

## One Eye But No Vision: Cave Fish With Induced Eyes Do Not Respond to Light

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**ABSTRACT** One of the most intriguing questions in evolutionary biology is the degree to which behavior is a necessary consequence of morphology. We explore this issue by examining phototactic behavior in epigeal (eyed surface-dwelling) and troglomorphic (blind cave) forms of the teleost *Astyanax fasciatus* whose eyes were modified during embryogenesis by removing one or both lens vesicles from the epigeal form or by transplanting the lens vesicle from an epigeal fish into the optic cup of a blind cave form. Lens removal results in eye degeneration and blindness in adult epigeal fish, whereas lens transplantation stimulates growth of the eye, inducing the development of optic tissues in the normally eyeless adult cave fish. Photoresponsiveness was examined by placing fish in an aquarium with one half illuminated and the other half dark and scoring their presence in the illuminated or dark half. Both the eyeless epigeal fish and cave fish with induced eyes are indifferent to the illumination whereas the surface forms are scotophilic, suggesting that optic development and phototactic behavior are decoupled. *J. Exp. Zool. (Mol. Dev. Evol.)* 300B:72-79, 2003. © 2003 Wiley-Liss, Inc.

### INTRODUCTION

The process leading to the reduction or elimination of phenotypic features among animals living in environments such as caves, abyssal zones, or remote islands continues to be a source of controversy. In the case of hypogean (cave, artesian, phreatic) fishes, more than 150 years of heated discussions have yet to definitively identify the mechanism underlying such troglomorphic changes when a population is isolated from and then subject to selective pressures different than its ancestors (Romero, 2001).

Romero ('84b) proposed selective forces generated by behavior as the main explanation for reduction and/or elimination of phenotypic features among hypogean fishes. Morphological evolution mediated by behavior has also been reported for damselfishes (McPeck, '95). Changes in behavior independent of changes in morphology during the process of hypogean colonization by fish have also been reported (Romero, '84a, '85b; Green and Romero, '97).

Given that a troglomorphic feature (eyelessness) is independent of a related behavioral response (to light), then if a naturally occurring troglomorphic fish (blind, depigmented) regains its visual appara-

tus, does it also regain its photoresponsiveness? Reversibility in evolution has been documented (Teotonio and Rose, 2000) but the related reversibility at the level of mechanism is largely unexplored.

The Mexican blind cave tetra (*Astyanax fasciatus*) provides an excellent model for studying these evolutionary phenomena. It is a freshwater characid teleost with a broad distribution in the New World and has both epigeal (surface) and hypogean (subterranean) populations. Most hypogean populations can be found in caves of the San Luis de Potosí area, east-central Mexico (ca. 22°05' N, 99°00' W). Not all the cave populations of this area display the same degree of morphological divergence from the epigeal forms. Most have reduced eyes (generally not externally visible), loss of pigmentation (including totally depigmented), and an increased number of taste buds compared to the surface form (Wilkins, '88), a set of characters called troglomorphic. Other caves, however, contain only individuals with full eyes

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and pigmentation whereas some are inhabited by phenotypically intermediate forms (see Romero and Paulson, 2001, and references therein).

The surface and cave forms interbreed in both natural and laboratory conditions, producing fertile hybrids with a phenotypically intermediate form in the  $F_1$  generation, and with an  $F_2$  generation (after self-cross) whose individuals range from an almost completely blind and depigmented form to an almost eyed and pigmented one. Breeding, electrophoretic, and karyotypic studies support the contention that the cave and epigean forms are the same species (Wilkens, '71; Avise and Selander, '72; Kirby et al., '77). The two forms differ not only in their morphology, but also in their behavior. Unlike the surface normally eyed form, the blind form never schools and is constantly active. Although the blind form produces an alarm substance, it does not respond to it. The two forms also differ in the angle of inclination used when feeding from the bottom (see references for *Astyanax fasciatus* listed in Romero and Paulson, 2001). Differences in phototactic responses among different populations have been reported as well (Romero, '85b).

Although adult troglomorphic *A. fasciatus* lack functional eyes, small eye primordia are formed during embryogenesis. They arrest later in development, degenerate, and sink into the orbit. The anterior structures of the eyes (cornea, iris, etc.) are absent or rudimentary but the optic cup is formed as is the retina, although lacking differentiated photoreceptor cells. These degenerate eyes are covered by a flap of skin. If, however, a lens is taken from a surface fish embryo and transplanted into the optic cup of a cave fish embryo, then an eye with restored cornea, iris, and retinal photoreceptor cells is formed, indicating that cave fish optic tissues have conserved the ability to respond to lens signaling. Conversely, if the lens vesicle of an epigean fish that usually develops normal eyes is extirpated, the eye fails to develop a pupil, cornea, anterior chamber, and iris, and its retina is small and distorted (Yamamoto and Jeffery, 2000). Genetic analysis indicates that multiple genes regulate eye degeneration; molecular studies suggest that *Pax6* may be one of the genes controlling cave fish eye degeneration (Jeffery, 2001; Strickler et al., 2001).

Most studies of cave dwelling species highlight morphological regression of the eyes and pigmentation. Considerably less is known about the connections between the degenerate eyes and the brain and the condition of the visual centers

themselves in *Astyanax* cave fish. Although the optic tectum is extremely reduced in cave fish (Peters et al., '93), nothing is known about the condition of the visual centers and retinotectal projections in cave fish with induced eyes, a prerequisite for understanding their phototactic behavior. Here we examine whether normally eyeless cave fish (troglomorphs) with an induced eye respond to light in the same fashion as epigean eyed forms or as blind cave fish.

## MATERIALS AND METHODS

### Fish

The *Astyanax fasciatus* referred to as "surface fish" are normally eyed laboratory raised progeny of fishes originally collected in a spring at Balmorhea State Park, Texas and in various streams and springs in Tamaulipas and San Luis Potosí, Mexico (Dowling et al., 2002). The *Astyanax fasciatus* referred to as "Pachón cave fish" are eyeless laboratory raised progeny of a collection made at La Cueva de El Pachón, Mexico (Jeffery and Martasian, '98). Those called simply "cave fish" are eyeless forms purchased from a pet store. Eyeless cave fish are also commonly referred to as "blind cave fish."

We used 21 individual *Astyanax fasciatus* of six different kinds in the behavioral experiments.

1. Three were normal surface fish with both eyes (2S).
2. Three were surface fish with one normal eye and one whose lens vesicle was surgically excised during early development (1S; one with a remaining right eye only and two with a left eye).
3. Two were surface fish without normal eyes, both lens vesicles having been bilaterally excised during early development (0S).
4. Six were blind and normally eyeless Pachón cave fish but with one eye induced by transplantation of surface fish lens vesicle into a cave fish optic cup during early development (1C; three with a left eye and three with a right eye).
5. Four were normal blind Pachón cave fish without eyes (0C).
6. Three were blind cave fish purchased from a pet store (aquarist) without induced eyes (0A). The origins of types 1 and 5 as well as the procedures for producing types 2, 3, and 4 are described in Yamamoto and Jeffery (2000).<sup>1</sup> The

<sup>1</sup>Y. Yamamoto and W. Jeffery kindly provided us with the surgically altered fish as approved under University of Maryland IACUC R-99-25B.



aquarist cave fish (OA) were used because a) they are widely available in pet shops and therefore frequently employed as experimental subjects and b) previous studies (Romero, '85b) revealed them to respond somewhat differently to light than do blind cave *Astyanax* from natural populations.

Fish were housed in communal tanks, one for each of the six types. Communal tanks were covered by opaque shields on all sides, so fish were in continual darkness except for brief periodic intervals when the tanks were cleaned. Each fish was individually identified by its unique standard length (SL) measured to the nearest 0.1 mm using digital calipers (Table 1).

### Behavioral experiments

Tests were designed to ascertain phototactic responsiveness by examining and recording the location of fish in experiments that exposed them to lit and unlit halves of an aquarium. The experiments were conducted consecutively in a 110 l (76 × 47 × 31 cm) tank masked by opaque cardboard on its vertical sides and top except for

one half of one of the long sides and the contiguous top half of the tank. Each experiment consisted of two trials of one fish, the first using infrared (IR) illumination and the next using normal tungsten 'white' (W) light. Infrared illumination was produced by the infrared source of light built into the 0-lux capable videotape camera (SONY HandyCam Vision CCD-TRV99) used to record fish position in the tank. The camera was placed at 2 m from the aquarium glass and directed horizontally at the unmasked section of the glass. The white light source was a 120 V, 115 W, standard tungsten light bulb (Ace brand from Ace Hardware) placed 25 cm above the surface to avoid heating. Like all tungsten lamps, the majority of energy emitted was in the infrared region of the spectrum, so both sources were infrared emitters with peak energy in the 900 ± 100 nm range, but the IR source lacked the visible component and had little energy below 800 nm. While videotaping under white light, the IR light on the camera was turned off. IR was therefore present under both conditions as a control in the illuminated portion of the choice chamber and the difference in illumination, visible light, is the experimental variable.

The experimental tank was therefore a light/dark choice chamber when illuminated through the vertical side (IR) or from above (W). The interior was spatially uniform, without any added substrate (e.g., sand or gravel) or internal objects (e.g., air stone or filtration device). The same tank was used for all the experiments and was filled to within 5 cm of the top using conditioned tap water that had been treated with an anti-chlorine solution at least 48 hours beforehand and continuously aerated thereafter. No aeration or filtration was provided during the experiments, but active filtration occurred for 5 min between experiments to mix and re-aerate the water as well as help remove any accumulated material (both standard aquarium filter floss and activated charcoal were in the filtration device); the filtration apparatus was removed before initiating the next experiment.

With normal room illumination, each subject was removed from its home tank using a minnow net and then placed in the experimental choice tank. The experimenters turned on the video camera and its IR light source and then turned off the normal room illumination and departed the room. Fish were then videotaped for 35 to 38 minutes. The experimenters re-entered the room, turned on the normal illumination briefly during which period they switched the camera from IR to normal mode, extinguishing the IR source, and

TABLE 1. Results of photore sponsiveness testing of each fish under white and infrared illumination

Fish #	Treatment	S.I.(IR)	S.I.(WL)	Std. Length mm
1	2S	-0.067	-0.667	52.55
2	2S	-0.067	-0.867	61.06
3	2S	0.200	-0.333	64.26
4	1S	0.000	-1.000	50.77
5	1S	0.000	-1.000	49.91
6	1S	0.267	-0.133	55.06
7	0S	-0.067	0.067	57.91
8	0S	0.000	-0.267	61.33
9	1C	-0.267	-0.267	52.90
10	1C	-0.067	0.200	46.81
11	1C	0.067	-0.533	52.81
12	1C	0.067	-0.133	47.28
13	1C	-0.200	-0.267	39.55
14	1C	-0.467	0.000	55.11
15	0C	-0.067	0.267	45.26
16	0C	0.067	-0.067	48.65
17	0C	0.133	-0.533	47.76
18	0C	0.200	-0.067	46.20
19	0A	-0.067	0.200	54.16
20	0A	-0.133	-0.400	57.90
21	0A	0.000	-0.067	60.71

Scotophobic indices for each fish under infrared illumination and white light [S.I.(IR) and S.I.(WL)], grouped by treatment. S.I. ranges from -1 (maximally scotophilic) to +1 (maximally scotophobic). The first character of the treatment (0, 1, or 2) is the number of eyes after removal, induction, or unmanipulated; the second character indicates the origin of the fish—surface and normally eyed (S), cave and normally eyeless (C), and aquarist piscicultural stock that is normally eyeless (A).



turned on the overhead W light. They departed, turning off the room illumination, and videotaping resumed for another 35 to 38 minutes.

The location of fish in the lighted or dark half of the aquarium was scored from the tape recording 30 times at 1 min intervals from the last 30 minutes of recording of each trial. Each subject was thereby permitted to habituate for at least 5 min before its behavior was scored, a period that previous studies had revealed to be sufficient for these fish to resume their normal behavior after a brief bout of rapid swimming throughout the tank typical of being moved to a new environment (Goodrick, '62; Romero, '85b; but see also Langecker, '92). A fish was scored as being in the illuminated compartment if its head was visible on the videotape. In each experiment the infrared trial preceded the white one so as not to incur visual pigment bleaching effects.

Temperature can affect phototactic responses (Brett, '79), so water temperature was measured before and after each experiment. Because temperature varied less than 0.1°C between trials and less than 1°C across all the experiments and Johnson ('67) found that both blind cave and epigeal *Astyanax* are indifferent to changes of up to 7°C, we assume temperature did not influence the results of our experiments.

#### Statistical analysis

We adopt the most conservative approach for the behavioral analysis, using each fish under each lighting condition to develop a single result rather than using each location score as an independent event. Thus we are not concerned with whether our intervals for scoring are so close together that temporally adjacent records may not be independent events (cf. Green and Romero, '97, to see how a runs test permits dealing with data of that type).

The scores for each fish in each lighting condition are transformed into a 'Scotophobic Index' [S.I.(IR) or S.I.(WL)] using a simple formula modified from Romero ('85b). The S.I. is calculated as  $2 * [(number\ of\ scores\ in\ the\ lit\ portion / total\ scores\ in\ the\ lit\ plus\ in\ the\ dark) - \frac{1}{2}]$ . The value will range from -1 to +1, with the maximum scotophobic value of +1 occurring if all the scores have recorded the fish in the lit half, and -1 if the fish was never recorded there. A value of zero (neutral) will be calculated if 15 of the 30 scores are in the lit portion. The twenty-one indices so calculated for each lighting

condition are then subjected to a one-factor ANOVA and to post-hoc tests as appropriate using SYSTAT 10.

For this initial analysis, an alpha of 0.10 is used as the criterion for statistical significance. This 'generous' value is conservative with respect to the biology involved—if there is some reasonable possibility that our treatment groups differ in their photoresponsiveness, we want to reject the null hypothesis and explore the data further. In that sense, it is similar to using a generous alpha for screening purposes in public health analyses and in exploring for potential pharmacological effects of newly examined compounds. If the null hypothesis of no effect of the treatment is rejected and further testing is warranted, the refined hypotheses are examined using a traditional alpha of 0.05.

#### RESULTS

First photoresponsiveness to infrared light is examined. If scotophilia is detected, it could point to infrared sensitivity, a side bias under these experimental conditions, or to an artifact produced by surgery. Such a result would call into question the interpretation of any scotophilia exhibited under white light. The S.I.(IR) for the 21 fish ranged from -0.47 to 0.26 with a mean of -0.02, almost neutral (Table 1). Using the six basic experimental conditions as factors (normal surface fish=2S, surface fish with one normal eye=1S, surface fish without a normal eye=0S, blind Pachón cave fish with one induced eye=1C, normally eyeless Pachón blind cave fish=0C, and normally eyeless blind cave fish from aquarist piscicultural stock=0A), the ANOVA gave no evidence of any effect of these treatments ( $F=1.52$ ;  $df=5,15$ ;  $p=0.242$ ) (Fig. 1). We therefore conclude that if there are any effects exhibited under white light, they cannot be interpreted as artifacts or biases caused by some aspect of the treatment or testing procedures, but can only be produced by responses to white light under these same conditions.

When responses to white light are examined, we see a range of the S.I.(WL) from -1 to 0.27, with a mean of -0.28 (i.e., scotophilic) (Table 1). Using the same six experimental conditions as factors in an ANOVA of the white light Scotophobic Index, it appears that the factors have an effect on scotophobia to white light ( $F=2.54$ ;  $df=5,15$ ;  $p=0.074$ ). To examine whether this result might be an artifact of potential lack of normality of the



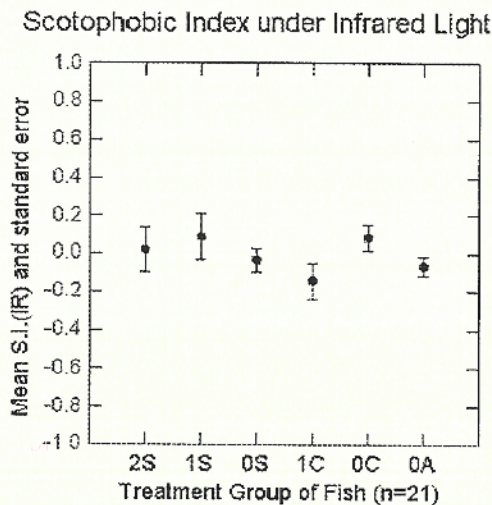


Fig. 1. Blind cave fish (*Astyanax fasciatus*) were scored for presence in the portion of an aquarium illuminated by infrared light over a 30 min period, yielding a Scotophobic Index for each fish [S.I.(IR)] in the six treatment groups. The other half of the tank was dark. S.I. ranges from -1 (maximally scotophilic) to +1 (maximally scotophobic). Means and their standard errors are shown for each treatment group. The first character of the treatment is the number of eyes after removal, induction, or unmanipulated; the second character indicates the origin of the fish - surface and normally eyed (S), Pachón cave and normally eyeless (C), and aquarist piscicultural stock that is normally eyeless (A). Eyes of the fish (n=6) in group 1C were induced. For 2S, n=3; 1S, n=3; 0S, n=2; 0C, n=4; 0A, n=3.

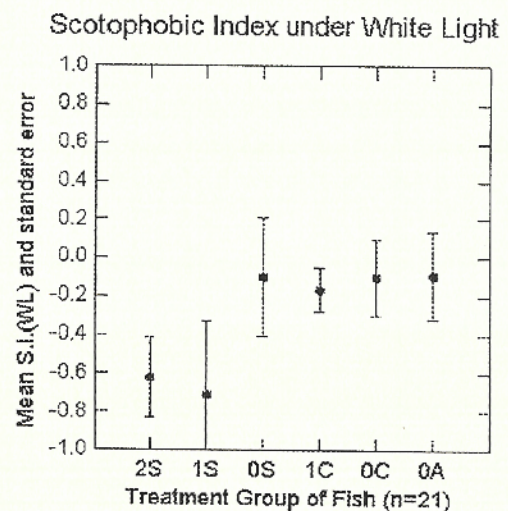


Fig. 2. Blind cave fish (*Astyanax fasciatus*) were scored for presence in the portion of an aquarium illuminated by white light over a 30 min period, yielding a Scotophobic Index for each fish [S.I.(WL)] in the six treatment groups. The other half of the tank was dark. S.I. ranges from -1 (maximally scotophilic) to +1 (maximally scotophobic). Means and their standard errors are shown for each treatment group. The first character of the treatment is the number of eyes after removal, induction, or unmanipulated; the second character indicates the origin of the fish - surface and normally eyed (S), Pachón cave and normally eyeless (C), and aquarist piscicultural stock that is normally eyeless (A). Eyes of the fish (n=6) in group 1C were induced. For 2S, n=3; 1S, n=3; 0S, n=2; 0C, n=4; 0A, n=3.

data, we conduct a Lilliefors one-factor Kolmogorov-Smirnov test and find that we cannot reject the null hypothesis of normality ( $p=0.44$ ). Looking at the mean index for each condition, it is apparent that the results fall into two groups - scotophilia for the surface fish with at least one eye and near neutrality for all the others (Fig. 2).

To determine whether our visual impression has merit, we conduct a post-hoc test by lumping the six experimental conditions into three groups determined by *a priori* assumptions. The first group will be those conditions that contain putatively sighted fish (surface fish with both eyes and with one normal eye=2S+1S) under the assumption that at least one eye is necessary for photoresponsiveness, the second group is blind and blinded cave fish (both our normal Pachón laboratory stock and those from aquarist breeding stock as well as surface animals without normal eyes=0C+0A+0S) under the assumption that photoresponsiveness will be absent in eyeless fish, and the third group is the fish with experimentally

induced eyes (1C) so as to permit comparing them with the other groups.

The ANOVA using these three experimental conditions reveals a strong effect ( $F=7.48$ ,  $df=2,18$ ;  $p=0.004$ ) and the Scheffé pairwise comparison reveals that the surface fish with one or two eyes differ from both eyeless fish ( $p=0.006$ ) and those with an induced eye ( $p=0.028$ ) whereas the latter two do not differ from each other ( $p=0.901$ ; Fig. 3).

In summary, then, we find that fish with experimentally induced eyes are not distinguishable in scotophobic photoresponsiveness from blind and blinded fish in that they are close to neutral (unresponsive) whereas surface fish, even with only one normal eye, are scotophilic (i.e., negatively scotophobic).

## DISCUSSION

This paper reports the first behavioral studies on naturally blind cave fish with experimentally



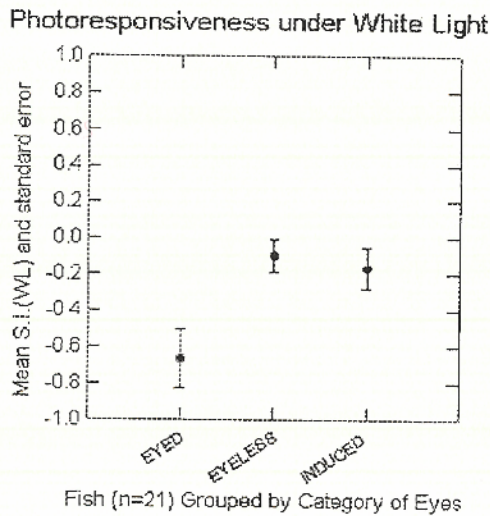


Fig. 3. Scotophobic indices for 21 blind cave fish (*Astyanax fasciatus*) scored under white light as shown in Figure 2 grouped as eyed (2S, 1S; n=6), eyeless (0S, 0C, 0A; n=9) or with induced eyes (1C; n=6). Means and their standard errors are shown for each grouping category.

induced eyes. We show here that cave fish with an induced eye do not respond to light despite rescued optic morphology. These results suggest that regressive changes have evolved beyond the level of the eye in the cave fish visual system. They also support previous studies in this species indicating that morphology and associated behavior may occur independently (Romero, '84a,b, '85a).

To interpret this lack of responsiveness fully will require an examination of the morphology and neural activity of the complete optic system. Preliminary investigation of the developmental morphology of the larval fish optic system by injecting DiI into the developing eyeballs of larval 2S surface and 0C Pachón cave reveals no difference in the movement of the dye from the site of injection along the optic nerves, suggesting that the presence of retinotectal projections in larvae of both forms cannot be ruled out by this technique and warrant further investigation (pers. comm., Y. Yamamoto and W. Jeffery). In adult 1S fish, preliminary histology reveals the thickness of the optic nerve from the degenerate eye appears to become reduced as does the contralateral optic tectum to which it projects. In adult 1C cave fish, however, the thickness of the optic nerve from induced eyes is slightly larger than from (normal) degenerate eyes but the optic tectum seems to be unchanged (pers. comm. Y. Yamamoto and

W. Jeffery), again offering no evidence that permits ruling out the possibility of retinotectal projections being formed. Consistent with the possibility that if neural connections between the eye and the brain are in fact established, neural signals may be available for them to transport, other preliminary studies indicate that weak electroretinogram signals may be produced by the induced eye of 1C cave fish (W. Hodos, pers. comm.).

If it is determined that cave fish develop retinotectal projections that are enhanced after an eye is induced by embryonic lens transplantation, it would then be reasonable to suggest that information indicating at least the presence or absence of light can move through the optic nerve to the tectum. We would then be able to conclude that the absence of responses to light in blind cave fish with induced eyes cannot be attributed to absence of retinal signals from these eyes, if that initial indication is borne out, nor to lack of a pathway for neural information.

Even if neural signals are transmitted from the induced eyes through the optic system to the brain, the lack of visual activity during development may yield a permanent sensory deficit. Cooper et al. (2001), examining epigeal and cave crayfish, showed, for example, that development of the visual apparatus in the epigeal form can be strongly influenced by whether they were raised under dark or light conditions, complementing the classic studies of vision in mammals emphasizing the importance of early visual experience for perceptual capabilities and responsiveness as adults. In addition, even if it is found that retinotectal projections are formed, it would require additional investigation to determine if they map the retina onto the tectum in the normal fashion so as to permit image formation. Image formation is clearly not a prerequisite for scotophilic photore sponsiveness, however, as this behavior is exhibited in other taxa of eyeless blind cave fish, possibly mediated by the pineal organ (Langecker, '92; Green and Romero, '97).

Our results suggest that eye induction in cave fish does not result in any detectable changes in their behavioral response to light. Romero ('84a,b, '85a) observed individual *A. fasciatus* that were morphologically identical to those of the epigeal population actively entering a cave in Costa Rica, both for feeding and avoiding predation. Unlike typical epigeal *A. fasciatus*, however, these animals did not form schools while in the cave, thereby behaving like typical cave dwellers.



Failure to school cannot be accounted for solely by the darkness and therefore reduction or absence of vision, since schooling does not depend on vision but can be mediated entirely by the lateral line system; artificially blinded fish can school (Pitcher et al., '76). This observation on lack of schooling in the cave entrants therefore suggests that the shift in behavior from that typical of epigeal forms to that typical of cave dwellers is decoupled from morphological changes and cannot be accounted for by a reduction of the attributes required to express the surface-typical behavior. As an example from another sensory system, consider that many cave fishes release an 'alarm substance' as do their epigeal ancestors (Pfeiffer, '66). But, in contrast to the surface fish that are subject to a variety of predation pressures, the cave fishes, with few or no known predators, either do not respond to it or show a much reduced response although they have similar or even enhanced chemosensory capabilities (Fricke, '87; Romero and Paulson, 2001), again consistent with decoupling lack of expression of a behavior in cave fish from absence of its morphological prerequisites.

In trying to explain scotophilia among cave-dwelling *Astyanax*, Breder and Rasquin ('47) suggested it serves as a mechanism facilitating the return to safety of animals that accidentally exit from caves. But many cave fish species with reduced eyes are not scotophilic (see Romero, '84a,b; Green and Romero, '97) and many eyeless cave fishes are responsive to light (probably mediated by the pineal organ or extrapineal sensors, cf. Langecker, '92). Breder and Rasquin's hypothesis linking retention of reduced eyes with an advantage to photoresponsiveness therefore does not offer a universal explanation. It seems at least equally plausible that photoresponsiveness of cave dwellers may simply be a disappearing relict from their epigeal ancestors rather than having an adaptive function maintained by natural selection (Green and Romero, '97; Romero, '85b) and that the degree to which it persists varies among species. The same can be said of circadian rhythms among hypogean fishes (Lamprecht and Weber, '92).

Although the most obvious regressive feature of *Astyanax* cave fish is eye reduction, there are other morphological traits that are enhanced or reduced in this species (Wilkens, '88; Jeffery, 2001). Among the latter is the optic tectum, which contains the visual center in the brain. The optic tectum is extremely reduced in size in adult cave fish (Peters et al., '93). If it is confirmed that the optic tectum

is not markedly changed in cave fish with induced eyes and enhanced retinotectal projections, it would suggest that reduction in the visual centers occurred independently of eye degeneration during cave fish evolution. It would be interesting to see if a complete cave fish visual system might be induced by transplanting both surface lens and optic tectum primordia into a cave fish host and then to examine photoresponsiveness.

It has been long known that phenotypic plasticity as a function of illumination affects troglomorphisms (e.g., pigmentation patterns of epigeal and cave populations of certain fishes; Rasquin, '47, '49). The ability to separate evolved troglomorphism from its experimental 'reversal' by using the induced optic structure and measuring its associated functional photoresponsiveness offers a new system that can permit characterizing and disarticulating the elements associated with the evolutionary loss of form and function.

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