In the four-eyed fish (*Anableps anableps*), the regions of the retina exposed to aquatic and aerial light do not express the same set of opsin genes

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The four-eyed fish, ***Anableps anableps***, has eyes with unusual morphological adaptations for simultaneous vision above and below water. The retina, for example, is divided such that one region receives light from the aerial field and the other from the aquatic field. To understand better the adaptive value of this partitioned retina, we characterized photoreceptor distribution using *in situ* hybridization. Cones expressing *sws1*, *sws2b* and *rh2-2* (i.e. UV, and short wavelength-sensitive) opsins were found throughout the retina, whereas cones expressing *rh2-1* (middle wavelength-sensitive) were largely limited to the ventral retina and those expressing *lws* (long wavelength-sensitive) opsins were only expressed in the dorsal retina. We next asked when this pattern evolved relative to the 'four-eyed' morphology. We characterized opsin expression in *Jenynsia onca*, a member of the sister genus to *Anableps* with typical teleost eye morphology. In *J. onca*, *sws1*, *sws2b*, *rh2-2* and *rh2-1* opsins were expressed throughout the retina; while *lws* opsins were not expressed in the ventral retina. Thus, the change that coincides with the evolution of unusual anablepid eye morphology is the loss of *rh2-1* expression in the dorsal retina, probably to accommodate increased *lws* opsin expression. The retinal area that samples aerial light appears not to have changed with respect to photoreceptor transcription.

Keywords: fish; visual pigment; wavelength sensitivity; intraretinal variability; spectral tuning; retinal topography

1. INTRODUCTION

The four-eyed fish, genus *Anableps*, presents a unique opportunity for studying how visual pigments play a role in adaptation to heterogeneous spectral environments and diverse visual tasks. *Anableps anableps*, typical of its genus, has large eyes extending above its head, allowing it to see above the waterline while keeping the rest of its body, and half of each eye, underwater. A pigmented band across the midline of the cornea creates distinct dorsal and ventral pupils, while an ovoid lens has evolved from the typical spherical lens of the fish to optimize simultaneous focusing of aerial and aquatic light [1]. The dorsal region of the retina receives upwelling light filtered through water and dissolved solutes that alter the spectral content. The ventral retina receives aerial light unfiltered by water. Thus, the eyes receive both broad-spectrum light and dimmer, narrow-spectrum light, but in separate regions of the retina (figure 1). This represents an exaggerated example of the spectral heterogeneity common to surface-dwelling fish, as upwelling light is filtered to a greater degree by water than downwelling light in near-surface aquatic environments. Previous work in fish has shown differences in sensitivity or opsin expression between regions of the retina, but the functional significance of intraretinal variability in fishes remains unclear ([2,3] but see [4]).

Vertebrate vision is dependent upon five families of G-protein-coupled receptors collectively called visual opsins that are expressed in retinal rods and cones. The common ancestor of all vertebrates possessed two short wavelength-sensitive (*sws1* and *sws2*), one middle wavelength-sensitive (*rh2*) and one long wavelength-sensitive (*lws*) cone opsin subfamilies [5]. Owing to the readily measurable connection between genotype (opsin gene sequence) and phenotype (wavelength of maximum sensitivity), much work has been done to correlate opsin gene repertoire, sequence and expression patterns with ecological factors [6]. One of the more consistent correlates identified is the spectral composition of ambient light [7,8]. However, so far, these associations have not been observed in the same species and at the same time in development. With the four-eyed fish, we can examine opsin expression in regions of the retina exposed to different spectral environments in the same individual at the same time.

We used *in situ* hybridization with opsin riboprobes to map cone opsin expression in the retina of *A. anableps*. This was repeated for *Jenynsia onca*, a species with normal eye morphology in the sister genus to *Anableps*. Recently, both species have had their cone opsin repertoires characterized, revealing nine genes in *A. anableps*, (one *sws1*, two *sws2*, two *rh2* and four *lws* genes), and eight in *J. onca* (one less *lws* gene) [9,10]. By studying *J. onca*, we have inferred ancestral expression patterns and identified changes that have evolved in concert with the unique eye morphology of *A. anableps*.

2. MATERIAL AND METHODS

The *in situ* hybridization procedure was adapted from a previously described protocol ([11,12] and see methods in the electronic supplementary material). Briefly, unique digoxigenin-labelled riboprobes, 352–792 bp in length, were designed from the *A. anableps* genes: *sws1*, *sws2a*, *sws2b*, *rh2-1* and *rh2-2*. Both *A. anableps* and *J. onca* have recent duplications in the *lws* subfamily. Thus, owing to the high degree of sequence similarity among the *lws* paralogues, unique riboprobes could not be produced. Rather, in this study we used one riboprobe designed to bind all *lws* paralogues. Both eyes from *A. anableps* (two adults and two juveniles) and *J. onca* (three
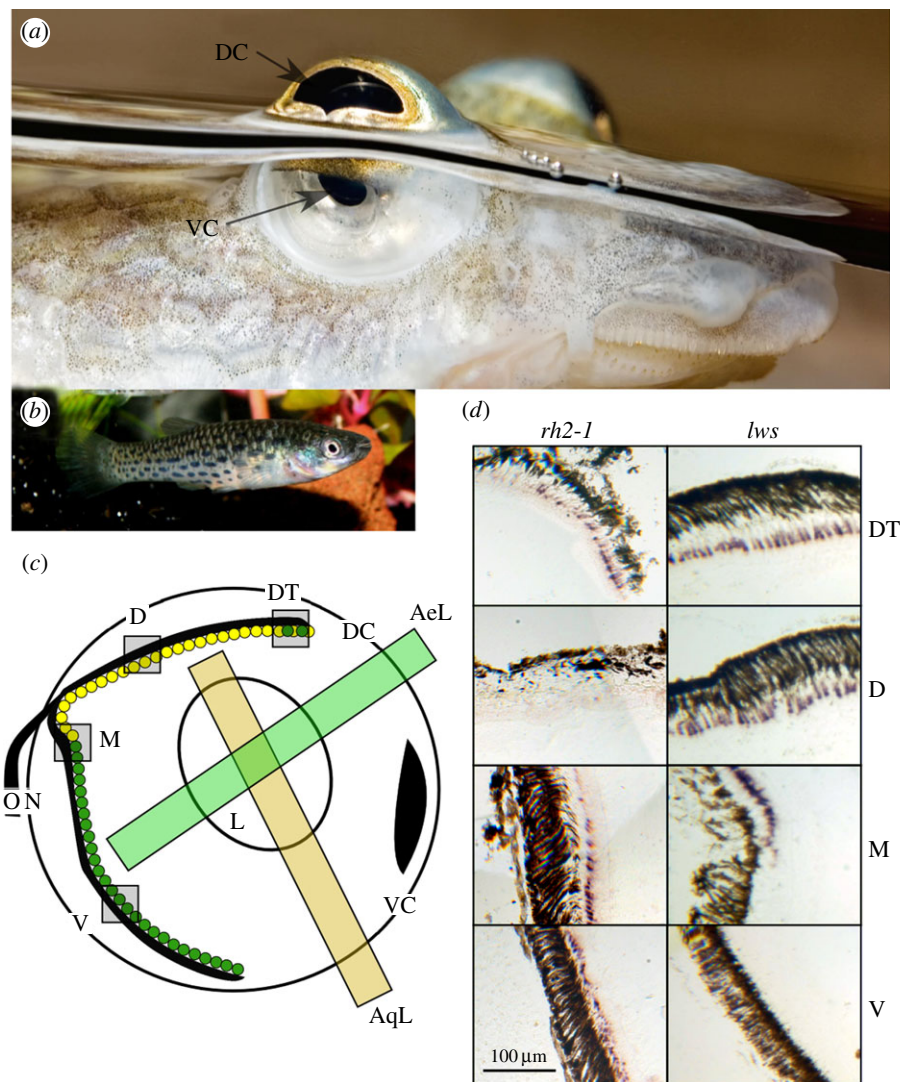
Figure 1. Comparison of anablepid eye morphology. (*a*) *A. anableps* eye at the water surface. The dorsal cornea receives light from the aerial environment, while the ventral cornea receives light from the aquatic environment. DC, dorsal cornea; VC, ventral cornea. Photo by Andreas Werth. (*b*) *Jenynsia onca* female. The eye is morphologically normal in this species. Photo by Leo van der Meer. (*c*) A schematic of a sagittal section of an *A. anableps* eye. Light paths are indicated. Green and yellow dots indicate *rh2-1* and *lws* gene expression, respectively. AeL, aerial line-of-sight; AqL, aquatic line-of-sight; L, lens; ON, optic nerve; DT, dorsal tip of the retina; D, dorsal retina; M, medial retina; V, ventral retina. (*d*) *In situ* hybridization images of adult *A. anableps* labelled with the *rh2-1* or *lws* riboprobe. Cone cells expressing the gene of interest are purple. The brown area is retinal pigment epithelium.

adults) were prepared and sectioned in the sagittal or transverse plane. Riboprobes were used individually on serial sections and overall expression patterns were inferred by comparing sections of both orientations and making use of the optic nerve as a landmark. Additional experiments on a third adult *A. anableps* were performed to confirm results for *lws* and *rh2-1* riboprobes. Riboprobe specificity was confirmed with a riboprobe-RNA dot blot assay (data not shown).

3. RESULTS

In situ hybridization was used to characterize the topography of cone photoreceptor subtypes. *Anableps anableps* and *J. onca* exhibited uniform distributions of cones expressing *sws1*, *sws2b* and *rh2-2* (figure 2 and figures in the electronic supplementary material). Neither species exhibited detectable *sws2a* expression in the retina. Species-specific differences were noted for the *rh2-1*-expressing cones. In *J. onca*, *rh2-1* had uniform expression across the retina in all sections, whereas adult *A. anableps* had *rh2-1* in a large

number of ventral cone cells and in a small patch of cones at the dorsal tip of the retina. This pattern was observed in all adults. In juveniles, *rh2-1* expression was confined to a smaller number of cells in the ventral half of the eye, or was entirely absent. The *lws* riboprobe, which was designed to bind all *lws* paralogues, revealed inter-individual and interspecific differences in *lws* cone photoreceptor distributions. In *J. onca*, *lws*-expressing cones were limited to the dorsal retina in two fish (a male and a female) and to a transverse streak in the middle of the eye in one male. In *A. anableps*, *lws* cones were detected only in the dorsal half of the retina. This pattern appears to be the inverse of the *rh2-1* cone distribution (figure 1).

4. DISCUSSION

Anableps anableps' remarkable eyes simultaneously sample photons from terrestrial and aquatic habitats.
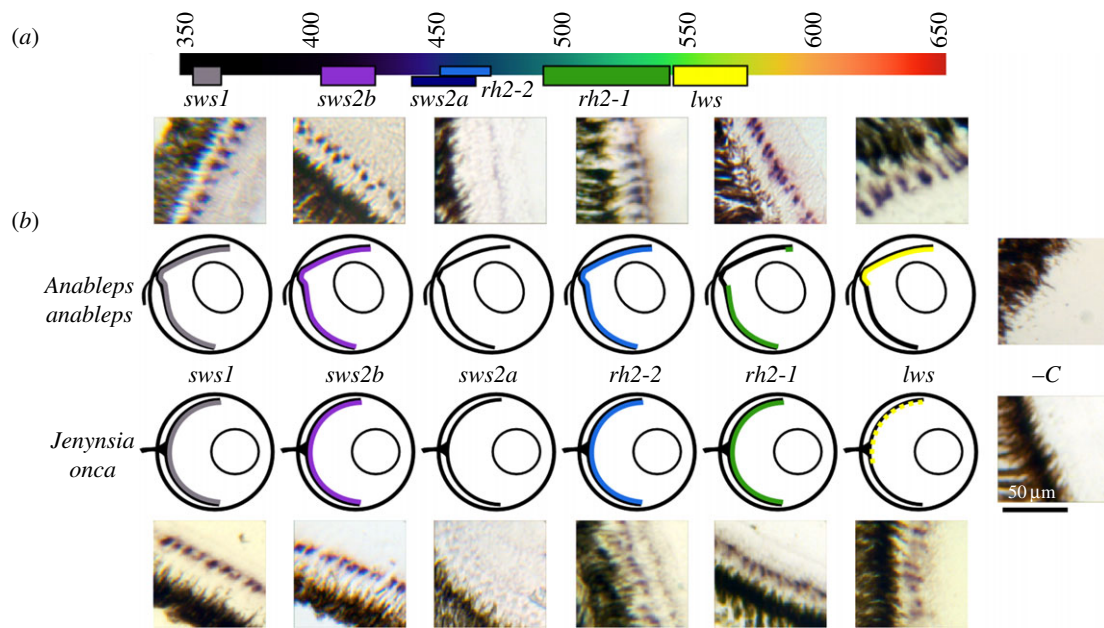
Figure 2. Summary of expression domains for each gene tested in *A. anableps* and *J. onca*. (*a*) The visual spectrum as seen by humans with putative spectral sensitivity for each opsin tested. Numbers are in nanometres. (*b*) Schematic of eyes with dorsal on top, ventral on bottom. Coloured areas indicated expression, while dotted bars indicated areas of polymorphic expression. Cropped microscope images are retinal sections probed using each riboprobe. Cone cells expressing the gene of interest are purple.

In situ hybridization with six riboprobes demonstrated that *A. anableps* and the closely related *J. onca* express at least five cone opsins (*sws1*, *sws2b*, *rh2-1*, *rh2-2* and *lws*), possibly more given the redundancy of our *lws* probe.

The wavelength of maximal sensitivity for a visual pigment (and consequently the photoreceptor expressing it) can be determined by microspectrophotometry (MSP) or *in vitro* protein reconstitution [13,14]. By comparing maximal sensitivity data from *A. anableps* and its relatives obtained using these techniques, we have endeavoured to assign specific opsin genes to cone cell spectral sensitivities: *sws1* (356–365 nm, UV), *sws2b* (405–425 nm, violet), *rh2-2* (452–472 nm, blue), *rh2-1* (492–539 nm, green) and *lws* (543–576 nm, yellow) (figure 2a and discussed in supplementary materials). While these represent the value for individual opsin proteins, multiple proteins may be found co-expressed in a single cone, causing intermediate sensitivity values for the photoreceptor.

Early MSP work showed no difference in the prevalence of different cone cells between retinal halves [15]. However, our *in situ* hybridization experiments, which are better suited to assess photoreceptor distributions because they sampled more photoreceptors by several orders of magnitude, did detect differences: the dorsal retina, used for aquatic vision, has cone photoreceptors that express *sws1*, *sws2b*, *rh2-2* and *lws*, while the ventral retina, used for aerial vision, expresses *sws1*, *sws2b*, *rh2-2* and *rh2-1* (figure 2). From these observations, we predict that wavelength sensitivity differs in the dorsal and ventral regions of the retina.

The pattern in *A. anabelps* of *lws*-positive cones only in the dorsal region of the retina and *rh2-1*-positive cones only in the ventral retina appears to have evolved at the same time as the unusual eye morphology. *rh2-1*

is expressed throughout the eye in *J. onca* and in another close relative, the guppy (*Poecilia reticulata*; D. J. Rennison 2011, personal communication). *rh2* and *lws* genes are typically expressed in double cones, a two-cell complex characterized by a joint inner membrane and neighbouring outer segments. Thus, the reduction in *rh2-1* transcripts in the dorsal region of the eye in *A. anableps* may be a trade-off that permits an increase in *lws* expression. Previous work has shown increases in long wavelength sensitivity in the dorsal retina through differences in chromophore use in frogs [16]. Here, we show evidence for a convergent adaptation mediated by changes in gene expression.

The reduction of *rh2-1* expression and retention of *lws* expression in the dorsal retina suggest that *A. anableps* has enhanced sensitivity to long wavelength light (543–576 nm) in the aquatic field of view. This enhanced sensitivity could be advantageous in the brackish waters of the mangrove forests and river deltas that *A. anableps* inhabits, as these often contain dissolved organic matter that shifts light abundance to longer wavelengths [17]. Light measurements taken in similar mangrove habitat showed that downwelling light is most prevalent at approximately 500 nm, while upwelling light peaks at approximately 580 nm [4]. We propose that, by the differential expression of *rh2-1* and *lws*, *A. anableps* is better able to match its double cones to the background light in each field of view.

Archerfish (*Toxotes chatareus*) experience a similar light environment to *A. anableps* and have had their vision studied using MSP [4]. They were found to have double cones in the dorsal retina that were most sensitive to the most prevalent wavelengths of upwelling light. However, unlike *A. anableps*, double cones in the ventro-nasal retina in archerfish were shifted to even longer wavelengths, suggesting that the ventral exclusion of *lws* opsin expression seen in three

surface-dwelling, cyprinodontiformes fish has not occurred. This implies that other features beyond light environment, such as phylogeny or the visual tasks required, may affect opsin expression.

While the guppy expresses *rh2-1* throughout the retina, recent *in situ* experiments in this species show many more *lws*-expressing cone cells in the dorsal retina than in the ventral retina, similar to *J. onca* (D. J. Rennison 2011, personal communication). Thus, it appears that the pattern we observe in *A. anableps* (non-overlapping *lws* and *rh2-1* opsin expression domains) represents an exaggeration and the fine-tuning of what may be a common trend in surface-dwelling fish (although see [4]). Interestingly, it appears that the distinct opsin expression domains are driven primarily by the muddy water environment in which *A. anableps* lives. Opsin expression in the ventral retina, which in *A. anableps* is exposed to aerial light, is the same in the near surface-dwelling *J. onca* and *P. reticulata*. Thus, evolution of morphology has allowed for aerial vision, while gene expression has permitted fine-tuning of aquatic vision in this remarkable fish eye. Behavioural tests on wavelength sensitivity and discrimination in the aerial and aquatic fields of view may shed light on how these differences in expression domains influence vision.

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