

Responses to light in two blind cave fishes (*Amblyopsis spelaea* and *Typhlichthys subterraneus*) (Pisces: Amblyopsidae)

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Synopsis

Explanations for the phenotypical features resulting from colonization of subterranean environments have always been a source of controversy. Although a great number of cave organisms are blind, they nevertheless display responses to light. The interpretation of this phototactic responsiveness in cave-dwelling animals may provide clues on the general issue of evolution of behavior in parallel with specialized structures. We studied the phototactic responses in two amblyopsid fishes of North America and found responses to light only in the species reported to have a functional pineal organ. Our findings are consistent with the hypotheses that (1) adaptation to the cave environment is a gradual process and (2) responsiveness to light in cave fishes may best be understood as a relict character, one that exists in an environment where it may never be expressed.

Introduction

The study of morphological and behavioral adaptations by surface organisms to the subterranean environment continues to be a source of controversy (Culver 1982, Culver et al. 1995). The two major issues are (1) the process of cave colonization and (2) the course of phenotypic changes so common among subterranean organisms, particularly the reduction or disappearance of features.

Cave colonization

There are many examples of obligate and facultative subterranean organisms with putative surface ancestry. Barr (1968), Poulson (1963) and Vandel (1965) argue that extant troglobites (obligatory subterranean organisms) descended from ancestors preadapted to, and that could exploit, the cave envi-

ronment due either to nocturnal habits or to possession of highly developed non-visual sensory systems. Other theories on the origin of cave populations postulate that accidental entry into caves led to permanent entrapment (Barr 1968). Another theory proposes directional ('regressive') evolution on the assumption that cave animals represent 'dying phylogenetic lines' that seek refuge in caves (see Barr 1968). All these hypotheses lack experimental support (Romero 1985a).

Phenotypes

A parallel issue is the reduction of structures and simplification of behavioral patterns that characterize cave dwellers. Wilkens (1993) proposes that neutral evolution can account for the reduction or disappearance of characters in cave-dwelling organisms. Others contend that selection, rather than

genetic drift, accounts both for the enhancement of some features (such as non-visual sensory structures) and for the reduction and disappearance of others (such as eyes and pigmentation) (Jernigan et al. 1994, Jones & Culver 1989, Jones et al. 1992).

Evolutionary changes from epigeal to troglotic existence are still matters of controversy supported more by conjecture than by evidence (cf. Culver et al. 1995, Kane & Richardson 1985, Sket 1985). The most enlightening evidence would be revealed by examining stages intermediate between ancestral forms and blind depigmented ones (Romero 1984). Only a few taxa provide the opportunity for such empirical evaluation, including the freshwater fish *Astyanax fasciatus* and the amblyopsid fish family (Poulson 1963, Langecker & Longley 1993). In this paper we focus on comparing responses to light in two of the amblyopsids, *Amblyopsis spelaea* and *Typhlichthys subterraneus*.

General questions

Constant total darkness is the most important feature distinguishing cave from surface environments. The behavioral and morphological attributes of troglotes must therefore be examined with respect to this feature. The key questions are: (1) Do animals that live exclusively in total darkness their entire lives still retain photosensory capabilities? Do they respond to photostimuli? (2) If there are phototactic responses, what are their underlying morphological and physiological bases? (3) How can phototactic responsiveness in cave-dwelling animals be interpreted from an evolutionary standpoint?

Issues regarding the extent of responsiveness to light in cave dwellers are addressed by Vandel (1965) who surveyed the relevant literature on cave animals. He found that twenty species were reported, on evidence of very uneven quality, to be scotophilic (i.e., preferred to remain in the dark portion of a light/dark choice chamber) whereas only ten were indifferent to light. Romero (1984), in a later review of the cave fish literature, found that scotophilia is associated with eleven species of cave fishes, whereas five are indifferent to light. One spe-

cies, *Astyanax fasciatus*, is scotophilic in some populations and indifferent to light in others (Romero 1985b). Langecker (1992) found references to fourteen scotophilic cave fish species.

Poulson (1963) notes that in the six species of the family Amblyopsidae, *Chologaster cornuta*, a cave, spring, and nocturnal epigeal (surface) fish, and *C. agassizi*, a facultative cave dweller, are scotophilic, although he does not cite any experimental evidence. Among other Amblyopsidae, *Amblyopsis spelaea* is reported to be scotophilic (Eigenmann 1909, Payne 1907) and *Typhlichthys subterraneus* to be indifferent to light (Eigenmann 1909, Verrier 1929). We agree with Thinès (1969) that the experimental procedures and data analyses for both these species are far from satisfactory.

Goals

In this paper we examine experimentally if *A. spelaea* and *T. subterraneus* exhibit preferences in a light/dark choice chamber. We then discuss phototactic responsiveness in cave fishes in terms of reports on the underlying structures and from an evolutionary perspective.

Material and methods

Fish

A. spelaea and *T. subterraneus* represent different stages of cave adaptation in amblyopsids along the range from surface-like to fully cave-adapted. *T. subterraneus* has been characterized as a species much more morphologically adapted to the cave environment than is *A. spelaea* (Bechler 1983, Cooper & Kuehne 1974, Woods & Inger 1957). These are uncommon species, rarely kept alive in captivity. Our subjects are three individuals of each species that were collected in the field and maintained in the laboratory described by Bechler (1983) who lent us the fish. The nature of phototactic responsiveness in cave fish changes with age (Langecker 1992, Romero 1985b), so we measured their length (the only external indicator of age) to

the nearest 0.1 mm using vernier calipers. The standard length and locality for each individual are listed in Table 1.

Procedure

Simple choice tests were designed to ascertain the extent and direction of phototactic responses. Each experiment consists of two trials of one fish, one using white light and the other using darkroom red light. Two experiments were conducted simultaneously in two identical 160 l fiber-glass-lined opaque wooden aquaria covered with flat pieces of cardboard that had been painted dull black and rendered opaque. Only half of a tank's surface was covered, thus forming a light/dark choice chamber when illuminated from above. The bottom of each tank was covered with sand. Tanks were filled to a depth of ca. 20 cm with previously aerated water from a subterranean spring. No aeration was provided during the experiments. Using minnow nets, individual fish were placed into each tank from a home tank for each experiment. Tanks were rotated to exchange illuminated and dark sides after each experiment.

A light source was fixed 53 cm above the surface of the water of each tank to avoid heating it. The lamps used as stimuli and to observe phototactic responses were commercial tungsten 'white' (General Electric 150 W, 130 V) and darkroom 'red' (Westinghouse 25 W Ruby, 120 V). The red light permitted seeing a fish's location while being conservative with respect to any results, i.e., differences in the re-

sponses of fish under the experimental bright white light vs. dark shadow choice and the dim red light vs. dark shadow choice are expected to be less pronounced than between a white light vs. dark and dark vs. dark pair of choices. As an indication of relative brightness, we measured the white light intensity to be 80 fc and the red's 6 fc using a photographic light meter at the surface of the water directly beneath the light source.

Because temperature can affect phototactic responses (Brett 1979), water was maintained between 10° and 13°C, a range much smaller than that noted in the only report on the thermal sensitivity of changes in behavior by blind cave fish. Johnson (1967) found that *Astyanax*, as well as its epigeal form, is indifferent to changes of up to 7°C. A small electric fan placed 0.50 m from each tank circulated air between the light and the surface of the tank, thus preventing the air from being heated by the incandescent source. Temperatures were recorded to the nearest half degree from a surface and a bottom thermometer at the beginning of the first trial and end of each trial (Table 2).

Chemicals released by fish during an experiment may affect the behavior of fish in subsequent experiments, so the tank water was replaced after each experiment. The surface of the sand was also swept after each experiment using the water-extracting hose that was connected to the pump emptying the tank.

The location of fish was recorded at one min intervals beginning one min after it was placed in the tank and continuing to the end of a 30 min trial. A fish was scored as in the light compartment if its

Table 1. Size and collection locality of subjects.

Experiment	<i>Amblyopsis spelaea</i>		<i>Typhlichthys subterraneus</i>	
	SL ¹	Locality ²	SL ¹ ²	Locality ²
1	55.1	Kentucky	48.3	Crittendon, Kentucky, near Tennessee
2	66.1	Orange Country, Central Indiana	47.2	Baugus Cave, Decatur County, Tennessee
3	71.7	Orange County, Central Indiana	33.9	Branch Cave, Perry County, Tennessee

¹ Standard length in mm.

² As noted by Bechler (1983).

head was visible in that compartment to the observer who watched from 1 m directly above the uncovered half of the tank. The two trials in each experiment were run consecutively with only a short interval between them to record the water temperature and change the lamps. In each experiment the 'red' trial preceded the 'white' one so as not to incur visual pigment bleaching effects. Two experiments were run simultaneously, one in each tank, one on each species, one by each author. The experiments started at 14:00, 16:10, and 18:20 h.

The primary null hypothesis is that there is no difference in phototactic response under the bright white light vs. darkness condition when compared with the dull red light vs. darkness condition. It is examined by testing if the locations of fish (light or dark portion) illuminated by bright white light does not differ from their locations when illuminated by dim red light.

For each species, the proportion of scores in which fish were located in the illuminated half under red light is calculated by simply taking the sum of such observations for all three individuals and dividing by the 90 scoring periods, 30 for each fish. This proportion is taken to be the expected probability of location in the illuminated half under white light under the null hypothesis of no difference. This expected probability for each species is then used to calculate the 95% confidence limits for evaluating the combined scores obtained under white light for all three fish of each species, and hence the complementary region of rejection for the null hypothesis ($\alpha = 0.05$). These 95% confidence limits are calculated using the normal ap-

proximation to the binomial probability distribution (Sokal & Rohlf 1995).

Because the null hypothesis is based on the performance of individuals as an indicator of photoresponsive capability of a species, it can be rejected if the behavior of even a single fish (i.e., one white light trial) lies outside the confidence interval. Experimentwise confidence limits are therefore calculated for each species to take account of the three replicates. To achieve the same overall Type I error rate of $\alpha = 0.05$, we calculate the corrected and more conservative 98.3% one-trial confidence limits for each species and use the complementary regions of rejection to examine the results of each experiment separately.

A second, but closely related, hypothesis is that fish are entirely indifferent to the experimental illumination. In this case, they would be equally likely to be located in the dark or light portion of the tank for both red and white light. To examine the null hypothesis that fish are indifferent to light, the same statistical test procedures, both for overall species scores and for individual experiments (correcting for replicates), are undertaken using the theoretical 0.5 expected probability of location in the illuminated half. Red and white illumination vs. dark are examined separately.

The appropriateness of one minute as the interval for position samples is evaluated by testing if the position scored at any one sample point is independent of those scored in neighboring samples. If not, the intervals are too short and the scores are inflated by a lack of independence. This issue is examined using tabulated critical values for the runs test (Rohlf & Sokal 1981).

Table 2. Water temperature ($^{\circ}\text{C}$) measured at surface (S) and bottom (B) of aquaria at beginning and end of 30 min trials under experimental white and control red lights.

	<i>Amblyopsis spelaea</i>						<i>Typhlichthys subterraneus</i>					
	Expt.1		Expt.2		Expt.3		Expt.1		Expt.2		Expt.3	
	S	B	S	B	S	B	S	B	S	B	S	B
Begin red	10	10 $\frac{1}{2}$	11 $\frac{1}{2}$	10	12	12	10	10 $\frac{1}{2}$	11 $\frac{1}{2}$	10	12	12
End red and begin white	10 $\frac{1}{2}$	9	11 $\frac{1}{2}$	10	13	10	10 $\frac{1}{2}$	9	11 $\frac{1}{2}$	10	13	10
End white	11	9	11 $\frac{1}{2}$	10	12	9	11	9	11 $\frac{1}{2}$	10	12	9

Results

A. spelaea exhibits a scotophilic phototactic response under white light (Table 3). Looking at each experiment separately, the degree of deviation from the expected values for each fish does not fall outside the confidence limits. Although statistical significance is therefore not reached for any of the fish considered individually, for all three experiments fish were scored less in the illuminated half of the tank under bright white light than under dim red. When we examine the total score for all three trials under white light (the three fish combined), we see that it does fall below the lower confidence limit generated for comparing their behavior score with that under red light. It also falls below the lower confidence limit based on the expectation of no effect of illumination (50% expectation). We there-

fore reject both the null hypothesis of no difference in behavior under red vs. white light ($p < 0.05$) and the null hypothesis of indifference to white light ($p < 0.05$).

T. subterraneus is altogether indifferent to the white light by any measure ($p > 0.05$).

Neither *A. spelaea* nor *T. subterraneus* demonstrate any effect of red light on their location in the illuminated vs. dark half of the tank.

If the behavior of a fish scored at one time contaminates the score in the next interval, then the data are not independent as required by our analysis. If, for example, a fish rests in one portion of the tank for a full 15 minutes and then changes to the other half, the data will exhibit only two runs of location scores of the same kind, and we would reject the hypothesis that two runs of 15 scores each is a random sequence. In this case we would conclude that

Table 3. Proportion of 30 samples at one-minute intervals that a fish is scored in the half of an aquarium illuminated by red or white light.

Experiment	<i>Amblyopsis spelaea</i> Illumination				<i>Typhlichthys subterraneus</i> Illumination			
	Red	Runs ¹	White	Runs	Red	Runs	White	Runs
1	16/30	16	10/30	13	16/30	10	13/30	14
2	13/30	18	10/30	13	10/30	5	16/30	8
3	17/30	23	14/30	19	16/30	14	12/30	16
Total	46/90		34/90		42/90		41/90	

Hypothesis: location of fish is not affected differently in white light compared to red light.

Total white score 95% confidence interval:¹

36.7 – 55.3

32.7 – 51.3

Single experiment white score 95% confidence interval:²

8.8 – 21.9

7.5 – 20.5

Hypothesis: location of fish is not affected by either red or white light illumination.³

Total illuminated score, 95% confidence interval, both species:

35.7 – 54.3

Single experiment score, 95% confidence interval, both species:

8.5 – 21.5

¹ A run is a sequence of consecutive like-scored samples.

² The 95% confidence intervals around the total scores of 46 and 42 for red are based on the normal approximation to the binomial distribution ($z = 1.960$). If the observed total score for either species under white light falls outside this interval, then the hypothesis for that species stating white light has no difference in effect from red light may be rejected at $p < 0.05$.

³ The experimentwise confidence intervals are calculated at 98.3% to reflect an overall 95% confidence interval ($z = 2.387$). If the score for white light on any single experiment falls outside this interval, then the hypothesis for that species stating white light has no difference in effect from red light may be rejected at $p < 0.05$.

⁴ The 95% confidence intervals, both overall and experimentwise, for the hypothesis that illumination has no effect is calculated by using the theoretical expectation of 0.5 probability of presence in the illuminated half of the tank. If the overall score or that for any single experiment falls outside the relevant limits, then the hypothesis that the location of fish of that species is not affected by the illumination may be rejected at $p < 0.05$.

a one minute sampling interval is too small. The outcome of the runs test on our data does not reveal any contagion ($p > 0.05$) among the sample scores for *A. spelaea* and hence shows that our one minute sampling intervals are appropriate for examining this species under these experimental conditions.

The data for *T. subterraneus*, however, reveal contagion in the red trial of experiment 1 and in both trials of experiment 2 ($p < 0.05$). This kind of non-independence for three of our six trials and occurring in two out of three subjects strongly implies that the one minute intervals are too short for this less active species. We therefore first determine if analysis using a longer interval between scores of the fish renders them suitably independent. The same data are reanalyzed using only every other score, thus doubling the interval. Rather than arbitrarily using either only the even or only the odd alternate scores, we examine in Table 4 both ways of utilizing half the original data. The outcome of the

runs test now indicates that the data are independent ($p > 0.05$) and we conclude that two-minute interval scoring is appropriate.

Next we must determine if our results differ when analyzed using samples separated by this longer interval. When the results for this uncontaminated set of scores are examined using the same kind of confidence-limit analytic procedures, our conclusions remain unchanged. There is no effect of either red or white light on the behavior of *T. subterraneus*.

Discussion

It has been argued that phototactic responses in cave fishes are mediated by remnants of the eye structure and by the pineal organ (or epiphysis). McNulty (1978a,b) found that the pineal organ of the blind *T. subterraneus*, unlike that of the microphthalmic yet scotophilic *Chologaster agassizi*,

Table 4. Proportion of 15 samples at two-minute intervals that a fish is scored in the half of an aquarium illuminated by red or white light.

Experiment	<i>Typhlichthys subterraneus</i> ^a							
	Begin at first minute Illumination				Begin at second minute Illumination			
	Red	Runs ^b	White	Runs	Red	Runs	White	Runs
1	8/15	10	8/15	9	8/15	5	5/15	10
2	6/15	5	9/15	6	4/15	4	7/15	6
3	7/15	8	4/15	9	9/15	7	8/15	12
Total	21/45		21/45		21/45		20/45	

Hypothesis: location of fish is not affected differently in white light compared to red light.

Total white score 95% confidence interval:¹

14.4 – 27.6

Single experiment white score 95% confidence interval:²

2.4 – 11.6

Hypothesis: location of fish is not affected by either red or white light illumination.³

Total illuminated score, 95% confidence interval:

15.9 – 29.1

Single experiment score, 95% confidence interval:

2.9 – 12.1

^a The same data represented in Table 3 for this species are used but employing scores only from every other interval.

^b A run is a sequence of consecutive like-scored samples.

¹ The 95% confidence interval around the total score of 21 for red is calculated as per footnote #1 of Table 3.

² The experimentwise confidence interval is calculated as per footnote #2 of Table 3.

³ The 95% confidence intervals, both overall and experimentwise, are calculated as per footnote #3 of Table 3.

is poorly developed and functionally ineffective. Our finding of non-responsiveness to illumination in *T. subterraneus* is consistent with the view that the pineal organ must remain functional for blind fish to exhibit phototactic responses to light. Although the pineal organ of *A. spelaea* has not been investigated, our results, as well as its general morphological features described by Poulson (1963) and by Woods & Inger (1957), lead us to predict that the pineal organ is photosensitively functional. Langecker (1992) summarizes reports that extraocular, extrapineal photoreception (EOPP) has been demonstrated in both troglitic and epigeal amphibians and fish, however, so we cannot dismiss the possible role of EOPP in this study.

Our finding of phototactic responsiveness in *A. spelaea* parallels the findings of morphological and taxonomic study of the Amblyopsidae, namely that there are numerous forms that differ in only small degrees, ranging from sighted epigeal forms to troglitic eyeless cave dwellers. Our behavioral result supports the contention derived from these other studies that evolutionary adaptation to the cave environment is gradual.

There is growing evidence that behavioral patterns such as phototactic responses are present only in those cave fishes that are closely related to their surface ancestors (Langecker 1992, Parzefall 1992, Romero 1985b). Although facultative behavioral traits are traditionally considered rather plastic in nature, the presence of scotophilia in an exclusively cave dwelling organism may best be understood as a relict character, one that exists in an environment where it may never be expressed.

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