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and
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INTRODUCTION

SINCE THE DISCOVERY in 1936 of blind cave characins derived from the surface-dwelling *Astyanax mexicanus* (Fillipi), they have been the subject of continual study from many viewpoints. These included investigations that were concerned with ecological, behavioral, physiological, morphological, and histological characteristics of these cave fish. It appeared, from the observations of Gresser and Breder (1940), Breder and Gresser (1941a), and Breder (1944) on adult cave fish (*Anoptichthys*) that the embryonic optic vesicle underwent some invagination and differentiation. This was indicated because such eye elements as lens, pigment epithelium, some sensory retinal layers, some optic nerve fibers, and outer mesodermal components were present in variable amounts. However, it was not known whether the adult optic failure was a consequence of some interference with the chain of inductions involved in eye organogenesis or the causes were of a degenerative nature.

Until recently all investigations on these cave characins were limited to mature animals. Kühn and Kahling (1954) and Lüling (1953a, 1953b, 1955a, 1955b) studied the histological aspects of the progressive eye degeneration in larval and young adult *Anoptichthys* from La Cueva Chica. These papers were published after the comparative optic developmental analyses to be described in the present paper were initiated. The above-mentioned authors were aware of the fact that at two days after hatching, the eye size of the Chica larvae was already smaller than in the related eyed characin larvae of *Hemigrammus caudovittatus* of comparable age. However, they concentrated their studies on the phases of eye degeneration and did not investigate early embryonic developmental processes.

Up to the present time these cave characins are the only known cave fish capable of prolonged survival and breeding under aquarium conditions. They therefore presented the interesting opportunity for an embryological analysis of their inherited blindness. The genetic analysis of the eye reduction in these characins was recently completed, but only two short reports of the results have been published (Sadoglu, 1956, 1957). It will be of interest to correlate the results of these genetic studies with the embryological findings.

Presumably, because of the difficulty in breeding most blind cave species under laboratory conditions, very few embryological studies on cave vertebrates have been published. Eigenmann (1909) described the embryonic development of the eyes in the oviparous blind cave teleost *Amblyopsis spelaeus*. He obtained the eggs for his studies from females brought to the laboratory from their cave habitat. The eggs were then accessible, because in this species the gill cavity serves as a repository during the two months of egg development. When freed from this cavity, by a lifting of the edge of the operculum, the eggs survived and were reared successfully in an aquarium. Comparison of living and fixed whole and sectioned embryos of *Amblyopsis* with embryos of the eyed teleost *Cymatogaster* indicated that the eye of the early embryo of this cave fish had already reached its full adult size. The extreme shallowness of the formed optic cup, the irregular formation of the choroid fissure, and the failure of the lens cells to develop beyond their embryonic condition all contributed to the degenerated state of the eyes in this species. Eigenmann found an early retardation of cell division in lens and retina, as well as morphogenetic and histogenetic retardation in these structures. *Amblyopsis* is thought to have been a cave inhabitant for a much longer period of time than *Anoptichthys*, as it was demonstrated that members of the latter genus had taken up cave dwelling relatively recently (Breder, 1943a). It is shown in the present paper that the eyes of *Anoptichthys* were also retarded in cell division, and in some aspects of morphogenesis and histogenesis, but the retardation was generally not so extensive as in *Amblyopsis*, and eye growth was not inhibited entirely during embryonic and early larval stages.

Prior to Eigenmann's studies, Schlampp (1892) described eye development in the cave-dwelling European salamander, *Proteus*

anguineus. In this species Schlampp observed that normal eye cup invagination occurred, but he described a premature migration of mesoderm that pushed in between the eye cup and outer ectoderm and prevented further lens development beyond a solid sphere of epithelial cells. Stockard (1909) observed that in embryos of the myxinoid *Bdellostoma stouti*, only a small anterodorsal region of the irregularly shaped eye cup came in contact with the future lens ectoderm, so that only a small conical lens bud formed. Immediately after this the eye cup lost contact with the lens bud, and no further development of the lens occurred; then mesenchyme grew in between the eye cup and the outer ectoderm. Stockard therefore attributed the reduced eyes of the adult myxinoids to this early failure of contact between optic cup and lens ectoderm, and he was impressed with the way in which lens failure in these embryos was similar to the experimentally produced lens inhibition in amphibian embryos, as investigated by Lewis (1906–1907). Lewis had determined that the size of the early lens stages was dependent in part on the actual area of contact between the optic vesicle and outer ectoderm, and in those experimental embryos in which only a small eye vesicle had formed, mesenchyme was more prone to grow in between this vesicle and the outer ectoderm and prevent normal contact. Inductive influences from the optic vesicle, either very early or later acting, are now known to be essential for the normal lens differentiation of most species that have been studied, and prolonged contact between these two structures has been shown to insure proper lens development. The importance of induction in lens differentiation is not diminished by the several known cases of differentiation of the lens independently, following complete extirpation of the optic rudiment (*Rana esculenta*; Spemann, 1938), or when isolated from the optic vesicle (in *Salmo* and *Fundulus*, described by Mencl and Werber, as mentioned by Twitty, 1955), as there has been evidence to indicate that the archenteron roof (Mangold, 1931) and the head mesoderm (Liedke, 1951) may provide early inductive influences for the lens epidermis.

The cave characins used for these studies inhabit limestone caves located in a dry valley in the State of San Luis Potosi. The underground drainage of this valley is into the Rio Tampaon, where the eyed ancestral river fish, *Astyanax mexicanus* (Fillipi), live. Although five different caves in this vicinity were found to be inhabited by blind fish, descendants from only two of these caves were available for these embryological studies. *Anoptichthys jordani* Hubbs and Innes from La Cueva Chica, the cave closest to the river, were found to show much individual variation in eye reduction. The fish from Cueva de los Sabinos developed eyes that were more uniformly and further reduced. This latter cave is located over 14 miles up the valley from the Chica cave.

In the laboratory of the Department of Fishes and Aquatic Biology at the American Museum of Natural History, extensive studies to examine further the mode of evolution of these cave fish have been and are still being carried out (Breder and Rasquin, 1943, 1947; Rasquin, 1949a, 1949b; Rasquin and Rosenbloom, 1954). Experimental studies on the eyed ancestral river fish are in progress to elucidate further relationships and divergencies between the cave and eyed species. The embryological studies reported in the present paper will be useful in this broader project of research on these teleosts.

As the absence of lens fibers in the eyes of blind cave vertebrates appears to be an almost universal condition (Schlampp, 1892; Eigenmann, 1909; Breder and Gresser, 1941a; Breder, 1944; and Lüling, 1955b), and the lenses of Chica and Sabinos larvae also failed to form fibers, several histochemical tests were initiated to determine if a chemical deficiency could be demonstrated that could help account for the structural failure. Although little was known of the chemical processes involved in the formation of the highly refractive lens fibers, it had been found that in amphibians the pattern of differences in concentration of ribonucleic acid (RNA), glycogen, and the number of mitoses in parts of the exterior wall of the eye vesicle seemed to be responsible in part for the way in which the lens fibers start growing (Woerdeman, 1953a). Osawa (1951), Takata (1952), and Rickenbacher (1952) had shown that the amphibian eye vesicle received an accumula-

tion of RNA which diminished after the formation of the lens, and this accumulation of RNA was localized on the side of the optic vesicle that comes into contact with the lens ectoderm. Similar results were obtained by McKeehan (1956) in studies on the distribution of RNA during lens induction in the chick. The histochemical localization of glycogen and RNA in the developing optic structures of the river and cave fish embryos and young larvae was therefore undertaken in order to compare the relative concentrations of these substances. As protein-bound sulfhydryl groups are known to be of great importance in protein synthesis (Barrnett and Seligman, 1952) and in morphogenetic processes (Brachet, 1950), these groups were also histochemically localized in the optic and associated structures of these characins.

In view of the preceding considerations, the investigation of the embryonic and early larval optic development in these three related characin fishes was undertaken. Some observations on egg viability were also made in the course of the solution of the many initial problems involved in the spawning and rearing of sufficient numbers of the fragile, transparent, adhesive eggs, which are approximately 0.5 mm. in diameter, under aquarium conditions. Then followed the comparison of the basic stages in the development of the cave and river fish and the examination of histological and some histochemical aspects of eye morphogenesis and differentiation.

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MATERIALS AND METHODS

ALL THE MATURE FISH used in this study were descended from cave or river-dwelling ancestors. Some of these mature fish were obtained from previous spawnings in the laboratory, but the majority of them were received in the laboratory as young adults or as young, immature fish, which were laboratory bred to sexual maturity. At the fish-culture establishment in Florida, where most of the fish were obtained, food, temperature, sunlight, and water were most favorable for spawning and breeding. Under laboratory conditions spawning frequency was low, especially during the winter months. Laboratory temperatures varied from 21° to 27° C., although spawning was most frequent when the temperature was maintained at 27° C. Tanks were kept in three different rooms. One room had no artificial light, only daylight. In another room artificial fluorescent overhead lights were kept on for 12 hours and off for 12 hours daily. The third room was a temperature-controlled darkroom.

Adult males have hook-like processes that have developed on the anal fin rays. This male secondary sex characteristic and the slimmer male body contour served to distinguish males from females. Mature fish were selected from large stock tanks and were removed to smaller aquaria. Two active males were isolated with each ripe female, in a spawning tank of 15-gallon capacity, and of standard angle-iron frame construction. Approximately 100 such groups were set up over a period of 16 months. Floating *Nitella* plants in tanks in the light, and dried Spanish moss in the darkroom tanks, were used to catch and conceal eggs and larvae. It was necessary to provide concealment for the eggs, because the parents were prone to eat them. This was especially true of the cave fish, which are known to be tank scavengers. Tanks used for spawning were left free of sand and gravel and were filled with half tap water and half conditioned water (ph 7 was most satisfactory) before each new group was put inside. After a spawning, the parents were removed to another tank, and all the visible eggs were distributed in finger bowls of conditioned water. The concealed eggs that remained in the tank were allowed to hatch, and their development was compared with the eggs that had been removed. Observations on gross morphology were made on living eggs and larvae, and photomicrographs were taken of all available stages.

The mature fish were fed a balanced daily diet of a standard dried food (Aronson's formula, 1949) which contained 12 per cent protein, 2 per cent fat, and 32 per cent carbohydrate, and was composed of dried shrimp, liver, chopped lettuce, spinach, Pablum, and salt. Semi-weekly supplements of live tubificid worms were fed when available.

The artificial induction of spawning by the injection and implantation of pituitary glands from freshly killed carp was tried during the winter months. The carp were obtained from the shop of a local fish merchant where they had been kept under crowded conditions, so that their gonadotrophins were of questionable potency. Pituitaries were dissected out rapidly and were placed in 0.6 per cent saline solution, prior to their use. Glands were never kept longer than 60 minutes before they were injected. One-third of a carp pituitary gland, cut so as to include some part of the transitional lobe in each piece, was the most effective dosage. Pituitaries were either macerated in 1 cc. saline solution and the suspension injected into the body cavity, or they were implanted directly into an incision in the body wall. Both males and females were so treated, and the sexes were isolated for several days before they were placed in spawning tanks. Many females became abnormally enlarged by this treatment but failed to spawn. Stripping of such ripe females was attempted, and artificial fertilization with a freshly prepared sperm suspension from testes dissected from pituitary treated males was tried, following the technique of Rugh (1948). These methods never were successful, however, presumably because in these characins only naturally ovulated eggs can be fertilized, as has been the experience of previous investigators. Some stripped eggs became water hardened by the swelling of the vitelline membrane, but they failed to develop.

The following fixatives were used for the morphological and histological studies on these eggs and larvae: 10 per cent formalin; Stockard's solution; Carnoy's solution; absolute alcohol; Bouin's solution; equal parts of Bouin's and dioxan. These fixatives were used for from two to 24 hours. When dioxan was used the yolk remained soft, and sectioning could be done without the dissecting out of the yolk. Eggs and larvae were kept soft and well preserved in cellosolve (ethylene glycol mono-ethyl ether) for days or months after fixation. Dehydration in two changes of pure dioxan, left for two hours each, was followed by short infiltration in a mash of xylol (5 cc.), paraffin (20 cc. of tissue mat with a low melting point), and dioxan (25 cc.), as recommended by Rugh (1948). After two more pure paraffin baths, tissue was embedded in a watchglass. Serial sections of from 3μ to 7μ were stained with Harris' hematoxylin and eosin, Masson's ponceau-acid fuchsin-fast green, Mayer's hemalum, and toluidine blue. Whole mounts were also prepared and were stained in Mayer's hemalum or hematoxylin.

For histochemical localization of glycogen, eggs and larvae were fixed in ice-cold picro-alcohol-formalin (85 parts of 96% alcohol saturated with picric acid, 10 parts of 40% formalin, and five parts of acetic acid) for two hours, dehydrated in dioxan, and carried through infiltration and embedding, as described above. Paraffin sections, coated with 1 per cent celloidin, were stained by the Best-carmine technique, as described by Pearse (1953). Control sections were pre-treated with saliva at room temperature for 30 minutes, washed thoroughly, then stained as described above. Some slides were also subjected to the lead-tetra-acetate Schiff technique for polysaccharides (Shimizu and Kumamoto, 1952) recommended for the demonstration of small amounts of glycogen.

For the demonstration of ribonucleic acid, eggs were fixed in absolute alcohol for one hour, and then were carried through the usual histological techniques. Paraffin sections were incubated at 37° C. for one hour in a 1 per cent aqueous (glass-distilled water) solution (adjusted to ph 6) of crystalline ribonuclease (obtained from General Biochemicals, Inc., Ohio) in which proteolytic activity was destroyed by prior heating to 90° C. for three minutes in a water bath. Control slides were either incubated in distilled water or stained without prior incubation. All slides were stained in a saturated aqueous solution of toluidine blue o (certified) for 20 minutes. The basophilic material removed by this enzymatic digestion can be considered to be ribonucleic acid (Brachet, 1953; Pearse, 1953).

Protein-bound sulfhydryl groups were localized by the method of Barrnett and Seligman (1952) in paraffin sections of trichloracetic acid fixed eggs (24 hours fixation). The slides were incubated for one hour at 50° C. in a buffered (Michaelis barbital buffer, ph 8.5) alcohol solution of 2,2'-dihydroxy-6,6'-dinaphthyl disulfide reagent. This reagent forms a colorless substance with the active sulfhydryl groups in the sections. Tetrazotized diorthoanisidine in 0.1 M Sorenson's phosphate buffer, ph 7.4, stains the colorless product. The staining reaction was blocked in control sections by pre-treating the slides with 0.1 M iodoacetate at ph 8 for 20 hours at 37° C.

RESULTS

SPAWNING AND EGG VIABILITY

THE RIVER FISH were found to spawn always in the early hours of the morning and were never observed during the actual spawning process. Pre-spawning behavior in these eyed fish was simple, as is typical of most characins, and involved rapid and prolonged chasing of the female by the males. The cave fish usually spawned in mid-morning, but were observed spawning in mid-afternoon as well. The spawning behavior in the Chica fish has been described by Lüling (1954), and both this form and the Sabinos fish were observed to follow more or less the same pattern of movements during pre-spawning and spawning activities. These activities involved a speeding up of their normal wandering movements. As expected, during this chasing, the fish lost their mates frequently. As a result, it was common to see a frustrated male rapidly swimming up and down around one end of a tank, while the female was doing the same thing at the opposite end of the tank.

No cave fish ever spawned in the tanks kept in the darkroom, and Rasquin and Rosenbloom (1954) also reported that no river fish ever spawned in darkness. The temperature in the darkroom was never more than 2° to 3° C. below that in the rest of the aquaria. On two occasions it happened that cave fish, left in the darkroom for several weeks, spawned 24 hours after having been removed to the lighted aquarium. The significance of these paradoxical results cannot be ascertained without additional experiments.

Table 1 lists the number of naturally and artificially obtained spawnings that were used for these studies. As sexually mature Chica fish were not available until almost the end of the spawning season, it was fortunate that one Chica spawning was obtained at that time. The three artificially induced spawnings occurred in fish that had been treated one to eight days previously with carp pituitary implants. Although 50 fish were treated with pituitary implants or injections, only nine fish responded to the pituitary treatment. Ten fish that were injected intraperitoneally with pituitary suspension became hyperemic and died. Successful induction of spawning seemed to be dependent on the nearness of the coming characin spawning season and the breeding condition of the carp from which the pituitary glands were obtained, in addition to many uncontrollable factors.

The approximate numbers of eggs obtained from the normal and artificially induced spawnings are also listed in table 1, with the approximate percentage of eggs that were viable through hatching. The high viability in the same Chica stock was confirmed by Sadoglu (unpublished observations) in her genetic studies on these cave fish. As two of the naturally obtained Sabinos spawnings each contained no more than 50 eggs, the total number of Sabinos eggs studied was about 600. The low viability of the Sabinos eggs appeared in part to be related to the failure of the eggs to survive certain critical periods during their development. In three

TABLE 1

NUMBER OF SPAWNINGS STUDIED AND VIABILITY OF THE EGGS

	Astyanax	Chica	Sabinos
Number of spawnings obtained naturally	6	1	4
Number of spawnings artificially induced by pituitary implants	2	—	1
Total number of spawnings studied	8	1	5
Approximate number of eggs obtained from a normal spawning	500	300	200
Approximate number of eggs obtained from artificially induced spawning	250	—	100
Approximate total number of eggs studied	3500	300	600
Approximate percentage of viable eggs per spawning	Over 90%	Over 90%	50% or less

of the five Sabinos spawnings studied, many of the non-viable eggs appeared to undergo early cleavage, but after the stage of eight to 16 cells some of the blastomeres began to separate from one another. As cleavage proceeded, bleb-like transparent or somewhat opaque extrusions appeared on the surface of the blastodisc and yolk. The number and size of these blebs were variable. Many of these eggs then cytolyzed and became completely opaque, but some of them continued to develop with the blebs still attached, and as blastulae they often had, adhering to the surface of the egg and within the chorion, small accessory aggregates. During gastrulation a large number of these eggs cytolyzed and died, others gastrulated abnormally, so that the advancing germ ring failed to encircle the yolk completely. Surviving gastrulae continued to develop and hatched into some normal and some rather strang-looking larvae. No dark eye pigment was grossly visible in three- to four-day old larvae from such a spawning. After 14 days, and again after about 30 days, the majority of the surviving larvae died. Unpublished observations of Sadoglu on eggs from the same Sabinos stocks also showed the existence of these critical stages.

In an attempt to control the surface blebbing described in these non-viable Sabinos eggs and to increase their viability, these eggs were reared in solutions with added calcium in different forms. Groups of 10 eggs from the same spawning, in early cleavage or blastula stages, were placed in 20 cc. of each of the following solutions: 0.25 per cent calcium carbonate; standard Holtfreter's solution; Holtfreter's with double concentrations of calcium chloride; and controls in conditioned water. Also, to determine if a photolytic effect was responsible for these changes, groups of eggs were placed in the above solutions in a temperature-controlled darkroom. No differential effects of these solutions were observed in light or dark, and none of the above treatments enhanced the viability of such defective eggs.

DEVELOPMENTAL STAGES: COMPARATIVE ANALYSES

Normal stages, based on the appearance of gross morphological changes in living and fixed river-fish eggs and larvae, were arbitrarily designated and are listed in table 2. The time represents the number of hours after spawning when the stage was completed, at water temperatures of from 24° to 27° C. Photomicrographs of these normal stages of river-fish development are found in plates 11 to 13. The cave-fish eggs and larvae were staged in accordance with the normal morphological stages of river-fish development. Photomicrographs of cave-fish living stages are found in plates 14 and 15 (Chica series) and plates 16 and 17 (Sabinos series).

The unfertilized eggs of the river and cave fish were so much alike that they could not be distinguished from one another. These eggs were demersal, adhesive, transparent, colorless, roughly spherical, and rather regular in size, and averaged 0.5 mm. in diameter. As is indicated in the section on Materials and Methods, some of the stripped unfertilized eggs became activated and showed some swelling of the egg membranes after a short period in tank water, but they failed to develop further.

Newly fertilized eggs were available only from the cave fishes, so the following description of stage 1 was based on Chica and Sabinos eggs, but it is almost certain that it also applies to the river-fish eggs. The fertilized eggs were surrounded by a rigid, sticky chorion, inside of which was a very small perivitelline space. This space enlarged shortly after fertilization, when the fertilization membrane became raised up from the surface of the yolk. The protoplasm was now concentrated in the upright animal pole, in the form of a small flattened blastodisc, so that at this time the yolk was still visible over most of the surface of the egg. A micropyle was present at the vegetal pole.

In order to get some idea of the size relationships among these different eggs during their development, the vertical diameter of the egg proper was computed from photomicrographs of living eggs (table 3). Each measurement in this table represents a different egg. It can be seen from table 3 that

TABLE 2

NORMAL STAGES OF EMBRYONIC DEVELOPMENT IN *Astyanax*

Stage No.	Description	Time, in Hours
1	Fertilized egg	0
2	Cleavage and blastula	3
3	Early gastrula	
4	Mid-gastrula	4
5	Early yolk plug	5
6	Neurula	
7	Optic buds	4– 6
8	Closure of blastopore	6– 8
9	Lens vesicle	8
10	Olfactory vesicle; auditory vesicle; mesodermal somites; brain constrictions	8–10
11	Midbrain expansion	11
12	Myotomes; elongate tail; gut anlage	12–14
13	External-eye pigment; swim-bladder anlage	15–18
14	Epineural and yolk-sac melanophores; hatching	24
15	Retinal pigment	48
16	Pectoral fins	72

the Chica eggs and embryos consistently showed a slightly larger egg size, up to stage 12, than the river fish or Sabinos eggs, but enough measurements for each stage were not available for statistical analysis. In the river fish there appeared a more or less regular increase in germ size during development, which was paralleled by an almost regular decrease in volume of the perivitelline space, as the enlarging embryo filled the chorion. This inverse relationship was not so regular in the cave-fish eggs, and in the Sabinos eggs the perivitelline space was unusually small in the early stages. A small perivitelline space was observed in the Sabinos egg in an abnormal cleavage stage, with partly separated blastomeres, as seen in plate 16, figure 1. A small perivitelline space was also apparent in plate 16, figures 2 and 3, of late blastula and early gastrula Sabinos eggs, respectively, with some cytolyzed cells in the perivitelline space at the former stage and a collapsed chorion at the latter stage. Therefore in the three Sabinos eggs in which small perivitelline spaces were observed developmental abnormalities were prevalent.

During stage 2 (pl. 11, fig. 1), as the eggs underwent cleavage and blastulation, the shape of the river-fish eggs varied from spherical to ovoid, as a result of the elongation of the vertical egg axis (through the animal and vegetal poles), so that the magnitude of the perivitelline space also varied. During this vertical elongation of the river-fish egg, there was evidence of lines of tension in the stretched chorion, which were not so apparent in the cave fish eggs.

By stage 3, the early gastrula (pl. 11, fig. 2; pl. 14, fig. 1; and pl. 16, figs. 2 and 3), the outer edge of the blastodisc had thickened to form the germ ring, which gradually moved down to encircle the yolk mass. In sections through this stage the blastodisc was seen to be made up of several layers of large, irregu-

TABLE 3

EGG SIZE (IN MILLIMETERS) OF RIVER AND CAVE FISH IN STAGES 1–12

Stage No.	Vertical Egg Diameter		
	Astyanax	Chica	Sabinos
1	0.50	0.50	0.50
2	0.72	0.86	0.83
3	0.69	—	0.78
4	0.72	0.88	—
5	0.83	—	0.78
7 and 8	1.01	1.06	0.91
9	1.01	0.98	—
10	0.96	1.06	—
11	1.00	1.04	—
12	1.09	—	0.98

larly shaped cells with many mitotic figures and large, deeply stained nuclei. No separation was seen yet between the chorda, mesoderm, or endoderm regions. Central periblast tissue immediately surrounded the yolk, and the more concentrated marginal periblast was prominent at the inner edge of the germ ring.

At mid-gastrula, stage 4, the advancing germ ring had reached about one-half of the way around the yolk (pl. 11, fig. 3; pl. 14, fig. 2). The future embryonic axis was now visible as a thickened ridge prominent at anterior and posterior dorsal regions of the blastodisc. In sections through some Sabinos embryos (from the 4/12/54 spawning) at this stage, there were three kinds of nuclei present which differed in staining reaction. These nuclei were either homogeneously acidophilic, almost entirely unstained except for nuclear membrane, or half of the nucleus was clear and unstained and the other half was acidophilic. This condition of the nuclei was not present in sections of the other cave fish or in the river fish. The significance of such a series of stained nuclei could not be determined. There was a possibility that this was the beginning of the gastrulation failure which the Sabinos eggs were prone to, as described in the previous section. It was during stage 5 when most of the Sabinos eggs cytolyzed and failed to gastrulate normally.

By stage 5, the early yolk plug (pl. 11, fig. 4), the extra-embryonic germ-ring tissue had expanded over three-quarters of the yolk mass, and the anterior end of the embryonic shield, which was to form the future head end of the embryo, had almost reached the animal pole of the egg. In sections through the embryonic axis the dorsal neurectoderm could be differentiated from the ventral chorda-mesoderm, but these layers were still in close contact. Towards the end of this stage the dorsal neural cord invaginated below the surface, and the future epidermal layer could be distinguished from the neural ectoderm. Excess amounts of marginal and central periblast tissue were found in sections of Sabinos eggs at this stage. Normally marginal periblast is confined within the limits of the lips of the germ ring, and this was true in Chica and river-fish eggs. But in eggs from two different Sabinos spawnings (7/23/54 and 8/16/54) patches of periblast tissue were found in the perivitelline space at many different regions around the outside of the blastoderm, as seen in plate 18, figures 1 and 2. Excess marginal periblast was found at the region of the dorsal lip (pl. 18, right side of fig. 3). Central periblast was over-abundant at the animal pole, as seen in plate 19, figures 1 and 2, where many of the blastoderm cells above the periblast showed regions of cytoplasmic degranulation. The periblast tissue was made up of a syncytium of very large, irregular, vesicular, mostly clear nuclei, with or without stained nuclear membranes, and granular, dense, basophilic cytoplasm. Some of these nuclei had a coarse chromatin network, with occasional nucleoli. No mitotic figures were found in any of this periblast tissue, although blastoderm cells showed mitoses. Many yolk globules were incorporated within the periblast tissue. This stage was found to be critical for most Sabinos eggs, so that it is very possible that this proliferation of excess periblast tissue was another manifestation of some morphogenetic derangement in this cave form. The histological structure of normal periblast tissue in river fish and Chica eggs resembled the above description.

Neuralization was well under way by stage 6, and the future head and caudal ends of the embryo had expanded and thickened. The yolk plug was barely visible in the river fish, but in the Chica embryos a large yolk plug was still visible. This may have been indicative of some slowing down of epibolic expansion at this time. No Sabinos embryos in this stage were available.

Solid optic buds appeared by stage 7, as outgrowths from the primitive forebrain region. By this stage the head end of the embryo had reached over the animal pole of the egg, and the advancing extra-embryonic membranes had stretched down towards the future blastopore region. In the river-fish embryos the extra-embryonic tissue on the side of the egg opposite the embryo below the head appeared raised away from the surface of the yolk (pl. 11, fig. 6, right side), so seemed to be separated from the yolk by a sizable space. This raised position of the extra-embryonic layers was not apparent in the cave-fish embryos in this or in later stages (compare pl. 12, figs. 1 and 2, with pl. 14, fig. 4, and pl. 16, fig. 5). The outpushing of

the optic buds was delayed some two hours in the Chica and Sabinos embryos, so that it was six to eight hours after spawning before this stage was completed.

Closure of the blastopore, stage 8 (pl. 12, fig. 1), was also delayed in these cave embryos by some two hours, and, by the time the lips of the blastopore came together at eight to 10 hours after spawning, as many as 12 mesodermal somites had formed in the trunk region (pl. 14, figs. 4, 5). The head end of the cave embryos had reached the egg equator by the time the blastopore closed (pl. 16, fig. 5).

Mesodermal somites were not prominent grossly in river-fish embryos until stages 9–10 (pl. 12, fig. 2), when lens, olfactory, auditory, and Kupfer's vesicles were all present, and brain constrictions could be seen. In the cave embryos the mesodermal somites were well developed by the time the lens vesicles appeared at 10 hours after spawning.

Towards the end of stage 10 melanophores were first found between the brain and the invaginated optic vesicles in sections through the head of the river-fish embryos. No pigment granules were found in cells that were thought to be neural crest cells, located at the dorsolateral borders of the diencephalon. At other levels of the neural tube no pigmented neural crest cells were seen either. No melanophores were present in the vicinity of the optic vesicles and the brain in the cave-fish embryos at this stage. Not until stage 14 or later did the head mesenchyme cells between the optic vesicles and brain of the cave-fish become pigmented, and then they contained only sparse concentrations of pigment granules.

By stage 11, the expanded optic lobes were prominent in river-fish embryos, but were not enlarged in Chica or Sabinos embryos. In the region where the optic lobes should have been prominent, just posterior to the optic cup, there seemed to be only an empty space in the photographs of the living cave-fish embryos (compare pl. 12, fig. 3, with pl. 14, fig. 5, and pl. 16, fig. 6). In histological sections through these clear regions in the cave-fish embryos the optic lobes were found to be flattened dorsally, and some central regions were absent. This accounted for the poorly visible optic lobes. A thickened rim around the outside of the eye cups in the cave-fish embryos was also observed at this stage. This thick outer rim was seen in histological sections to have formed from the eye-vesicle tissue that failed to invaginate.

By stage 12, myotomes were seen in the posterior trunk and tail regions of river-fish embryos (pl. 12, fig. 4) and were already apparent in more anterior trunk regions of cave-fish embryos (pl. 14, fig. 6; pl. 17, fig. 1). Tail-bud elongation had not yet started in these cave-fish embryos, although elongate tails were formed in the river fish by this stage. Anteriorly, the lateral walls of the hindbrain had thickened, and a dorsal cavity was visible in the midbrain and hindbrain.

At stage 13, punctate melanophores with sparsely scattered, brown, pigment granules were visible grossly around the outside of the optic cups of river-fish embryos, and also along the ventrolateral border of the developing gut wall. No melanophores were seen externally in Chica embryos until after hatching, at stages 14–15, and even by the third larval day no brown pigment was seen around the eyes of most Sabinos larvae.

In all three forms, by stage 14 (pl. 13, figs. 1 and 2; pl. 15, fig. 1; and pl. 17, fig. 3) hatched larvae were found, either moving rapidly along the surfaces of the glass or attached by the sticky dorsal adhesive gland to the sides of the tank or to plants. No significant differences in total length of the preserved larvae were found at this stage, and none were apparent at later stages. These measurements were not tabulated, because enough preserved larvae were not available at each stage for statistical evaluation. At stage 14 the total length of the larvae ranged from 2.0 to 2.9 mm. The river-fish larvae, with prominent and numerous stellate melanophores on the yolk sac, around the eyes, and in the dorsal epineural head and trunk regions, could be distinguished from the poorly pigmented or unpigmented cave-fish larvae. Some punctate yolk-sac and eye melanophores were found in the Chica larvae by this stage, but they were less numerous than in the river-fish larvae. Dorsal epineural melanophores were not seen until stage 15, in Chica larvae, and then were present only in limited numbers. But even on the second larval day many Chica larvae still had no melanophores around

the eyes or in the other regions. No dorsal epineural or yolk-sac melanophores were seen in any Sabinos larvae at this or at later stages.

On the second larval day, stage 15, the larvae ranged in total length from 2.9 to 3.2 mm. Dense retinal pigment now darkened the eyes of the river-fish larvae (pl. 13, fig. 3), and densely concentrated melanophores lined the pericardium. The eyes of Chica and Sabinos larvae were not conspicuous grossly because they generally lacked external pigment at this stage (pl. 15, fig. 2; pl. 17, figs. 4, 5), and they were smaller than in the river fish, with correspondingly reduced pupil and lens size. Semicircular canals and visceral arches were well developed, and most other structures showed more or less parallel gross development in the eyed and cave fish at this stage.

In three-day old larvae, stage 16, pectoral fins and opercular structures were formed, and the swim bladder was filled with air (pl. 13, fig. 4). These larvae ranged in total length from 3.1 to 4.0 mm. In some Chica and Sabinos larvae the dorsally visible auditory structures appeared more expanded than in the river fish, but this finding needs further confirmation. A gradient in eye size, which ranged from the normal-sized eyes of the river fish to the smaller eyes of the cave-fish larvae, was apparent at this stage. This was similar to the known geographical gradient of these cave fish in their natural habitats, with respect to the distance of the caves from the river where the normal eyed fish live. In plate 20, figure 1, the large, densely pigmented eyes of the river-fish larvae of stage 16, and in figure 2 of the same plate the small, lightly pigmented eyes of the Sabinos larvae of the same stage, can be seen. No pupils were grossly visible in some Sabinos larval eyes at this stage. In sections through this stage it was observed that the anterior eye chamber was expanded in river-fish larvae, but was not formed in Sabinos larvae until the fourth to fifth larval days. The anterior eye chamber was formed in the Chica larvae by the fourth larval day.

By the fourth larval day the eyes of the cave fish began to sink underneath the skin. This process did not occur with any regularity. In some Sabinos larvae the eyes were sunk in ventrally more than dorsally. At this stage the larvae ranged in total length from 3.5 to 4.2 mm. Retinal pigment was prominent in most Chica larvae (pl. 15, fig. 3).

The enlargement of the posterior eye chamber occurred by the fifth larval day in river fish and in some cave larvae. In the cave larvae the shape of the eyes was altered by what appeared to be a closing in of the lips of the eye cup as the eye sank underneath the skin. There was much individual variation in size, shape, and position of the eyes in different cave larvae, and in both eyes of the same larva. In general there appeared less variability in embryonic eye size and more eye symmetry in the more reduced eyes of the Sabinos cave fish than in the eyes of the Chica cave fish. It was common to find that the eye on one side of a larva had sunk deeper under the skin than the eye on the opposite side. As far as

TABLE 4

DIFFERENCES IN TIME OF APPEARANCE OF OPTIC AND OTHER STRUCTURES OBSERVED DURING THE DEVELOPMENT OF RIVER AND CAVE FISH

Structure	Time, in Hours		
	Astyanax	Chica	Sabinos
Optic buds	4– 6	6– 8	6– 8
Closed blastopore	6– 8	8–10	8– 10
Lens vesicle	8	10	10
Mesodermal somites	8–10	8–10	8– 10
Eye cup	8–10	10+	10+
Optic-lobe expansion	11	14+	14+
Elongate tail	12–14	14+	14+
Lens fibers	24–48	None by 120 hrs.	None by 120 hrs.
Anterior eye chamber	72	96	96–120

TABLE 5

DIFFERENCES IN TIME OF APPEARANCE OF PIGMENT IN EYE AND BODY REGIONS OF RIVER AND CAVE FISH

Location of Pigment	Time, in Hours		
	Astyanax	Chica	Sabinos
Between eye cup and brain	10	24	48+
Externally around eye	15–18	24–30	72
Dorsal epineural regions	24	24–48	None
Yolk sac	24	24–48	None
Retinal pigment epithelium	48	48–72	72
Choroid	72	72+	72
Iris argentea and stroma	72	72+	72–120

could be determined, this occurred with equal frequency on left and right sides of these cave larvae; however, a larger sample would be needed to verify this statement. At this stage several river-fish larvae with one normal-sized eye and one small eye were observed. These larvae appeared normal otherwise. After the fifth larval day the pupils in many cave larvae closed, and the eye chambers were obliterated partly or completely.

Table 4 summarizes some of the differences in the time of appearance of optic and other structures observed during the development of the river and cave fish. In table 5 the differences in the time of appearance of pigment in the eye and body regions of the river and cave fish are listed. These results indicate that a delay of both blastopore closure and tail-bud elongation accompanied the delayed optic and pigmentary development in the cave fish.

HISTOLOGICAL AND HISTOCHEMICAL STUDIES

Optic Cup Formation

RIVER FISH: At stage 7 the solid optic buds extended out to the outer head epidermis. Most of the outer bud wall was in contact with the outer epidermis.

By stage 9 a small cavity had formed in the early optic vesicle, and invagination had proceeded to some extent (pl. 20, fig. 3). The optic-vesicle cells had elongated and were oriented around the vesicle so that their inner ends more or less converged towards the vesicle center (pl. 20, fig. 4; pl. 21, fig. 1). Two kinds of optic-vesicle cells were found, those with and those without elongated cytoplasmic processes. These processes were on the inner ends of the cells so that they approached the center of the vesicle. The nuclei of both kinds of optic-vesicle cells were deeply basophilically stained and granular and were peripherally located (on the side away from the opticoele). The cytoplasm of the optic-vesicle cells was homogeneous and deeply eosinophilic, except for the paler stained cytoplasmic processes. The forebrain cells were oriented perpendicular to the optic vesicles. In the vicinity of the invaginating vesicle and eye-stalk cells the forebrain cells had elongated somewhat, as if they had been pulled by the apparent tension exerted during the invagination process. A small lumen had also formed in the forebrain, opposite the opticoeles. Mitoses were numerous in the optic and forebrain cells.

Histochemical tests at stage 9 indicated that very little glycogen-positive cytoplasm was present in the optic-vesicle or forebrain cells, except for some lightly stained positive cytoplasm in the elongated processes of some of the vesicle cells. In these same sections darker, more densely concentrated, glycogen-positive granules were present in the yolk-sac endoderm and mesoderm cells. As for protein-bound sulfhydryl groups, they appeared sparsely distributed in the cytoplasm and nuclei of the optic-vesicle cells, as in all other embryonic cells at this stage, including neu-

ral, epithelial, chordamesodermal, and mesenchymal cells. The yolk spheres were more intensely stained than the embryonic regions. Study under the oil-immersion lens showed that some of the elongated cytoplasmic processes of the optic-vesicle cells contained a more dense concentration of sulfhydryl groups than the other optic regions. No special distribution of cells with sparsely reactive or more densely concentrated sulfhydryl groups was observed in the optic cells, and no apparent pattern of staining differences along the exterior wall of the vesicle was noted. As expected, ribonucleic acid was widely distributed and densely concentrated in most embryonic tissues. Localization of RNA was almost exclusively in the cytoplasm. No prominent differences between incubated and non-incubated controls were apparent, except that the incubation produced a lighter shade in some areas of toluidine-blue-stained cytoplasm. The densest concentrations of RNA-positive granules were found in the cytoplasm of the neural tissue, with high concentrations also present in the somite mesoderm, notochord, embryonic endoderm, and periblast cytoplasm. In the optic vesicles the opticoele was surrounded by densely blue cytoplasmic granules that were digested by ribonuclease. The rest of the optic vesicle was also filled with positive RNA granules, as was the outer wall of the vesicle, in contact with the presumptive lens epidermis. Epidermal and extra-embryonic, yolk-sac cells and the yolk spheres were less reactive than the other regions.

By stage 10 the eye cup was further invaginated. The pigment epithelial cells, with large, round nuclei and without cytoplasmic processes, could be distinguished from the sensory cells, which contained elongated ovoid nuclei and cytoplasmic processes. The nuclei of the sensory cells were located principally along the wall adjacent to the pigment epithelium. The cytoplasm in the invaginated region of the eye cup was eosinophilic, and many yolk spheres were incorporated within these cells. The eye cup remained in close contact with the diencephalon (pl. 21, fig. 2), which was now thickened laterally. The third ventricle had a diamond-shaped contour in cross section. The lateral angles of this lumen were located about opposite the center of the invaginated region of the eye cup. The retinal sensory cells were oriented more or less parallel with the diencephalon cells. Many mitoses were still found in the optic cup and diencephalon cells, indicative of the active growth and proliferation still going on in these regions.

At stages 12–13 the optic cup appeared fully invaginated, and some sensory retinal cells had started to differentiate.

The optic cup in the newly hatched larvae at stage 14 was still in close contact with the diencephalon (pl. 21, fig. 3). Pigment granules were not yet accumulated in the cells of the thin, outer, epithelial layer of the retina. The thick differentiating sensory layers of the retina contained four to six rows of basophilic, elongated nuclei oriented with their long axes parallel with the diencephalon cells (pl. 21, fig. 4). These retinal nuclei were capped on the side towards the lens by basophilic cytoplasmic processes. Many mitoses were still found in the sensory retina and diencephalon. The dorsal and ventral lips of the optic cup were of about equal size.

Histochemical tests at stage 14 indicated that there was still very little glycogen-positive cytoplasm in the optic-cup sensory cells or diencephalon. In these same sections some positive-glycogen granules were present in developing optic nerve fibers and corneal epithelial cells. In other sections of the same larvae, yolk-sac endoderm, developing gut, and mesodermal somites contained positive glycogen granules. Protein-bound SH groups were still sparsely distributed in the cytoplasm and nuclei of the optic-cup cells. Around the nuclei of some of the sensory cells and in the cytoplasm located at the center of the invaginated region of the retina some more reactive, positively stained granules were found. Positive RNA granules were still found in large concentration in the cytoplasm of the optic-cup sensory cells and diencephalon cells. The cytoplasm that immediately surrounded the sensory-cell nuclei was more basophilic than the elongated cytoplasmic processes.

CAVE FISH: The solid optic buds at stage 7 appeared smaller than in the river fish, and only a small region of the outer bud wall was in contact with the outer epidermis. This region was in the dorsal part of the outer bud wall in the Chica embryos and in the central

part of the outer bud wall in the Sabinos fish.

Very little optic invagination had occurred by stage 9, and there was little evidence of a cavity forming in the optic buds. No lumen had yet formed in the forebrain. No sensory cells with cytoplasmic processes as described for the river fish were observed. Few mitoses were seen in the optic and forebrain cells.

Histochemical tests at stage 9 were not carried out for glycogen, because appropriately fixed sections were not available. Protein-bound SH groups appeared sparsely distributed in the nuclei and cytoplasm of the optic buds of the Chica fish, as in river fish, although the nuclei appeared slightly more reactive than the cytoplasm. No elongated cytoplasmic processes were formed in the cave-fish optic cells. Some of these processes in the river fish contained a more dense concentration of SH groups than the other optic regions. As in the river fish, no special distribution of cells with reactive SH groups was observed in the optic cells. No Sabinos sections at this stage were available for SH localization. In the Chica embryos in the vicinity of the barely visible opticoele there were a few scattered cells that were negative for RNA, although the rest of the optic-bud cytoplasm was RNA positive. In the small region where the exterior wall of the optic bud reached the outer epidermis the cytoplasm was filled with positive RNA granules. No Sabinos sections in this stage were available for RNA localization.

In stage-10 Sabinos embryos some optic-vesicle invagination had occurred (pl. 22, fig. 1). The pigment epithelium cells could be distinguished from the sensory cells. The retinal sensory cells were oriented as in the river fish, more or less parallel with the diencephalon cells. Few mitoses were seen in the optic and diencephalon cells. The eye vesicles appeared to be crowded both dorsally and ventrally because of deep epithelial clefts, not apparent in the river-fish embryos. These clefts seemed to have cut off some of the optic stalk ventrally, and dorsally may have inhibited the migration of neural crest cells. The crowded position of the eyes of the Sabinos embryos may be related to the reported failure of the head to become raised above the surface of the yolk. The cavity of the diencephalon was small, irregular, and not of diamond-shaped contour as in the river fish. The lateral walls of the diencephalon were not differentially thickened. No Chica sections in this stage were available for histological study.

In Chica embryos of stages 12–13 (pl. 22, fig. 2) the optic vesicles showed signs of an irregular invagination that had not progressed so far as in river-fish embryos of stage 10. In many of these Chica embryos the optic vesicle on one side was invaginated farther than the vesicle on the opposite side. It appeared that invagination had taken place only in those regions of the outer wall of the eye vesicle that had been in contact with the outer epidermis. This produced a small optic cup, and the vesicle tissue that was not invaginated seemed to become part of the pigment epithelial layer. Cellular differentiation in retinal and pigment epithelial layers had occurred to a limited extent, but primarily in the vicinity of the invaginated regions. In this limited region of invagination the sensory nuclei were oriented along the wall adjacent to the pigment epithelium, and the cytoplasm occupied the invaginating region, as in the river-fish embryos of stage 10. In the vicinity of the optic stalk there were some elongated cells with large, basophilic nuclei and cytoplasmic processes as observed in river fish of stage 9. The ventrolateral part of the stalk had started to invaginate. The third ventricle of the diencephalon had an irregular contour, and in many larvae this lumen had not yet formed. There seemed to be some relationship between the extent of optic-cup invagination and the size of the central cavity of the diencephalon. Few mitoses were found in retinal or diencephalon cells.

The optic cup in the newly hatched larvae at stage 14 was smaller than in the river fish, and did not appear in most sections to maintain so close a contact with the wall of the diencephalon as in the river fish. The number of rows of retinal nuclei was irregular and usually fewer than in the river fish. Basophilic cytoplasmic processes were differentiated in the sensory cells, but these processes were not always found on the side of the nuclei. The dorsal lips of the eye cups appeared much thicker than the often incompletely formed and undifferentiated ventral lips. Few mitoses were seen in the retinal or diencephalon

cells. Some ventral lip cells in the Chica larvae showed mitoses, so that growth may have resumed in this region. At this stage the eye cups of the cave fish (Chica, pl. 23, fig. 1; Sabinos, pl. 23, fig. 2) resembled those of the river fish more than at any previous or any later stage, despite the differences just pointed out. Soon after stage 14 the lips of the cave-fish eye cups started to close in, the eye chambers failed to develop completely, lens-fiber differentiation did not take place, and regressive changes appeared in the retina. As a result of these alterations the eye cups of the larvae of the river fish appeared distinctly different from those of the larvae of the cave fish.

Histochemical tests at stage 14 indicated that glycogen-positive granules were highly concentrated in the optic-cup sensory cells and diencephalon, although this condition was not found in the river fish. Numerous carmine-stained granules were found around the nuclei of the retinal sensory cells and diencephalon cells of the cave larvae, especially in those diencephalon nuclei at the distal border of the gray matter. In saliva-treated controls no carmine-stained granules were present in the structures enumerated above. These glycogen-positive granules located in the optic structures appeared even more densely concentrated in the Chica larvae than in the Sabinos larvae. The Chica yolk-sac endoderm cells contained positive glycogen granules, as in the river fish, but the conspicuous absence of glycogen-positive granules was noted in the yolk-sac endoderm cells of the Sabinos larvae. Protein-bound SH groups were sparsely distributed in the cytoplasm and nuclei of the optic-cup cells. Around the nuclei of some of the sensory cells more reactive granules were seen, but they were not found in the cytoplasm at the center of the invaginated region of the retina where they were found in river fish. Positive RNA granules were found in large concentration in the cytoplasm of the optic-cup sensory cells and diencephalon cells, as in the river fish. Also, the cytoplasm that immediately surrounded the sensory-cell nuclei was more basophilic than the elongated cytoplasmic processes.

COMPARISON: The histological appearance of stages in the formation of the optic cup of the river and of the cave fish is summarized in table 6. This table indicates that at the earliest appearance of the optic rudiments at stage 7, the optic buds of the cave fish were smaller than those of the river fish. This size discrepancy remained throughout the development of the optic cup. Mitoses were numer-

TABLE 6

COMPARISON OF OPTIC-CUP FORMATION IN RIVER AND CAVE FISH

Stage	Histological Characteristics	River Fish	Cave Fish
7	Solid optic buds	Large	Small
	Area of contact of outer bud wall with outer epidermis	Extensive	Small dorsal or central area
9	Opticoele	Small	Smaller or absent
	Optic vesicle invagination	Some	Little
	Cytoplasmic processes on some optic vesicle cells	Yes	No
	Mitoses	Many	Few
10	Opticoele	Enlarged	Small
	Optic vesicle invagination	Well under way	Just started
	Pigment epithelium	Yes	Yes
	Sensory cell orientation	Yes	Yes
	Mitoses	Many	Few
12–13	Optic invagination	Complete	Incomplete
14	Optic cup	Large	Small
	Rows of retinal nuclei	4–6	Irregular, smaller number
	Dorsal and ventral lips	Same thickness	Dorsal thicker
	Mitoses	Many	Few

ous in the cells of the optic and brain structures of the river fish during their formation but were scarce in these structures of the cave larvae. The delayed invagination of the optic vesicle in the cave larvae appeared to be related to the small area of contact of the outer-eye bud wall with the outer-head epidermis. The incomplete optic invagination in many of the young cave larvae resulted in the formation of optic cups with thickened dorsal lips and poorly developed ventral lips. At stage 14 the eye cups of the cave larvae resembled those of the river fish more than at any earlier or later stage, so that the differences in growth, morphogenesis, and differentiation were not very great up to that time.

The results of the histochemical localization of glycogen, protein-bound SH groups, and RNA in the developing optic regions of river and cave larvae are summarized in table 7. These results indicate that the faster and greater optic growth in the river fish was accompanied by an apparent depletion of glycogen from the optic cells. The intense glycogen reaction in the cave larvae seemed to indicate a glycogen retention in the optic structures. During the differentiation of the optic cup in the river fish the SH groups showed an increased intensity of histochemical reaction not observed in the incompletely differentiated optic cells of the cave fish. RNA distribution was similar in the optic cup of river larvae and in that of cave larvae.

Lens Formation and Differentiation

River Fish: At stage 9 the lens anlage had just started to thicken and was seen as a densely stained row of high cuboidal epithelial cells. These lens cells were in close contact with the outer nucleated wall of the eye vesicle. The lens-cell nuclei were large and ovoid, with deeply basophilic, granular chromatin and several large nucleoli. The lens-cell cytoplasm was granular and deeply eosinophilic. Mitoses were numerous in the lens cells. Histochemical studies at this stage demonstrated very little glycogen-positive cytoplasm in the lens cells. Protein-bound SH groups were sparsely distributed in the cytoplasm and nuclei of the lens cells, as in the optic-vesicle cells. RNA-positive cytoplasm filled the lens cells.

By stage 10 the thickened lens vesicle had started to invaginate, but remained in close contact with the outer epidermis and the invaginating wall of the retina (pl. 21, fig. 2). The one to two rows of basophilic, elongating nuclei of the lens cells were oriented parallel with the retinal nuclei and were located on the side of the lens adjacent to the retina. Mitoses were still numerous in the lens cells.

By stages 12–13 the lens appeared fully invaginated, but contact was maintained with the outer epidermis and the retina.

At stage 14 most of the outer wall of the lens was detached from the outer epidermis. Contact of the inner lens wall with the retina was maintained. Two zones of lens nuclei appeared, an outer and an inner zone. The nuclei in the outer zone resembled the stage-9 lens nuclei with deeply basophilic granular chromatin. The inner-zone nuclei were changing in preparation for lens-fiber formation.

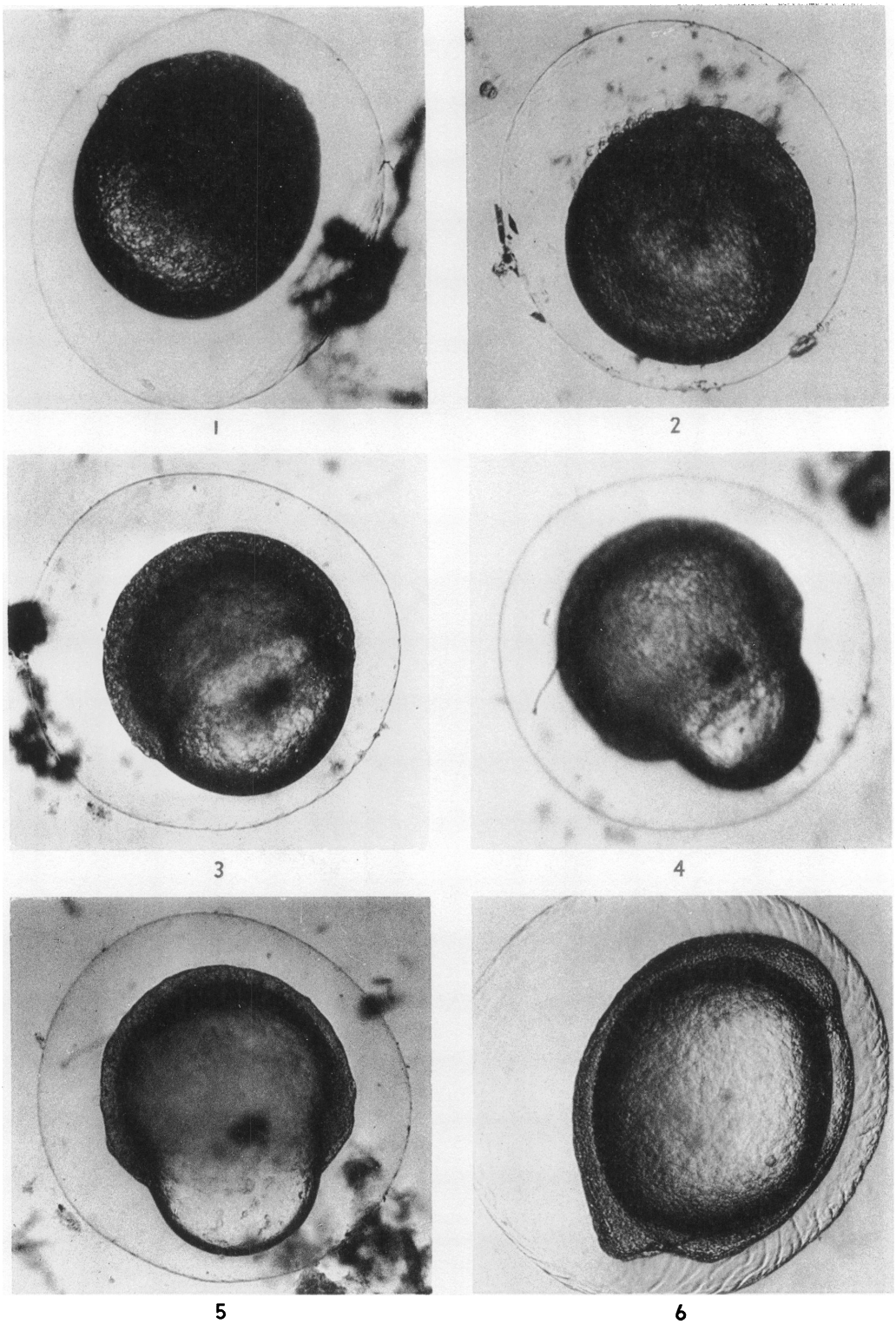
TABLE 7

Histochemical Reactions in Developing Optic Regions of River and Cave Fish

Substance	Intensity of Histochemical Reaction		
	Astyanax	Chica	Sabinos
Glycogen in optic cup and lens cells (stage 14)	+[a]	+++	++
SH groups in optic-vesicle cells	+	V	0
SH groups in optic-cup and lens cells	++	+	+
RNA in optic-vesicle cells	+	V	0
RNA in optic-cup and lens cells	+	+	+
Ribonuclease-resistant basophilia in fiber-forming lens cells	+	−	−

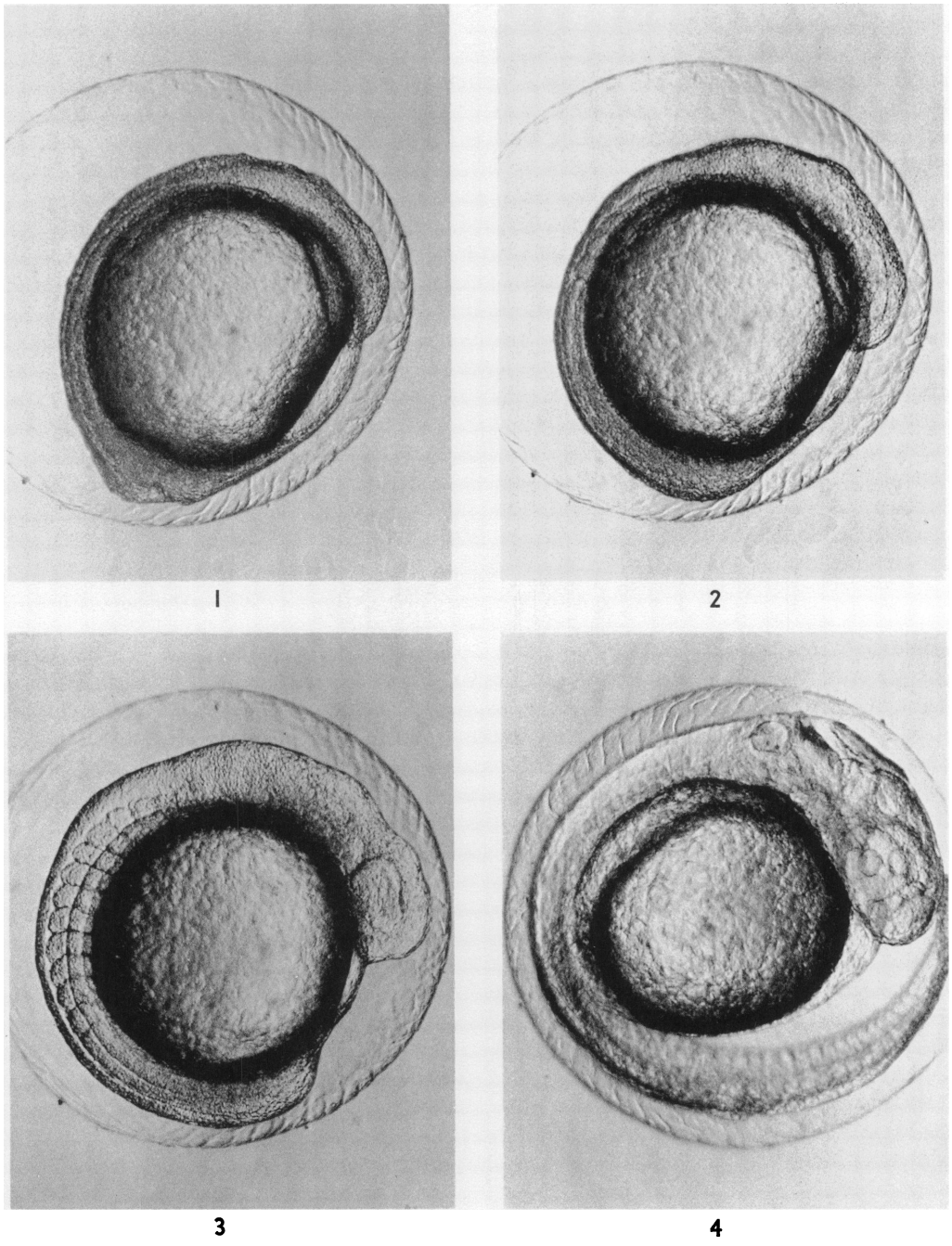
[a] *Symbols:* +, weak; ++, moderate; +++, strong; −, negative; 0, no sections available; V, variable, not uniformly positive.

PLATES 11–27



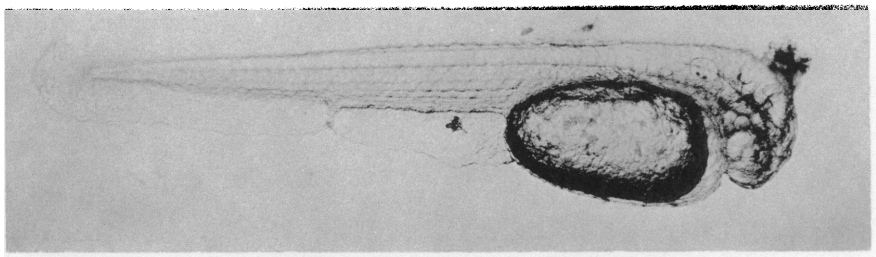
DEVELOPMENT OF EGGS OF RIVER FISH

1. Stage 2, blastula. × 55
2. Stage 3, early gastrula. × 55
3. Stage 4, mid-gastrula. × 55
4. Stage 5, early yolk plug. × 55
5. Stage 5, yolk plug. × 55
6. Stage 7, optic buds. × 55



DEVELOPMENT OF EMBRYOS OF RIVER FISH

1. Stages 8 and 9, closure of blastopore and lens vesicle. × 55
2. Stage 10, olfactory and auditory vesicles, mesodermal somites, brain constrictions. × 55
3. Stage 11, midbrain expansion. × 55
4. Stage 12, myotomes and elongate tail. × 55



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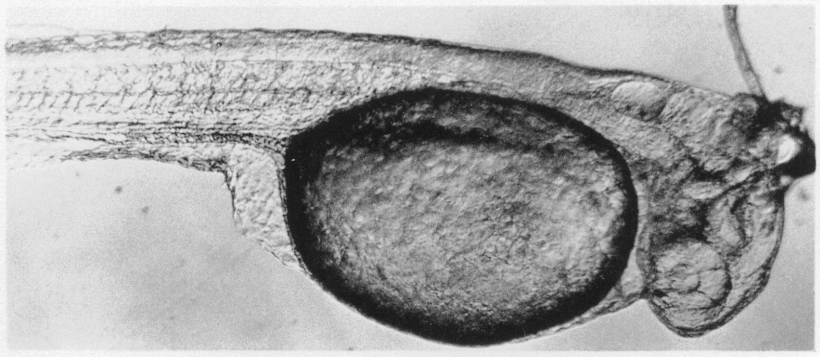

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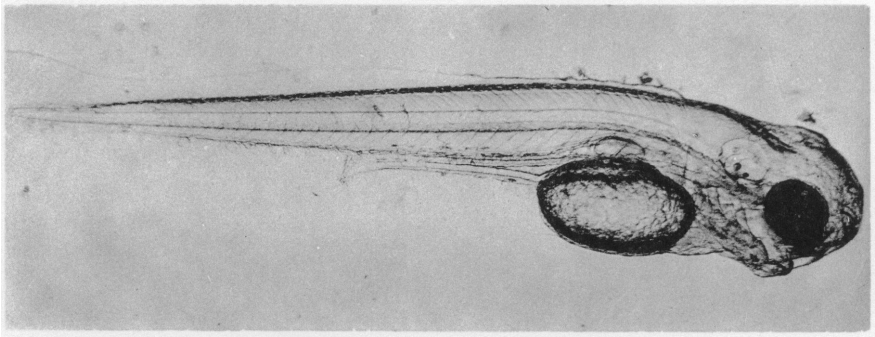

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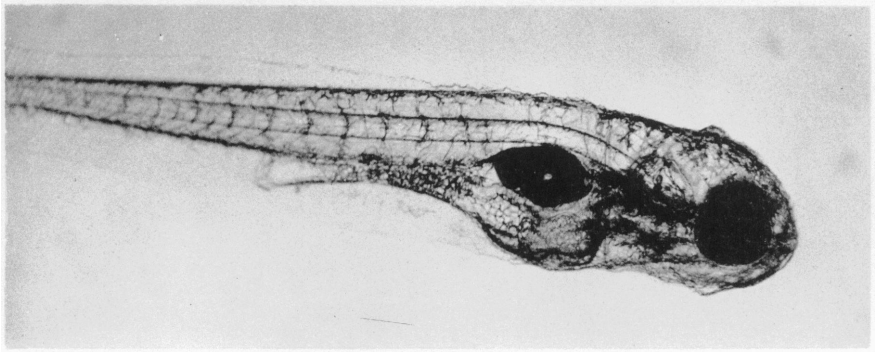
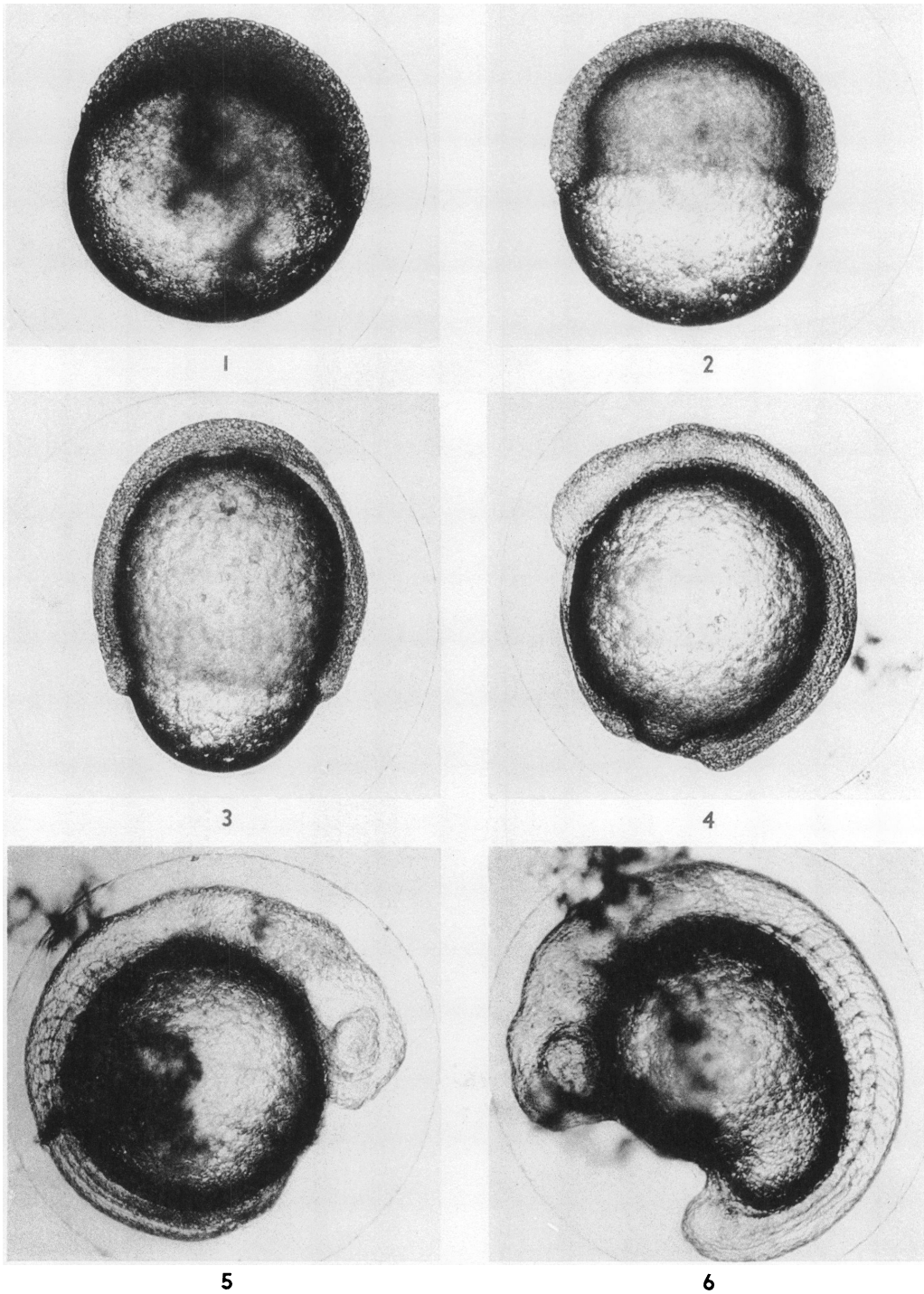


4

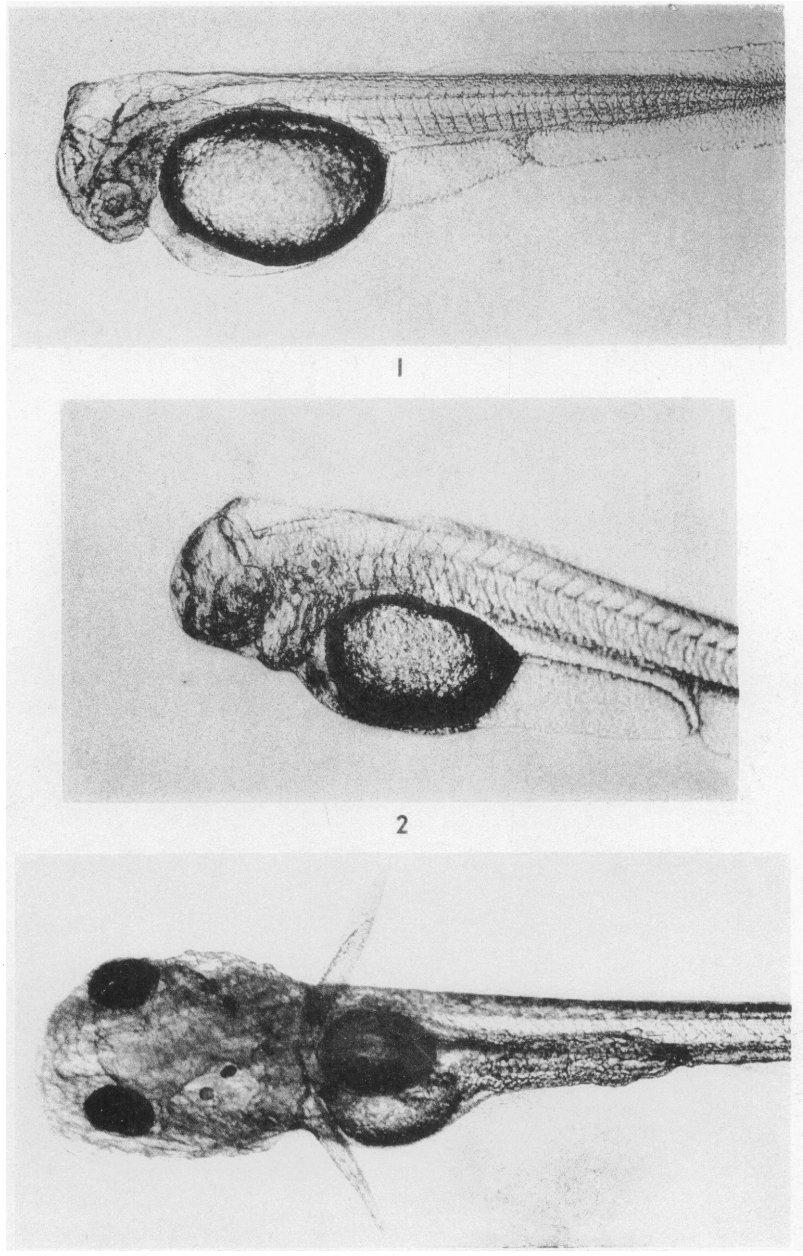
DEVELOPMENT OF LARVAE OF RIVER FISH

1. Stage 14, hatched larva. × 30
2. Stage 14, hatched larva. × 55
3. Stage 15, second day of larval development, retinal pigment. × 30
4. Stage 16, third day of larval development, swim bladder filled with air. × 30



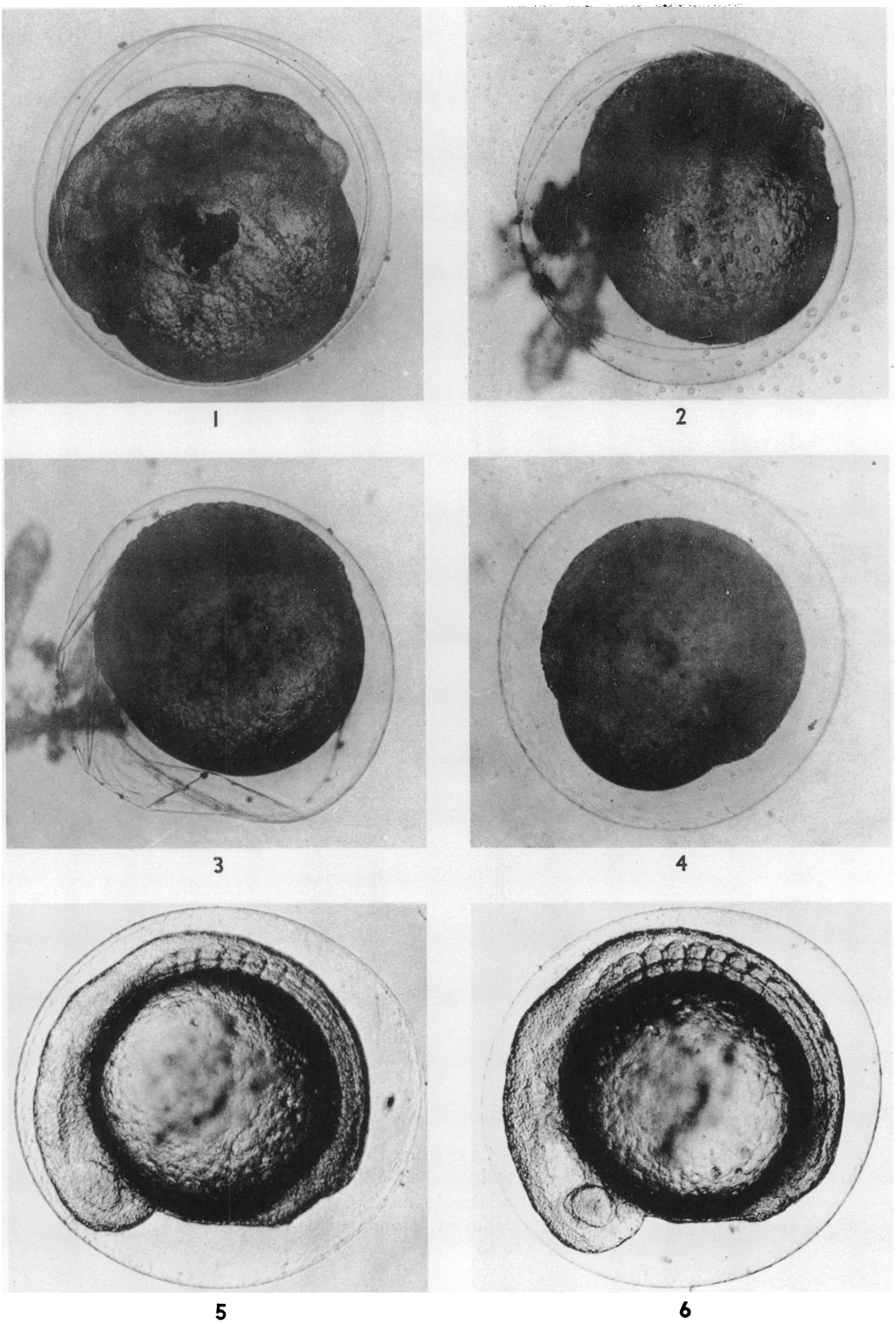
DEVELOPMENT OF EGGS AND EMBRYOS OF CHICA CAVE FISH

1. Stage 3, early gastrula. × 50
2. Stage 4, mid-gastrula. × 50
3. Stages 5–7, yolk plug, neurula, and early optic buds. × 50
4. Stages 7–8, blastopore approaching closed position, optic buds. × 50
5. Stages 8–10, closure of blastopore, lens vesicle, mesodermal somites. × 50
6. Stages 11–12, myotomes present but tail still in bud stage, some midbrain expansion. × 50



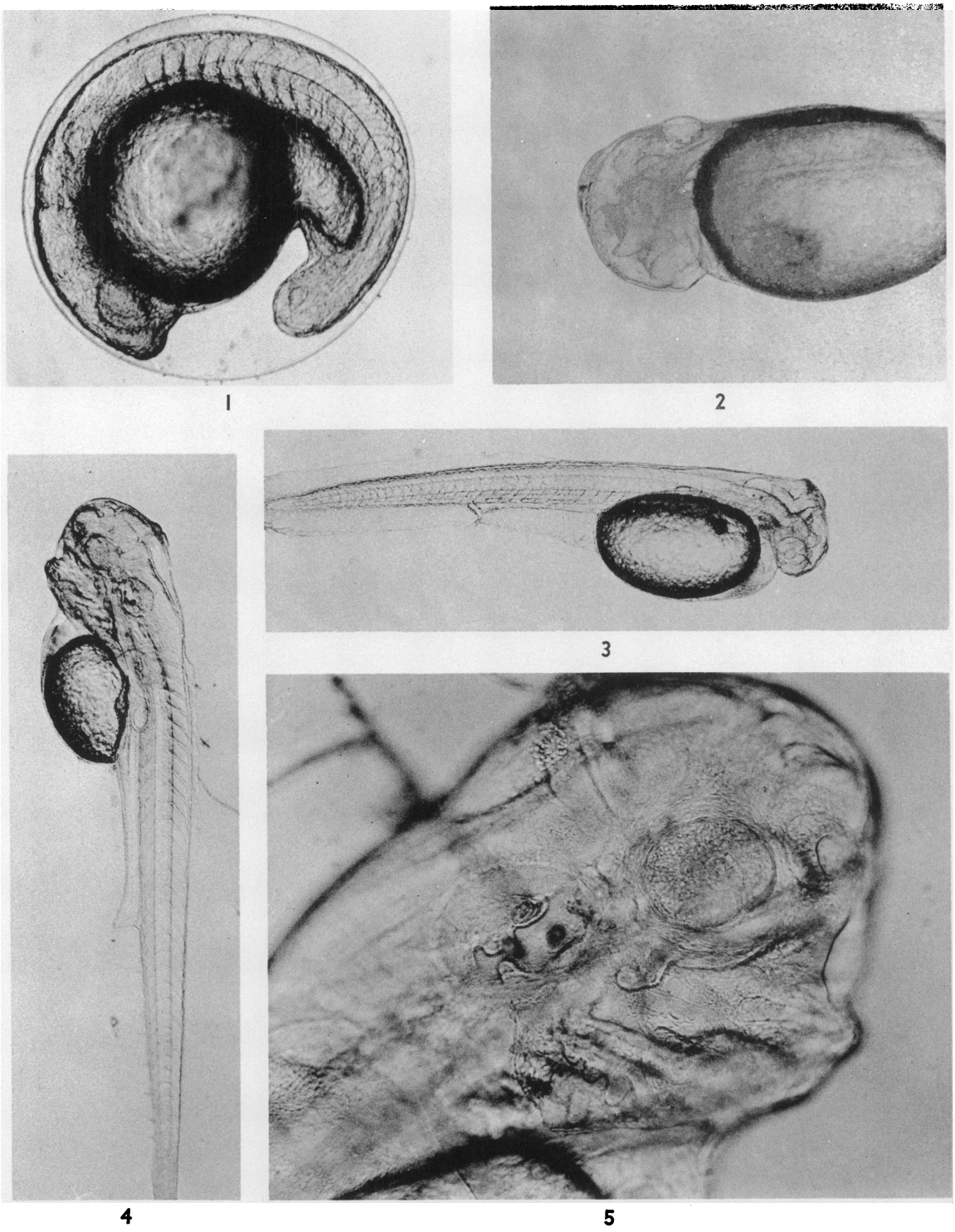
DEVELOPMENT OF LARVAE OF CHICA CAVE FISH

1. Stage 14, hatched larva. × 40
2. Stage 15, second day of larval development, no retinal pigment yet. × 40
3. Fourth day of larval development, prominent retinal pigment, auditory structures, and pectoral fins. × 40



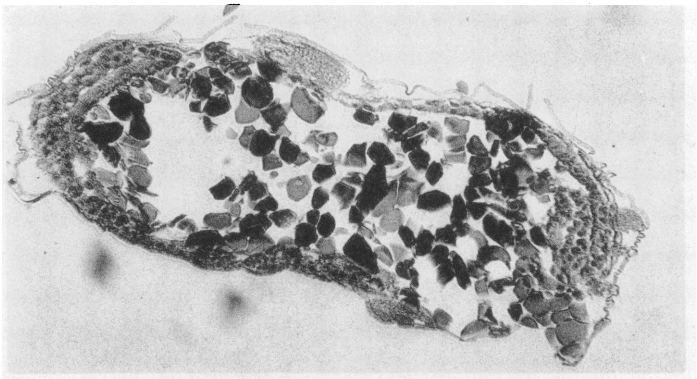
DEVELOPMENT OF EGGS AND EMBRYOS OF SABINOS CAVE FISH

1. Stage 2, abnormal cleavage stage with separated blastomeres. × 55
2. Stages 2–3, late blastula with cytolyzed cells on right side of blastodisc. × 55
3. Stage 3, early gastrula with collapsed chorion. × 55
4. Stage 5, yolk plug. × 55
5. Stages 8–10, blastopore almost closed, optic and lens vesicles, mesodermal somites. × 55
6. Stages 10–11, optic lobes faintly distinguishable posterior to eye vesicles. × 55



DEVELOPMENT OF EMBRYOS AND LARVAE OF SABINOS CAVE FISH

1. Stage 12, myotomes, some tail-bud elongation. × 55
2. Stage 14, abnormal hatched larva. × 40
3. Stage 14, normal hatched larva. × 30
4. Stage 15, second day of larval development, no retinal pigment. × 30
5. Stage 15, second day of larval development. × 125



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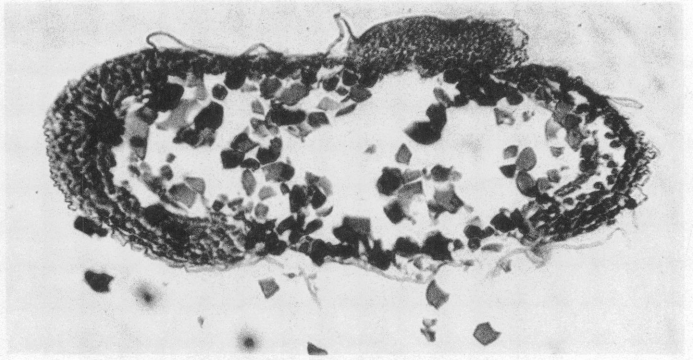

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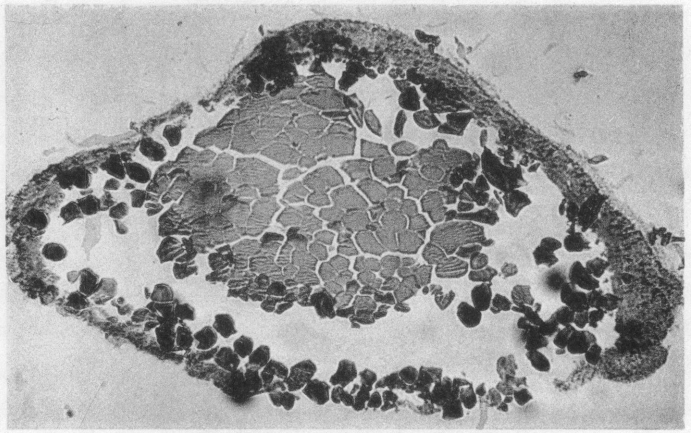


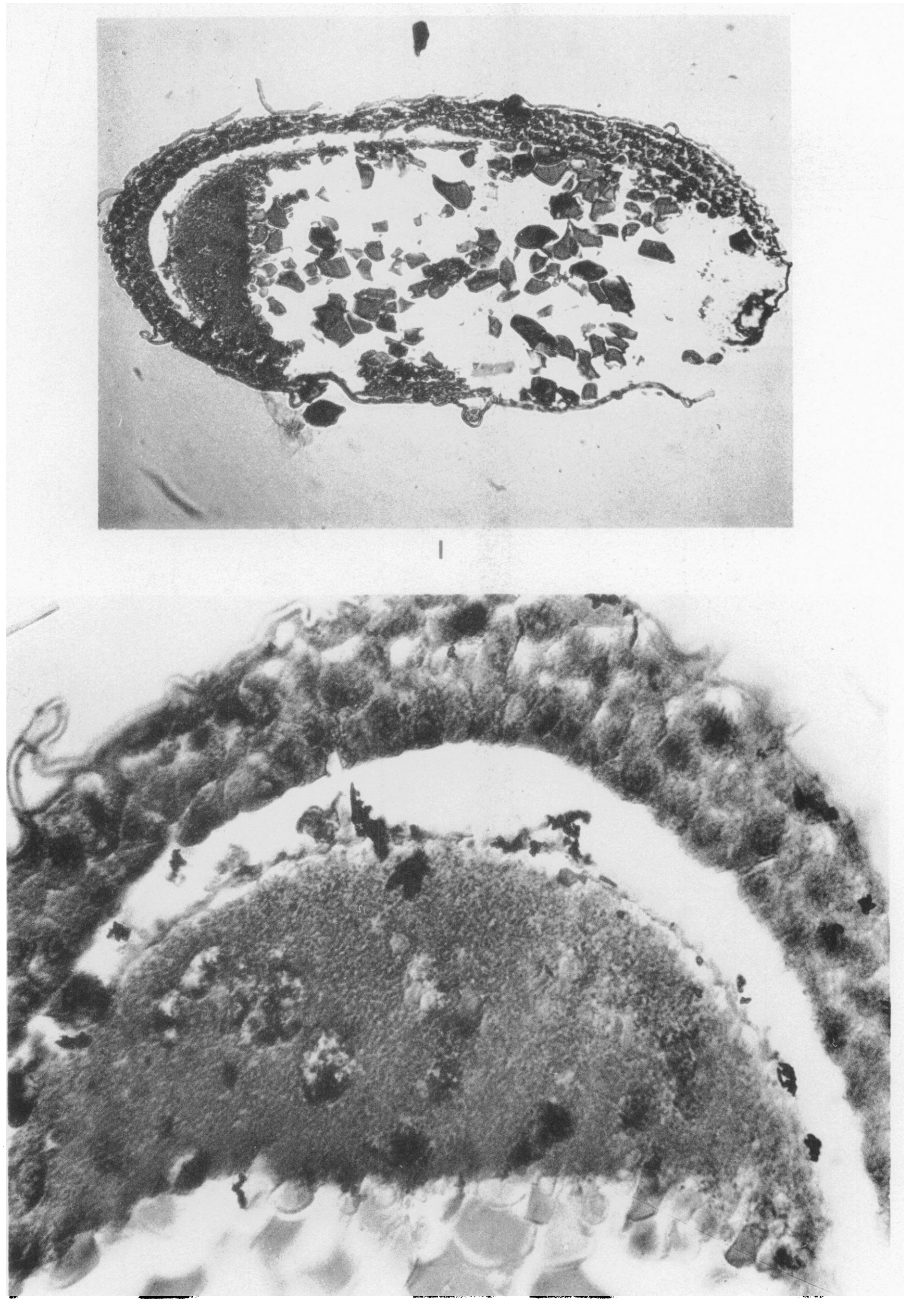
3

SECTIONS THROUGH EGGS OF SABINOS CAVE FISH AT STAGE 5 WITH EXCESS PERIBLAST TISSUE

1. Cross section through equatorial region of an egg from the 7/23/54 spawning, with periblast tissue within perivitelline space. × 75

2. Cross section through equatorial region of an egg from the 8/16/54 spawning, with periblast tissue within perivitelline space. × 75

3. Section through vegetal pole of egg and caudal embryonic region of embryo from 7/23/54 spawning, with excess marginal periblast at region of dorsal lip, seen on right side of photograph, and a thick layer of central periblast surrounding yolk. × 75



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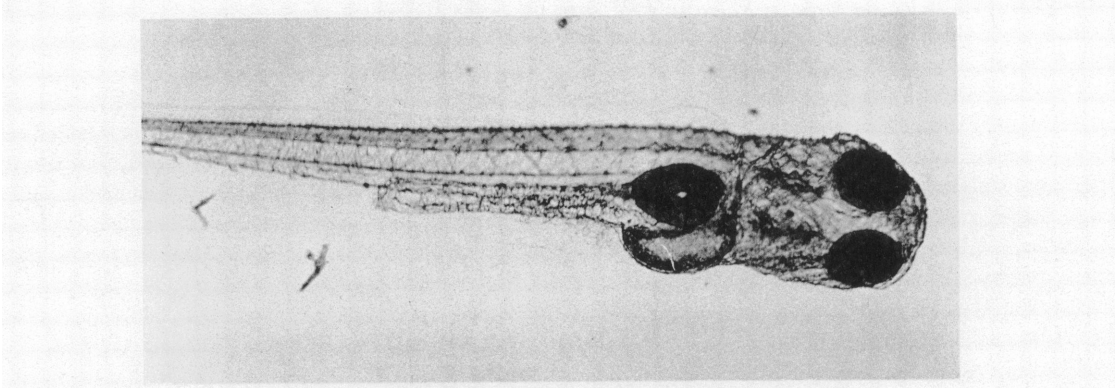
2

1. Sagittal section through animal pole of egg of Sabinos cave fish at stage 5 from 8/16/54 spawning. Excess central periblast tissue can be seen in vicinity of animal pole, located to left of yolk in this photograph. × 75

2. High-power view of animal pole of egg with excess central periblast tissue seen in above photograph. Structure of periblast syncytium can be seen clearly. × 650



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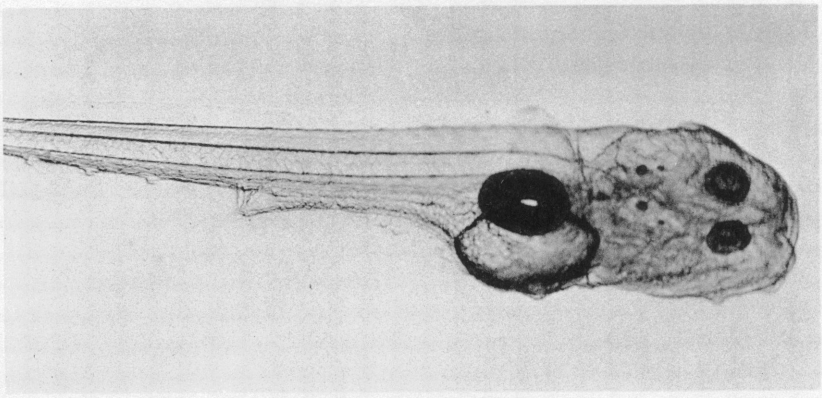

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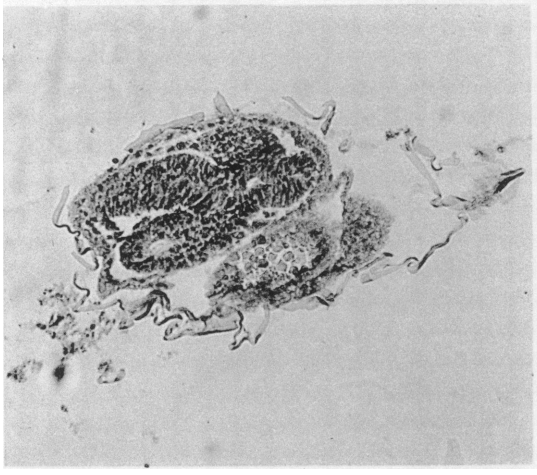

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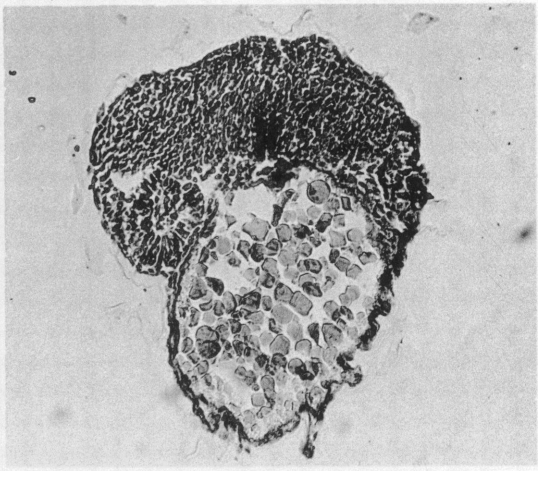
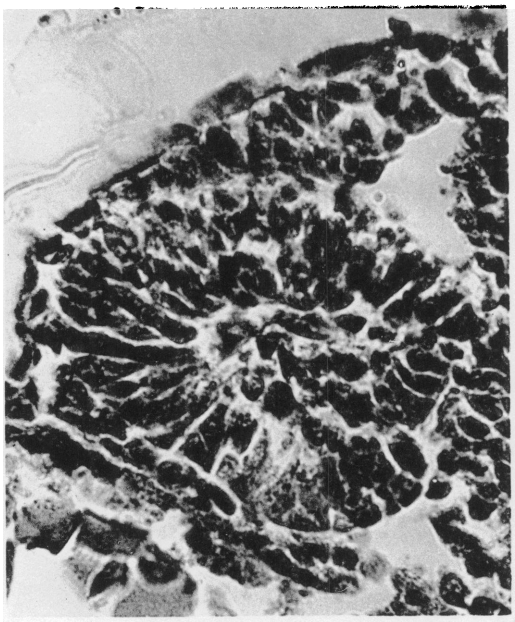


4

1. Stage 16, larva of river fish, showing large, densely pigmented eyes. × 40
2. Stage 16, larva of Sabinos cave fish, showing small eyes with some retinal pigment. × 40
3. Stage 9, embryo of river fish, horizontal section through optic vesicles and forebrain. × 75
4. Stage 9, embryo of river fish, sagittal section through optic vesicle, forebrain, and yolk sac. × 75



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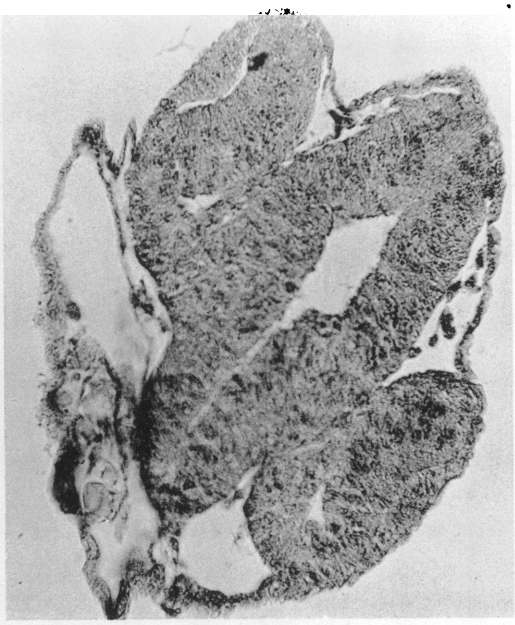

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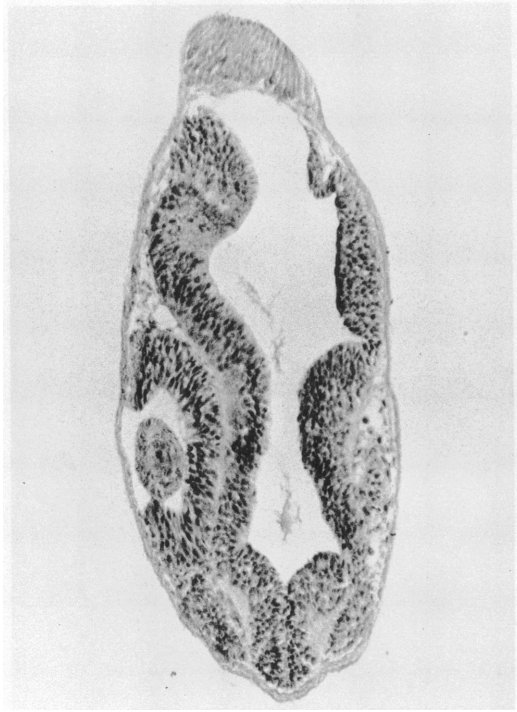

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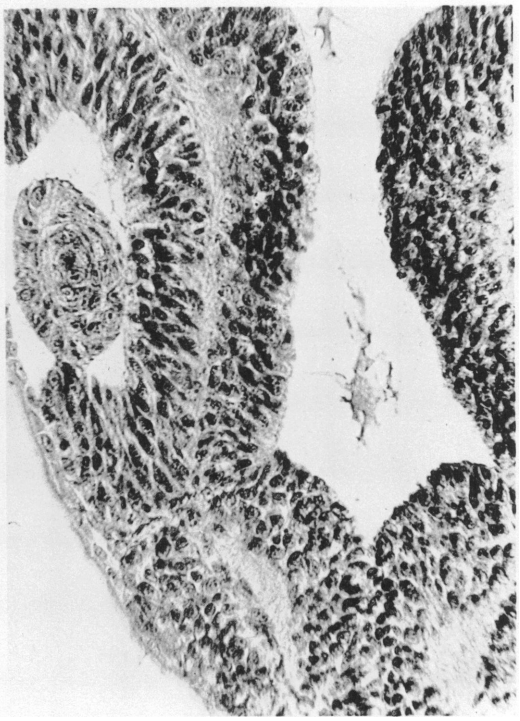


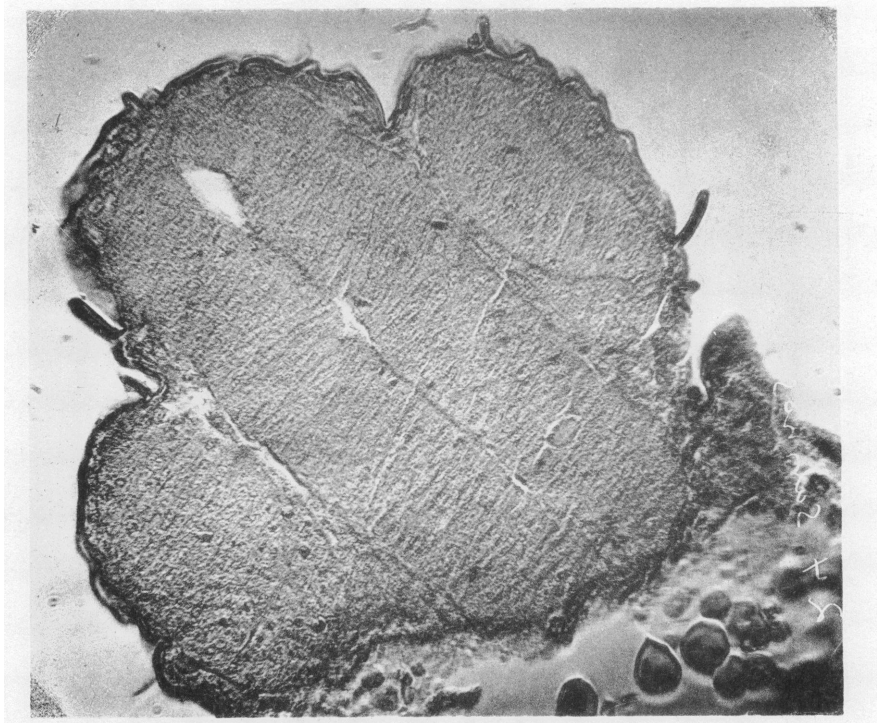
4

1. Stage 9, high-power view of optic vesicle seen in sagittal section of embryo of river fish shown in plate 20, figure 4. × 650

2. Stage 10, cross section through eye cup, lens vesicle, and diencephalon of embryo of river fish. × 360

3. Stage 14, cross section through eye cup, lens, and diencephalon of river fish on the first day of larval development. × 130

4. Stage 14, high-power view of eye cup, lens, and diencephalon seen in 3. × 360



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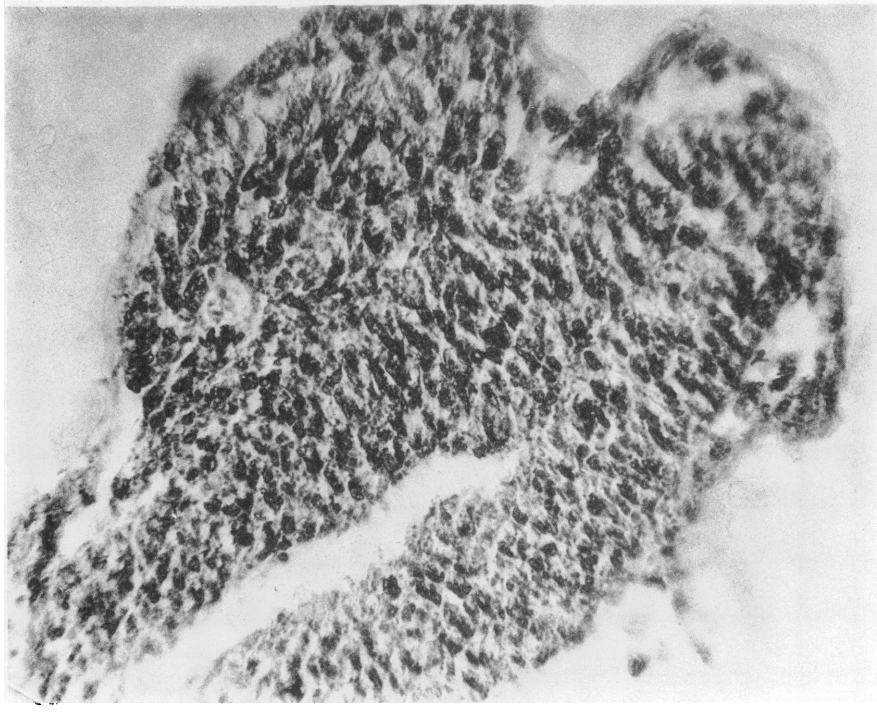
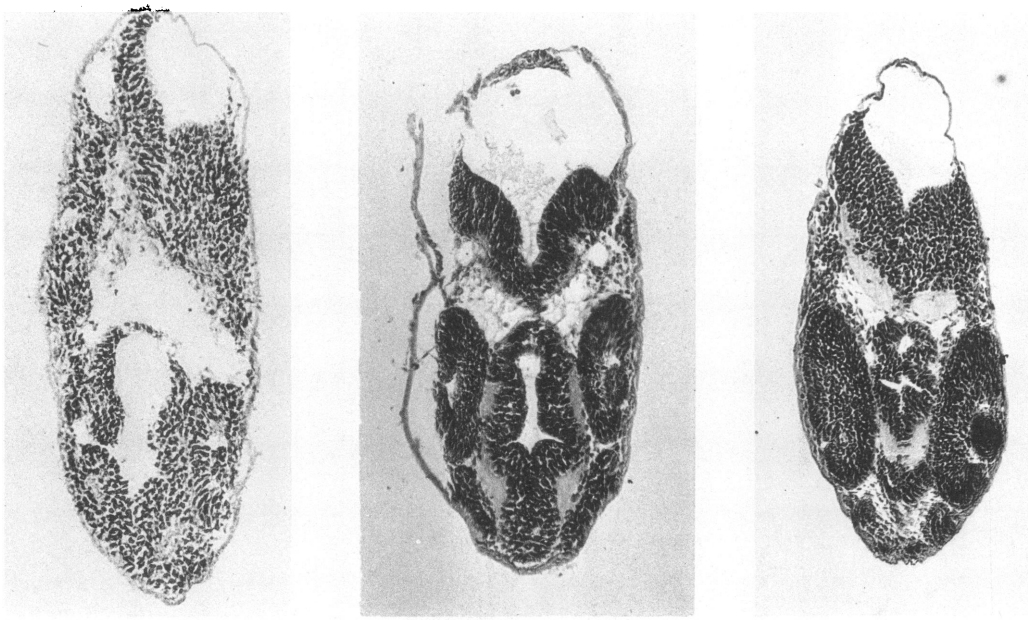


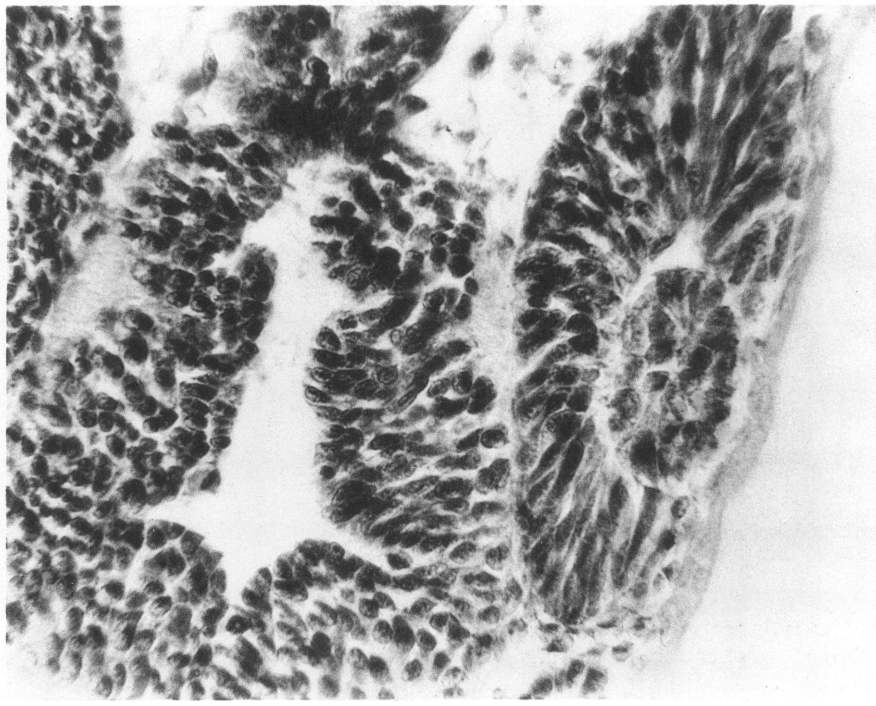
2

SECTIONS THROUGH EMBRYOS OF CAVE FISH

1. Stage 10, Sabinos embryo, cross section, showing epithelial clefts dorsal and ventral to eye vesicles, lens vesicles prior to invagination, and diencephalon with a small cavity. × 360

2. Stages 12–13, Chica embryo, horizontal section showing irregular, partly invaginated optic vesicle, dorsolaterally located lens placode, and irregular contour of diencephalon cavity. × 500





4

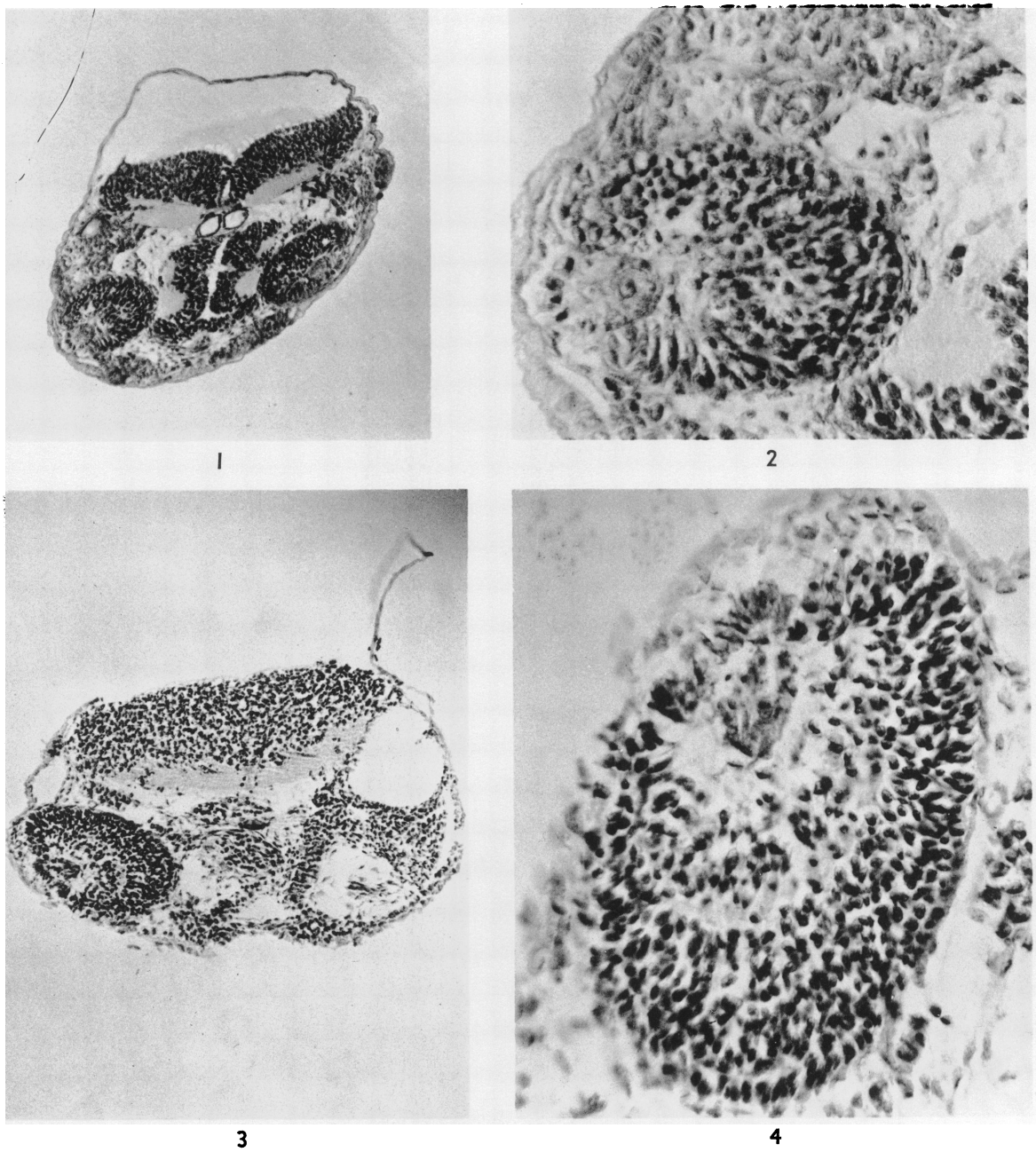
CROSS SECTIONS THROUGH STAGE-14 LARVAE OF CAVE FISH AND OF RIVER FISH

1. Section through head of Chica larva, showing small optic cup and close association of retinal dorsal lip with lens. No lens fibers are differentiated. × 130

2. Section through head of Sabinos larva, showing small optic cup and lens without fiber differentiation. × 130

3. Section through head of river-fish larva, showing large optic cup and basophilically stained lens fibers. × 75

4. Section through optic cup, lens, and diencephalon of Sabinos larva, showing irregular number of rows of retinal nuclei, poorly structured ventral lip, and absence of two distinct zones of lens nuclei. × 580



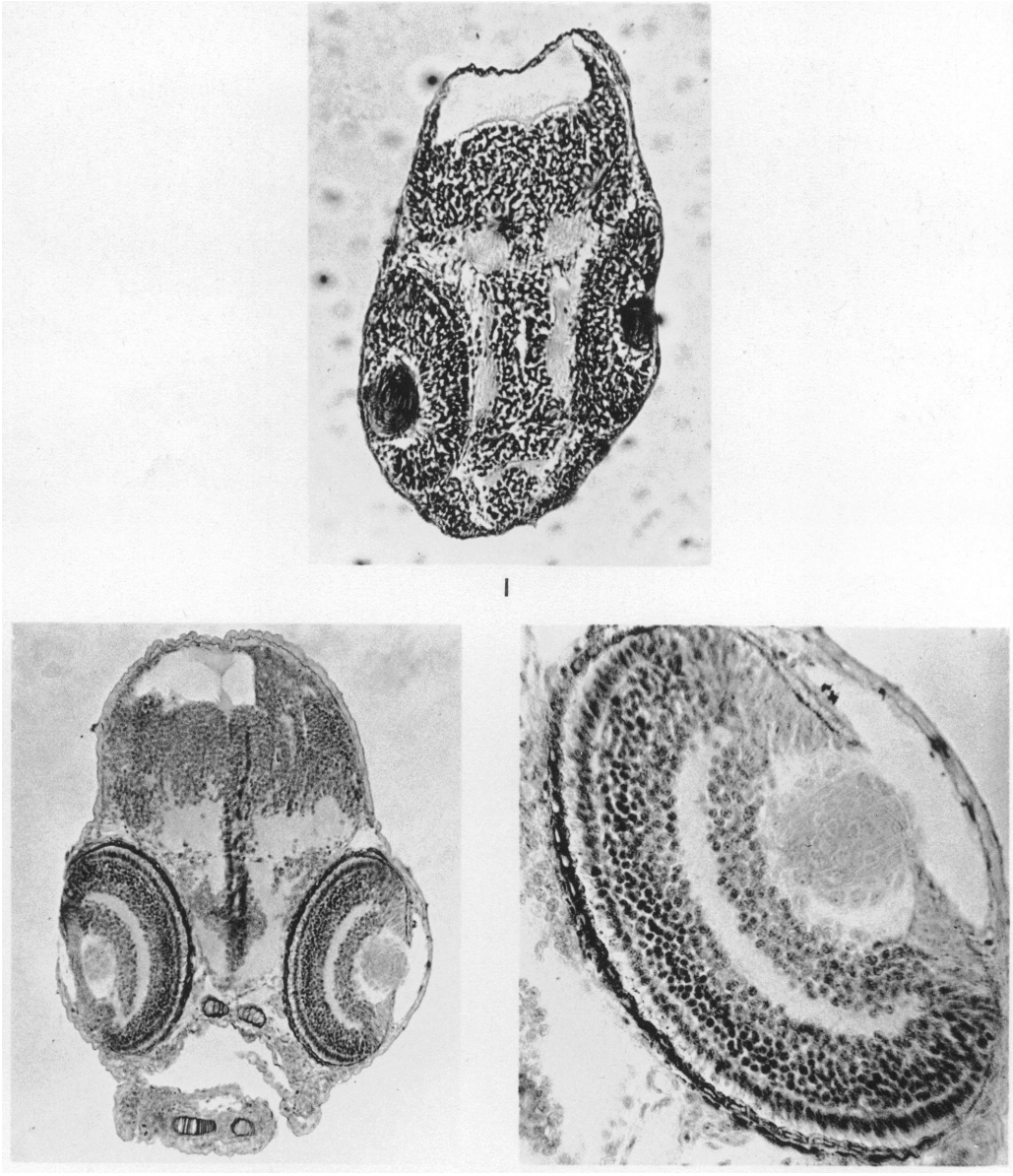
CROSS SECTIONS THROUGH STAGE-15 LARVAE OF CAVE FISH AND OF RIVER FISH

1. Section through head of Sabinos larva, showing small, closed-in optic cup and lens and adjoining parts of diencephalon. × 130

2. High-power view of section of Sabinos eye cup and lens seen in 1, showing undifferentiated regions and empty spaces in retina, and appearance of two zones of lens nuclei. × 580

3. Asymmetrical section through hindbrain and diencephalon of Chica larva, showing expanded auditory labyrinth on one side and small optic cup and lens on other side. × 75

4. High-power view of section of Chica eye cup and lens seen in 3, showing differentiated layers visible in central parts of retina, and two zones of lens nuclei. × 500



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3

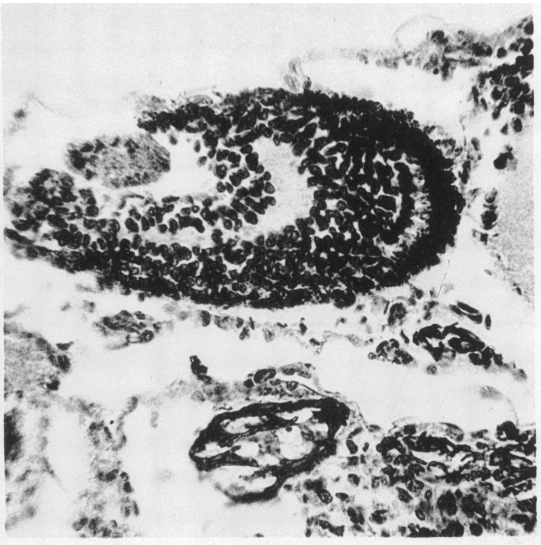
CROSS SECTIONS THROUGH LARVAE OF RIVER FISH

1. Section through head region of stage-15 larva, showing large optic cup and lens. The outer, oriented row of basophilic rod and cone nuclei, the characteristic lens fibers, and the adjoining parts of the diencephalon can be seen. × 75

2. Section through head region of stage-16 larva, showing differentiated layers of the retina and adjoining brain regions. × 130

3. High-power view of eye cup and lens seen in 2. The non-visual parts of the eye can be seen, including the choroid, iris, cornea, annular ligament, and anterior eye chamber. × 360

1



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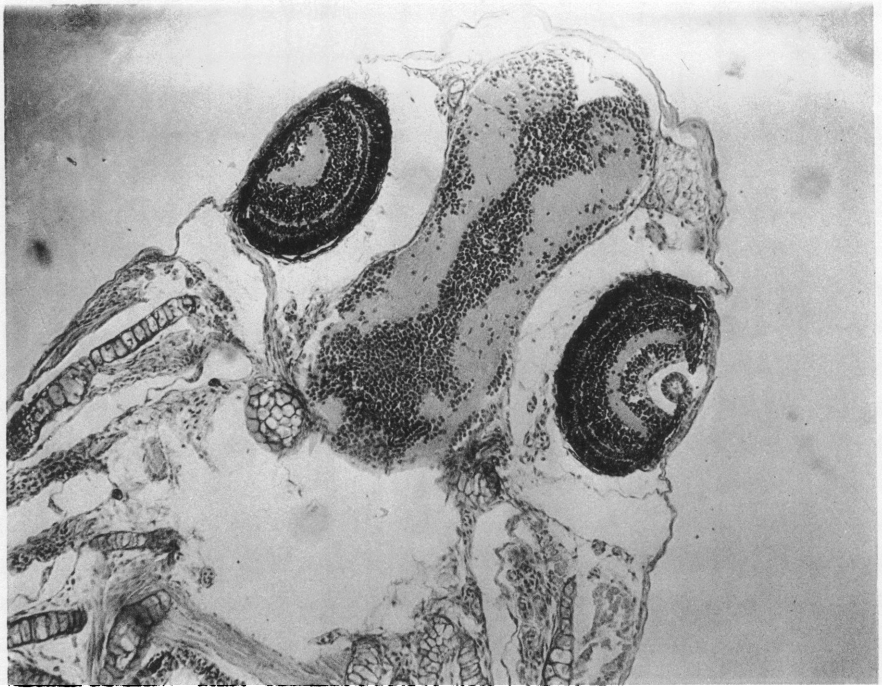
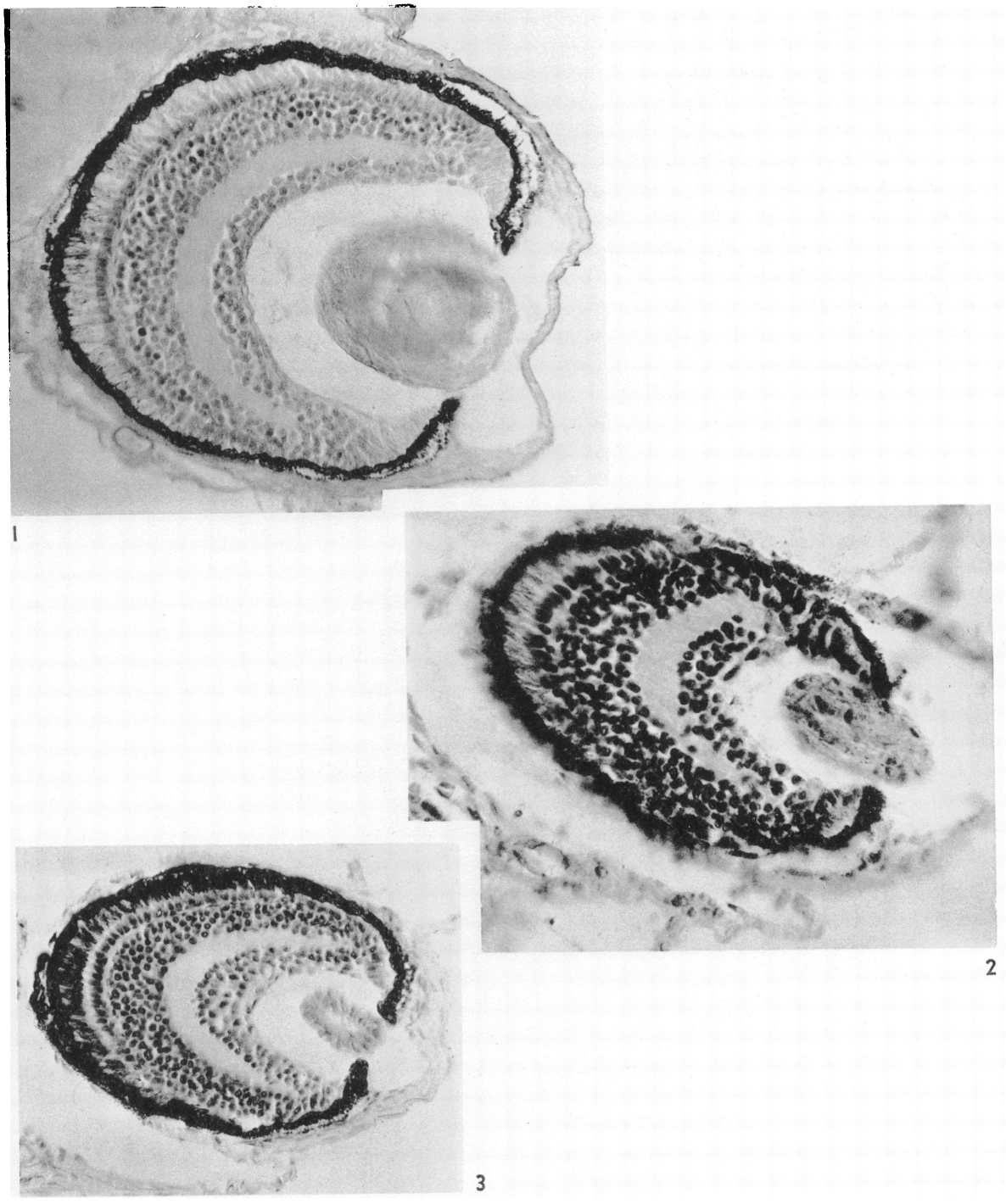


3

1. Cross section through eye cup, lens, and diencephalon of river-fish larva on fifth day of larval development. The posterior eye chamber can be seen. × 130

2. Cross section through eye cup and lens of larva of Chica cave fish on fourth day of larval development. Dense pigment can be seen in pigment epithelial cells. Central retinal regions were well differentiated, but dorsal and ventral regions were poorly defined. × 360

3. Horizontal section through eyes and diencephalon of larva of Sabinos cave fish on fifth day of larval development. Many empty spaces can be seen in nuclear layers of retina. The corneal region appears thickened. × 75



CROSS SECTIONS THROUGH EYES ON FIFTH DAY OF LARVAL DEVELOPMENT

1. Eye of river fish, with little migrating pigment between rods and cones, well-developed retinal layers in dorsal, central, and ventral regions, fibrous lens, and thin cornea. × 360

2. Eye of Chica cave fish, showing compressed eye shape, large rod and cone nuclei differentiated only in central retinal regions, non-fibrous lens, and densely pigmented iris. × 500

3. Eye of Sabinos cave fish, with much migrating pigment between rods and cones, differentiation of rod and cone nuclei extending to dorsal and ventral retinal regions, non-fibrous lens, and thickened cornea with annular ligament cells along inner border. × 360

The nuclei of these fiber-forming lens cells lost their granulation and became almost clear, except for one deeply stained chromatin mass that remained for a time. The inner-zone lens cells became elongated and then concentrically arranged (pl. 21, fig. 4). The numerous, densely basophilic, retinal nuclei seen in the central parts of the innermost retinal layer in this illustration may be indicative of some retinal contribution to the process of lens-fiber formation. Before the end of this stage lens fibers were prominent and were distinguished by their rather homogeneous, basophilic-staining properties (pl. 23, fig. 3). In later stages the outer-zone nuclei formed the cuboidal epithelial capsule that surrounded the lens.

Histochemical studies at stage 14 indicated that there was very little glycogen-positive cytoplasm in the lens cells. Protein-bound SH groups appeared in slightly greater concentration than previously around the nuclei of the lens cells, as around the nuclei of some of the retinal cells. Reactive granules were also concentrated around the nuclei of the fiber-forming lens cells. The lens-cell cytoplasm showed a reaction indicative of sparsely distributed, protein-bound, SH groups. In the central fiber-forming lens cells ribonuclease digestion failed to eliminate completely the basophilic staining of the cytoplasm. This indicated that another basophilically stained component in addition to RNA was present.

CAVE FISH: At stage 9 no thickening was apparent in the future lens epidermal cells. Few mitoses were seen in these cells.

By stage 10 lens thickening had occurred in the Sabinos embryos (pl. 22, fig. 1), but invagination of the lens vesicle did not appear to have started. Few mitoses were seen in the lens cells. The lens vesicle seemed to be smaller than in the river fish. No Chica sections in this stage were available. In the Chica embryos at stages 12–13 thickening of the lens ectoderm had formed a lens placode made up of a double row of high cuboidal cells. This placode was located dorsolateral to the eye vesicle (pl. 22, fig. 2). No invagination of these placodes was yet apparent, and few mitotic figures were found in these lens cells.

By stage 14 lens invagination had occurred. The lenses were smaller than in the river fish in harmony with the smaller eye cups. Two zones of lens nuclei as observed in the river fish at this stage were not seen. No nuclear degranulation of inner lens cells had occurred (pl. 23, fig. 4). In the lens cells of the Chica larvae some mitoses were seen, and the lens appeared to be in close association with the dorsal lip of the optic cup. Because of this proximity it often appeared that the lens had actually formed from the dorsal lip cells. Some of the cells in the Chica lens resembled sensory retinal cells with cone-shaped outer segments more than epithelial cells. Some elongation of Sabinos lens cells occurred and some partial coiling of these elongated cells around two or three different centers in one lens was observed. But most of the lens cells were not concentrically oriented, and no typically structured lens fibers with homogeneously stained, basophilic cytoplasm were formed in the cave larvae.

Histochemical studies at stage 14 indicated that glycogen-positive granules were highly concentrated in the cytoplasm of the lens cells. Protein-bound SH groups were sparsely distributed in the nuclei and cytoplasm of the lens cells. In the cytoplasm of those Sabinos lens cells that showed an abortive effort to form lens fibers reactive granules were more concentrated. RNA was present in the lens-cell cytoplasm. No ribonuclease-resistant, basophilic cytoplasm was found in the lens cells, although this reaction occurred in the fiber-forming lens cells of the river fish.

At stage 15 in some lenses of both Chica and Sabinos larvae two zones of nuclei were seen. In the outer zone each nucleus contained a large, deeply basophilic-stained, chromatin mass. In the inner zone the nuclei were paler stained and showed some nuclear degranulation (pl. 24, figs. 1, 2, 3, and 4). In the Sabinos larvae the elongation of most of the inner-lens cells failed to follow this appearance of two zones of nuclei, and no homogeneously basophilic-stained lens fibers formed. The elongation and partial coiling of some of the inner-zone lens cells described at stage 14 appeared to be an abortive effort at fiber production. In the Chica larvae some of the lens cells that more resembled sensory retinal cells with elongated cytoplasmic processes became oriented in a spiral pattern and homogeneously basophilic. This, too, ap-

peared to be an abortive effort at fiber production.

After stage 15 no further differentiation of the lens cells took place, and no typical lens fibers appeared. By the fourth and fifth larval days pycnotic lens nuclei were present, with irregularly shaped, dense, chromatin masses. Lens-cell boundaries were not visible, and the inner regions of the lens contained a palely stained granular syncytium. The lens remained as a small, epithelial vesicle.

COMPARISONS: The histological appearance of stages in the development of the lens of the river and of the cave fish is summarized in table 8. This table indicates that the lenses of the cave fish showed delayed thickening, invagination, and differentiation when compared with those of the river fish. The lenses of the cave larvae were smaller than those of the river fish, and at all stages showed relatively few mitoses. Differentiation of characteristic lens fibers failed to occur in the cave larvae, although some of the pre-fiber-forming differentiation processes were observed. The results of the histochemical localization of glycogen, protein-bound SH groups, and RNA in the developing lenses of river and cave larvae are summarized in table 7. These results indicate that the intensities of the histochemical reactions in the lens cells corresponded with the intensities of the reactions in the optic-cup cells. As in the optic cup, in the faster-growing lenses of the river fish the glycogen reaction was less intense, and the SH reaction was more intense, than in the slower-growing cave-fish lenses. RNA distribution in the lenses of the river and of the cave larvae was similar in the stages prior to fiber differentiation. In the fiber-forming lens cells in the river fish ribonuclease-resistant, basophilic cytoplasm was found.

DIFFERENTIATION OF THE VISUAL LAYERS OF THE RETINA

RIVER FISH: At stage 15 many of the flattened pigment epithelium cells contained numerous pigment granules concentrated within the cytoplasm, but not yet dispersed between the rods and cones. In the sensory retina the outermost layer of rods and cones was made up of an oriented row of ovoid basophilic nuclei (pl. 25, fig. 1). Cone-shaped basophilic segments adjacent to these nuclei were located on the side towards the pigment epithelial layer in central retinal regions, but were found on the side towards the eye chambers around the lips of the eye cup. The external limiting membrane was indistinctly seen just inside the layer of rods and cones. The outer nuclear layers consisted of four to five rows of closely packed, basophilic nuclei, separated from the three to four rows of the inner nuclear layers by a narrow layer of fibers that later formed the outer fibrous layer. From the narrow, inner fibrous layer that bordered on the lens, fibers were seen that traversed the ventroposterior region of the inner nuclear layers. These fibers joined with fibers of the outer fibrous layer, at the level of the optic stalk, and formed the optic nerve which progressed along this stalk towards the brain. Many mitoses were still found in the retina.

TABLE 8

COMPARISON OF LENS FORMATION IN RIVER AND CAVE FISH

Stage	Structure	River Fish	Cave Fish
9	Lens anlage	Thickened; many mitoses	Not thickened; few mitoses
10	Lens vesicle	Invagination started; many mitoses	No invagination; few mitoses
12–13	Lens vesicle	Fully invaginated	Not invaginated
14	Lens size	Large	Small
	Nuclear zones	Outer and inner zones	No zonation
	Inner-zone nuclei	Degranulated	No nuclear degranulation
	Inner-zone cells	Elongated; oriented concentrically; formed lens fibers	Some cell elongation; some concentric orientation; no lens fibers formed
15	Lens capsule	From outer zone cells	Not apparent
15+	Lens differentiation	Completed	Incomplete; no lens fibers formed

By stage 16, on the third larval day, the pigment epithelial cells were so densely packed with pigment granules that the cell nuclei were obscured. A few crystalline-like pigment granules from the pigment epithelium were found between the outer segments of the rods and cones. The outer segments of the rod and cone layer were eosinophilic (myoid and ellipsoid regions could not be distinguished). The external limiting membrane, with its small, flattened, ovoid nuclei, separated the rod and cone layer from the outer nuclear layers. The outer fibrous layer had widened, and separated the six to seven rows of the outer nuclear layers from the three to four rows of the inner nuclear layers. The inner fibrous layer was still made up of a very narrow band of fibers. Some small, ovoid nuclei of the internal limiting membrane were seen, and some ganglion cells with large, pale-stained nuclei were found just inside this membrane. Most of these differentiated layers of the retina are visible in plate 25, figures 2 and 3.

Most of the changes observed by the fifth larval day were concerned with the further development of the non-visual parts of the eye, and are described in the next section. All the visual layers of the retina, with the exception of the ganglion and nerve-fiber layers, were differentiated and clearly visible by this time (pl. 26, fig. 1). Few of the pigment epithelial granules had migrated out between the rod and cone segments. The inner and outer segments of the rods and cones had elongated. The inner segments were acidophilic, granular, ovoid, and were located just mesial to the small, clear, hemispherical vacuole that adjoined the peripheral nuclei of the rods and cones. The outer segments were also acidophilic, but in most regions they were obscured by the few migrating pigment epithelial granules. The outer and inner nuclear layers showed some thinning out as the fibrous layers widened.

CAVE FISH: At stage 15 there were few pigment granules in the pigment epithelial cells of both Chica and Sabinos larvae. In the rest of the Chica retina structural differentiation of the various layers was often good in central and some dorsal areas. In plate 24, figures 3 and 4, the outer layer of rods and cones, and the nuclear and fibrous layers, can be made out but with some difficulty. Empty spaces indicative of degenerative changes were apparent in the outer and inner nuclear layers. Few mitoses were found in the retina. In some of the Sabinos retinas a more even distribution of differentiated regions was noted, although this was not apparent in the section shown in plate 24, figures 1 and 2. Undifferentiated regions were seen in many sections, and empty spaces were present in the nuclear layers. Few mitoses were found in the retina.

At stage 16, on the third larval day, there were few pigment granules in the pigment epithelial cells of Sabinos larvae. The other retinal layers appeared differentiated, but they were not clearly visible in the sections available. No sections of Chica retinae were available at this stage. On the fourth larval day pigment granules were densely concentrated in the pigment epithelial cells of Chica retinae (pl. 26, fig. 2). The other layers were well differentiated and clearly visible in the central regions of the retina, but in dorsal and ventral areas the retinal layers had coalesced and could not be distinguished. Many more empty spaces were seen in the outer and inner nuclear layers.

By the fifth larval day pigment granules filled the pigment epithelial cells of the Chica and Sabinos retinas. Many migrating pigment granules extended into the processes of the pigment epithelial cells between the segments of the rods and cones. This was most pronounced in the Sabinos eyes. In the Chica retina the layers were well differentiated in the central regions, but in dorsal and ventral areas coalescence of the retinal layers had taken place, as described for the fourth larval day. This appeared to worsen as the lips of the eye cup moved closer together. This closing in of the eye cup caused the nuclear layers to appear thicker than in the river fish, where they were spread out and seemed thin. Empty spaces were more numerous than on the previous day in the Chica nuclear layers. The internal limiting membrane also appeared thicker than in the river fish, with pale, acidophilic, cytoplasmic areas. It was difficult to determine if the pale cytoplasm was part of the inner fibrous layer or the internal limiting membrane. In the Sabinos retina the various layers were well differen-

tiated and clearly visible in dorsal, central, and ventral regions. Although the lips of the eye cup had started to close in, coalescence of the layers was not observed. Many empty spaces were found in the nuclear layers (pl. 26, fig. 3). The thickened internal nuclear membrane with pale acidophilic cytoplasm, as described in the Chica retina, was also seen in the Sabinos sections.

COMPARISONS: In table 9 the stages in the differentiation of the visual layers of the retina of river and of cave fish are compared. This table indicates that the differentiation of the retinal layers occurred at about the same time in the river and in the cave larvae. The extent of the differentiated area was limited to the central parts of the retina in the Chica larvae. In the Sabinos larvae this range extended beyond the central regions to the dorsal and ventral parts of the retina, but nevertheless did not approach the extensive distribution of the differentiated layers found in the retina of the river fish. The many empty spaces found within the nuclear layers of the cave retinas were not present in the river fish, and were considered signs of degeneration. These empty spaces increased as the cave-fish larvae developed further. The many migrating pigment granules from the pigment epithelium cells found between the rods and cones of the cave larvae, and especially in the Sabinos retina, were not seen in the river fish. This dispersed position of the pigment granules resembled the typical light-adapted state known to be found in the retinas in which photomechanical responses are operative.

Most of the deficiencies described in the above paragraphs found during the differentiation of the retinal layers of the cave larvae when compared with those of the river fish can be seen in the sections through the eyes of river and cave larvae on the fifth day of larval development shown in plate 27, figures 1, 2, and 3.

DEVELOPMENT OF NON-VISUAL PARTS OF THE EYE

RIVER FISH: At stage 16, on the third larval day, the choroid argenteal layer was formed. The cells of this layer could not be clearly distinguished because of the thin network of sparse pigment granules that seemed to obscure the cell boundaries. Occasional small nuclei were seen within this layer. Just inside the argentea, at the outer border of the retinal pigment epithelium cells, were found clear vacuolated structures that may be part

TABLE 9

COMPARISON OF THE DIFFERENTIATION OF THE VISUAL LAYERS OF THE RETINA IN RIVER AND CAVE FISH

Larval Day	Visual Layers	River Fish	Cave Fish
Second	Pigment epithelium	Much pigment	Little pigment
	Outer rod and cone nuclei	Oriented in dorsal, central, and ventral eye-cup regions	Oriented only in central and some dorsal regions
	Nuclear and fibrous layers	Differentiated in dorsal, central, and ventral eye-cup regions; no empty spaces in nuclear layers; many mitoses	Only differentiated in central and some dorsal regions; many empty spaces in nuclear layers; few mitoses
Third	Pigment epithelium	Much pigment	Little pigment
	Nuclear and fibrous layers	Differentiated around entire eye cup; no empty spaces	Only differentiated in central regions; many more empty spaces
Fifth	Ganglion cells	Formed	Not formed
	Pigment epithelium	Few migrating granules between rod and cone cells	Many migrating granules between rod and cone cells
	Retinal layers	All regions clearly differentiated and visible	Only central regions clearly visible; other regions show merged, poorly defined layers, with many empty spaces

of the choroidal choriocapillaris layer. These vacuolated structures are barely visible in plate 25, figure 2, but can be seen more clearly in plate 25, figure 3, at the outer border of the retinal pigment epithelium. The choroid argentea was continuous with the lightly pigmented argentea of the iris. Outside the iris argentea was an outer mesothelial layer with flat, nucleated, lightly basophilic-stained cells. Just within the argentea was the fibrous stroma, not yet vascular, with densely pigmented cells along the inner border that adjoined the retinal part of the iris. These pigmented stromal cells appeared to be continuous with the rest of the retinal pigment epithelial layer. The innermost region of the iris was formed from the extended lips of the retinal layers of the eye cup and appeared still to be proliferating. Many mitoses and large nucleoli were seen in these undifferentiated optic-cup cells that formed part of the iris. The cornea was made up of an outer, very thin, darkly stained epithelium, inside of which was a narrow, fibrous layer with occasional flat, elongated nuclei. This fibrous layer probably represented the scleral part of the cornea. A second, somewhat thicker, fibrous, corneal layer, the developing substantia-propria of the autochthonous layer, also contained some elongated, ovoid, flattened nuclei, and was found inside the thin scleral part of the cornea. Vacuoles that were present between each of these proprial cells were the corneal lamellae. The innermost layer of the cornea was a thin membrane, Descements mesothelium, that contained flattened nuclei. The annular ligament cells appeared in the angle between the iris and the cornea, and seemed to be continuous with Descements mesothelium. These ligament cells, believed to be of mesodermal origin, contained elongated, ovoid, lightly stained nuclei. Most of the above structures can be seen in plate 25, figure 3.

On the fifth larval day the pigment granules in the choroid argentea had increased, and some fibrous components of the choroid were seen. No choroid gland or vascular choroid elements were formed. In the iris, the cells formed from the rim of the retina were no longer proliferating, and the nuclei in this region contained no prominent nucleoli. The stromal region of the iris had widened, and the iris argentea was more densely pigmented. The inner fibrous layer of the cornea was now very thin in that region of the cornea opposite the lens, so that corneal induction appeared to have been completed. Many more annular ligament cells were seen in the angle between the iris and cornea. The posterior eye chamber was expanded (pl. 26, fig. 1).

CAVE FISH: In the Sabinos larvae on the third larval day few pigment granules were seen in the choroid and iris argenteal layers and iris stroma. No evidence of any proliferation of the retinal component of the iris was seen, as no mitoses or large nucleoli were seen in these cells. The corneal dermal component was not entirely free of gland cells found in other regions of the dermis. No corneal lamellae were formed between the proprial cells. The outermost corneal epithelium and innermost mesothelium were not deeply basophilically stained. Some annular ligament cells were present, but they were not clearly visible, because no anterior eye chamber had formed.

No sections of the Chica larvae on the third larval day were available, but by the fourth larval day the iris argentea and stroma were pigmented. The lips of the eye cup had started to close in, so that the retinal component of the iris was poorly defined and crowded. There was little evidence of any proliferative activity in the retinal region of the iris. In the choroid the argentea was pigmented, and the choriocapillaris region appeared crowded and narrow. The annular ligament cells were numerous and were not confined to the angle between the iris and the cornea, but were spread over much of the inner layer of the cornea and inner layer of the retina. In the cornea, no gland cells were present in the dermal component. Some corneal lamellae were present between the proprial cells. Basophilic staining of the outermost corneal epithelium and innermost mesothelium was not intense.

By the fifth larval day in Chica and Sabinos larvae the iris and choroid argenteal layers and the iris stroma were pigmented (pl. 27, figs. 2, 3). The stromal region of the iris in the Chica larvae was so densely pigmented that the stromal spaces were not visible. No choroid gland was seen, but some

vascular elements of the choroid and iris were seen in the cave larvae. The cornea in the Chica larvae was thinned out centrally, where the inner fibrous layer was narrowed, opposite the lens, but the outer epithelium and inner mesothelium were thicker and less basophilic than in the river fish. In the Sabinos larvae the cornea remained thick in all regions. Some gland cells were still found in the dermal component. No corneal lamellae were seen between the proprial cells. The innermost mesothelial layer of the cornea in the Sabinos larvae appeared to contribute to what appeared to be additional annular ligament cells (pl. 26, fig. 3). These cells were found within the now formed anterior eye chamber, just underneath the cornea, and around the lens in the posterior eye chamber. The cells resembled elongated epithelial cells with spindle-shaped, basophilically stained nuclei. The annular ligament cells in the Chica larvae were also spread out along the inside of the cornea and inner layer of the retina but were not so numerous as in the Sabinos larvae.

COMPARISONS: In table 10 the stages in the development of the non-visual parts of the eye in river and in cave fish are compared. This table indicates that by the fifth larval day the iris and choroid in the cave fish were fairly well developed. This was found despite the somewhat delayed appearance of pigment in these structures and the absence of any proliferation of the retinal rim of the iris in the cave larvae. The presence of some gland cells in the cornea, and the failure of the fibrous layer of the cornea to become thin, indicate that corneal induction was not completed in the cave fish. The spreading annular ligament cells were also characteristic of the cave larvae.

APPEARANCE OF DIENCEPHALON, OPTIC LOBES, AND OPTIC NERVE

RIVER FISH: As indicated in the section on the development of the optic cup, the forebrain was still solid at stage 7 when solid optic buds were present. By stage 9 a lumen had formed in the forebrain, opposite the opticoeles. Many mitoses were found in the forebrain cells. At stage 10 the diencephalon was differentially thickened laterally, mitoses were found in the diencephalon cells, and the third ventricle of the diencephalon had a characteristic, diamond-shaped contour in cross section (pl. 21, fig. 2). The optic lobes

TABLE 10

COMPARISON OF THE DIFFERENTIATION OF THE NON-VISUAL PARTS OF THE EYE IN RIVER AND CAVE FISH ON THE THIRD TO FIFTH LARVAL DAYS

Non-Visual Parts	Components	River Fish	Cave Fish
Iris	Argentea and stroma	Pigmented by 3d day	Pigmented by 4th–5th day
	Retinal rim	Proliferating on 3d day	Not proliferating on 3d, 4th, or 5th day
	Stromal spaces	Formed on 5th day	Obscured by dense pigment
	Vascular elements	Not present by 5th day	Some formed by 5th day
Choroid	Argentea	Pigmented on 3d day	Little pigment until 4th–5th day
	Choriocapillaris	Wide, vacuolated region	Narrow, vacuolated region
	Vascular elements	Not present by 5th day	Some formed by 5th day
Cornea	Outer epithelium	Thin, darkly stained	Thick, palely stained
	Outer fibrous layer	Narrow, no gland cells	Narrow, some gland cells
	Inner fibrous layer	Thick, with corneal lamellae; thinned out by 5th day	Thick, few corneal lamellae; remained thick by 5th day
	Descements mesothelium	Thin	Thick
Annular ligament	Nuclei	Lightly stained	Darkly stained
	Location of cells	Confined to angle between iris and cornea	Not confined, but spread out to other regions

had started to enlarge. By stage 14, two to three layers of optic-nerve cells with thin, spindle-shaped nuclei and fibrous cytoplasmic processes were seen, growing out from ventral regions of the optic cup. These fibrous cells were approaching the ventroposterior region of the diencephalon. Many mitoses were present in the cells along each side of the neurocoele of the diencephalon and mesencephalon. The optic chiasma was developing in the hypothalamic region of the diencephalon, and the pineal cells were proliferated slightly in the dorsal epithalamic region. By stage 15, on the second larval day, the optic-nerve fibers had reached the brain and were seen to cross over to join with the ventrolateral regions of the diencephalon of the opposite side of the head. In cross section the optic chiasma region was located several sections anterior to where the optic-nerve fibers left the retina. Numerous mitotic figures were still found in the diencephalon. The diencephalic white matter had increased. The dorsal regions of the diencephalon, as seen in plate 25, figure 1, did not appear to be enlarged, as in the cave fish, from the innervation from the lateral-line sensory organs. These lateral-line organs were barely visible in the lateral epidermis dorsal to the eyes in the river fish. The optic lobes had increased in size, and by the third larval day they had expanded anteriorly and posteriorly to overlie most of the diencephalon. On the fifth larval day the optic-nerve fiber bundle, with its wavy, acidophilic fibers, did not appear to have increased in thickness and was found as in the earlier stage to decussate ventral to the diencephalon. Dorsally the diencephalon was covered by a thin layer of skin. The dermal region of the skin was basophilically stained and contained occasional dermal and subdermal melanophores. The pineal gland was underneath the epidermal and dermal layers of the skin and was found between the anterior regions of the optic lobes. The expanded optic lobes, although traversed by some fibrous tracts, primarily contained gray matter. The typical pattern of fibrous layers seen in the optic tectum of older fishes had not differentiated at this time.

CAVE FISH: No lumen formed in the forebrain until stage 10, when a small, irregular-shaped cavity appeared in the diencephalon (pl. 22, fig. 1). This cavity was not of typical diamond-shaped contour, and the lateral walls of the diencephalon were not differentially thickened. In some Chica larvae no third ventricle had formed in the diencephalon by stage 12. Few mitoses were found in the diencephalon cells. The start of optic-lobe enlargement was not observed. At stage 14 some nucleated, non-fibrous, optic-nerve cells were seen leaving the centromedial region of the retina. The lateral walls of the diencephalon were differentially thickened, although few mitoses were present in the cells along each side of the third ventricle. In some larvae there seemed to be a posterior displacement of the diencephalon, so that this region was seen to be overlain dorsally by the middle parts of the hindbrain, instead of by the midbrain and anterior parts of the hindbrain, as in the river fish. This, however, may have been the effect of an irregular plane of the section. There was little evidence of optic chiasma formation. The optic lobes had started to expand laterally, but they were dorsally flattened and seemed to be pushed somewhat anteriorly by the anterior parts of the hindbrain. By stage 15 some narrow bundles of optic-nerve fibers had reached the diencephalon, but in most cases just nuclei without fibers were present. Some crossing over of optic-nerve cells to the opposite side of the diencephalon was seen, but this optic chiasma region showed a more posterior position than in the river fish. Few mitoses were present in the diencephalon, and little white matter had formed in this region. The dorsal region of the diencephalon in the section of the Sabinos larvae shown in plate 24, figure 1, appeared to receive the innervation of some of the lateral-line sensory structures. These lateral-line organs can be seen in this illustration in the lateral epidermis dorsal to the eyes. The lateral-line sensory organs in the cave larvae gave evidence of more extensive development than in the river fish. This problem will be examined further in a future study. The optic lobes were still dorsally flattened. At stage 16 many cells that resembled connective-tissue cells seemed to have infiltrated the optic-nerve fibers. This condition worsened by the fourth to fifth larval days when blood cells were found mixed with the connective-tissue cells so

TABLE 11

COMPARISON OF THE DEVELOPMENT OF DIENCEPHALON, OPTIC LOBES, AND OPTIC NERVE IN RIVER AND CAVE FISH

Stage	Structure	River Fish	Cave Fish
9	Forebrain lumen	Formed	Not formed
	Forebrain cells	Many mitoses	Few mitoses
10	Third ventricle	Diamond-shaped contour	Small, irregular shape
	Diencephalon walls	Differentially thickened laterally	Not differentially thickened laterally
	Diencephalon cells	Many mitoses	Few mitoses
	Optic lobes	Began to enlarge	No enlargement
14	Optic nerve cells	Nuclei and fibers	Nuclei, few fibers
	Optic nerve exit	Ventral retina	Central retina
	Diencephalon cells	Many mitoses	Few mitoses
	Optic chiasma	Visible	Not visible
	Optic lobe expansion	Dorsally and laterally	Just laterally
15	Optic nerve	Many fibers; complete decussation	Few fibers; some decussation
	Optic chiasma	Anterior to exit of optic nerve from retina	Posterior to exit of optic nerve from retina
	Diencephalon	Many mitoses	Few mitoses
	Optic lobes	Expanded dorsally and laterally	Flattened dorsally, expanded laterally
5th larval day	Optic nerve bundle	Free of connective tissue and blood cells	Infiltrated by connective tissue and blood cells
	Optic lobes	Dorsal tectal white matter	No dorsal tectal white matter

that the view of the optic nerve was obscured. Regular connections of the optic-nerve elements to the brain were no longer seen. On the fifth larval day, in the skin dorsal to the diencephalon in the Sabinos larvae, the dermal layers were not basophilically stained, the mesenchyme was thinner, and no dermal or subdermal melanophores were present. In the Chica larvae the dermal layers were basophilically stained, and some subdermal melanophores were found. The optic lobes in Chica and Sabinos larvae were still very flat dorsally, but lateral regions appeared normally expanded. The pineal gland was not covered over by dermal mesenchyme.

COMPARISONS: In table 11 the stages in the development of the diencephalon, optic lobes, and optic nerve in river and in cave fish are compared. This table indicates that in the cave fish these regions were not so well developed as in the river fish. In the cave fish few mitoses were seen in the brain cells, and little fibrous tissue formed in the brain and optic nerve.

DISCUSSION

COMPARATIVE STUDY of the embryonic development of these fish has further demonstrated the genetic continuity of the two blind cave fish with the eyed river-dwelling fish. All three forms developed more or less at the same rate, except for the somewhat delayed appearance of the optic primordia and associated structures and optic and body pigment in the two cave varieties.

Now that it has been shown that the earliest direct indication of any optic differences in the cave-fish embryos is present in the optic-bud stage, it is of interest to try to determine, from the embryological evidence, when in ontogeny the mutated genes in the cave fishes may have produced their primary effects. The developmental alterations observed in the Sabinos fish prior to the stage when the eyes formed provided some support for the theory that the defective genes were operative very early in development and that the subsequent eye defects were secondary effects. These early changes included: a significantly reduced egg viability; a relatively small perivitelline space; an often collapsed chorionic membrane; bleb-like extrusions on the blastodisc and yolk surface; failure to survive certain critical periods as cleavage, gastrulation, and some later larval stages; delayed blastopore closure; and excessive concentrations of periblast tissue near the end of gastrulation. The reduced egg viability could have occurred as a result of all the other early changes enumerated above. Almost all these defects could be ascribed to some genetically produced weakness in the chemical and physical structure of the egg cortex in this cave fish. Recent studies have demonstrated the importance of the teleost egg cortex in fertilization and activation changes (Kao and Chambers, 1954; Kao, Chambers, and Chambers, 1954; Yamamoto, 1954a, 1954b), in blastodisc formation (Lewis, 1943a, 1943b), and in morphogenetic processes involved in gastrulation (Trinkhaus, 1951a, 1951b). Sol-gel changes and permeability properties of the egg cortex, and the maintenance of the internal hydrostatic pressure of the egg by the perivitelline colloid, are all of significance during embryonic development and could have been disturbed in the Sabinos eggs.

Early Chica development did not show most of these abnormalities described for the Sabinos eggs, except for the somewhat delayed blastopore closure and the failure of the head extra-embryonic membranes to become raised away from the yolk surface. The Chica studies, therefore, would support the theory that the defective genes were operative just shortly before their effects were apparent and that their action was initially on the early optic anlagen. In both cave forms the optic buds appeared some two hours later than in the river fish. Sections through the developing eyes shortly after this stage indicated that active mitoses were in process in the river fish, but there were few, if any, mitotic figures in the cave-fish optic sections. Mitotic division in the optic structures of the cave-fish embryos all but ceased shortly after these rudiments first appeared. This could mean that there was a decreased activity of the genes that controlled the growth rate of the eyes (supposedly via control of local metabolic processes). As a result of this retarded growth, the outer wall of the cave-fish optic vesicles was seen to come in contact with only a small area of the outer epidermis, and the lens vesicle that then formed there was correspondingly small. The incomplete eye cup and lens invaginations were probably causally related, and the failure of lens-fiber formation and proper corneal differentiation followed the early retardation. Lüling (1955b) determined from growth studies on young Chica fish that the eyes exhibited weak negative allometric growth, whereas in the related characin *Hemigrammus caudovitattus*, the eyes showed positive allometric growth.

Optic failure in these Mexican cave characins could also have been caused by a reduction in the quantity of the presumptive eye-forming region, and this would imply an earlier action of the mutated genes than the preceding theory. De Beer (1951) refers to such a reduction as "rudimentation," and this results in the formation of vestigial structures and also involves negative allometry. To determine if there occurred a reduction in the

chemical or structural components of the presumptive eye-forming regions of these cave species, it would be necessary to carry out experimental operative studies on the eggs and embryos. An immunobiological approach to these problems along the lines of the studies of Woerdeman (1955) on lens proteins would be of interest, and would determine if there also may have occurred a reduction in the organ-specific proteins formed in the presumptive eye-forming regions. Ten Cate and van Doorenmaalen demonstrated (1950) the existence of lens proteins even before the lens rudiment of the chick embryo appeared.

The alterations described above in the early development of the Sabinos eggs, prior to the formation of the optic structures, require some further discussion. Although Sadoglu (1956), in a preliminary report of the genetic analysis of the inherited defects in these Mexican cave characins, stated that under normal breeding conditions she had no difficulty in obtaining all kinds of crosses, it should be pointed out that the Sabinos × Sabinos mating also produced a high percentage of non-viable eggs in her studies, as in the embryological analyses. It was considered that light may have produced defects in the protoplasm of these Sabinos eggs, as, for instance, in the eggs of *Lampetra fluviatilis* raised in the light. In these lampreys Damas (1948) observed lesions in the superficial protoplasm of the blastomeres that resulted in the formation of a large dorsal protuberance in the marginal zone of these eggs, which then resembled exogastrulae. When Damas raised these eggs in darkness, close to 90 per cent formed normal larvae. When Sabinos eggs were transferred to the dark shortly after they were spawned, the blebs and gastrulation failure still occurred in eggs from a so-called "defective" spawning. Perhaps some other deficiency in the chemical and physical conditions of aquarium existence could have contributed to these changes. The cave waters are known to contain high concentrations of calcium carbonate, and excess amounts of this salt may be essential for normal development of the cave species. Yamamoto (1954a, 1954b) has shown that the time required for the completion of the cortical changes that result in the formation of the perivitelline space in the activation and fertilization of goldfish eggs was dependent on the salt concentration of the media. The addition of calcium carbonate to the conditioned water, or raising the eggs in Holtfreter's solution with double concentrations of calcium chloride, failed to enhance the viability of the "defective" Sabinos eggs. Yamamoto (1954a, 1954b) also observed that when unfertilized goldfish eggs were first activated in fresh water some five minutes before being inseminated, only a few eggs became fertilized and developed into embryos. It is possible that in the Sabinos eggs fertilization was somewhat delayed after the females spawned the eggs into the water, and this delayed fertilization could have contributed to the low viability of these eggs. This delay could easily occur in these blind fish, as males and females were frequently observed to lose each other during pre-spawning activities.

If the fertilization and activation processes were interfered with in the Sabinos eggs, permeability properties may not have been normally established. Kao, Chambers, and Chambers (1954) indicated that in *Fundulus* and trout eggs activation resulted in a rapid decrease in the permeability of the surface of the egg proper. It would be of interest to carry out some permeability studies on these characin eggs, as in the cave waters the salt concentration was much higher than in the river. Because free calcium ions appeared to be necessary for the wave-like breakdown of cortical alveoli that occurs on fertilization and activation of *Oryzias latipes* eggs (Yamamoto, 1954b), and these calcium ions were shown to be released from the cortical protoplasm, it is possible that this release was interfered with in the Sabinos eggs. The separation of the blastomeres observed in some Sabinos eggs could also indicate a calcium lack, because calcium normally promotes cell adhesiveness.

Normal morphogenesis of the teleost egg seems to be somewhat dependent on the contractile tension of the gelated egg cortex. Lewis (1943b) described the continual contraction of this outer gel layer during the formation of the blastodisc in the egg of *Brachydanio rerio*. The reversible changes in the shape of *Astyanax* eggs from spherical to ovoid observed during development were probably also dependent on a gelated egg cor-

tex. Trinkhaus (1951a) demonstrated that the central periblast tissue of *Fundulus* eggs does not possess the contractile tension of the gelated egg cortex, and, when exposed by the removal of some of the blastodisc, it formed a bulging surface. He further showed that randomly located protrusions of the periblast and the yolk gel layer formed over the surface of the yolk when these eggs were placed in solutions of oxalate and citrate in distilled water, or in sea water free of calcium ions. He attributed these protrusions to a solation of the cortical gel layer, in the absence of the needed calcium ions. These periblast protrusions experimentally produced in *Fundulus* eggs appear similar to those that formed in Sabinos eggs and may also have been caused by a solated cortical gel layer. Again, a lack of needed calcium ions is implicated.

All these abnormalities in early Sabinos development may have been peculiar to the strain of fish used and may not be of general occurrence in the rest of this group of cave fish. Although it was indicated how optic involvement could have occurred following these early morphogenetic disturbances, the close similarity between the optic defects in the two cave forms would point to a common origin of these eye defects, from mutations that directly affected either presumptive eye-forming tissue or early eye rudiments. It would be of interest to observe eggs from other Sabinos strains, or perhaps from some newly collected cave individuals, to see if these eggs also have a high percentage with a weakened cortical structure.

The vertebrate lens, as the rest of the vertebrate eye, develops more or less in the same way in all vertebrate classes (Walls, 1942; Dalcq and Pasteels, 1955). The steps that led to lens fiber formation in *Astyanax* corresponded closely to the classical descriptions of lens differentiation in amphibians and birds (Spemann, 1938; Woerdeman, 1950; McKeehan, 1951). In the cave-fish larvae the lens was retarded in the amount of mitotic division and in fiber differentiation, so that lens development never proceeded beyond the embryonic vesicle stage. In some cave-fish larvae there was evidence of an aborted fiber formation in the reduced lenses, with some elongated lens epithelial cells oriented concentrically. But the strongly refractive lens fibers typically found in the vertebrate lens never formed in the Chica and Sabinos larvae. Although no significant histochemical differences in the lens-forming cells of eyed and blind fish were determined, some ribonuclease-resistant, basophilically stained, lens-cell cytoplasm was found only in *Astyanax*, indicative of some protein-polysaccharide complex not produced in the cave-fish lens.

In the development of the chick lens, Weiss (1950) and McKeehan (1951) have indicated the importance of close contact and adhesion between the elongated lens epidermal cells and the subjacent oriented retinal cells. When cellophane strips were placed between the chick retina and lens epithelium, the typical nuclear orientation in the prospective lens cells was abolished, but only in that region in which contact was blocked. Direct contact between the optic vesicle and the lens-forming epidermis had also been demonstrated to be of importance in amphibians (Lewis, 1906–1907; Spemann, 1938; and Woerdeman, 1950). This relationship of close contact that normally ensues between retinal and lens-forming cells does not preclude the ability of the lens to self-differentiate when the early lens primordium is isolated (Woerdeman, 1950; McKeehan, 1954).

It is assumed that the accumulation of specific lens proteins takes place very early in the cells of isolated lens primordia, before the cells were removed. Immunological studies by Ten Cate and van Doorenmaalen (1950) on chick embryos, with the use of organ-specific antisera, demonstrated the presence of lens specific antigens in the lens placode cells. Flickinger, Levi, and Smith (1955) used non-organ specific antisera on the lenses of adult frogs, chicks, and cattle in precipitin tests with various embryonic and adult-tissue, test-antigen preparations, and also in tests on frog and chick-embryo cultures. These authors grossly confirmed the earlier results of Ten Cate and van Doorenmaalen, but were able to demonstrate the presence of a serologically detectable lens antigen only shortly after the lens had formed, rather than at the placode stage. In a preliminary report on the detection of lens antigens in frozen sections of eyes from mice one day old, Clayton and Feldman (1955) implied that lens-specific

proteins were localized in other eye regions, as well as in lens cells. These frozen sections were treated with antisera labeled with radioactive isotopes, and from differences in the density of the grains in different parts of autoradiographs made from the treated sections, it was found that the pigment epithelium regions, the outer parts of the rods, and the fibrillar layers of the retina all showed strong activity, although the reactions of the lens cells were the highest.

It cannot be determined, in the absence of experimental studies, whether the cave-fish lens was just retarded temporarily or was permanently inhibited in its development. It is reasonable to assume that both factors were involved, and along with the growth retardation and inhibition of differentiation there must also have occurred a failure in the formation of specific lens proteins essential for fiber formation. The shorter period of contact and the smaller area of contact between the lens epidermis and the retarded optic vesicles in the cave-fish embryos may have been insufficient for the syntheses of these needed proteins in the lens cells, or for the possible transfer of enzymes from the optic vesicle. The histochemical localization of glycogen, protein-bound sulfhydryl groups and ribonucleic acid failed to reveal any significant chemical lacks in the cave-fish lens cells, other than the absence of ribonuclease-resistant, basophilic cytoplasm in their lens cells. Future studies along experimental and immunochemical lines may reveal more clearly defined differences in lens-specific proteins of the eyed and blind fish.

Beckwith (1927) pointed out the reciprocal influence of the absence of the lens on the failure of the eye cup to develop normally in *Amblystoma punctatum*. When the *Amblystoma* lens rudiment was extirpated, the choroid fissure did not close, the vitreous humor did not develop, and the eye cup was collapsed and folded. In some cases the entire optic cup was found everted into the pupil, when a lens structure was lacking. In many of the cave-fish larvae the pupil was closed and the eye chambers were often obliterated after the lens failed to differentiate normally. It would be interesting to determine by surgical and tissue-culture studies the following: the capacity of the cave- and river-fish lenses to self-differentiate; the extent of development of the cave-fish optic cup when the river-fish lens anlage was transplanted to the cave-fish eye chamber; and the development of the cave-fish lens rudiment when transplanted to the river-fish eye chamber. Stone and Sapir (1940) successfully re-implanted a lens segment into *Fundulus* of 50–70 mm. in size. The segment taken from the original fibrous lens included a portion of the lateral surface, and this segment was replaced partly under the pupillary margin of the iris. New lens fibers formed in the graft after 17 days. The failure of any lens regeneration in eyes from which the lens was surgically extirpated, and no grafted tissue was re-implanted, indicated that in this teleost some chemical or physical stimuli from the small, grafted, lens segment was essential for any lens regeneration. However, the fact that some lens regeneration did take place in *Fundulus* under the influence of the piece of re-implanted lens tissue is encouraging and may also be subsequently found to take place in other teleost species. Rasquin (1949a) transplanted entire *Astyanax* eyes from young individuals into the eye sockets of the blind cave-derived forms, but in most cases the transplants were pushed out and never became vascularized. These experiments should be repeated on early larval cave and eyed forms, and eye segments rather than whole eyes should be used, if possible. Only from studies of this kind would it be possible to determine the competence of the cave-fish optic tissues for differentiations that normally fail to take place because of some inhibition and retardation.

The process of lens regeneration from the dorsal rim of the iris of most urodele amphibians has recently been reviewed by Reyer (1954). In some Chica larvae the lens appeared to have formed from the dorsal part of the optic cup, with which it was in very close contact. If this actually occurred, the process would be related to Wolffian regeneration of the lens. However, Reyer (1954) indicated that in these urodeles, normal lens formation from the dorsal iris in an eye in which no embryonic lens had ever developed cannot take place until an early larval stage. Sections through embryonic Chica eyes indicated that the lens rudiment in the ectoderm had already showed some thickening. The larval

lens vesicle may have been somewhat displaced to a more dorsal position in relation to the retina and appeared to be part of the dorsal rim of the retina.

Embryological studies on other vertebrates with hereditary anopthalmia or other hereditary optic degenerative conditions have been reported for dogs (Parry, Tansley, and Thomson 1953), rats (Bourne, Campbell, and Tansley, 1938), and mice (Bonnevie, 1934; Chase and Chase, 1941; and Tansley, 1951, 1954). In the condition of hereditary retinal degeneration described in these mammals, normal retinal differentiation first occurred. This was later followed by the gradual appearance of pycnotic cells in the outer nuclear layers. This degenerative condition progressed until all the rods were destroyed, and secondary changes in pigment epithelium, choroid, and inner layers of the retina then occurred. The retinal degenerative changes were constant and regular and were considered to be produced by a direct effect of the mutant gene. The secondary conditions were found to be more variable and were thought to be indirectly related to the original mutation. In the Mexican cave fish the retinal degeneration itself appeared to be the secondary effect of genes that produced earlier optic defects. Degenerative retinal changes were seen before retinal differentiation was completed. In these fish the mutant genes seemed to have acted even earlier than in the mammalian species. In an anopthalmic strain of mice in which 90 per cent of the individuals were completely eyeless on both sides and only 10 per cent developed different grades of small eyes, Chase and Chase (1941, 1945) determined that a growth inhibition of the optic vesicle was responsible for the later optic failure. If the eye vesicle was large enough to reach the outer ectoderm and induce a lens, then an eye that was normally formed but small in size developed. But if a lens was not induced the eye cup collapsed, the choroid fissure failed to close, and the optic nerve and optic tracts failed to develop. These authors indicated how extensive were the pleiotropic effects of one gene complex that acted on the early optic vesicle. The changes in this abnormal strain of mice resemble the optic alterations described in the Mexican blind cave embryos and larvae. In these cave fish it also appeared that the primary effects of the mutant genes resulted in the retarded growth of the optic vesicles, with many secondary optic defects. Chase and Chase (1941) found, however, from calculations of the mitotic index in these inhibited eyes, that, after the critical period during which the optic inhibition occurred, the parts of the eye that were already formed continued to grow at a normal rate. This was not true of the parts of the retarded cave-fish eyes, for after the initial growth retardation the later stages contained very few mitotic figures in these optic structures. The optic growth retardation therefore extended far beyond the optic-vesicle stage.

Although no trace of the early blebs could be found in sections through the embryonic or larval stages of Sabinos fish, it is very possible that the blebs were responsible for some of the later developmental anomalies in this species. Bonnevie (1934) attributed the eye and foot abnormalities in a strain of abnormal mice to the secondary effects of an embryonic bleb formation. The bleb fluid in these mice was demonstrated to have originated from a high concentration of cerebrospinal fluid expelled through an opening in the myelencephalon. This fluid moved underneath the embryonic epidermis and when it became concentrated at any one point the pressure of the bleb fluid mechanically obstructed many embryonic processes. The blebs observed in the cleavage and gastrula stages of Sabinos eggs may have disrupted normal morphogenetic movements, as pointed out above in the Discussion. But their origin was not the same as in the abnormal mice, and their harmful effects, if any, were not clearly demonstrable.

The cave fishes used in these embryological studies did not appear to show homogeneously reduced optic and pigmentary systems. Sadoglu (1956) in her genetic studies used many cave fish from the same stock and concluded, from the diversity of optic and pigment reduction in her F_1 phenotypes obtained from the same cross, that heterozygosity for these defects appeared to exist in these mature cave fishes. The parents of the Chica spawning studied embryologically showed asymmetrical optic reduction, with the eye cyst on one side of the head not grossly visible and a surface rudiment still visible on the

other side. These parents phenotypically resembled those Breder and Gresser (1941a) classified as group 2. At that time marked asymmetry of morphological and functional development in adults of that group was observed, but as Breder (1943a) has indicated, later collections revealed changes in the phenotypic ratios of the different groups. Although it was not possible to determine what post-larval optic changes would have occurred in the Chica embryos and larvae, it also appeared that these F_1 Chica fish probably were most like those in group 2. They were not similar to Breder and Gresser's group 3 (1941a), because the Chica fish classified in this group were said to be indistinguishable from the normal river fish, except for frequent micro-ophthalmia. By the second to third days of larval development it was already apparent that the developing eye cups of F_1 Chica fish deviated structurally from those of *Astyanax*. These Chicas may undergo post-larval optic involution and degeneration that could result in the disappearance of most eye elements that were differentiated, in which case they would be classified in group 1. However, it appears most likely that these Chica larvae resembled group 2a, in which Breder and Gresser described covered, sunken eyes, with the globe developed but malformed in size and shape, the retina either partly or completely deficient in sensory elements, the lens small without fibers, the pupil irregular or absent, and a shallow anterior chamber. It therefore appeared that post-larval changes in these Chica fish consisted mainly in the sinking in of the partly differentiated but shallow eye cup under the surface of the skin.

Sadoglu (1956, 1957) carried out genetic studies on Chica and Sabinos cave fish, as well as on fish from the Pachon cave, which is over three times as far from the river as the Sabinos cave. Sadoglu observed that there was a greater variation in eye size in the F_1 Chica fish than in the Sabinos fish or in the Pachon F_1 descendants. The embryological studies also revealed less variability in embryonic eye size and more eye symmetry in the more reduced eyes of the Sabinos cave fish. This increased stability of eye size in the fish from the two caves farthest from the river may indicate that some gene exchange between *Astyanax* and Chica populations may have been taking place, whereas the other caves were too far away from the river for this to occur. *Astyanax* larvae with one eye of normal size and the other greatly reduced were obtained on several occasions, so that the normal eyed fish also contained an asymmetry component. A possible insight into where this asymmetry factor may operate can be seen from the studies by Lehmann (1928) on the production of asymmetrically developed amphibian neural plates after a lateral injury was made to part of the invaginating chorda-mesoderm. It appeared that in these amphibians the reduction of the underlying chorda-mesoderm produced a decreased development of the medullary plate on the deficient side only. In the optic-vesicle stage the Chica embryos had formed a forebrain with ectoderm and lumen of asymmetric contour which markedly resembled the experimentally produced asymmetry in the amphibians. It may be that there was also some mesodermal involvement in the production of the cave-fish optic defects, but a more complete series of early stages would have to be studied to determine if this existed.

The failure to demonstrate the presence of many glycogen-positive granules in the developing optic structures and associated brain parts of the river-fish embryos and young larvae, and the relative abundance of such granules in these structures of the young cave-fish larvae, were of special interest. These results can be interpreted in either of the following ways: the relative absence of glycogen in these cells of the river fish may mean that most of this carbohydrate was used up during the optic evagination and invagination, whereas the glycogen in the retarded optic and brain cells of the cave-fish larvae failed to be utilized or depleted; or it may be considered that metabolism in the cave-fish optic structures at that particular stage was more rapid than in the river fish, so that more glycogen was needed and stored, as well as combusted. The first interpretation sounds more probable in view of the demonstrated retardation in growth and differentiation of the optic structures in the young cave-fish larvae. There was no morphological or histological evidence for the second interpretation. No river-fish stages between stage 9

and stage 14, the two that were analyzed for glycogen distribution, were available for glycogen localization, so it was not known if there was any appreciable glycogen storage in the optic and diencephalon cells of the river fish during that period of development. If it should be found that at a stage of river-fish development shortly before stage 9, or between stages 9 and 14, a glycogen storage in the optic cells occurs comparable to that demonstrated at stage 14 in the cave-fish larvae, then the condition in the cave larvae would not appear to be atypical. Until a more complete series of stages can be histochemically analyzed, the possibility cannot be excluded that the abundant concentration of glycogen in the cave larvae represented a temporary storage, and depletion was to have occurred subsequent to the stage that was studied. This glycogen retention observed in the cave-fish larvae may have been caused by a lack or inhibition of certain enzymes or other co-factors needed for glycogenolysis, and it would be of interest to explore this possibility. The young cave-fish larvae may have partially utilized some other metabolic pathway for the energy needed for these developmental processes, such as protein or fat breakdown. That the adult cave fish differed metabolically from the eyed river fish was shown by the excess subdermal fat deposits found in the cave varieties, and the differences in thyroid activity of the cave and eyed fish when kept in light or darkness (Rasquin, 1949b).

Recently a new theory has been advanced to explain the process of regressive evolution in cave animals (Heuts, 1951, 1953a, 1953b). This theory is based on biometric, ecological, and some oxygen-consumption studies on almost exclusively adult populations of wild and laboratory-maintained, African, blind, cave cyprinids of the genus *Caecobarbus*. Heuts concedes that adaptive hereditary changes caused by a selective factor are responsible for the regressive alterations in these cave fish. He assumes that the presence of small amounts of food in the closed cave environment is the selective factor responsible for these optic and pigment hereditary changes in that this low food concentration can be related to the reduced growth and metabolic rates and the loss of internal stability. Although, as has been illustrated (Breder, 1953; Thines, 1956), the cave characins and *Caecobarbus* show many morphological and behavioral similarities, the latter fish shows more advanced optic and pigmentary regression than the former. In the characins the eye retardation and delayed time of appearance of melanin pigmentation occurred long before any larval feeding began, as demonstrated in the present report, so that differences in the growth of these structures could not be ascribed to the lack of food. It should be pointed out that the food supply in the Mexican caves is known to be ample and was presumed to be so when these cave forms evolved. It would be interesting to study chemically the distribution of metabolic stores in the eggs of the cave and eyed forms to determine if significant differences exist.

Woerdeman (1953a) indicated that the way in which the amphibian lens fibers start to grow was dependent in part on differences in the concentration of glycogen in the cells along the outer wall of the eye vesicle. In his early studies Woerdeman (1933) demonstrated by the use of Lugol's iodine solution that the cells along the floor of the prosencephalon and those that evaginated to form the optic vesicles contained more glycogen than the rest of the forebrain wall. During the formation of the lens the glycogen was found to disappear from the central regions of the retina, which were in contact with the lens ectoderm, and to become aggregated in the lens cells. These experiments were carried out on axolotl embryos, and as far as could be determined these results have never been repeated or corroborated on other species. In *Astyanax* this disappearance of glycogen, as such, from the optic cells was not demonstrated. The absence of much glycogen-positive cytoplasm in the optic cells of the stages of *Astyanax* that were studied therefore cannot be considered comparable to Woerdeman's results.

The absence of glycogen-positive granules in the yolk-sac, endoderm cells of stage-14 Sabinos larvae may be of some significance. The origin of the glycogen in the various neural embryonic and larval cells is not certain. It is not known if this endogenous glycogen is produced in the neural cells themselves from

glucose or is synthesized in other trophic tissues and then transferred to the neural cells. If the yolk-sac endoderm is involved in this process of glycogen synthesis and storage, the absence of glycogen from those regions of Sabinos larvae may be related to the storage of much glycogen in the optic and associated brain cells of this species. Daniel (1947) found glycogen in all the layers of the yolk sac of *Salmo* and was almost certain that the yolk-sac cells were involved in the formation, storage, and regulation of glycogen. Daniel also found many fat globules in the yolk-sac cells. No special fat stains were used on these characins, but it would be of interest to compare the distribution of fatty materials in the developmental stages, in view of the demonstrated excess deposition of fat in the adult blind cave fish (Rasquin, 1949b).

Ribonucleic acid was histochemically localized in the cytoplasm of almost all optic-vesicle, optic-cup, and lens-vesicle cells in the three characin fish studied. No differential concentrations of ribonucleic acid were observed between the cells along the outer wall and those along the inner wall of the eye vesicles, as described in amphibians by Woerdeman (1953a), Takata (1952), and Rickenbacher (1952), and in the chick embryo by McKeehan (1956). In the optic vesicles of the Chica embryos there were a few scattered cells around the small opticoele that showed no basophilic-stained cytoplasm, which may be indicative of some early failure in these vesicles. The central, fiber-forming, lens cells in young river-fish larvae contained ribonuclease-resistant, basophilic-stained cytoplasm not found in the cave-fish lenses. Takata (1952), in histochemical studies on lens regeneration in adult *Triturus pyrrhogaster*, determined that the regenerating lens fibers showed weak basophilic-stained but ribonuclease-resistant cytoplasm. Rickenbacher (1952), in a histochemical study of the nucleic acids in eye development of some amphibians and chicks, also determined that the secondary lens fibers that formed stained intensely with pyronin but were not decolorized by ribonuclease, and considered that these fibers contained a chondroitinsulfuric acid, a component of a mucopolysaccharide-protein substance. Jackson (1955) found that fibrogenic cells of cultures of chick-embryo bone, tendon, and cornea also contained basophilic but ribonuclease and desoxyribonuclease-resistant cytoplasm that was periodic acid-Schiff positive, and he considered this evidence of a mucopolysaccharide-protein material. Some such carbohydrate-protein substance may be needed for lens-fiber formation, and it may be that the polysaccharide component was responsible for the resistance of the basophilic cytoplasm of the central, fiber-forming, lens cells of the river fish to the ribonuclease. This polysaccharide component may have been lacking in the cave-fish lens cells that failed to form true lens fibers, possibly because most of the carbohydrate precursor, which may be glycogen, remained in the optic cup and diencephalon.

It is uncertain if ribonucleic acid is a nonspecific biochemical accompaniment of lens-fiber differentiation, or if, as McKeehan (1956) postulated, the RNA acts as a specific mediator of the inductive effect. In the chick-embryo lens McKeehan observed that the accumulation of RNA coincided with the first evidence of lens differentiation. However, in the developing lenses of the river and cave fish no significant increases of the relative RNA concentration were observed. Brachet (1953) pointed out that the use of toluidine blue as the basic dye for the cytochemical detection of RNA does not give very accurate results when the tissues contain much RNA. In these characin embryos and larvae RNA was densely concentrated in most tissues, especially in the neural structures, so differences, if they did exist, were not made apparent by this staining procedure.

As for the distribution of protein-bound sulfhydryl groups in the optic structures, it was very difficult to determine the significance of the small differences observed, because in general the histochemical reactions were indicative of sparse, widely separated, sulfhydryl groups. There was some indication of a greater concentration of positively stained granules in the cytoplasm of some optic-cup cells located at the center of the invaginated region of the retina of river-fish larvae of stage 14 than were found in similar regions of comparable cave-fish larvae.

Future histochemical studies that would demonstrate protein-bound sulfhydryl groups

in the eyes of later larval stages of these characins would be of interest, in view of the evidence discussed by Sidman and Wislocki (1954) for the location of the sulfhydryl groups on the visual proteins. These investigators found that in the immature photoreceptor cells of the young kitten, these sulfhydryl groups were not yet prominent, but they probably appeared at the time visual function was attained, when the visual pigments developed.

These embryological studies have therefore demonstrated that the optic failure in the cave fish was apparent as early as the optic-bud and optic-vesicle stage, at which time the eye rudiments that developed were smaller, and poorly structured when compared with those of the normal-eyed river fish. The secondary inductions of lens and cornea that followed were also defective in the cave species, and a normal fibrous lens and transparent cornea never formed. The development of small eye cups in the cave larvae appeared to be related to the few mitoses in the optic and lens rudiments and incomplete eye cup and lens invaginations. Some metabolic differences between the eyed and cave forms were implied by the results for glycogen distribution in the optic and associated brain regions, but further studies are needed to determine specifically how widespread these differences are and how they may be involved in the optic and other morphogenetic differences in the cave species. The numerous morphological, histological, and histochemical similarities observed during the development of the eyed and cave fish would seem to indicate that the modifications involved in the evolution of these cave fish were of relatively small magnitude. Future studies will show if these small changes dealt with changes in the requirements for carbohydrate metabolism during embryonic, larval, and perhaps even during adult life.

SUMMARY AND CONCLUSIONS

1. COMPARATIVE MORPHOLOGICAL, histological, and histochemical studies were made of optic development in the eyed, river-dwelling characin *Astyanax mexicanus* and in two of its blind cave-derived forms from the Chica and Sabinos caves in Mexico.

2. Eggs were obtained from natural and artificially induced spawnings in the laboratory. Egg viability was high in the eyed river fish and in the fish from the Chica Cave, which was close to the river. Egg viability was low in the fish from the Sabinos Cave, which was far from the river.

3. Many Sabinos eggs failed to survive certain critical periods of development. During these crises the following abnormalities were seen: separation of blastomeres, bleb-like extrusions on blastodisc and yolk surface, and excessive concentrations of periblast tissue that protruded into the perivitelline space. All these changes appeared to involve some weakness in the structure of the egg cortex in the strain of Sabinos fish studied.

4. Unsuccessful attempts were made to increase the viability of the Sabinos eggs by raising them in solutions with added calcium carbonate or calcium chloride.

5. The unfertilized eggs of the river and cave fish were indistinguishable from one another. Fertilized eggs reached more or less closely comparable stages of development at the same time. In the cave fish the time of appearance of the following were delayed: optic primordia and associated brain structures, eye and body pigmentation, blastopore closure, and tail-bud elongation.

6. The optic rudiments of the cave fish from the time of their earliest appearance and throughout their development were smaller than those of the river fish. In the smaller optic structures of the cave fish mitoses were scarce, invagination was delayed and often incomplete, and areas of differentiation were limited or absent.

7. It appeared that the small-sized eye buds in the cave fish failed to provoke adequate lens induction for lens-fiber differentiation. Characteristic lens fibers, as are typically found in the vertebrate lens, never developed in the cave larvae. In some cave fish there was evidence of an aborted fiber formation in the reduced lenses.

8. Corneal induction in the cave larvae by the poorly differentiated lenses was insufficient for the thinning out of the fibrous layers of the cornea.

9. Degenerative changes, such as the appearance of empty spaces in the retinal nuclear layers and the presence of pycnotic nuclei in the reduced lenses of the cave larvae, were seen shortly after the partly successful (retinal) or unsuccessful (lens) differentiation processes were completed.

10. Many secondary optic defects observed in the cave larvae, such as the closing in of the lips of the eye cup and the obliteration of eye chambers and pupillary openings, appeared to follow the growth retardation of the optic rudiments.

11. The following histochemical results in the cave fish were contrary to the findings in the river fish:

A. In the retarded optic structures of the cave larvae there appeared a much greater concentration of glycogen-positive granules in the cytoplasm than were found in the well-developed optic cup, lens, and diencephalon of the river fish. These results can be interpreted as evidence of a possible decreased utilization and an increased retention of glycogen in the optic regions of the cave fish.

B. Protein-bound sulfhydryl groups in the cave larvae failed to show the increased intensity of reaction observed in the optic cup and lens of the river fish during the stage of lens differentiation.

C. In the fiber-forming lens cells of the river fish a ribonuclease-resistant, basophilically stained, cytoplasmic component, considered to be possibly a polysaccharide, accompanied ribonucleic acid. No such histochemically localized component was demonstrated in the lens cells of the cave larvae.

12. These embryological studies have therefore demonstrated that optic deficiency in the cave fish was a consequence of interference at two or more points with the chain of inductions involved in eye organogenesis, followed by degenerative changes in the small, improperly formed eyes. Some metabolic differ-

ences between the river and cave larvae were implicated by the results for glycogen distribution in the optic and associated brain regions. Further studies are needed to determine how widespread these differences are, and how they may be involved in the optic and other morphogenetic variations in the cave fish.

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