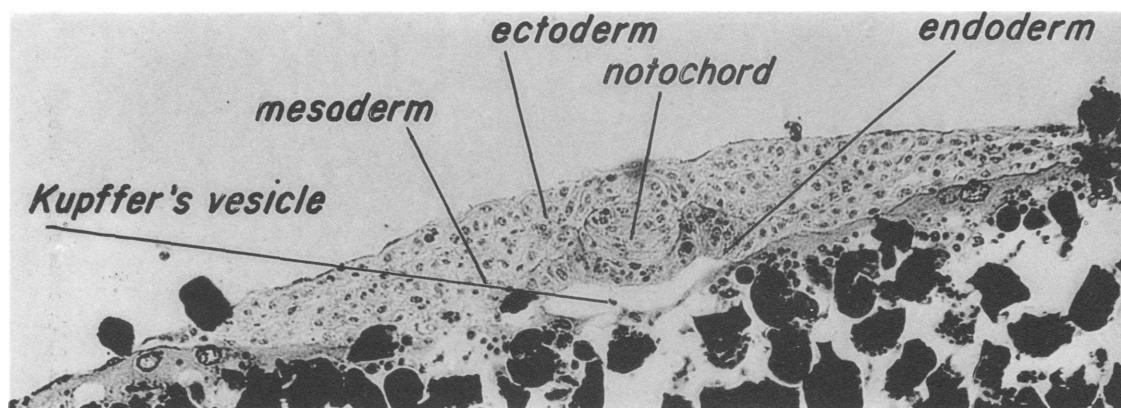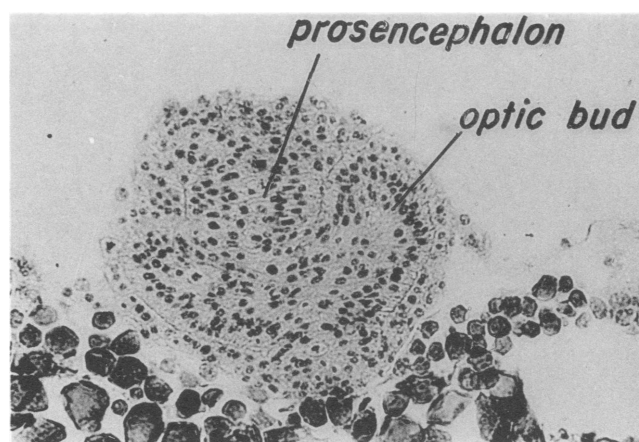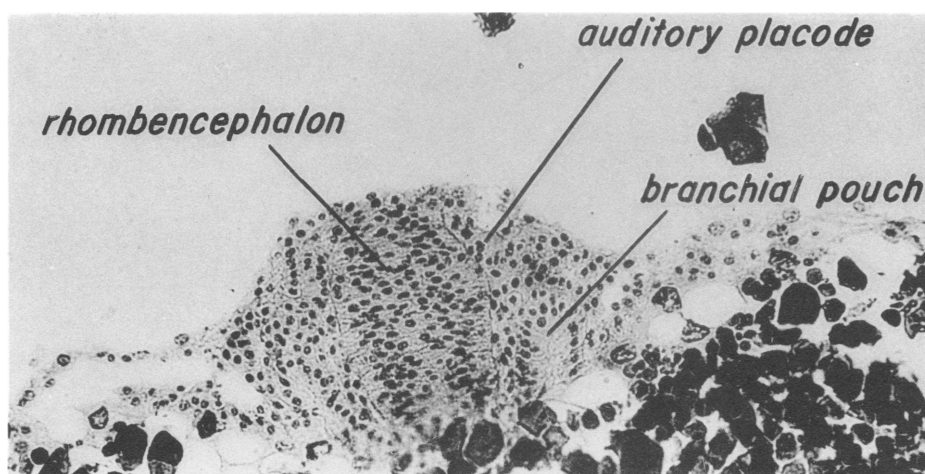
Y 3

1. Stage 12, head fold, cross section through Kupffer's vesicle. ✕ 440. 2. Stage 13, optic buds, cross section through optic buds. ✕ 440. 3. Stage 13, optic buds, cross section through auditory placode. ✕ 440
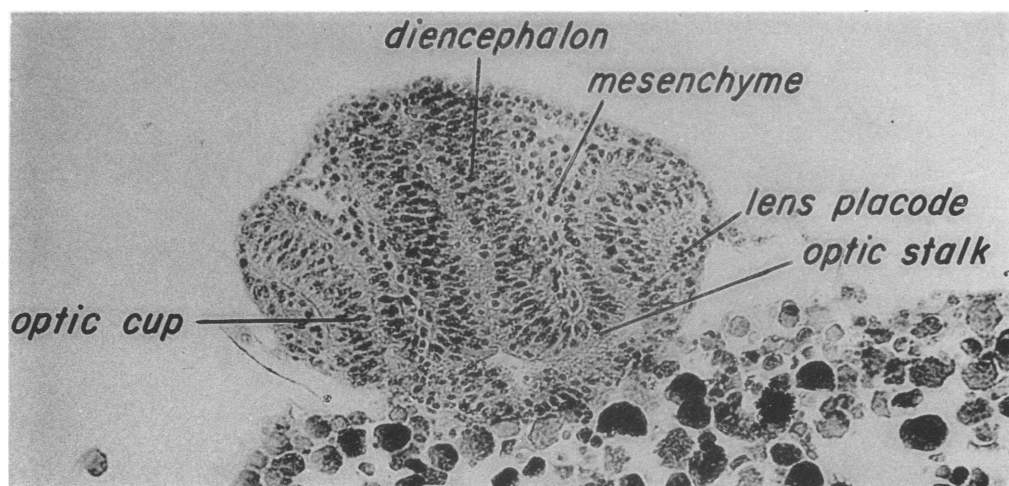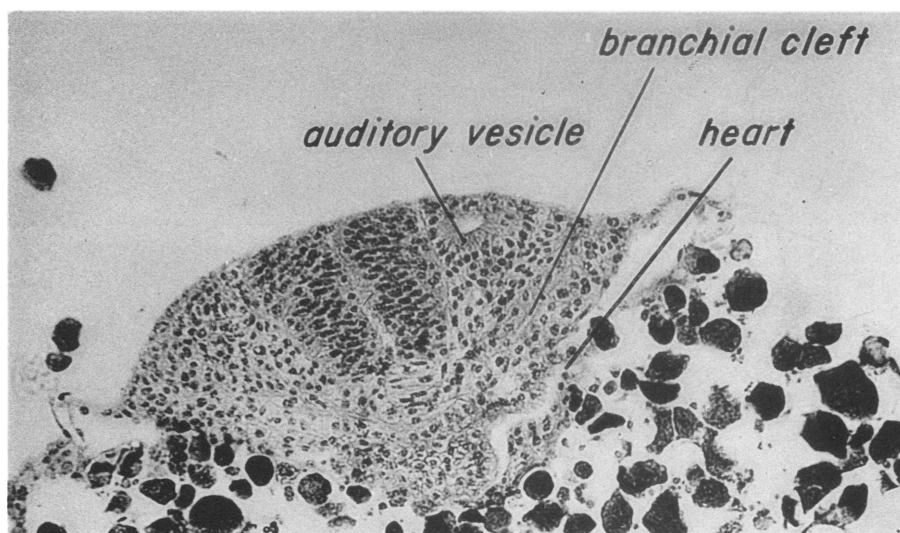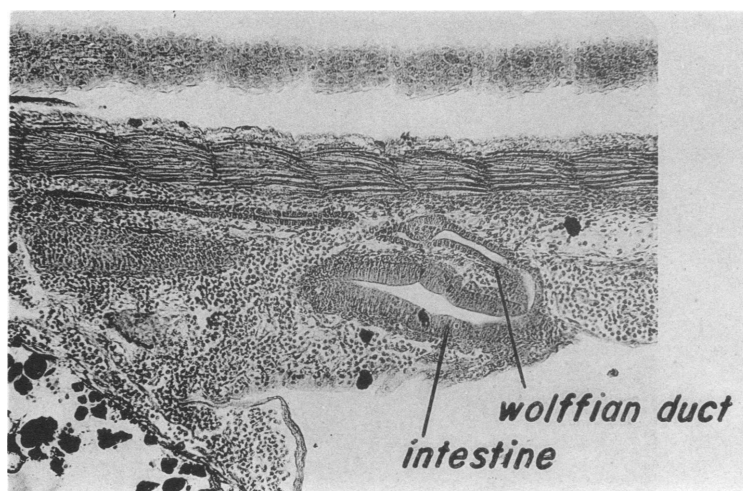
1. Stage 15, heart beat, cross section through optic vesicles. × 440.   2. Stage 15, heart beat, cross section through branchial cleft. × 440.   3. Stage 17, otoliths in ear vesicles, longitudinal section through anal region. × 100
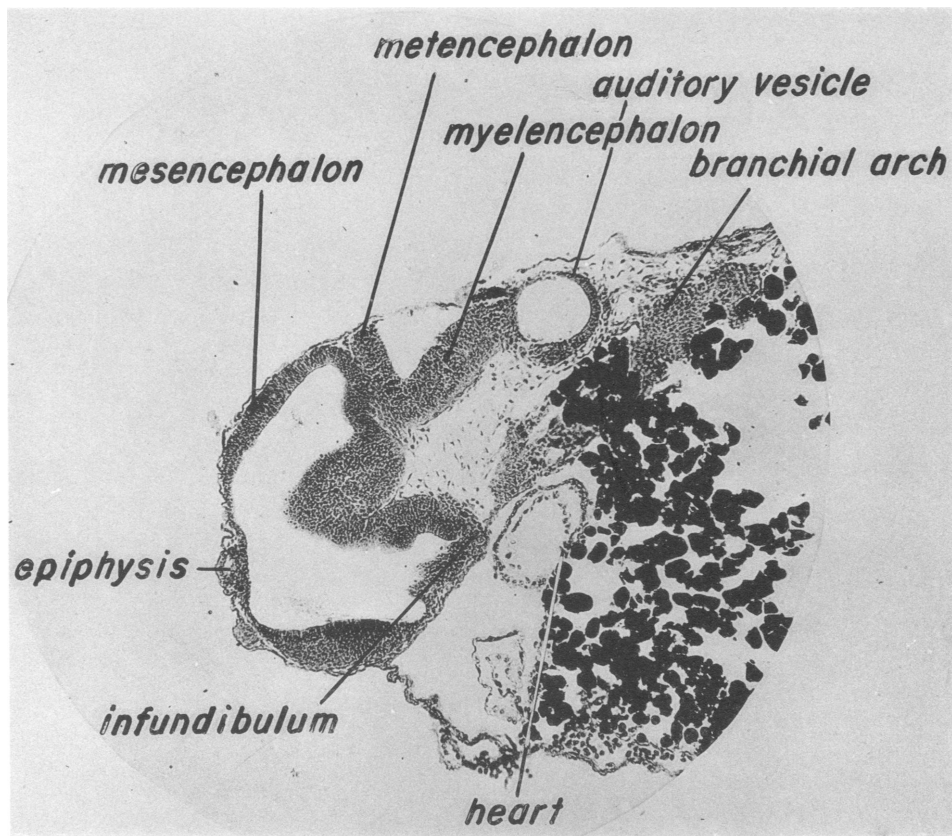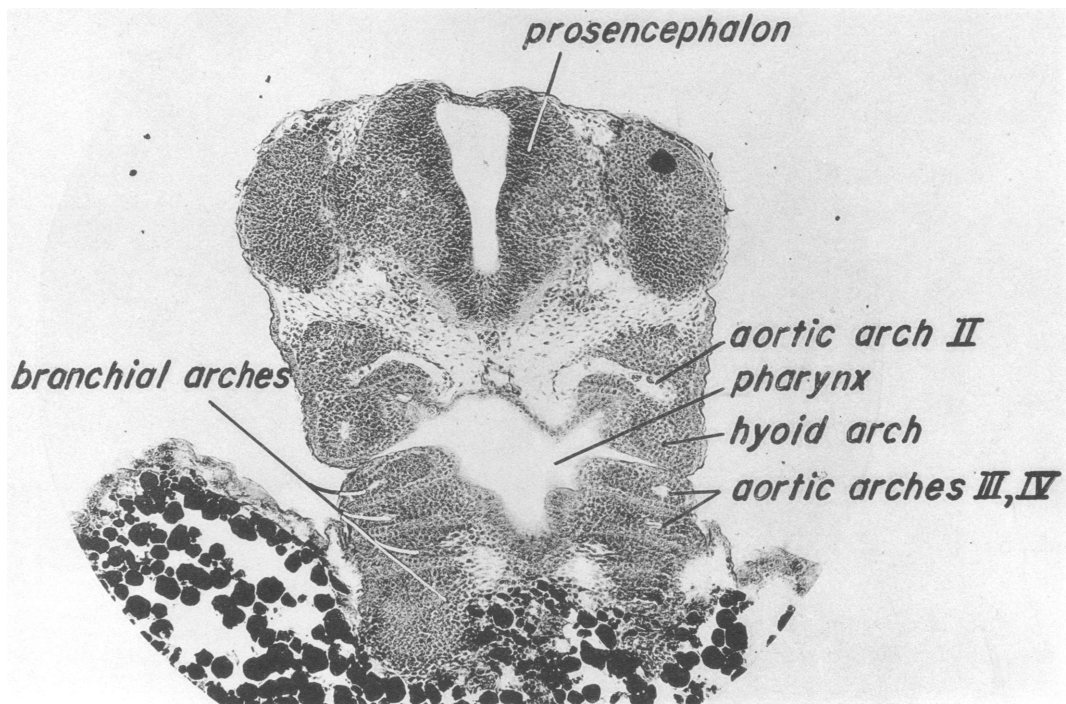
1. Stage 17, otoliths in ear vesicles, longitudinal section through anterior end. × 100. 2. Stage 19, pectoral fin buds, horizontal section through branchial arches. × 100
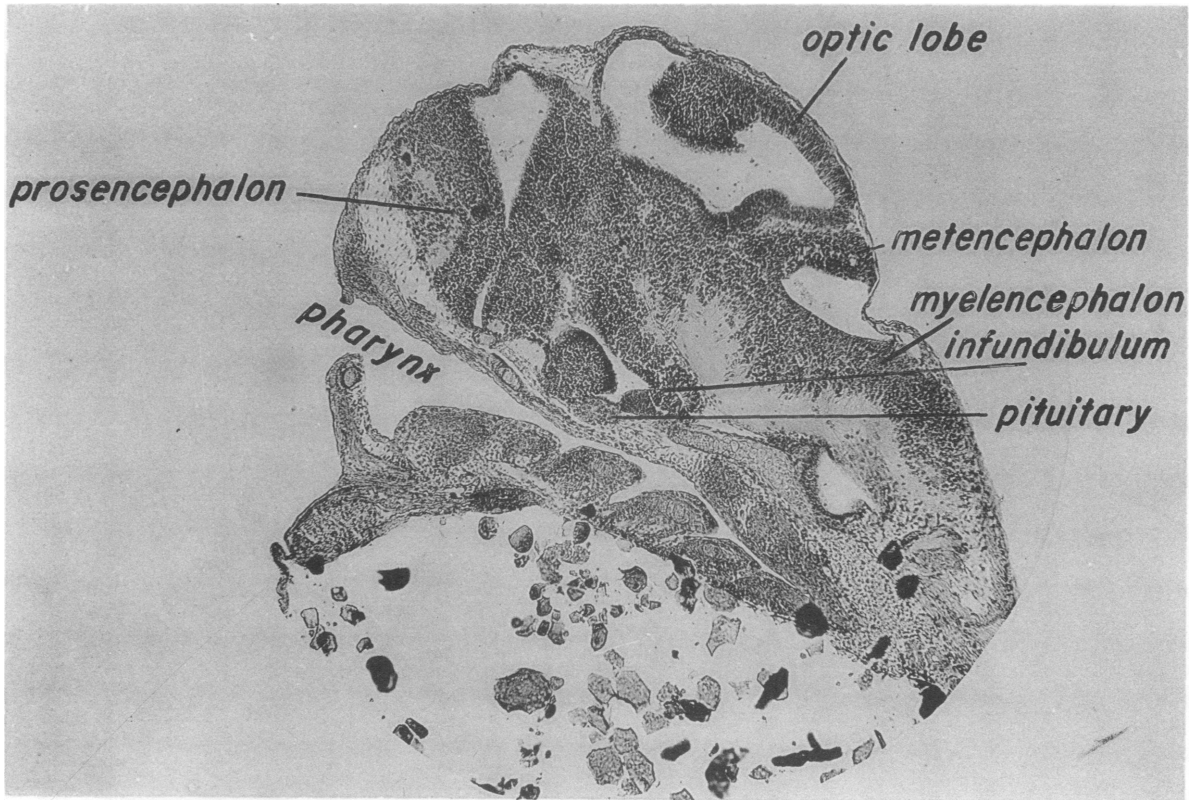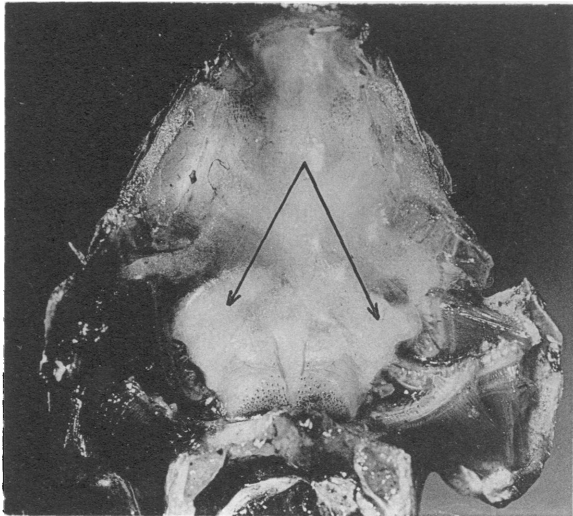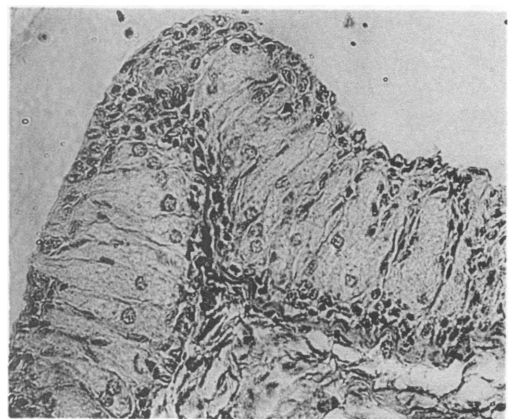
1. Stage 23, mouth open, cross section through anterior end. × 100. 2. Ventral view of upper palate showing pharyngeal glands. × 4. 3. Cross section through pharyngeal glands. × 440

TABLE 6

SURVIVAL OF EMBRYOS IN BACTERICIDAL SOLUTION

Stage Introduced	No. Surviving to Various Stages in Bactericidal Solution					No. Surviving to Various Stages in 40 Per Cent Sea Water (Control)				
	15	17	19	21	24	15	17	19	21	24
2	10[a]	[b]			10	10				10
2	10	7	4	0	0	10		9	8	8
2	10	8	2	—[c]	1	10	7			7
4	10	8	3	1	1	5				4
5	9		8		7	7	6	4		4
6	9	0			0	9	8	7	6	6
6	8	0			0	6	5	4		4
6	10	9	8		8	8				8
6	10	9			9	10				10
7	6	0			0	9				8
7	0				0	10				10
7	0				0	2				2
7	10	8	6		6	10	8	7		7
7	6				6	9	8		7	7
7	10	8			8	9		8	7	7
Means	7.9	5.5	4.3	3.9	3.7	8.3	7.7	7.2	6.9	6.9

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in number of survivors.
[c] Dash indicates no observation at this stage.

TABLE 7

SURVIVAL OF EMBRYOS IN LYSOZYME SOLUTIONS

Concentration	Stage Introduced	No. Surviving to Various Stages in Lysozyme Solutions					No. Surviving to Various Stages in 40 Per Cent Sea Water (Control)				
		15	17	19	21	24	15	17	19	21	24
1/50000	2	10[a]	8	0		0	8	[b]			8
1/100000	5	0				0	10				10
1/100000	9	10	6	—[c]	0	0	10				10
1/150000	8	10	10	8	0	0	10	8	6		6
1/200000	8	8	7	0		0	10	10	—	9	9
1/300000	5	5	0			0	3				3
1/500000	10	6			6[d]	0	9				9
1/1000000	8	10	8	0		0	10				10
Means		7.7	5.9	2.2	0.7	0	8.9	8.7	8.4	8.3	8.3

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in the number of survivors.
[c] Dashes indicate no observations at this stage.
[d] Embryos hatched prematurely and were abnormal.

TABLE 8

Survival of Embryos in Methylene Blue

Concentration	Stage Introduced	No. Surviving to Various Stages in Methylene Blue Solutions					No. Surviving to Various Stages in 40 Per Cent Sea Water (Control)				
		15	17	19	21	24	15	17	19	21	24
1/1000	2	10[a]	—[b]	5	[c]	5	6	6	6	5	4
1/1000	4	9	9	—	5	4	8	6	—	4	4
1/1000	7	10	5	5	0	0	10				10
1/1000	8	0				0	9	—	—	—	7
1/2000	8	0				0	10	10	—	7	7
1/4000	8	0				0	7				7
1/5000	4	7	6	—	1	1	8	6	—	4	4
1/5000	4	1	0			0	8	6	—	4	3
1/7500	5	0				0	2				2
1/10000	1	7	4	—	2	0	8	8	—	4	4
1/10000	6	8		8	4	0	7				7
1/10000	6	10	—	6	3	3	8	—	5		5
1/10000	7	4	2	—	1	0	4	4	—	1	0
1/10000	7	9	—	5		5[d]	8	—	6		6
1/10000	7	9	—	4		4	10	—	4		4
1/10000	7	5	3			3	6	3	—	—	2
1/10000	7	7		6	2	0	10				10
Means		5.6	4.8	3.7	2.0	1.5	7.6	7.0	6.4	5.4	5.1

[a] All experimental and control tests started with 10 embryos.
[b] Dashes indicate no observations at this stage.
[c] Blank spaces indicate no change in number of survivors.
[d] Embryos hatched and died.

blue[1] in aquarium water ranged from 1/1000 to 1/10000. Seventeen groups of embryos were treated in the various solutions, eight being treated in solutions containing 1/10000 methylene blue and four being treated in solutions containing 1/1000 methylene blue. Survival rates (table 8) were somewhat better than in aquarium water, although they were erratic. The mean survival was 1.5 embryos. All of the embryos, however, exhibited anomalies. Many, after hatching, showed abnormalities in mouth and gill action. The mouth remained open continuously and the usual rhythmic respiratory movements were not seen. Pericardial edema was prevalent. It was thought that the solutions might be toxic to embryos after long immersions. However, transferring the embryos after stage 19 to aquarium water did not mitigate any of the abnormalities, nor did it enhance survival rates.

[1] Methylene blue, National Aniline, C. I. No. 922, 85 per cent dye content.

Methylene blue, aside from being mildly germicidal, is also a vital dye and a mediator in biological oxidations (Barron, 1939). It was possible that these properties and not the bactericidal properties might have been instrumental in maintaining the embryos. Janus Green,[2] a vital dye, and cytochrome c,[3] a biological mediator, were tested. Three groups were treated in solutions of aquarium water containing 1/1000, 1/2000, and 1/10000 Janus Green B, and four groups were immersed in solutions of aquarium water containing 4/100000 or 8/1000000 of cytochrome c. The results were negative. The embryos died in Janus Green prior to stage 15. Many stopped developing shortly after immersion. In cytochrome c the embryos were dead by stage 19, following the same pattern as embryos in aquarium water.

[2] Janus Green B, National Aniline, C. I. No. 133, 54 per cent dye content.
[3] Supplied by Dr. M. J. Kopac of New York University.

TABLE 9

SURVIVAL OF EMBRYOS IN AQUARIUM WATER IMMERSED ONCE A
DAY FOR A 10-MINUTE PERIOD IN SEA WATER

Stage Intro- duced	No. Surviving to Various Stages After Immersion in Sea Water					No. Surviving to Various Stages Immersed in Aquarium Water (Control)					No. Surviving to Stage 24 in 40 Per Cent Sea Water (Control)
	15	17	19	21	24	15	17	19	21	24	
1	10[a]	7	4	1	1	10	3	0		0	0
3	7	7	5	2	0	8	3	0		0	10
3	7	4	[b]	3	3	6	2	0		0	1
4	9	8	5	5	0	8	6	0		0	0
5	1	0			0	0				0	5
6	8	7	4	0	0	10	7	4	0	0	4
6	9	8	6	1	0	3	0			0	5
6	8	6	4	3	0	6	5	2	0	0	8
7	10	9	8	8	0	7	4	3	0	0	8
10	10	9	7	4	0	7	4	0		0	3
Means	7.9	6.5	4.7	2.7	0.4	6.5	3.4	0.9	0	0	4.4

[a] All experimental and control tests started with 10 embryos.

[b] Blank spaces indicate no change in number of survivors.

In many of the experiments up to this time abundant growths of fungus were found on the embryos. In five instances fungus had established itself on embryonic chorions as early as stage 14, but the embryos did not die until stage 19. Several embryos in this stage exhibited muscular twitches while a fungus mycelium was growing on the chorions. These observations suggested that fungus might be destroying the embryos. A number of methods for fungus control were therefore tested.

Sodium propionate,[1] a mold inhibitor, is used extensively by the baking industry. Three groups were treated in solutions of aquarium water containing 1/100, 2/1000, and 1/5000 sodium propionate. The embryos died in this medium, and a granular fungus growth, morphologically different from the usual growth, was seen on the chorions.

SURVIVAL OF EMBRYOS DIPPED
INTO SEA WATER

Since fungus was never observed in the sea-water dilutions, an experiment was designed whereby the embryos were dipped into sea

[1] DuPont, "Mycoban," A0-1150, courtesy of E. I. du Pont de Nemours and Company.

water. Ten groups, reared in aquarium water, were immersed once per day for a 10-minute period into 100 per cent sea water. Fresh sea water for the dip was used at the beginning of each experiment. At the end of the 10-minute period the embryos were rinsed in aquarium water and were then returned to the original bowl of aquarium water. Special control embryos were handled in similar fashion, except that aquarium water was substituted for the sea-water dip. The results are summarized in table 9. In spite of the fact that survival to hatching was negligible (mean survival was 0.4 embryo), it was significant that more embryos dipped into sea water survived to later stages of development than the corresponding controls. Indications are that the ultimate high mortality rate may have been due to a physiological disturbance of the embryos as they were plunged into solutions having markedly different osmotic pressures.

SURVIVAL OF EMBRYOS DIPPED INTO A
FORMALDEHYDE SOLUTION

In order to eliminate possible osmotic factors, a very dilute formaldehyde solution was tried, since formalin dipping as a fungus control method is successfully used by the fish

hatcheries (Watanabe, 1940; Nakamura, 1948; Burrows, 1949). Ten groups of embryos, reared in aquarium water, were immersed once per day for a 10-minute period in a special aquarium water solution containing 1/1000 formaldehyde. This solution was prepared anew each day. Embryos were rinsed in aquarium water, prior to being returned to the original medium. A similar procedure was followed for an additional group

SURVIVAL OF EMBRYOS IN STERILE AQUARIUM WATER

Since the formaldehyde dip sterilized the embryos, an attempt was made to rear them in sterile aquarium water. Water taken from *Tilapia* stock tanks was sterilized at 15 pounds pressure for 20 minutes. Fifty cubic centimeters of this water was placed into sterile, 8.5-cm., glass-covered storage dishes.

TABLE 10

SURVIVAL OF EMBRYOS IN AQUARIUM WATER IMMERSED ONCE A DAY FOR A 10-MINUTE
PERIOD IN A 1/1000 SOLUTION OF FORMALDEHYDE

Stage Intro- duced	No. Surviving to Various Stages After Immersion in Formaldehyde Solution					No. Surviving to Various Stages Immersed in Aquarium Water (Control)					No. Surviving to Stage 24 in 40 Per Cent Sea Water (Control)
	15	17	19	21	24	15	17	19	21	24	
4	10[a]	8	[b]		8	0				0	9
4	10		9		9	0				0	9
6	5	1	0		0	0				0	9
7	8				8	5	2	0		0	5
7	10	8			8	6	3	0		0	9
7	8[c]	7	6		6	3	3	0		0	10
7	9[d]	7	4	3	3	3	0			0	10
8	8[c]	8	7		7	1	0			0	9
10	10	9			9	7	5	4	0	0	8
10	10				10	7	5	3	0	0	10
Means	*8.8*	*7.6*	*6.9*	*6.8*	*6.8*	*3.2*	*1.8*	*0.7*	*0*	*0*	*8.8*

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in number of survivors.
[c] Two out of 10 embryos accidentally destroyed during dipping before stage 15.
[d] One out of 10 embryos accidentally destroyed during dipping before stage 15.

of controls, where aquarium water was substituted for the formaldehyde dip. The results summarized in table 10 demonstrate the success of the technique. A mean survival at hatching of 6.8 embryos was obtained.

Immediately after being dipped into 1/1000 formaldehyde several embryos were placed on plates containing nutrient agar or corn meal agar. No bacterial growths were noted on the nutrient agar plate and no fungus growths were observed on the corn meal agar plate, indicating that the formaldehyde solutions were both bactericidal and fungicidal.

Embryos, prior to being immersed in the sterile medium, were dipped into a 1/1000 solution of formaldehyde for 10 minutes. They were transferred with sterile pipettes into the experimental medium and remained in it until they hatched. To avoid contamination, dead embryos were not removed. The striking results of this technique are summarized in table 11. The mean survival at hatching was 8.2 embryos, just 0.3 embryo lower than the mean survival in 40 per cent sea water. The embryos remained in the sterile medium until their yolk sacs were absorbed. After they finally died of starvation

TABLE 11
Survival of Embryos in Sterile Aquarium Water

Stage Introduced	No. Surviving to Various Stages in Sterile Aquarium Water					No. Surviving to Various Stages in Untreated Aquarium Water (Control)					No. Surviving to Stage 24 in 40 Per Cent Sea Water (Control)
	15	17	19	21	24	15	17	19	21	24	
2	8[a]	7	[b]		7	X[c]					8
3	10	9			9	7	4	1	0	0	10
3	10				10	5	0			0	10
3	10				10	10	6	3	0	0	10
3	8	7	—[d]	6	6	X					6
5	6				6	0				0	8
5	10				10	4	0			0	10
6	10	—	9		9	X					8
7	8	6	5		5	8	5	0		0	6
7	10				10	X					10
8	10				10	X					10
8	10				10	X					10
8	8	7	6		6	X					10
8	9	—	—	8	7	X					8
8	10	7	6		6	X					10
8	7	6			6	X					6
8	10				10	X					10
8	10	9	8		8	X					9
9	9				9	6	3	0		0	10
10	10				10	9	7	2	0	0	10
Means	9.2	8.6	8.4	8.3	8.2	7.0	3.6	0.9	0	0	9.0

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in the number of survivors.
[c] X indicates no tests performed.
[d] Dashes indicate no observations at this stage.

the fry did not disintegrate for two months, at which time they were discarded.

SURVIVAL IN STERILE AQUARIUM WATER PLUS CULTURE OF FUNGUS

Since sterile aquarium water kept fungus and bacteria growths to a minimum, it seemed desirable to determine which agent or agents actually killed the embryos. Experiments were designed to answer this question.

A culture medium was prepared with 950 milligrams of Bacto-corn meal agar and 2.2 grams of Bacto-agar in 100 cc. of distilled water. The solution was autoclaved and poured into Petri dishes. After the medium hardened, embryonic chorions were placed on the corn meal agar plates. Three days later, bacteria appeared around the chorions.

About one week later, fungus[1] appeared at the periphery of the bacterial colonies. The fungus was serially transferred to several plates until the fungus culture had uniform morphological characteristics.

In the first experiment a small piece of agar about ½-inch square containing the fungus culture was introduced into sterile aquarium water. In addition a piece of sterile corn meal agar about ¼ inch square was added to

[1] The fungus was identified by Dr. Helen Simpson Vishniac as belonging to the Fungi Imperfecti, Order Moniliales. It was tentatively assigned to either the genus *Dactylium* of the family Moniliaceae or *Fusarium* of the family Tuberculariaceae. Sexual reproductive structures are necessary characteristics for complete identification. Unfortunately this fungus did not reproduce sexually on the agar plates.

the medium on the opposite side of the bowl. Five groups of embryos, previously dipped in a 1/1000 solution of formaldehyde for 10 minutes, were introduced into these media. To avoid contamination dead embryos were not removed. A summary of the results is presented in table 12. The mean survival of the five groups at hatching was 7.8 embryos. When the piece of sterile agar was removed from the medium at the termination of the water). Colonies appeared in about six hours, and they were serially transferred on nutrient agar plates until colonies of uniform appearance were established. The culture was kept in nutrient agar at 5° C. as a stock culture.

At the beginning of each experiment a bacterial smear was introduced into nutrient broth. On the following day a loopful of the broth was placed in 0.5 cc. of sterile distilled water. This was then introduced into

TABLE 12

SURVIVAL OF EMBRYOS IN STERILE AQUARIUM WATER PLUS PURE CULTURE OF FUNGUS

Stage Intro- duced	No. Surviving to Various Stages in Sterile Aquarium Water Plus Pure Culture of Fungus					No. Surviving to Various Stages in Sterile Aquarium Water (Control)					No. Surviving to Stage 24 in 40 Per Cent Sea Water (Control)
	15	17	19	21	24	15	17	19	21	24	
2	10[a]	8	[b]		8	8	7			7	8
8	10				10	10				10	8
8	10	9	—[c]	—	8	10	9	—	—	8	6
8	7	6			6	9	—	—	8	7	8
8	9	8	7		7	10	7	6		6	9
Means	9.2	8.2	8.0	8.0	7.8	9.4	8.4	8.2	8.0	7.6	7.8

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in number of survivors.
[c] Dashes indicate no observations at this stage.

experiment and was placed on a corn meal agar plate, a fungus mycelium appeared. This indicated that the fungus was capable of dispersing through the solution and that the fungus was not able to attack and kill the embryos directly. No fungus growth was ever noted on the embryos.

SURVIVAL IN STERILE AQUARIUM WATER PLUS CULTURE OF BACTERIA

Chorions and embryos were placed in test tubes containing sterile nutrient broth (8 grams of Bacto-nutrient broth in 1000 cc. of distilled water). One day later a loopful of the broth containing bacteria[1] was transferred to a nutrient agar culture medium (23 grams of Bacto-nutrient agar in 1000 cc. of distilled

the sterile medium. Five groups of embryos, which had been immersed in a 1/1000 formaldehyde solution for 10 minutes, were placed in these bacteria-contaminated media. The results are summarized in table 13. No embryos survived to stage 15,[2] whereas the controls in sterile water had many survivors.

Four groups of embryos were treated in 40 per cent sea water containing a loopful of the

obtained in maltose, tryptone, and Koser citrate media and in plates containing casein. Starch plates were hydrolyzed. In the methylene blue reductage test, the bacteria reduced the methylene blue in six hours, and the casein was completely digested in 48 hours. The M.R.V.P. test produced split results; the methyl red was negative, but the bacteria were positive for acetyl methyl carbinol. Nitrates and lead acetate gave positive results. The bacteria were facultative anaerobes, gram-negative, rod-shaped, and motile. They were tentatively identified as a species of *Pseudomonas*, which are pathogenic.

[2] When the embryos were placed on nutrient agar plates, bacterial colonies grew around the embryos.

[1] Biochemical tests on the bacteria culture were performed through the courtesy of Dr. Ross Nigrelli. He found that the bacteria were proteolytic, fermented saccharose, and mildly fermented lactose. They were unable to deaminate amino acids. Negative results were

TABLE 13

SURVIVAL OF EMBRYOS IN STERILE AQUARIUM WATER PLUS PURE CULTURE OF BACTERIA

Stage Intro-duced	No. Surviving to Various Stages in Sterile Aquarium Water Plus Pure Culture of Bacteria					No. Surviving to Various Stages in Sterile Aquarium Water (Control)					No Surviving to Stage 24 in 40 Per Cent Sea Water (Control)
	15	17	19	21	24	15	17	19	21	24	
3	0[a]				0	10	[b]			9	10
8	0				0	10				10	10
8	0				0	8	7	6		6	10
8	0				0	10	7	6		6	10
8	0				0	10				10	10
Means	0				0	9.6	8.8	8.4		8.2	10

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in the number of survivors.

bacterial culture. All of the embryos survived, indicating that the sea water was bactericidal.

INVESTIGATION OF MECHANICAL FACTORS

SURVIVAL OF EMBRYOS CHURNED IN CIRCULATING WATER

In the oral cavity, the embryos are gently and continuously churned by the respiratory movements of the adult fish. The churning and the large amount of water flowing over the embryos were thought to be beneficial to embryonic survival. An experiment was therefore designed to simulate these conditions.

Two aquaria serving as reservoir tanks were utilized. Water in which the parents of the embryos had originally spawned was transferred to the reservoir aquaria in each experiment. The lower tank held a test tube containing the embryos. From the upper tank a jet of water was siphoned onto the embryos, mildly churning them. Water passed through the jet at the rate of 40 cc. per minute. It was then pumped by an all-rubber pump into the upper reservoir tank. Overflow from the upper tank fell through a glass tube into the lower tank, thus maintaining a constant level in both tanks. A special control group consisting of 10 embryos in a finger bowl was placed on the bottom of the lower reservoir tank in relatively non-moving water (fig. 3).

A significant number of survivors were found in 15 out of 17 separate tests (table 14). The mean survival at hatching for the entire experiment was 36 per cent. None of the special control embryos (same aquarium water but non-circulating) survived beyond stage 19. When the bowl containing the control embryos was elevated closer to the surface of the water no increase in survival occurred.

It was apparent at the conclusion of the experiment that this technique was partially effective in maintaining the embryos. However, the apparatus not only mechanically churned the embryos, but circulated a large volume of water as well. This suggested that in addition to the churning, other factors might have been responsible for embryonic survival: the large amount of aquarium water might have removed nitrogenous waste products or other toxic substances; the large quantity of water might have maintained the embryos by increasing the oxygen supply, or by increasing the availability of some other requirement.

SURVIVAL IN AQUARIUM WATER WITH ACTIVATED CHARCOAL

To test the first possibility, 0.8 gram, or 1.5 grams, of activated coconut charcoal[1] were added to stationary aquarium water to remove ammonia (McPherson et al., 1940), which might be deleterious to the embryos.

[1] Eimer and Amend, 6–14 mesh.
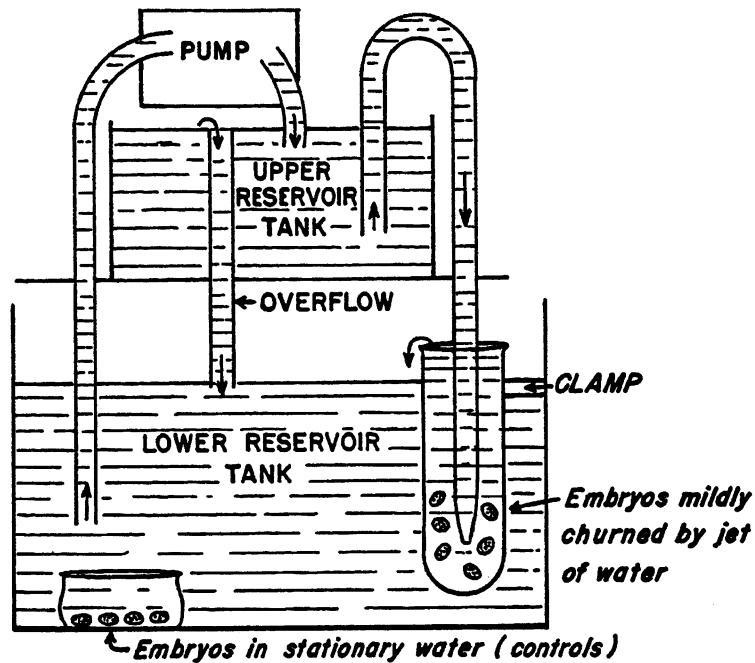
✗ Fig. 3. Apparatus used to test the effects of circulating
water over the embryos.

TABLE 14

SURVIVAL OF EMBRYOS IN CIRCULATING AQUARIUM WATER[a]

Stage Introduced	Initial No. of Embryos per Experiment	Per Cent of Experimental Survivors at Hatching (Stage 24)	Per Cent of Control Survivors at Hatching (Stage 24)[b]	
			In 40 Per Cent Sea Water	In Aquarium Water
1	85	0	70	0
2	50	30	100	0
2	40	35	100	0
2	35	54	0	0
2	85	17	90	0
2	72	39	50	0
2	90	0	60	0
5	70	57	80	0
5	70	40	40	0
5	55	60	90	0
5	40	26	0	0
6	65	53	90	0
7	40	68	70	0
7	70	27	90	0
7	70	71	70	0
7	40	25	100	0
10	50	30	100	0

[a] See figure 3 for diagram of apparatus.
[b] Controls in aquarium water and in 40 per cent sea water started with 10 embryos per bowl and were not churned.
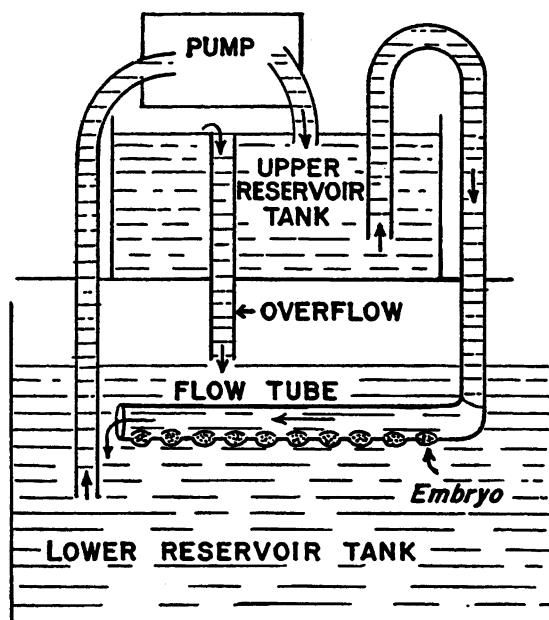
✕ FIG. 4. Apparatus used to test the effects of water flowing over stationary embryos.

TABLE 15

SURVIVAL OF EMBRYOS IN FLOW TUBE[a]

Stage Introduced	Per Cent of Survivors in Flow Tube to Stage 19	Per Cent of Survivors in 40 Per Cent Sea Water (Control) to Stage 24
2	0[b]	40
3	0	30
5	0	90
5	0	0
6	0	100
6	0	90
7	0	100
7	0	80
7	0	100
7	0	70
10	0	90

[a] See figure 4 for diagram of apparatus.

[b] Each control and experimental test started with 10 embryos.

A total of five groups were tested in these media. No embryos survived beyond stage 17. The controls in 40 per cent sea water and charcoal yielded a mean survival of 7.0 embryos at hatching.

SURVIVAL IN A FLOW TUBE

The second possibility was tested by means of a flow tube which was designed to permit a large quantity of water to flow over the embryos while they remained stationary. The tanks and pump of the previous experiment were similarly used. A series of 10 depressions, about 5 mm. apart, were blown in a glass tube 12 mm. in diameter. Each depression was approximately 3 mm. in depth. For rapid reference, the tube is called the flow tube (fig. 4). One embryo was placed in each depression of the flow tube by means of an elongate capillary pipette. The tube was placed in the lower reservoir tank, and a stream of water was very slowly siphoned into the tube from the upper reservoir tank. To prevent shifting the embryos out of the depressions the stream was regulated very carefully. Gradually the stream was increased until 40 cc. per minute flowed over the non-moving embryos.

In spite of the fact that the water flowed over the embryos at the same rate as in the previous churning experiment, no embryos in 11 groups survived beyond stage 19 (table 15). Luxuriant fungus growths appeared on the part of the embryos which were in juxtaposition with the glass tube, whereas no fungus appeared on that part of the embryo which was in constant contact with the flowing water.

SURVIVAL OF EMBRYOS AGITATED IN NON-CIRCULATING AQUARIUM WATER

A 500-cc., round-bottom flask was attached to a vertical shaking board similar to the board in the standard Van Slyke apparatus. The flask contained 200 cc. of aquarium water which was changed daily. Eight groups of embryos were tested under one series of conditions, and 17 groups were tested under a second series of conditions. In the first series the board moved through an arc of 10 degrees 90 times per minute. The apparatus was located in the greenhouse, and the flask containing the embryos was exposed all day to direct sunlight. Embryos remained in the flask until they hatched or died. Five out of eight groups yielded a number of survivors (table 16). The mean survival of the groups at hatching was 22 per cent. There was a

TABLE 16

NUMBER OF SURVIVORS IN SHAKER

Stage Introduced	Per Cent of Survivors in Shaker to Stage 24	Per Cent of Survivors in 40 Per Cent Sea Water (Control) to Stage 24
5	10[a]	100
5	60	100
7	0	80
7	90	100
7	0	100
8	0	10
10	10	90
10	40	90

[a] All experimental and control tests started with 10 embryos.

tendency for the embryos to hatch prematurely, suggesting that the agitation was causing a mechanical breakdown of the chorions.

In the second series, the board moved through an arc of 15 degrees 126 times per minute. The flasks were completely enclosed in a wooden box to protect the embryos from excessive sunlight. Control groups in 40 per cent sea water were subjected to similar conditions of agitation and light. In 14 out of 17 experimental groups (table 17) there were a number of survivors (mean survival at hatching was 58%). Breakdown of the chorion was noted in the experimental groups but it was never observed in the control groups, indicating that agitation did not cause the premature hatching.

INVESTIGATION OF ORAL CAVITY

ANATOMY OF ORAL CAVITY

The oral epithelium in the anterior part of the palate and on the surface of the lower jaw is smooth and grayish white, containing a few scattered macromelanophores. The floor of the mouth contains cartilages which serve as supporting rods for the branchiostegal membrane. This membrane expands to house the

TABLE 17

NUMBER OF SURVIVORS IN SHAKER (126 TIMES PER MINUTE)

Stage Introduced	No. Surviving to Various Stages in Shaker					No. Surviving to Stage 24 in 40 Per Cent Sea Water (Control)
	15	17	19	21	24	
5	0[a]	[b]			0	8
7	10				10	10
8	—[c]	0			0	6
8	10	—	—	—	8	10
9	10	—	9	—	4	5
9	10	—		—	6	8
9	10				9	10
9	10	—	—	—	2	10
9	10				10	10
10	10	—	—	—	3	10
10	—	—	0	—	0	7
10	9				9	10
11	10			9	8	5
11	10	6	—	2	2	5
11	10				10	10
11	10	9			9	10
12	10				10	9
Means	8.2	7.9	7.8	7.5	5.9	8.4

[a] All experimental and control tests started with 10 embryos.
[b] Blank spaces indicate no change in the number of survivors.
[c] Dashes indicate no observations at this stage.

incubating embryos. Posteriorly, a pair of unusual pharyngeal glands is found on the dorsal surface of the palate (pl. 47, fig. 2). They are attached to the pharyngobranchial cartilages of the first three gill arches and extend anteriorly and ventrad. The glands are situated lateral to the superior pharyngeal teeth and are connected by a median isthmus. The lateral parts of the glands are rugose; the more medial regions are smooth. Many small, highly refractive globules, not found in any other part of the epithelium, are visible throughout the glandular area. The glands are about 7 mm. wide (medial-lateral) and about 4 mm. long (anterior-posterior) in adults that measure 9 or 10 cm. in length.

A study of the pharyngeal glands in younger, smaller individuals revealed that they were not present in fishes smaller than 3.0 cm. In fishes ranging in size between 3.0 and 3.5 cm. refractive globules were noted, but the rugose part of the gland was absent. Fishes between 3.5 cm. and 4.0 cm. sometimes had the rugose area. The rugose region of the gland was invariably present in males and females longer than 4.0 cm.

The oral cavity of two other *Tilapia* species was examined. In *Tilapia sparrmani* and *T. zilli*, which are not oral incubators, no glands were present, but in a small region around the pharyngeal teeth, many refractive globules were noted. *Tilapia ovalis*, an oral incubator, displayed a large area of globules. Small pharyngeal glands were present, but the rugose area was not so well developed as in *Tilapia macrocephala*.

An entire head was serially sectioned at 6 μ and 10 μ and stained with Harris' hematoxylin and eosin or with a Masson trichrome stain. In addition pharyngeal glands were removed from five adult males and two adult females and stained with Harris' hematoxylin plus eosin and the Masson trichrome stain.

Basically, the pharyngeal glands and the oral epithelium consist of similar cells, namely, stratified squamous epithelium and mucous cells. In the general oral epithelium, the stratified squamous epithelium varies in depth from three to four cell layers in some areas to seven and eight cell layers in other areas. The mucous cells, many of which contain two nuclei, are ovoid to slightly columnar in shape and are scattered among the

stratified epithelial cells. In a few areas the mucous cells are found in patches.

In the epithelium of the pharyngeal glands the secretory cells are tall, columnar, and are very closely packed together (pl. 47, fig. 3). They are wedge-shaped in one plane, and the nuclei are crowded to one basal corner. Frequently two nuclei are found. Three distinct layers can be recognized in the epithelium of the pharyngeal gland, namely, a surface layer of stratified epithelium two to three cells in depth, then the compact columnar secretory cells, and thirdly a deep layer of stratified epithelium, approximately four cells in depth, which lies against a basement membrane. Adipose tissue is found beneath the basement membrane.

CAUTERY OF PHARYNGEAL GLANDS

Eight breeding pairs, which had good spawning records and which had consistently high brood survival rates for a period of two months, were selected for this study. The males were anesthetized in urethane, and the pharyngeal glands were cauterized with an electric cautery needle. To accomplish this, the operculum was lifted and held open with one prong of a pair of forceps while the other prong kept the gills flat against the body. The cautery was inserted until it touched the pharyngeal glands. The males were placed in a 2-gallon tank containing a 0.5 per cent salt solution for one week to facilitate recovery and were then returned to their respective aquaria. For two months the number in each brood was counted 10 days after fertilization, and the frequency of spawnings was checked. At the termination of the experiment the gland region was grossly inspected for possible regeneration of tissue. Four pairs of adults served as controls. In these the anterior-dorsal portion of the males' oral epithelium was cauterized.

A summary of the results is seen in table 18. The pre-operative groups of experimental animals had a mean of 37.7 embryos per brood after 10 days. After cautery the mean decreased to 26.1. The postoperative control males also showed a large decrease in brood size, indicating that the trauma of the operation may have caused the decrease in the numbers in each brood. It is of interest to note that among the experimental pairs, the

TABLE 18

CAUTERY OF PHARYNGEAL GLANDS IN MALES

| | Adult No. | Pre-operative | | Postoperative | | |
		No. of Spawnings	Mean No. of Embryos per Brood	No. of Spawnings	No. of Times Female Picked Up Eggs	Mean No. of Embryos per Brood
Experimentals						
	1	3	21	4	1	33
	2	5	40	3	0	55
	3	3	26	4	2	16
	4	3	39	1	0	0
	5	5	26	4	1	44
	6	3	61	1	1	0
	7	4	35	1	0	36
	8	5	51	3	1	25
Totals	8	31	299	21	6	209
Means		3.87	37.7	2.62	0.75	26.1
Controls[a]						
	1	2	54	2	0	27
	2	3	57	5	0	56
	3	5	63	0	0	0
	4	3	27	2	0	14
Totals	4	13	201	9	0	97
Means		3.25	52.5	2.25		24.3

[a] The antero-dorsal region of the oral epithelium was cauterized in the postoperative control males.

females picked up the eggs with much greater frequency than usually found under normal circumstances (Aronson, 1949).

The results of these experiments did not indicate that pharyngeal glands were instrumental in maintaining embryos in the mouth. However, these experiments are not considered critical, since the histological study showed that the gland is rather diffuse, while the cautery removed the rugose area only.

It was thought that the oral epithelium might secrete a protective coat of mucus found over the eggs and that this mucus might have bactericidal properties.

Therefore an attempt was now made to rear embryos in fish mucus and in gastric mucin in aquarium water. Three groups were immersed in solutions containing 50 cc. of aquarium water and body mucus scraped from two or three fish. The results were unsuccessful, and the embryos died prior to stage 19. The solutions of fish mucus became murky and foul-smelling in spite of the fact that they were changed daily. Of three groups of embryos placed in gastric mucin,[1] none survived. It was impossible to determine clearly whether or not the gastric mucin had gone into solution. Filmy white material was always present in the aquarium water even in the smallest concentrations of gastric mucin.

EFFECTS OF MUCOUS SMEARS ON
BACTERIAL PLATES

Colonies of the bacteria previously discussed were uniformly spread on nutrient agar plates as follows: A loopful of bacteria in nutrient broth was placed in 0.5 cc. of distilled sterile water. The mixture was poured into a sterile Petri dish. Liquid nutrient agar was poured onto the bacteria, and the bacteria were distributed uniformly over the

[1] Armour Company, No. 880309.

plate. A dull, grayish-looking mucus was scraped from the mouths of adult males and smeared onto the plates. Five smears were placed on each of 10 plates. In addition, body mucus was smeared onto two of the plates. Controls consisted of small pieces of sterile glass cover slips or of small slivers of paraffin.

The bacterial colonies at the end of eight hours were scattered over the surface of the plate. When each mucous smear was removed from the plate, no colonies were found growing in that area. However, the controls also inhibited growth of the colonies, leaving a clear region in the agar plate. At 12 hours, a few colonies were found in the deeper part of the agar beneath the smears, but no colonies were found directly underneath the mucous smears or directly below the glass or paraffin slivers of the controls. Hence these tests were not decisive.

DISCUSSION

ORAL INCUBATION IN *Tilapia macrocephala*, aside from protecting the embryos from predators, is shown to have an important additional function in embryonic survival. The experiments, as they developed, led to the conclusion that 40 per cent sea water, the most successful medium for extra-oral survival, was bactericidal to particular bacteria which attacked the embryos. It also became apparent that in most of the other experimental media counteracting influences were at work, namely, the bactericidal (or bacteriostatic) properties of the medium used and its toxic properties. This is advanced as the explanation for the failure or only partial success of numerous other substances tested. The bactericidal properties of salts are well known. Their use as a food preservative dates back to at least 1800 B.C. (MacCurdy, 1924), and there is evidence that it was used in prehistoric times (Tressler and Lemon, 1951). It is not surprising therefore that sea water protected the embryos against bacterial infection.

The fact that several of the other salt combinations tested were unsuccessful in maintaining the embryos is probably a reflection of the toxicity of these salt solutions. Loeb (1912) treated *Fundulus* embryos with a ⅝ molar solution of NaCl and found that after several days of treatment, the embryos were dwarfed and pale and the pericardium was usually puffy and prominent. Solutions of CaCl₂, ⅛ molar, proved to be highly toxic. Our finding that artificial sea water will not sustain *Tilapia* embryos indicates the presence of a toxic factor. On the other hand, it is well known that in addition to the complex of inorganic ions, sea water contains small quantities of a large number of organic compounds (dissolved, colloidal, and minute particles). These are not present in synthetic preparations. Hence we may advance an alternative hypothesis that the effectiveness of natural sea water in maintaining *Tilapia* embryos results from the presence of an organic substance having antibiotic properties. This difference in the effectiveness of natural and artificial sea water is by no means unique, for it is common knowledge among marine biologists that artificial sea water is not an adequate substitute for natural sea water for the maintenance of many forms of marine life. ZoBell (1946) emphasizes the existence of thermolabile organic substances in sea water which are bacteriostatic. ZoBell also observes that coastal sea water which is susceptible to terrigenous contamination may also contain bacteriophage active against various bacteria.

Several other substances having bactericidal (or bacteriostatic) properties were also tested. Aureomycin definitely seemed to disrupt development. Methylene blue, a mild bactericide (Mitchell, 1904; Marshall, 1920), yielded rather interesting results. In several of the experimental tests, many embryos survived to hatching and then suddenly died. The results of this experiment indicate that methylene blue was successful in keeping the harmful bacteria to a minimum but was eventually injurious to the embryos, particularly about the time of hatching. Even when the embryos were transferred from methylene blue into aquarium water after stage 19, or just prior to hatching, survival rates could not be increased.

Lysozyme which has antibacterial properties (Thompson, 1940; Feiner, Meyer, and Steinberg, 1946) and is found in nasal secretions, such as tears and saliva (Fleming, 1922), did not appear to damage the embryos, although it was unsuccessful in maintaining them. This suggests the possibility that the various bactericidal solutions, in addition to their various toxic properties, may not be sufficiently bactericidal to the particular species that attacks the *Tilapia* eggs.

Methylene blue is a vital dye and a mediator in biological oxidations (Barron, 1939). It was thought that this property might be instrumental in embryonic survival, since embryos treated with this substance developed further than the controls in aquarium water. However, tests with Janus Green B and cytochrome c were negative and did not support this proposition.

In the observations on embryos reared in aquarium water, it was noted that fungus grew in abundance on the chorions until the time of death, usually by stage 19. Some embryos exhibited muscular twitches even when there was a prolific growth of fungus on their

chorions, thus showing that the fungus attacked living embryos. However, biologists generally consider bacteria to be the primary invader and fungi a secondary invader. In this connection it should be noted that substances that are fungicidal are usually bactericidal.

Fungus infection has been an important problem in fish hatcheries, and methods of controlling this disease have been widely studied (Foster, 1936; Fish, 1938; O'Donnell, 1947; Nakamura, 1948; Burrows, 1949; Hoffman, 1949; Sharp, Bennett, and Sacugling, 1952). Formalin dipping has been developed by the fish hatchery investigators as an adequate fungus control method for salmon and trout (Watanabe, 1940; Burrows, 1949). It is interesting to note that Watanabe found that a weekly dip of salmon in 0.5 per cent formalin was also effective in combating a soft egg disease, which he states is caused by bacteria. Formaldehyde itself is highly toxic, but by diluting it sufficiently and by utilizing the technique of brief exposures, it was possible to eliminate its toxic effects to the embryos and at the same time to retain its bactericidal effectiveness. Our results with formalin were striking. Exposing the embryos to a 1/1000 solution of formaldehyde for only 10 minutes per day was almost as effective for embryonic survival as rearing them in 40 per cent sea water. This was an outstanding improvement over all other media previously tested. When embryos that had been previously dipped into the formalin solution were placed onto nutrient agar plates and corn meal agar plates, no bacterial or fungus growths were seen. This pointed the way to a series of critical experiments. Raising embryos in previously sterilized aquarium water was very effective, and this provided the approach to the problem of separating the effect of the fungus from that of the bacteria. The fact that all of the embryos died in water that had been sterilized and then inoculated with bacteria isolated from chorions is conclusive evidence that the bacteria were killing the embryos. This bacterium was found to be Gram-negative, rod-shaped, motile, and proteolytic, probably a species of *Pseudomonas*. Hence it is capable of attacking the chorions. Conversely, when the embryos were reared in sterile aquarium water inoculated with fun-

gus isolated from chorions, survival rates were high. This demonstrated that the fungus was a secondary invader.

The embryos reared in sterile aquarium water were not discarded after hatching. However, they were not fed and eventually died of starvation. Even after a period of two months, intact corpses were noted in the sterile media. Since *Tilapia* can be maintained solely on a diet of cooked food which can be readily sterilized, the above finding suggests a far easier technique for germ-free studies on fish than the method previously developed by Baker and Ferguson (1942). The technique of raising sterile embryos might also be of pertinent interest to fish hatchery investigators.

That the extra-oral mortality was due to bacterial infection and that our early success with sea water was due to its bactericidal properties having been shown, it was of interest to determine in what manner the process of oral incubation protected the embryos from the bacteria. Three particularly noteworthy features of oral incubation were investigated for their possible bactericidal properties: the slow but continuous churning of the embryo due to the respiratory movements of the mouth; the thin coating of mucus over the embryos and the presence of mucous cells in the oral epithelium; and the presence of prominent pharyngeal glands in the oral cavity.

It was shown that the survival in circulating aquarium water was due to the churning of the embryos in the water rather than to the quantity of water that flowed over them. Although the mean per cent of embryonic survival was increased when the embryos were agitated in non-circulating water, it was felt that the optimum mode of agitation was not achieved, since there were indications of mechanical injury to the embryos. The outstanding fact that the embryos did survive when agitated and did not survive in the flow tube indicated that the mechanical agitation was partially effective in keeping the bacterial growth to a minimum. An interesting comparison is the use of a ball mill as a standard technique for grinding bacteria. Also of interest is the fact that Gram-negative bacteria are more susceptible to mechanical injury than Gram-positive bacteria (Clifton, 1950).

Although the standard bacteriological tests that we employed were inadequate for a study of the bactericidal properties of oral mucus, it is felt that this should not be dismissed as a factor aiding the survival of the embryos. It is possible that the thin coating of mucous over the embryos mechanically protects them from bacterial invasion. In addition, the mucous may also be an active bactericide.

Special pharyngeal glands were discovered in the mouth of *Tilapia macrocephala*. Such glands have been previously described by Pellegrin (1903) in mouthbreeding species of *Pelmatochromis* and *Geophagus*. Pellegrin suggested that the glands may be related to oral incubation. Our findings that such glands are not present in the immature fish, are not found in the closely related non-mouthbreeding species *Tilapia sparrmani*, and are not found in other species of non-mouthbreeding cichlids support Pellegrin's view. On the other hand, the experiment designed to test this hypothesis yielded ambiguous results. Survival decreased equally in both experimental and control animals. It has been shown by histological examination that the boundaries of these glands are not sharply delineated, and it is possible that in the cautery experiments a sufficient quantity of glandular tissue remained to protect the embryos under the particular conditions of our aquaria. Thistlethwaite (1947) describes special modifications in the oral epithelium in the form of crypts in the mouthbreeding catfish, *Galeichthys felis*, which reach their height during incubation of the embryos.

If we accept the hypothesis of Breder (1933) and Myers (1938) that mouthbreeders evolve from non-mouthbreeding species which have elaborate patterns of parental care, one might conclude that with the genesis of mouthbreeding in *Tilapia macrocephala* the embryos, developing in a highly protected environment, lost their ability to survive extra-orally and became secondarily dependent upon this new environment. Conversely, one might view mouthbreeding in this species as a pre-adaptation to a new ecological niche in which protection against bacteria was essential to survival of the embryos. However, preliminary experiments and those now in progress show that, in the non-mouthbreeding *Tilapia sparrmani* and in certain other non-mouthbreeding cichlids, the embryos do not survive when the parents are removed. It is likely that even in these species protection against bacterial infection is necessary. Hence, as a third alternative hypothesis, mouthbreeding in *Tilapia* may be viewed as an adaptation for protection against predators, and any special adaptation, such as the pharyngeal glands, may be thought of as a new method for protecting the embryos against bacteria necessitated by the changed pattern of parental care.

SUMMARY AND CONCLUSIONS

EMBRYOS OF THE mouthbreeding cichlid fish *Tilapia macrocephala* do not survive, extra-orally, in aquarium water taken from the tank in which the spawning occurred. Experiments were performed to determine the role of oral incubation in embryonic survival. The approach used was to find methods of raising the embryos extra-orally and to relate extra-oral survival with the factors of survival in the milieu of the oral cavity (fig. 1). To facilitate this study, a series of embryological stages were described and defined from fertilization to hatching. Certain of the more obvious microscopic changes were described in conjunction with the gross descriptions of the stages.

The most effective experimental media were 40 per cent sea water and sterile aquarium water in which mean embryonic survival ranged between 80 and 85 per cent. The common factor found in these media was their capability of keeping the bacterial growth that killed the embryos to a minimum. The limited effectiveness of other experimental media, e.g., NaCl, artificial sea water, methylene blue, and lysozyme, was dependent on a balance between their toxic properties and their bactericidal (or bacteriostatic) properties. Fungus mycelia usually appeared on embryos raised extra-orally in aquarium water. However, it was demonstrated experimentally that the fungus was a secondary invader, since survival was high when embryos were raised in sterile aquarium water to which a fungus culture had been introduced. Conversely, no embryos survived when they were raised in sterile water to which a culture of bacteria had been introduced.

The churning action of the mouth was found to be one of the survival factors in oral incubation. When embryos were placed in a small quantity of aquarium water in a mechanical shaker, survival rates were good. On the other hand, embryos that remained stationary while a large quantity of water flowed over them never survived. These experiments show that the slow mechanical churning of the embryos in the mouth is à factor in survival, whereas the quantity of water flowing over the embryos during respiration of the parent is not critical. The oral epithelium may secrete mucus which protects the embryos. However, experiments designed to test the protective nature of mucus were not conclusive. Special pharyngeal glands, described in this paper, may secrete a bactericidal agent. Similar glands were found in *Tilapia ovalis* and have been described in two other mouthbreeding cichlids, while we were unable to find them in two non-mouthbreeding species of the genus *Tilapia*.

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