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Shadow response in the blind cavefish *Astyanax* reveals conservation of a functional pineal eye

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SUMMARY

The blind cavefish *Astyanax mexicanus* undergoes bilateral eye degeneration during embryonic development. Despite the absence of light in the cave environment, cavefish have retained a structurally intact pineal eye. We show here that contrary to visual degeneration in the bilateral eyes, the cavefish pineal eye has conserved the ability to detect light. Larvae of two different *Astyanax* cavefish populations and the con-specific sighted surface-dwelling form (surface fish) respond similarly to light dimming by shading the pineal eye. As a response to shading, cavefish larvae swim upward vertically. This behavior resembles that of amphibian tadpoles rather than other teleost larvae, which react to shadows by swimming downward. The shadow response is highest at 1.5-days post-fertilization (d.p.f.), gradually diminishes, and is virtually undetectable by 7.5 d.p.f. The shadow response was substantially reduced after surgical removal of the pineal gland from surface fish or cavefish larvae, indicating that it is based on pineal function. In contrast, removal of one or both bilateral eye primordia did not affect the shadow response. Consistent with its light detecting capacity, immunocytochemical studies indicate that surface fish and cavefish pineal eyes express a rhodopsin-like antigen, which is undetectable in the degenerating bilateral eyes of cavefish larvae. We conclude that light detection by the pineal eye has been conserved in cavefish despite a million or more years of evolution in complete darkness.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/3/292/DC1

Key words: pineal eye, shadow response, blind cavefish, behavior.

INTRODUCTION

The pineal gland, the only unpaired region of the vertebrate brain, has important roles in light detection, neuroendocrine secretion and circadian behavior (Collin, 1971). In amphibians, teleosts and reptiles, the pineal gland functions to detect light in larval or young animals before the development of acute vision. For example, in young *Xenopus laevis* larvae swimming behavior is controlled by the pineal gland (Roberts, 1978; Jamieson and Roberts, 2000). In mammals, the light sensing function of the pineal gland appears to be absent. Another role of the pineal gland is entrainment of circadian rhythms *via* secretion of melatonin (Zachmann et al., 1992a). To mediate its light-detecting function, teleost pineal photoreceptor cells express the opsin-related photon-transducing pigments VA opsin, parapinopson and ERrod-like opsin (reviewed in Foster et al., 2006). Because of its important role in light detection, particularly during ontogeny, the pineal gland is referred to as the third or pineal eye in some vertebrates.

Cave animals are important models for studying the role of the environment in generating evolutionary change because they are not usually exposed to light, which is one of the most pervasive environmental cues. As a consequence of evolution in complete darkness, many cave-adapted animals have lost or reduced their eyes, visually based behaviors, pigmentation and other traits that are essential for life in the surface environment (Culver, 1982). The evolutionary forces responsible for degeneration of the visual system are not completely understood. The most likely mechanisms are neutral mutation, in which eyes are thought to regress passively and gradually due to the accumulation of hypomorphic mutations in eye forming genes, or natural selection, in which the loss of eyes is mediated more rapidly by positive selection (Culver, 1982). Among the potential adaptive benefits of eye loss could be energy saving or compensatory trade-offs with other sensory systems (Jeffery et al., 2000; Jeffery, 2005; Protas et al., 2007).

We used the Mexican tetra *Astyanax mexicanus* to study the role of the dark cave environment in promoting phenotypic changes during evolution. *Astyanax* has two conspecific forms: an eyed epigean form (surface fish) and an eyeless hypogean form (cavefish) (Şadoğlu, 1957; Wilkens, 1988; Jeffery, 2001). At least 29 different cavefish populations are present in Mexican limestone caves (Mitchell et al., 1977), and some of these are likely to have evolved independently from the same or different surface fish ancestors (Dowling et al., 2002; Strecker et al., 2004). Cavefish embryos form an optic primordium consisting of a lens and optic cup, which begin to differentiate and grow (Cahn, 1958). During larval development, however, the cavefish eye begins to degenerate, gradually sinks into the orbit, and is covered by an overgrowth of epidermis and connective tissue (Wilkens, 1988; Langecker et al., 1993; Jeffery and Martasian, 1998). Although a few optic nerve fibers are formed, there is a substantial reduction in the cavefish optic tectum (Soares et al., 2004). Thus, vision does not develop, and cavefish instead rely on other senses, including the lateral line (Teyke, 1990; Montgomery et al., 2001), the gustatory system (Schemmel, 1967) and possibly olfaction (Yamamoto et al., 2003), to survive in the dark cave environment.

Astyanax surface fish have a pineal gland consisting of sensory cells, nerve cells, supporting cells and a cell-type resembling

phagocytes. Despite degeneration and the loss of visual capacity in the bilateral eyes, little or no morphological changes have occurred in the pineal eye between surface fish and cavefish (Grunewald-Lowenstein, 1956; Omura, 1975; Herwig, 1976; Langecker, 1992). For example, the photoreceptor segments of pineal sensory cells are still present in cavefish. The morphological differences that have been seen in the two forms of *Astyanax* appear to be quantitative rather than qualitative (Langecker, 1992). In addition, electrophysiological studies suggest the persistence of pineal photosensory function in blind cavefish (Tabata, 1982). As pointed out by Wilkens (Wilkens, 1988), however, the latter and other previous studies on pineal gland structure and function are likely to be compromised by analysis of a hybrid cavefish population. Thus, it is currently unknown whether the cavefish pineal gland can function in light detection.

We describe here behavioral studies comparing the light detecting function of the pineal gland in *Astyanax* surface fish and two divergent cavefish populations (Pachón and Tinaja cavefish), which exhibit a relatively high degree of eye degeneration and regression of surface-adapted features. Despite the absent of light in the cave environment, we demonstrate that both types of cavefish have retained the shadow response, a pineal governed activity (Jamieson and Roberts, 2000). Pinealectomy experiments confirm that the shadow response is controlled by the pineal eye in *Astyanax*. Consistent with light detection, we also show that the cavefish pineal eye expresses a rhodopsin-like antigen at similar levels to its surface fish counterpart. We propose several reasons for the conservation of pineal eye function in blind cavefish, which have evolved for a million or more years in complete darkness.

MATERIALS AND METHODS

Biological materials

These experiments were performed on laboratory-raised populations of *Astyanax mexicanus* De Filippi 1853. The original surface fish were collected at Balmorhea State Park, TX, USA, Pachón cavefish were collected at Cueva de El Pachón, Tamaulipas, Mexico, and Tinaja cavefish were collected at Sótano de la Tinaja, San Luis Potosi, Mexico. Fish were kept in the laboratory at 25°C on a 14 h:10 h light:dark photoperiod (Jeffery and Martasian, 1998; Jeffery et al., 2000). Embryos were obtained by natural spawning and raised at 22–25°C in the absence of light.

Shadow response assay

The shadow response was assayed in flat transparent plastic bottles (25 ml EasYFlasks™, NUNC, Rochester, NY, USA) that were cut at their shoulders to make rectangular chambers (7.5×7.5×3.1 cm; length×height×width). Each chamber contained 100 ml of conditioned water (conductivity approximately 600 μS) for normal larvae or zebrafish ringer (ZFR: 1.77 mmol l^{-1} $CaCl_2$, 116 mmol l^{-1} NaCl, 2.9 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes, pH 7.2) for operated larvae (see below). Standard 32 W fluorescent room lights positioned 3 m above the assay chambers provided even illumination. The assay chambers were shaded (light dimming) by insertion of an opaque board between them and the light source.

To quantify the shadow response, 20 surface fish or cavefish larvae, raised from embryos kept in constant darkness, were transferred to each assay chamber. The larvae were accommodated with even illumination for at least 30 min at room temperature prior to the assays. For each assay, larvae were introduced to the assay chamber, exposed to constant illumination for 3 min, then the chamber was shaded, and the numbers of larvae that swam upward from the bottom of the chamber to half the distance to the water surface (2.0 cm from the bottom) were counted during a 5 s shading period. This procedure was repeated at least two times with the same group of 20 larvae and the results were averaged. There was an interval of at least 1 min between each assay. This sequence of assay steps was subsequently repeated with at least four groups of 20 surface fish or cavefish larvae (at least 80 larvae per assay).

Fish swimming movements were video recorded using an infrared light (BL1960 Black light, Advanced Illumination, Rochester, VT, USA) and were captured by an infrared CCD camera (QICAM IR, Qimaging, Surrey, Canada) equipped with StreamPix software (NorPix, Montreal, Canada) at a rate of 10 frames s^{-1}. The public domain NIH ImageJ software (US National Institutes of Health, Bethesda, MD, USA) was used for video analyses. Statistical analysis was carried out by Student's unpaired *t*-test unless otherwise indicated.

Pinealectomy and eye removal

The procedure used for removing the pineal gland was modified from operations designed previously for lens deletion and transplantation (Yamamoto and Jeffery, 2000; Yamamoto and Jeffery, 2002). At 1.5 days post-fertilization (d.p.f.), surface fish and cavefish larvae (raised as described above) were washed for 10 min in calcium-free zebrafish ringer (CFZFR: 116 mmol l^{-1} NaCl, 2.9 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes, pH 7.2), rinsed in CFZFR (40°C) containing 0.2% EDTA, and embedded in 1.2% agar in CFZFR (40°C). After cooling to room temperature, the agar was cut into blocks containing individual larvae. The operations were done with sharp tungsten needles on larvae embedded in the agar blocks.

For pinealectomy, larvae were positioned on their side, a small opening was made in the dorsal epidermis above the brain, and the pineal gland was removed. Sham-operated control larvae had their dorsal epidermis opened but the pineal gland was not removed. For removal of a single eye, larvae were positioned on their side, the lens vesicle was deleted as previously described (Yamamoto and Jeffery, 2000; Yamamoto and Jeffery, 2002), and then the optic cup was removed by applying gentle pressure to the periocular area with the side of a tungsten needle. For removal of both eyes, larvae were placed ventral side up, and one or both optic cups were removed by the same procedure as described above. The experimental and control larvae were allowed to recover in ZFR for 3 h before performing the behavioral assays described above.

Rhodopsin immunocytochemistry

Surface fish and cavefish larvae were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed with $PBST_{BSA}$ (PBS plus 0.1% Triton X-100 and 2 mg ml^{-1} of bovine serum albumin), and stored in 100% methanol. Specimens were rehydrated with $PBST_{BSA}$, incubated in blocking solution (2% bovine serum albumin, 10% goat serum in 100 mmol l^{-1} maleic acid, pH 7.5, and 150 mmol l^{-1} NaCl) for 1 h, incubated overnight at 4°C in mouse anti-rhodopsin monoclonal antibody (RET-P1; Sigma, St Louis, MO, USA) diluted 1:1000 in blocking solution. After antibody treatment, the specimens were washed four times with the blocking solution and incubated in rhodamine-conjugated anti-mouse antibody (Chemicon, Temecula, CA, USA) diluted 1:100 in blocking solution. After washing four times in $PBST_{BSA}$, the specimens were viewed with a fluorescence microscope (Axioskop 2 with Axiocam, Zeiss, Göttingen, Germany).

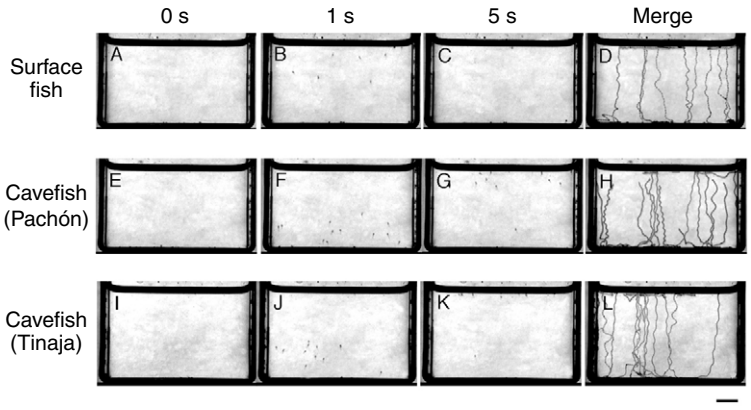

Fig. 1. The shadow response is elicited by shading 1.5 d.p.f. surface fish (A–D), Pachón cavefish (E–H), and Tinaja cavefish (I–L) larvae. Each frame shows an individual assay chamber. Video recordings were made through the side of the chambers at 0.05 s intervals for a total of 5 s. Examples of frames are shown at 0 s (A,E,I), 1.0 s (B,F,J), and 5.0 s (C,G,K). (D,H,L) Merged frames of all images from 0 to 5.0 s. Before shading (0 s), larvae were located at the bottom of the assay chambers. During shading (1.0 to 5.0 s) most larvae swam upwards until they reached the surface of the water. Scale bar, 1 cm.

RESULTS

Cavefish larvae exhibit a shadow response

We compared larval swimming behavior after light dimming in *Astyanax* surface fish with Pachón and Tinaja cavefish, genetically distinct cavefish lineages with highly regressed visual systems (Dowling et al., 2002).

In these experiments, 1.5 d.p.f. Pachón cavefish, Tinaja cavefish or surface fish larvae raised from eggs in complete darkness were placed in individual assay chambers and their swimming behavior was video recorded (Fig. 1; see supplementary material movies 1–3). During constant light or darkness, most cavefish and surface fish larvae remained at the bottom of the chambers (Fig. 1A,E,I). When the chambers were illuminated and then shaded from above, the larvae swam upward spirally from the bottom of the chamber in a synchronized manner and ceased swimming after they reached the water surface, where they often remained attached by their cement organs (Fig. 1B–D,F–H,J–L). Surface fish larvae usually reached the top of the chamber more quickly than either type of cavefish larvae. To quantify the shadow response, we counted the number of larvae that swam halfway to the water surface, which was indicative of them eventually reaching the top of the chamber. When quantified in this way, we found that about 50–70% of surface fish and both types of larvae exhibited a shadow response (Fig. 2, far left). Interestingly, at 1.5 d.p.f. more cavefish showed the shadow response than surface fish (P<0.01, N=180 for both Pachón and Tinaja cavefish compared to surface fish) (Fig. 1B,F,J). This difference was not observed at later developmental stages (Fig. 2). The results show that larvae of both cavefish and surface fish exhibit the shadow response.

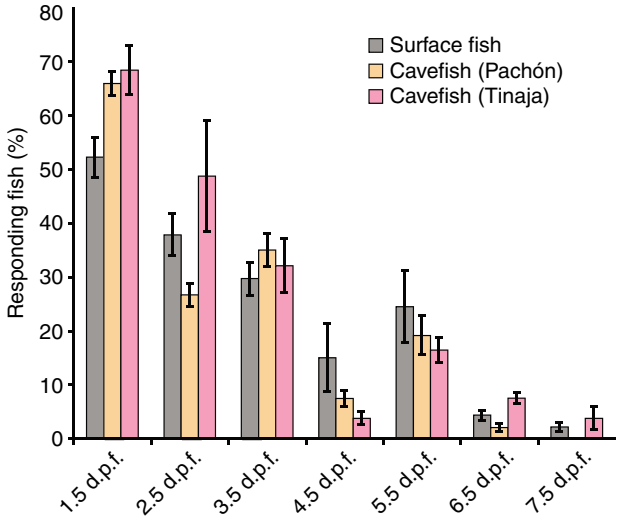

Fig. 2. Gradual reduction of the shadow response during surface fish, Pachón cavefish and Tinaja cavefish development. Values are means ± s.e.m., N=320.

Ontogeny of the shadow response

Prominence during early development and subsequent reduction is a characteristic of the larval *Xenopus* shadow response (Foster and Roberts, 1982). Therefore, we conducted shading experiments at intervals between 1.5 and 7.5 d.p.f. to determine the ontogeny of the shadow response in surface fish and cavefish. The results, quantified as described above, were similar to those described previously for *Xenopus* larvae (Foster and Roberts, 1982). The *Astyanax* shadow response was strongest at 1.5 d.p.f., diminished gradually at later stages, and was barely detectable by 6.5 or 7.5 d.p.f. (Fig. 2). In contrast to the results obtained at 1.5 d.p.f. (see above), at later developmental stages there were no significant differences in the light dimming response between cavefish and surface fish (F=0.13, P=0.88; Kruskal–Wallis test). However, overall reduction in shadow response intensity during ontogeny of surface fish and both types of cavefish was highly significant (P<0.001; surface fish N=120, Pachón cavefish N=120, Tinaja cavefish N=80) when pooled larvae were compared at 1.5 d.p.f. and 7.5 d.p.f., respectively. The results show that the ontogeny of the shadow response in *Astyanax* larvae resembles that described in *Xenopus* larvae.

Pineal opsin expression

The *Astyanax* pineal eye has been suggested to have a single photoreceptor type containing a unique opsin, potentially ERrod-opsin (Parry et al., 2003; Foster et al., 2006). Although the structure of the cavefish larval pineal eye has been examined previously and concluded to be remarkably similar to that of surface fish (Langecker, 1992), the cavefish pineal gland has not been shown to express opsin. To obtain this information and to develop a general marker for the *Astyanax* pineal eye, we used a mouse rhodopsin antibody to detect and compare opsin expression between 1.5 and 4.5 d.p.f. in surface fish and Pachón and Tinaja cavefish.

The pineal eyes of surface fish and both types of cavefish showed strong rhodopsin-like immunoreactivity at developmental stages between 1.5 and 4.5 d.p.f. (Fig. 3). The rhodopsin-like antigen was also observed in surface fish bilateral eyes beginning at 2.5 d.p.f.,

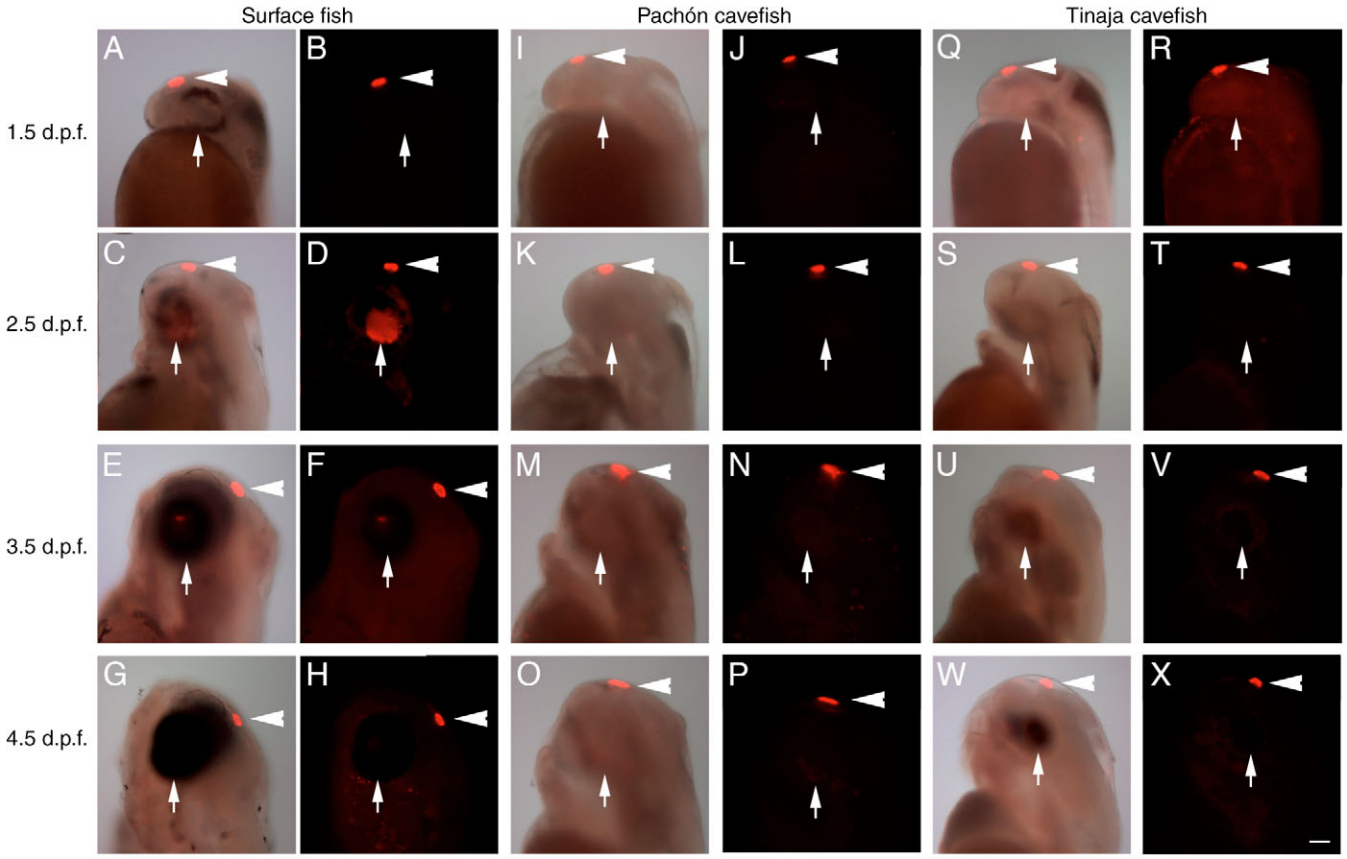

Fig. 3. Rhodopsin-like immunoreactivity in the pineal eye of 1.5 d.p.f., 2.5 d.p.f., 3.5 d.p.f. and 4.5 d.p.f. surface fish (A–H), Pachón cavefish (I–P), and Tinaja cavefish (Q–X) larvae. (A,C,E,G,I,K,M,O,Q,S,U,W) Bright field images with focus on the pineal eye. (B,D,F,H,J,L,N,P,R,T,V,X) Fluorescence images of the same specimens as those shown in bright field. Larvae are shown from the left side with the head at the top. Arrowheads mark pineal eye; arrows mark bilateral eye. Scale bar in X, 100 μm; magnification is the same in each frame.

but was later obscured by melanin deposition in the developing retinal pigment epithelium (Fig. 3E–H). In contrast, despite the absence of melanin pigment, the rhodopsin-like antigen was not detectable in the degenerating bilateral eyes of Pachón or Tinaja cavefish (Fig. 3I–X). This result is consistent with previous studies showing downregulation of opsin expression in the degenerating photoreceptor layer of the cavefish retina (Langecker et al., 1993; Yamamoto and Jeffery, 2000). We conclude that the developing cavefish pineal gland shows a rhodopsin-like antigen, which we then used as a pineal marker.

Role of the pineal eye in the shadow response

Pinealectomy experiments were conducted to determine whether the pineal eye is responsible for the shadow response. In these experiments, pinealectomies and control operations were done in 1.5 d.p.f. Pachón cavefish and surface fish larvae. In some larvae, the pineal was removed, in others the basic operation was conducted to remove the pineal but it was not excised (sham-operated controls), and in others one or both bilateral eyes were deleted instead of the pineal eye. After a recovery period of 3 h, the pinealectomized larvae, sham-operated control larvae, larvae lacking one bilateral eye, and larvae lacking both bilateral eyes (N=106, 103, 99 and 84, respectively) were assayed for the shadow response and video recorded as described above. After the conclusion of the assays, examples of each the four types of surface fish and cavefish larvae were fixed and processed for rhodopsin immunocytochemistry to determine whether they contained a pineal eye.

Fig. 4 shows video recordings of the shadow response in pinealectomized larvae, sham-operated control larvae, larvae lacking a single bilateral eye and larvae lacking both bilateral eyes. The sham-operated surface fish and cavefish larvae showed a shadow response similar to that described above (compare Fig. 4A,B with Fig. 1D,H). Likewise, larvae with one or both bilateral eyes removed also showed a shadow response resembling unoperated larvae and sham-operated controls (compare Fig. 4E–H with Fig. 1D,H). In contrast, the shadow response was abolished in most (but not all) pinealectomized surface fish and cavefish larvae (Fig. 4C,D). Quantification of these results confirmed that the shadow response in pinealectomized larvae was significantly reduced relative to sham-operated control larvae and larvae lacking one or both bilateral eyes (Fig. 5; P<0.01, N=205 surface fish and 187 cavefish).

Rhodopsin staining was used to determine the efficiency of these operations and to investigate why a few pinealectomized surface fish and cavefish larvae still showed a shadow response (Fig. 4C,D). In these experiments, examples of pinealectomized and control fish that had recently been assayed for the shadow response were selected individually, fixed and stained with rhodopsin antibody. All of the sham-operated control larvae (Fig. 6A–D), the larvae lacking a single bilateral eye (Fig. 6I–L) and the larvae lacking both bilateral eyes (Fig. 6M–P) that showed a positive shadow response (Figs 4

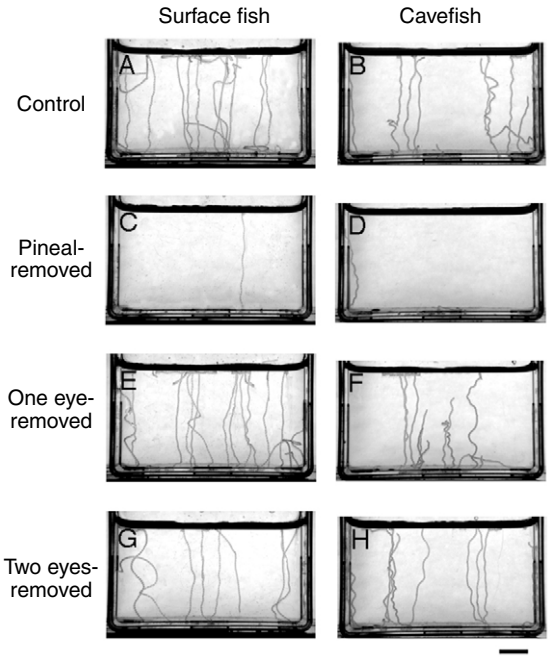

Fig. 4. The shadow response of surface fish (A,C,E,G) and Pachón cavefish (B,D,F,H) after pinealectomy or removal of one or both bilateral eyes. The frames show merged images at 0.05 s intervals from 0 to 5.0 s after the assay chambers were shaded. A normal shadow response is observed in sham-operated surface fish and cavefish controls (A,B) and after removal of one (E,F) or both (G,H) bilateral eyes from surface fish or cavefish larvae. In contrast, the shadow response is absent in most surface fish or cavefish larvae after pinealectomy (C,D). Scale bar, 1 cm. Other details are the same as in Fig. 1.

and 5), exhibited rhodopsin-like antigen in the pineal eye. In contrast, pinealectomized larvae without a shadow response did not have any indication of a pineal gland, as determined by the absence of rhodopsin-like antigen in the dorsal brain (Fig. 6E–H). However, each of the relatively small number of surface fish and cavefish larvae subjected to pinealectomy that did show a shadow response (see Fig. 4C,D) had rhodopsin-like antigen in the dorsal brain (Fig. 6Q–T). Thus, based on the persistence of rhodopsin-like antigen, we concluded that 'pinealectomized' larvae with a shadow response retained all or part of their pineal eyes. Together, the results demonstrate that the *Astyanax* shadow response is dependent on the presence of a pineal eye.

DISCUSSION

The bilateral eyes of cavefish have degenerated and become non-functional during about a million years of relaxed selection in absolute darkness (Avise and Selander, 1972; Chakraborty and Nei, 1974; Mitchell et al., 1977). In the present study, we have investigated whether the pineal gland, another sensory organ involved in light detection, is functional in cavefish. Using the *Astyanax mexicanus* system, which consists of conspecific eyed surface fish and blind cavefish (Şadoğlu, 1957; Wilkens, 1988; Jeffery, 2001), we show here that the pineal eye of young cavefish larvae can sense light to mediate a shadow response. To our knowledge, this is the first demonstration of a functional pineal eye in a blind cave vertebrate. In addition, our results reveal a classic shadow response in teleost larva, which is similar in many respects to the shadow responses of *Xenopus* (Roberts, 1978) and ascidian (Kajiwara and Yoshida, 1985) tadpoles.

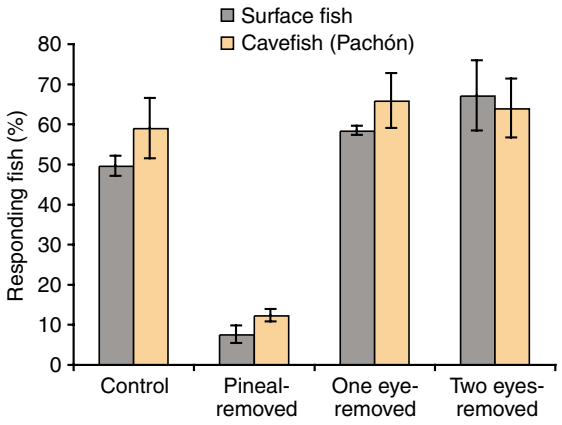

Fig. 5. Quantification of the shadow response after pinealectomy or bilateral eye removal in 1.5 d.p.f. surface fish and cavefish larvae. Values are means ± s.e.m., *N*=392.

Astyanax larvae exhibit a classic shadow response

The shadow response has been studied most extensively in *Xenopus laevis* tadpoles (Roberts, 1978). The present results demonstrate that *Astyanax* has a shadow response that is strikingly similar to that of *Xenopus* (Foster and Roberts, 1982; Jamieson and Roberts, 2000; Roberts, 1978). First and foremost, the shadow response is controlled by the pineal eye in both species. This conclusion is strongly supported by our pinealectomy results demonstrating that *Astyanax* surface fish or cavefish larvae with a complete or partial pineal eye exhibit a shadow response, whereas those lacking the pineal gland do not show a shadow response. Second, the behavioral components of the *Astyanax* shadow response resemble those of *Xenopus*. After shading, surface fish and cavefish larvae swim upward spirally and cease swimming after they reach the water surface, where they often remain attached by their cement organs. Third, the shadow responses of both species show similar kinetics. The *Astyanax* shadow response is elicited immediately after shading and usually completed in less than 10 s in surface fish, although there may be a longer response period in very young cavefish larva. The latter is the only substantial difference that we have noted in the surface fish and cavefish shadow responses. Fourth, the ontogeny of the shadow response is similar in *Astyanax* and *Xenopus*. In both species, the shadow response is strongest soon after hatching and then gradually weakens prior to disappearance during subsequent larval development. The eventual loss of the pineal-based shadow response appears to coincide with the maturation of functional bilateral eyes.

Shadow-like responses have been described in larvae of other teleosts, including flounder, sole and herring (Blaxter, 1968; Burke et al., 1995; Champalbert et al., 1991). They contrast with the effects of shading we have described in *Astyanax* in that they culminate in downward, rather than upward, swimming in the water column. Thus, predator avoidance behaviors may differ among teleosts, although the stimulators and mediators, light shading and pineal sensory activity, respectively, are probably the same.

The tadpoles of two ascidian species, *Ciona intestinalis* and *Ciona savignyi*, also show a shadow response (Inada et al., 2003; Kajiwara and Yoshida, 1985; Kusakabe et al., 2001). At about 3.5 h after hatching, *Ciona* larvae attain the capacity to swim upward after shading. The ascidian shadow response depends on expression of *Ci-opsin1*, the *Ciona opsin* homologue, in the larval ocellus, a potential homologue of the vertebrate pineal eye (Kusakabe et al.,

2001). Therefore, shadow responses evoked by shading the pineal eye or a homologous structure may have emerged early during chordate evolution, before the appearance of bilateral eyes, and are conserved in basal vertebrates, including teleosts.

The shadow response is conserved in cavefish

Because of their unusual environment, it was interesting to determine whether a pineal-based shadow response is conserved in cavefish. Cavefish normally live in absolute darkness. It would be predicted that the shading response, which may be costly to maintain and useless in a dark environment, would tend to be lost under these conditions. Contrary to this expectation, however, morphological studies suggest that cavefish larvae and adults retain a pineal gland with sensory cells containing normal appearing photoreceptor segments (Langecker, 1992), although prior to the present study there was no strong evidence that they are functional in light detection. In striking contrast to the pineal eye, although a few photoreceptor cells differentiate initially in the bilateral eyes of cavefish larvae, they subsequently degenerate and are not replaced in the vestigial eyes of adults (Langecker et al., 1993; Yamamoto and Jeffery, 2000; Strickler et al., 2007).

The present results demonstrate that the larval shadow response is conserved in cavefish. The observations and experiments that support this conclusion are as follows. First, light shading of

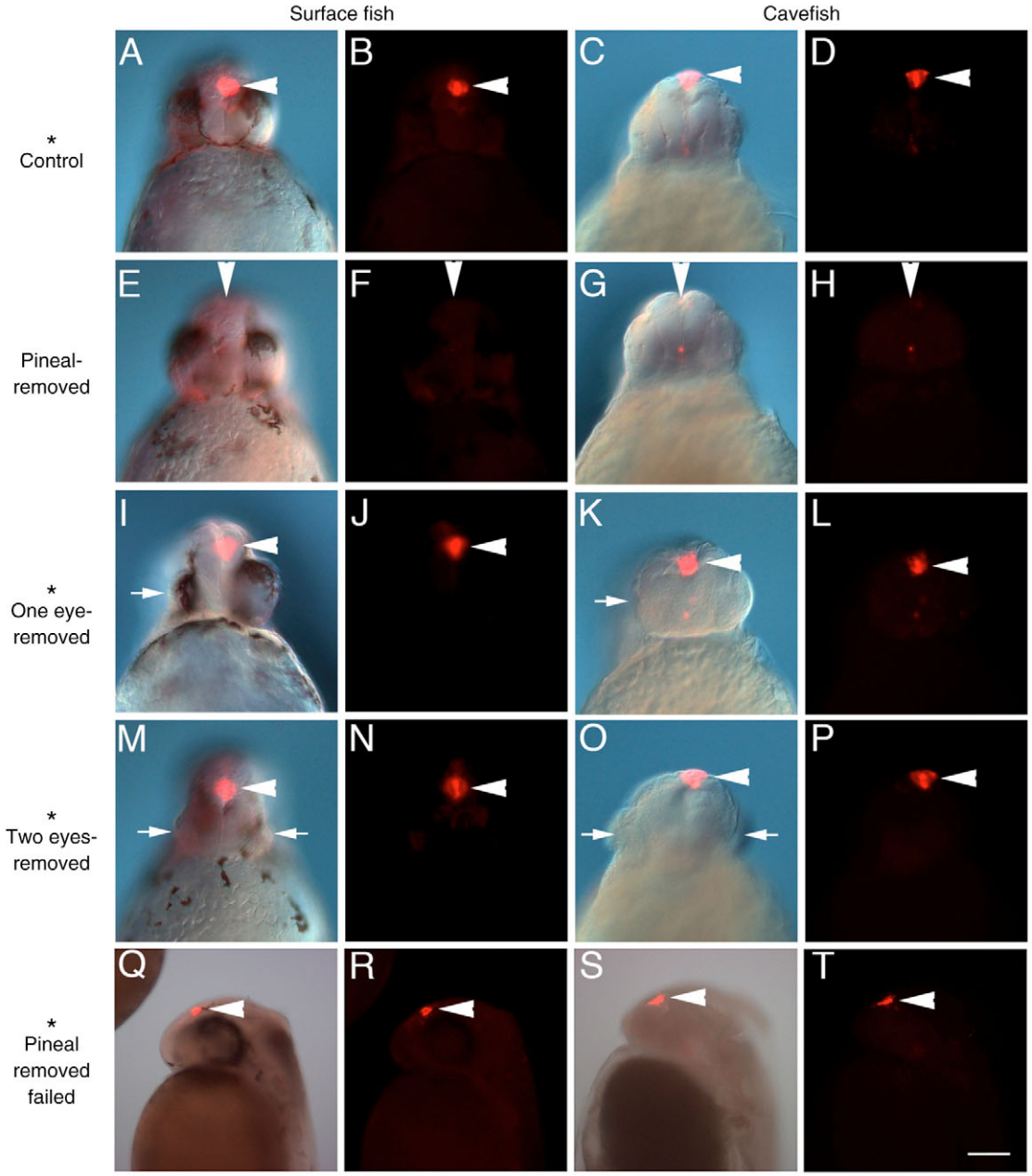

Fig. 6. Rhodopsin-like immunoreactivity in the pineal eye of 1.5 d.p.f. surface fish (A,B,E,F,I,J,M,N,Q,R) and Pachón cavefish (C,D,G,H,K,L,O,P,S,T) after pinealectomy or removal of bilateral eyes. (A–D) Sham operated controls. (E–H) Pinealectomy. (I–L) Removal of one bilateral eye. (M–P) Removal of both bilateral eyes. (Q–T) Failed pinealectomy. Image pairs show DIC image (left) and fluorescence images of the same specimens (right). Larvae are shown from the rostral (A–P) or left (Q–T) side. Arrowheads mark pineal eye or deleted pineal eye; arrows, deleted bilateral eye. Asterisks (far left) indicate fish of each class that exhibited a positive shadow response. Scale bar in T, 100 μm; magnification is the same in each frame.

cavefish and surface fish larvae elicit almost identical upward swimming behaviors. The ontogeny of the shadow response, which is prominent during early development and gradually recedes, is identical in cavefish and surface fish. Finally, pinealectomy shows that the cavefish shadow response is specifically elicited by the pineal eye, which expresses similar levels of opsin as its surface fish counterpart. Therefore, despite the likely cost of developing light detecting ability, the results imply that the pineal gland is still able to sense light in blind cavefish.

The conservation of pineal eye function has important implications regarding developmental and physiological interactions between the pineal and bilateral eyes in basal vertebrates. In these animals, the disappearance of light sensing ability in the pineal eye and the attainment of visual sensitivity in the bilateral eyes are temporarily correlated, suggesting that bilateral eye maturation might suppress pineal eye function. However, we have shown that light detection by the pineal eye decreases with the same kinetics during blind cavefish and sighted surface fish development. Thus, other factors, such as increased opacity in the cranium that may impede light penetration, may responsible for the diminishment of light detection by the pineal eye. Alternatively, minimal development of an organized retina, photoreceptor cells, and a ganglion layer with functional projections to the optic tectum prior to subsequent degeneration (Voneida and Sligar, 1976; Yamamoto and Jeffery, 2000; Soares et al., 2004), may be sufficient to inhibit pineal eye function during development.

Role of the cavefish pineal eye

To elicit a shadow response, at least two features must be retained during cavefish evolution: (1) the photosensitivity of the pineal eye and (2) a neural connection between the pineal eye and the motor system involved in swimming behavior. The development of both features probably requires appreciable investment of metabolic energy. Why then is the light sensing function of the pineal eye conserved in blind cavefish? Although we cannot answer this question with certainty, two possibilities are offered below.

The pineal gland consists of two parts, one with a role in light detection and the other devoted to neurosecretion, including melatonin production. The retina also produces melatonin but it is used and metabolized locally, whereas melatonin from the pineal gland is released into the blood and has a paracrine function (Falcón, 1999). Melatonin regulates daily variations in locomotor activity, sleeping, skin pigmentation (which is absent in cavefish), and seasonal growth and reproduction (Zachmann et al., 1992a). Among these periodic activities, seasonal growth and reproduction are particularly important in the cave environment, where influxes of new food resources may occur only once a year during seasonal flooding (Mitchell et al., 1977). In the absence of light, melatonin secretion by the pineal eye could depend on water temperature, which has been documented in controlling pineal secretion in other teleosts (Falcón et al., 1994; Zachmann et al., 1992b) and would be changed during flooding. Thus, the neurosecretory role of the pineal gland may be necessary for survival in cavefish.

The developmental processes responsible for the formation of a two-part pineal gland may be interrelated. If so, the photosensitive portion of the pineal, although seemingly useless in the cave environment, would be conserved due to developmental constraints. Supporting this idea, opsin genes are still expressed in the mammalian pineal gland (Blackshaw and Snyder, 1997), despite the fact that it lacks photosensitivity. In addition, the transcription factor cone rod homeobox (CRX/Otx), which controls opsin gene expression, may also regulate expression of the melatonin synthesis genes, *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), during pineal development (Asaoka et al., 2002; Furukawa et al., 1999; Li et al., 1998). Therefore, the cascade of regulatory gene expression leading to photoreception and melatonin synthesis may be integrated to an extent that it cannot be easily uncoupled during relatively short evolutionary intervals.

Finally, light detection by the larval pineal gland may be conserved because it is beneficial for survival in the cave environment. The cave systems inhabited by *Astyanax* cavefish can contain karst ‘windows’, areas of ceiling collapse that allow dim light penetration, and are subject to periodic episodes of extensive flooding (Mitchell et al., 1977). Floods could propel cavefish from the light-less cave interior to semi-lighted locations, such as near cave entrances or spring resurgences. Both scenarios could expose cavefish larvae to predation in lighted habitats. Conservation of the pineal eye could be used to avoid the potential threat of exposure to light and predation.

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