Reduced opsin gene expression in a cave-dwelling fish

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Regressive evolution of structures associated with vision in cave-dwelling organisms is the focus of intense research. Most work has focused on differences between extreme visual phenotypes: sighted, surface animals and their completely blind, cave-dwelling counterparts. We suggest that troglodytic systems, comprising multiple populations that vary along a gradient of visual function, may prove critical in understanding the mechanisms underlying initial regression in visual pathways. Gene expression assays of natural and laboratory-reared populations of the Atlantic molly (*Poecilia mexicana*) revealed reduced opsin expression in cave-dwelling populations compared with surface-dwelling conspecifics. Our results suggest that the reduction in opsin expression in cave-dwelling populations is not phenotypically plastic but reflects a hardwired system not rescued by exposure to light during retinal ontogeny. Changes in opsin gene expression may consequently represent a first evolutionary step in the regression of eyes in cave organisms.

Keywords: regressive evolution; vision; qPCR;
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1. INTRODUCTION

Regressive evolution—the loss or simplification of derived traits—is a widespread phenomenon, the understanding of which provides important insights into the consequences of natural selection and genetic drift (Porter & Crandall 2003; Wilkens 2007). Most of the research on regressive evolution has focused on embryonic eye and pigment development in the blind Mexican cave tetra, *Astyanax mexicanus* (Jeffery 2005). While this system provides a powerful model for elucidating the developmental mechanisms associated with regressive eye evolution (Jeffery 2005), we suggest that elucidating the initial mechanisms underlying regressive evolution may be facilitated by studying systems in which eye regression is less advanced and eyes are still functional.

This is the case in the Cueva del Azufre system in southern Mexico, where a small livebearing fish, the Atlantic molly (*Poecilia mexicana*), has colonized at

least two caves (Tobler *et al.* 2008a). Cave-dwelling *P. mexicana* occur parapatrically with their epigean ancestors, but despite spatial proximity and the lack of major physical barriers, gene flow between surface and cave populations as well as between different cave chambers is extremely low (Tobler *et al.* 2008a). Cave populations are morphologically distinct and exhibit reductions in pigmentation and eye size (Parzefall 2001). Unlike in other cave organisms (Langecker 2000), the eyes of cave-dwelling *P. mexicana* are fully functional, and fish readily respond to visual stimuli when given an opportunity (Tobler *et al.* 2008b). In this study, we compare the expression of opsin genes, which are responsible for visual sensitivity (Yokoyama 2000), in surface-dwelling *P. mexicana* to cave-dwelling conspecifics from two photically distinct cave environments: front cave chambers where natural skylights provide dim light, and deep cave chambers with perpetual darkness. We also compare photoreceptor cell densities between the two cave populations.

Theory predicts that natural selection favours the evolution of sensory systems that are locally adapted to ecological conditions (Endler & Basolo 1998). Locally adapted visual systems can increase an individual's sensitivity to available wavelengths of light in two ways: (i) changes in the amino-acid sequence of opsin proteins as a result of mutations in the opsin genes can 'tune' spectral sensitivities of photoreceptors to the local photic environment (Yokoyama 2000) and (ii) opsin genes may be differentially expressed to maximize an individual's sensitivity to particular wavelengths of light (Fuller *et al.* 2004; Parry *et al.* 2005). Functional changes in amino-acid sequences are absent in Atlantic mollies from different habitats, as cave and surface-dwelling fish have identical spectral sensitivities of photoreceptors (Körner *et al.* 2006). Hence, we focus on the latter mechanism in three opsin genes: short-wavelength-sensitive (*SWS1*) opsin coding for an ultraviolet-sensitive opsin protein, medium-wavelength-sensitive (*Rh2*) opsin coding for a green-sensitive opsin protein, and long-wavelength-sensitive (*LWS*) opsin coding for a red-sensitive opsin protein (Yokoyama 2000). Upregulating particular opsin genes—and hence increasing translation of corresponding opsin proteins in the retina—may increase an individual's sensitivity to particular wavelengths of light. Likewise, downregulation of an opsin gene may decrease sensitivity to certain wavelengths. Since individuals that occupy perpetually dark cave habitats have no use for vision, we predicted that opsin gene expression would be reduced in these populations relative to populations exposed to natural light because of either the fixation of now neutral mutations by drift, or natural selection on opsin gene expression (Jeffery 2005).

2. MATERIAL AND METHODS

All fish were captured near the village of Tapijulapa (Tabasco, Mexico). Cavefish were collected in the chambers V (in dim light) and X (in perpetual darkness) of the Cueva del Azufre. Surface fish were collected in a nearby creek (Arroyo Tres). Immediately after capture, individuals were sacrificed, measured (standard length and eye diameter) and eyes were extracted and preserved in RNAlater (Ambion, Inc.). Laboratory stocks of cave and surface fish were established in May 2007 and maintained as randomly outbred populations at the University of Oklahoma. All stocks were

exposed to identical environmental conditions and a natural sunlight cycle. Laboratory-reared fish were sacrificed and eyes were preserved as described above. For further information about field collections and husbandry of laboratory stocks, see electronic supplementary material.

RNA was isolated from retinal tissue using a TRIzol reagent (Invitrogen) isolation. Superscript III First-Strand Synthesis System (Invitrogen) was used to create cDNA. qPCR was conducted using Sybr Green (Invitrogen) chemistry and primers developed from sequences of *Xiphophorus* opsin genes (see electronic supplementary material). We compared relative eye size among populations using ANOVA. ANCOVA was used to investigate population differences in opsin expression patterns. Since the size of an eye potentially could affect the amount of mRNA present, eye size was used as a covariate in all analyses.

We quantified photoreceptor cell density of laboratory fish from cave chamber V and chamber X using immunohistochemistry. Eyes were enucleated from adult fish, fixed in 4 per cent paraformaldehyde in phosphate buffered saline and equilibrated in 30 per cent sucrose prior to cryosectioning. We used the monoclonal antibodies ZPR1 and ZPR3 (Vihtelic *et al.* 1999) to label an unknown epitope on the surface of red/green double cones and rhodopsin, respectively. Slides were counterstained with DAPI (Invitrogen) to label DNA (see electronic supplementary material). We estimated cone density based on photographs of transverse retinal sections near the optic nerve. Densities were compared between populations using a *t*-test. All analyses met the assumption of normality.

3. RESULTS

We found significant differences in eye size in wild-caught ($F_{2,21} = 31.359$, $p < 0.001$) and laboratory-reared fish ($F_{2,15} = 19.10$, $p < 0.001$), with surface fish having larger eyes than cave-dwelling fish (figure 1a). However, eye size did not significantly predict opsin gene expression (ANCOVA: $F_{1,20} \leq 0.173$, $p \geq 0.682$ for all three genes); hence we removed the covariate from the final population comparisons.

Among populations of wild-caught *P. mexicana*, we found significant differences in the expression of *SWS1* ($F_{2,21} = 5.206$, $p = 0.015$), *Rh2* ($F_{2,21} = 8.999$, $p = 0.002$) and *LWS* ($F_{2,21} = 4.608$, $p = 0.022$) (figure 1b). As predicted, the highest expression levels were observed in the surface-dwelling population, with similarly high levels of *SWS1* and *Rh2* expression in the population living in the front cave chamber. *LWS* expression, however, was significantly lower in this population. In fish from the deep cave chamber, the expression of all opsin genes was reduced (figure 1b). Comparisons of gene expression patterns among laboratory-reared fish revealed a similar pattern as in wild-caught fish (*SWS1*: $F_{2,15} = 3.93$, $p = 0.042$; *Rh2*: $F_{2,15} = 4.61$, $p = 0.028$; *LWS*: $F_{2,15} = 6.85$, $p = 0.008$). Laboratory-reared fish from surface-dwelling and front-cave populations had significantly higher expression of all three opsin genes than did laboratory-reared fish derived from the deep cave population (figure 1c).

Laboratory-reared fish from chambers V and X did not differ in the densities of cones in the retina ($t_9 = 0.257$, $p = 0.803$, figure 2). Furthermore, qualitative inspection of rod densities revealed no differences between the two populations (electronic supplementary material).

4. DISCUSSION

The regressive evolution of structures associated with vision in cave-dwelling organisms is the focus of much controversy because of the difficulties inherent in falsifying the null hypothesis of evolution by genetic
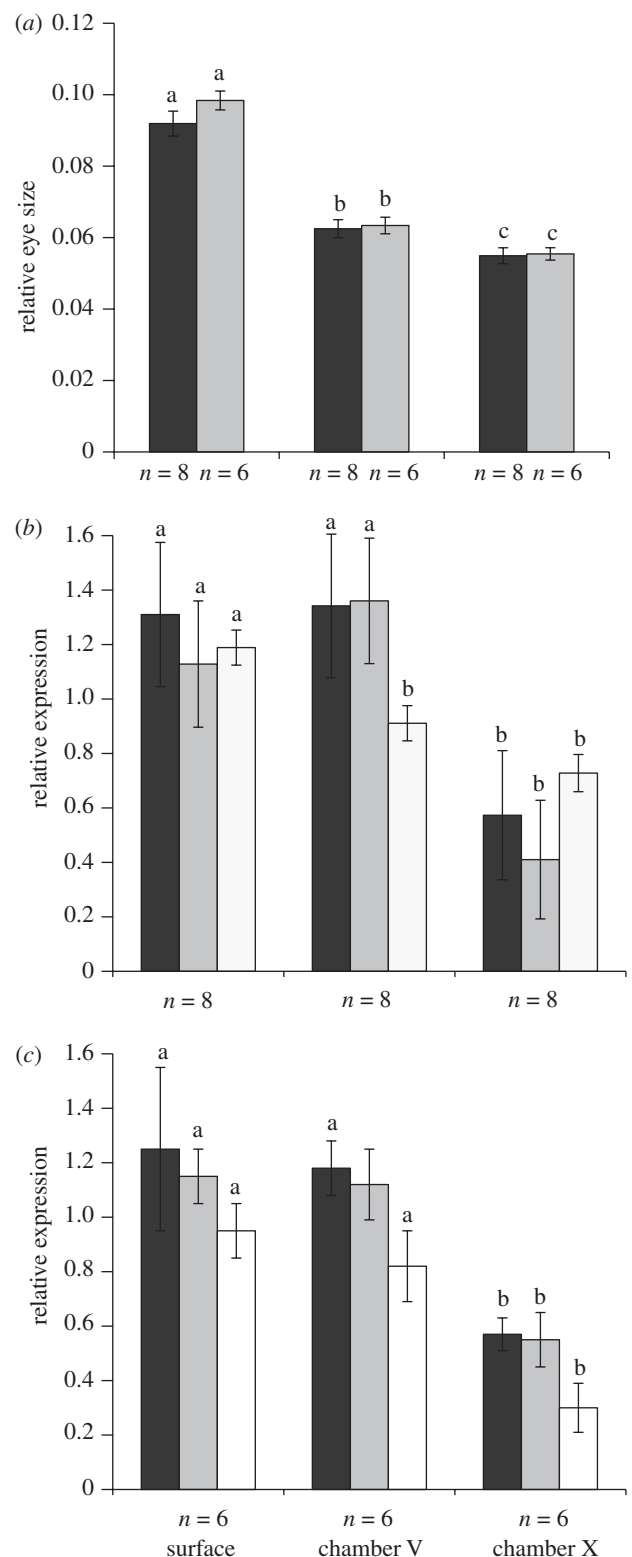
Figure 1. (a) Relative eye size (eye diameter/standard length) for wild-caught (black bars) and laboratory-reared (light grey bars) fish. (b,c) Relative expression levels of *SWS1* (black bars), *Rh2* (light grey bars) and *LWS* (white bars) for (b) wild caught and (c) laboratory-reared populations. Within groups ((a) wild-caught versus laboratory-reared) and (b,c) genes, bars with different letters above are significantly different ($p < 0.05$; Fisher's LSD post hoc comparisons based on ANOVA).

drift (Romero & Green 2005; Wilkens 2007). Most research has focused on extreme differences between sighted surface animals and their completely blind counterparts, and multiple genes are involved in the
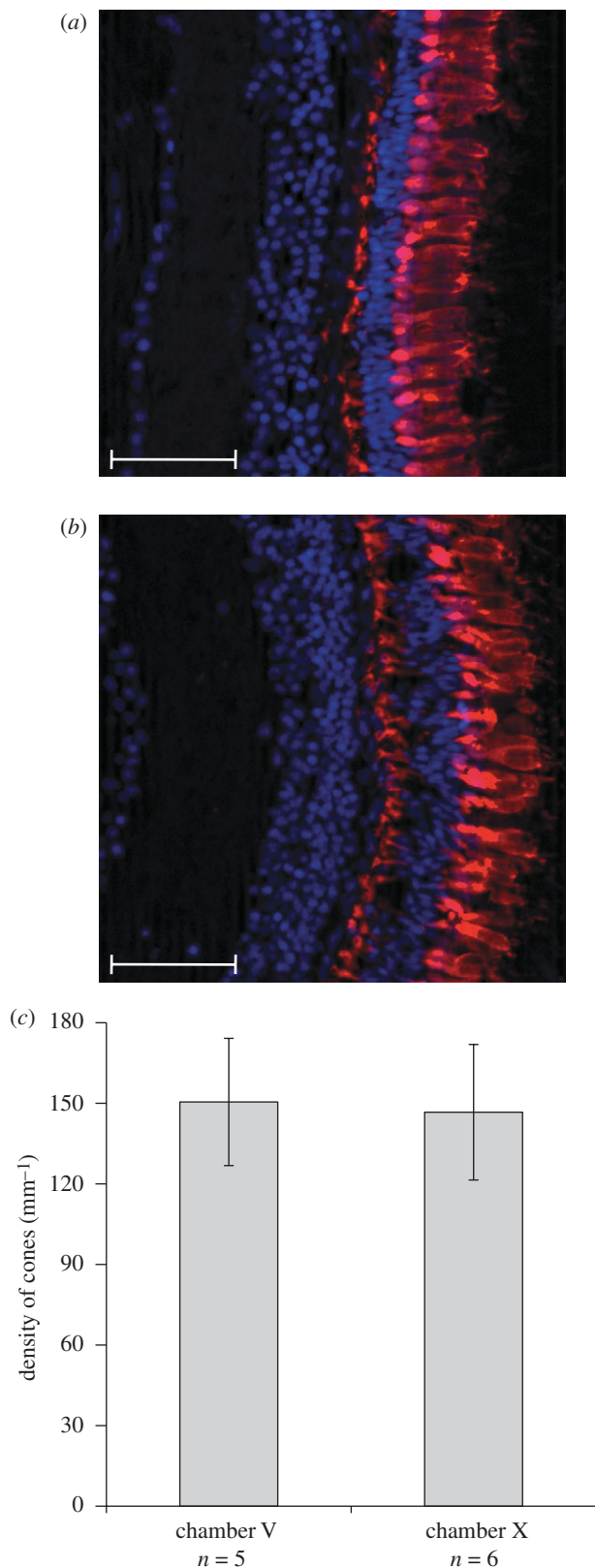
Figure 2. (*a*,*b*) Representative labelling of red/green double cones (red dye) in *P. mexicana* from the cave, (*a*) chamber V and (*b*) chamber X. The blue-dye-labelled cell nuclei. (*c*) Mean ($\pm$s.d.) red/green double cone density in fish from the two cave chambers. Scale bar, (*a*,*b*) 40 μm.

complex developmental pathways leading to eye regression (Jeffery 2005; Protas *et al.* 2007). Troglodytic organisms, which retain some visual function, may prove critical in understanding the mechanisms underlying the initial regression of visual pathways. We suggest that cave-dwelling Atlantic mollies are one such system. In this study, we found an overall reduction in opsin gene expression in mollies from perpetually dark cave chambers, which could not be explained by differences in eye size. Also, while surface and front-cave mollies have similarly high levels of *SWS1* and *Rh2* expression, front-cave mollies showed reduced expression of *LWS* opsin. The reduction in *LWS* opsin expression in individuals from the front cave chamber might be owing to the rapid attenuation of long-wavelength light characteristic of low-light conditions (Bradbury & Vehrencamp 1998).

To test whether opsin gene expression is a phenotypically plastic response to local photic environment (Fuller *et al.* 2004), we quantified opsin gene expression in fish derived from these same three natural populations that were born and reared in the laboratory under identical lighting conditions. We found similar opsin gene expression patterns in laboratory-reared fish as in wild-caught fish, suggesting that the reduction in opsin expression in wild-caught cave-dwelling mollies is not entirely phenotypically plastic. Instead, these results suggest heritable differentiation among populations. Changes in opsin gene expression may consequently represent a first evolutionary step in the regression of vision in cave organisms.

Retinal immunohistochemistry indicated that the decrease in opsin gene expression does not merely reflect a decrease in photoreceptor number, as the two cave populations—albeit different in gene expression—do not differ in cone and rod densities. Consequently, the structure of the retina appears to remain unchanged while the level of opsin gene transcription per photoreceptor has decreased.

Whether the heritable reductions of eye size (Plath *et al.* 2007) and opsin gene expression (this study) in cave mollies are driven by natural selection or genetic drift is as yet unclear. In other systems, however, there is mounting evidence that the regression of visual systems is adaptive and caused by antagonistic pleiotropic effects, where traits beneficial to survival in darkness are enhanced at the expense of others (Jeffery 2005; Protas *et al.* 2007). Specifically, the development of eyes has recently been shown to be dependent on the same regulatory genes as the development of jaws and taste buds; i.e. selection on the latter two structures may directly affect eye development (Yamamoto *et al.* 2009). Incidentally, cave mollies—like *Astyanax* cavefish—have also diverged in taste bud density (Parzefall 2001) and jaw morphology (Tobler 2008). This raises the question as to whether common developmental trade-offs underlie the convergent evolution of troglodytic phenotypes.

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