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Spectral tuning by opsin coexpression in retinal regions that view different parts of the visual field

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Vision frequently mediates critical behaviours, and photoreceptors must respond to the light available to accomplish these tasks. Most photoreceptors are thought to contain a single visual pigment, an opsin protein bound to a chromophore, which together determine spectral sensitivity. Mechanisms of spectral tuning include altering the opsin, changing the chromophore and incorporating pre-receptor filtering. A few exceptions to the use of a single visual pigment have been documented in which a single mature photoreceptor coexpresses opsins that form spectrally distinct visual pigments, and in these exceptions the functional significance of coexpression is unclear. Here we document for the first time photoreceptors coexpressing spectrally distinct opsin genes in a manner that tunes sensitivity to the light environment. Photoreceptors of the cichlid fish, *Metriaclima zebra*, mix different pairs of opsins in retinal regions that view distinct backgrounds. The mixing of visual pigments increases absorbance of the corresponding background, potentially aiding the detection of dark objects. Thus, opsin coexpression may be a novel mechanism of spectral tuning that could be useful for detecting prey, predators and mates. However, our calculations show that coexpression of some opsins can hinder colour discrimination, creating a trade-off between visual functions.

1. Introduction

Photoreceptors of most animals have long been thought to contain a single visual pigment [1]. A growing yet still relatively small number of exceptions have been documented in which a single mature photoreceptor coexpresses opsins forming spectrally distinct visual pigments, though in these exceptions the functional significance of coexpression is generally unclear [2–6]. In many of these cases spectrally divergent opsins are coexpressed, producing very broad photoreceptor absorbance. We report that in *Metriaclima zebra*, a cichlid fish, the coexpression of spectrally similar pairs of opsins results in spectral tuning to the light environment.

The cichlid fishes of Africa rapidly form new species [7], driven in part by diversity in spectral sensitivity and the colour signals that have evolved to be seen by these visual systems [8]. Visual sensitivity varies among cichlid species primarily as a result of differences in the expression, and also the sequences of the seven cone opsin genes that they share [9]. Double cones of African cichlids express four opsin genes, *RH2B*, *RH2Aβ*, *RH2Aα* and *LWS* [9,10]. Opsins are combined with the A_1 chromophore to form visual pigments in *M. zebra* and other Lake Malawi cichlids [9–12]. The wavelengths of maximum absorbance (λ_{max}) of the RH2B, RH2Aβ, RH2Aα and LWS visual pigments are 484, 519, 528 and 567 nm, respectively [9]. Quantitative PCR has shown that most cichlid species express two of these double cone opsins strongly [9,13]. The additional two double cone opsins are expressed at lower levels, but their retinal distributions are unknown. These additional opsins may be expressed individually in rare classes of double cones, or coexpressed with the more abundant opsins. Furthermore, the additional double cone opsins may be unevenly distributed across the retinas of cichlids. A number of animals, including Neotropical cichlids, alter expression of opsins

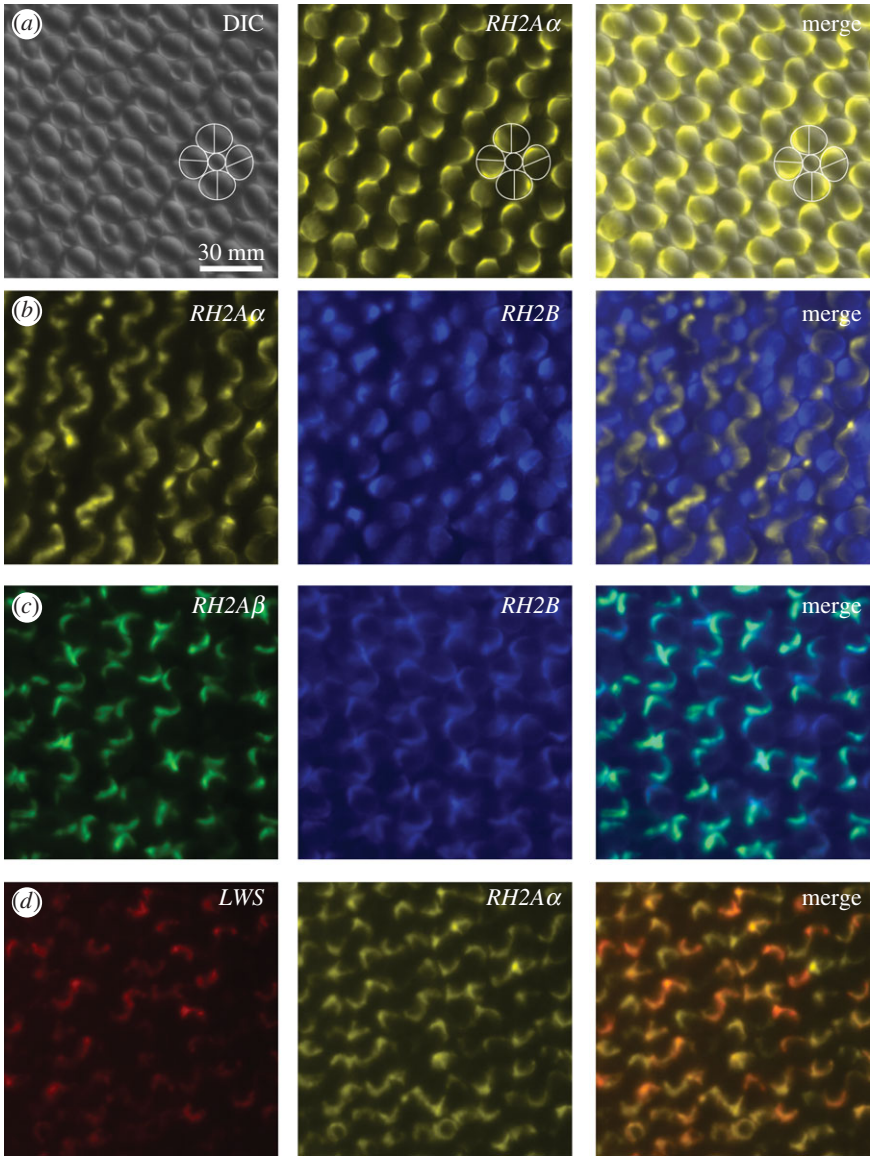

Figure 1. Opsin expression in *Metriaclima zebra* double cones revealed by *in situ* hybridization of whole retinas. (*a*) Differential interference contrast (DIC) image shows cone mosaic where every single cone (white circle) is surrounded by four double cones (white ovals), each composed of two members (separated by white lines). One member of each double cone expresses *RH2Aα*. (*b*) *RH2Aα* and *RH2B* are expressed in opposite members of nearly every double cone throughout the retina. (*c*) In the nasal retina, *RH2B*-containing members frequently coexpressed *RH2Aβ*. (*d*) In the ventral retina, *RH2Aα*-containing members frequently coexpressed *LWS*.

regionally within their retinas, though little is known about the function of this intraretinal variation [14–17].

To determine the intraretinal distribution of opsin coexpression, we performed fluorescent *in situ* hybridization (FISH) on whole retinas from both laboratory-reared and wild-caught *M. zebra* adults. In addition, we used mathematical models to examine the effects of opsin coexpression on two main visual functions: contrast detection and colour discrimination. We find that retinal regions viewing distinct backgrounds contain different pairs of coexpressed opsins. This mixing of visual pigments increases absorbance of the corresponding backgrounds, potentially aiding the detection of dark objects by luminance contrast. However, coexpression of certain opsins sacrifices colour discrimination, creating a trade-off between contrast and colour vision.

2. Material and methods

(a) *In situ* hybridization

All laboratory-reared *M. zebra* were a mixture of F_1 and F_2, descendants of fish captured at Mazinzi Reef in Lake Malawi. Fish were reared under standard fluorescent lighting (12 L : 12 D) and sacrificed between approximately 13.00 and 15.00 according to IACUC procedures. To examine opsin expression in the wild, we isolated and preserved retinas in the field from additional *M. zebra* that we captured at Mazinzi Reef.

We performed FISH [18,19] on whole retinas of adult *M. zebra* using pairs of opsin probes. Probes were designed to the 3′ untranslated region (3′UTR) (*RH2B*, *RH2Aα* and *RH2Aβ*) or the coding sequence (*LWS*). We confirmed probe specificity by dot-blot to sense strand RNA. Probe signals were amplified sequentially with tyramide signal amplification (TSA) labelled with either Alexa Fluor 488 or 594 (Invitrogen). Reversing amplification order between eyes for a given fish produced no noticeable effect. Using transmitted light we identified double cones in sampling areas (figure 1*a*), then quantified transcript distributions. All retinas were also surveyed globally for expression patterns and anomalies. To quantify the distribution of opsins expressed in separate cones, five laboratory-reared and three wild fish were examined for expression of *RH2B* and *RH2Aα*. For every fish, 50 double cones were examined in each of two sampling areas, one in the ventral retina and one in the dorsal retina. To quantify the distribution of opsin coexpression, five laboratory-reared individuals were probed by FISH for

RH2Aβ and *RH2B* and 11 were probed for *LWS* and *RH2Aα*. In each retina, a transect was delineated along the nasotemporal or ventrodorsal axis. The transect was subdivided into five regions of equal length, and in each region 50 double cones were examined for expression of the two genes. We quantitatively compared the pattern of LWS expression between wild and laboratory-reared fish that had significant levels of LWS. These included three wild fish and 9 of 11 laboratory fish. The percentage of double cone members expressing LWS was determined in each region of each fish retina. We used an arcsine transformation of these percentages, and then performed an ANOVA and used Tukey's test to compare treatment effects (laboratory versus wild) with $\alpha = 0.05$ (SAS 9.2, Cary, NC, USA).

(b) Microspectrophotometry

Because transcript abundance does not necessarily equate to protein concentration, we used microspectrophotometry (MSP) to measure spectral absorbance of double cones in five additional laboratory-reared *M. zebra*. Double cone absorbance was measured with MSP [20]. Cone λ_{max} and opsin mixture ratio were determined by fitting data to visual pigment templates [21] for single pigments (in which λ_{max} was free to vary) and for mixtures of two pigments (in which λ_{max} was specified for each pigment in the mixture and the percentage mixture varied) according to least squares. Absorbance of *M. zebra* LWS has not been measured, so we used the λ_{max} of *Dimidiochromis compressiceps* LWS (567 nm [10], amino acid identity = 99.7%). After measuring absorbance, we confirmed the presence of both pigments in putative RH2B/RH2Aβ-coexpressing cells by exposing them to 565-nm monochromatic light that would preferentially bleach the RH2Aβ pigment. At this wavelength, absorbance of RH2Aβ and RH2B was at 52 and 8% of their respective maxima.

(c) Contrast detection

To determine whether regional opsin coexpression spectrally tunes the *M. zebra* retina to its viewing backgrounds, we measured the angular distribution of radiance at Mazinzi Reef, the source of both our laboratory *M. zebra* population and of the wild fish we collected for FISH. Radiance was measured [22] toward open water (at depths of 3 m and 7 m between 11.00 and 12.30, respectively; azimuth approximately 270°) at altitudes spanning 0° (directly overhead) to 180°, in 15° intervals (probe full angle of acceptance = 7°). To compute background light absorption, we calculated absorptance of pure and mixed visual pigments from normalized absorbance (measured by MSP) [23], measured double cone outer segment length (26 µm) and specific absorbance at the peak (assumed 0.015 per micrometre [20,24]). Detection of a black (zero reflectance) object was modelled [25] for the horizontal view using Lake Malawi light attenuation [26]. For the overhead view, we estimated intervening light entering the visual path by subtracting the beam-attenuated spectrum from the diffuse-attenuated spectrum at a given depth [27]. We computed the diffuse attenuation coefficient from radiance previously measured in the lake at Zimbawe rock, at 0° just below the surface and at 7 m [28]. Modelling for both views used *M. zebra* lens transmittance [29], assumed contrast threshold = 0.02 [30], and encompassed the spectral range of *M. zebra* sensitivity, 350–700 nm [9,29]. Additional details are provided in the electronic supplementary material.

(d) Colour discrimination

To evaluate the effects of opsin coexpression upon colour vision, we used trichromatic colour space models assuming a depth of 3 m and following the methods of Dalton *et al.* [22] (also see the electronic supplementary material). These models incorporate measurements of nuptial colour reflectance [22], lens transmittance [29] and absorbance of visual pigments [9] previously obtained for *M. zebra*.

3. Results

(a) Do double cones coexpress multiple opsins?

The *M. zebra* retina forms a regular mosaic in which four pairs of double cones surround each single cone (figure 1*a*). Nearly all double cones expressed *RH2B* in one member and *RH2Aα* in the other (99.6 ± 0.9% of dorsal double cones, 97.2 ± 1.1% of ventral double cones, $n = 50$ double cones per region in each of five individuals; figure 1*b*). *RH2Aβ* and *LWS* were also expressed, but to a lesser degree, by all laboratory-reared *M. zebra* that we probed for these genes. These genes were expressed in double cones such that *RH2Aβ* was coexpressed with *RH2B* and *LWS* was coexpressed with *RH2Aα* (figure 1*c*,*d*).

Microspectrophotometry confirmed the coexpression of *RH2B* with *RH2Aβ* and *RH2Aα* with *LWS* (electronic supplementary material, table S1). All fish had some double cones where the λ_{max} of one member was determined to be between those of the visual pigments containing RH2B and RH2Aβ, indicating a mixture of these opsins ($\lambda_{max} = 496.7 \pm 7.7$ nm, 49% RH2Aβ, $n = 29$ cells; figure 2*a*). After measuring absorbance, we confirmed the presence of both pigments in 15 of these cells by preferentially bleaching the putative RH2Aβ pigment by exposure to 565-nm monochromatic light. The post-bleach λ_{max} of these cells consistently shifted toward the RH2B λ_{max} (figure 2*b*), indicating the presence of more than one pigment. By comparison, the 565-nm light did not alter the λ_{max} of a cell whose absorbance was consistent with pure RH2B (figure 2*c*,*d*). Therefore, these experiments confirm that *RH2Aβ* is coexpressed with *RH2B* and that both opsins form visual pigments in the same photoreceptor. We also found a class of double cone members whose absorbance ($\lambda_{max} = 536.3 \pm 0.6$ nm, $n = 3$ cells, two individuals) indicated a 7 : 3 mixture of RH2Aα and LWS. One of these double cones had an RH2B/RH2Aβ mixture in the opposite member.

The rest of the MSP data remained consistent with our FISH results. Although we focused on measuring opsin-coexpressing cells, we measured several double cones where absorbance indicated RH2B pigment in one member and RH2Aα pigment in the other ($n = 6$, four individuals). Some of these cells may have had low-level coexpression that was undetected owing to its minor effect on absorbance. When it was possible to measure the member opposite to one expressing *RH2Aβ*, its λ_{max} was consistent with pure RH2Aα pigment ($n = 17$) except in the one case where it indicated an RH2Aα/LWS mixture. Double cones in which RH2Aβ replaced RH2B were concentrated in one individual ($n = 5$ of the 7 cells total), in agreement with our FISH results.

(b) How are the double cone opsins distributed across the retina?

RH2Aβ and *LWS* were expressed in different regions of the retina where they formed separate gradients of frequency among double cones. *RH2Aβ* varied nasotemporally with expression most frequent in the nasal (anterior) regions (figures 1*c* and 3*c*), while *LWS* varied ventrodorsally with the majority of its expression in the ventral regions (figures 1*d*

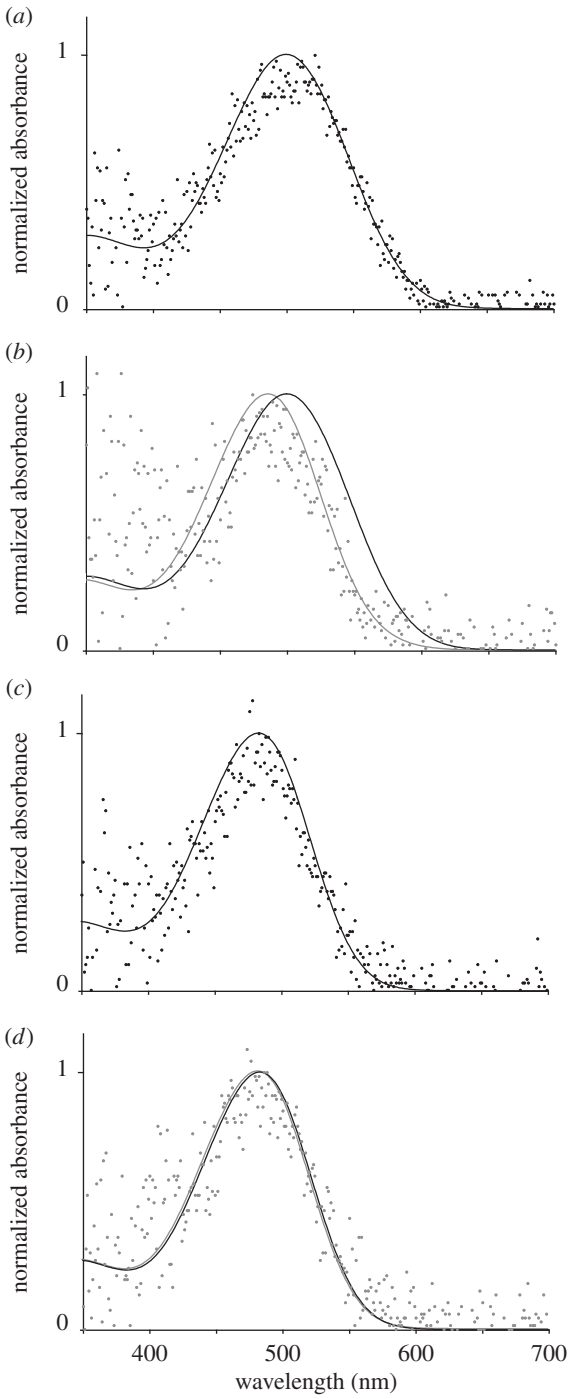

Figure 2. Double cone spectral absorbance measured by MSP. (*a*) Typical coexpressing cell's absorbance (circles), best fit by a template [21] (line) representing 44.1% RH2B, 55.9% RH2Aβ ($\lambda_{max} = 500.0$ nm). (*b*) Exposing cell in (*a*) to 565 nm light bleaches RH2Aβ pigment, lowering λ_{max} from 500.0 nm (black) to 486.0 nm (grey), and reducing RH2Aβ to 10.4% (RH2B = 89.6%). (*c*,*d*) Pre-bleach λ_{max} of pure RH2B cell (*c*; 483.0 nm) was unchanged by exposure to 565 nm light (*d*; black, pre-exposure; grey, post-exposure).

and 3*a*). *RH2Aβ* was expressed in nearly every double cone in the nasal region of three out of five laboratory-reared fish but was only rarely expressed in the temporal and middle retina of all fish (figures 1*c* and 3*c*). *RH2Aβ* typically was coexpressed in cells containing *RH2B*, although in one fish *RH2Aβ* expression replaced *RH2B* in the nasal region. *LWS* was concentrated in the ventral retina, coexpressed with *RH2Aα* (figures 1*d* and 3*a*). Thus, *RH2Aβ* and *LWS* were strongly expressed by a majority of laboratory-reared fish in specific retinal regions, typically in cells also containing *RH2B* and *RH2Aα*.

Expression of double cone opsins in the wild *M. zebra* was similar in most ways to that of laboratory-reared fish. *RH2B* and *RH2Aα* were expressed in opposite members of nearly every double cone in the wild *M. zebra* (98.7 $\pm$ 2.3% of double cones in dorsal and ventral regions, $n = 50$ double cones per region in each of three individuals). In addition, when inspecting retinas we found that 5 out of 12 wild *M. zebra* had abundant, widespread *LWS* expression in the ventral retina, but little, if any, in the dorsal retina. Quantifying *LWS* and *RH2Aα* in three of these wild fish confirmed that the ventro-dorsal gradient of *LWS*/*RH2Aα* coexpression exists in both wild and laboratory-reared fish (figure 3*a*,*b*). A statistical analysis confirmed that the percentage of double cone members expressing LWS differed significantly between retinal regions ($p < 0.0001$). According to a *post hoc* Tukey test, LWS expression was higher in the ventral and mid-ventral regions than in the mid, mid-dorsal and dorsal regions in both wild and laboratory-reared fish ($p < 0.05$). No difference was detected between wild and laboratory-reared fish in the intraretinal distribution of LWS ($p = 0.80$). The seven other wild fish had little or no LWS expression anywhere in the retina. Similarly, we did not find frequent *RH2Aβ* expression in any of the 16 wild fish we examined for this gene.

(c) Effect of regional opsin coexpression on contrast detection

The overhead radiance spectrum we measured in Lake Malawi was broader than both the blue–green horizontal spacelight (figure 4) and the red-enriched light from below, which was influenced by the brown rock substrate (electronic supplementary material, table S2). The nasal retina looks behind the fish, often viewing horizontal spacelight. *RH2Aβ* was coexpressed with *RH2B* in nasal regions of laboratory fish, and qPCR indicates that *RH2Aβ* is expressed by some wild *M. zebra*. The average RH2B/RH2Aβ mixture measured by MSP would absorb 7.5% more of Lake Malawi horizontal spacelight than pure RH2B. Adding RH2Aβ to RH2B yields a mixture with red-shifted absorbance that equals the sum of the weighted absorbances of the constituent pigments (figure 4*a*). Peak absorbance of a pigment mixture is lower than that of a pure pigment, but the RH2B/RH2Aβ mixture is better than pure RH2B in aligning with the brightest part of the background spectrum. Adding RH2Aβ to RH2B also makes the receptor more broadly sensitive at longer wavelengths. For wavelengths greater than 532 nm, absorbance of the RH2B/RH2Aβ mixture is higher than that of a pure pigment with the same λ_{max} (496.7 nm). At a depth of 3 m, the RH2B/RH2Aβ mixture absorbs 5.9–11.1% more of every background from zenith to nadir than RH2B alone. Similar to RH2B/RH2Aβ coexpression, replacing RH2B with RH2Aβ increases sensitivity to the entire range of backgrounds 3 m below the surface, suggesting this may be an alternative tuning mechanism.

Overhead radiance is viewed by the ventral retina, where *LWS* is coexpressed with *RH2Aα*. Our shallowest measurements (3 m) indicate that the RH2Aα/LWS mixture absorbs 2.0% more of the overhead radiance than pure RH2Aα. For overhead radiance previously measured in *M. zebra* habitat at a depth of 1 m [22], this gain increases to 5.0%. The RH2Aα/LWS absorbance spectrum is broader and extends to longer wavelengths compared with pure RH2Aα (figure 4*b*). Background matching by *LWS* coexpression diminishes as the

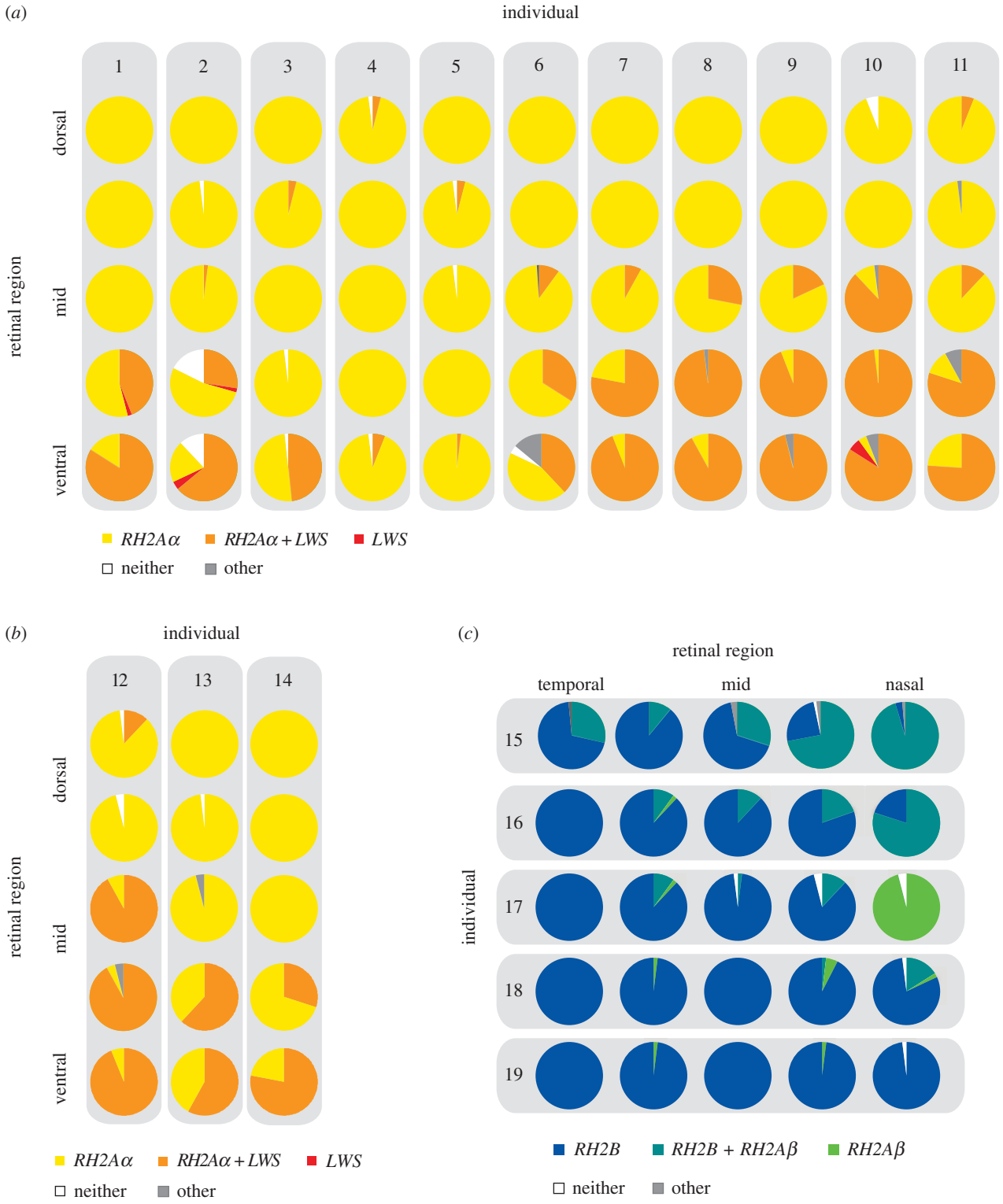

Figure 3. Frequency of coexpressing double cones by retinal region, revealed by dual-labelling *in situ* hybridization of whole *Metriaclima zebra* retinas. Gene pairs probed were *RH2Aα* and *LWS* (*a*,*b*) or *RH2B* and *RH2Aβ* (*c*). For each individual, double cones were examined in five regions, either along the ventrodorsal axis (*a*,*b*), or from the nasal to the temporal (caudal) retina (*c*). Pie graphs indicate percentage of double cones in which one member expressed a single gene or coexpressed both genes. 'Neither' refers to double cones in which neither member expressed either gene. Double cones in which both members expressed at least one of the two genes were classified as 'other'. Fish were either laboratory-reared (*a*,*c*) or captured wild and dissected in the field (*b*). We examined LWS expression in the retinas of nine additional wild-caught fish but did not quantify frequency of expression in these individuals. *LWS* expression in two of these fish appeared similar to that in (*b*), while it was absent in the seven remaining fish.

viewer descends or as the gaze moves toward the horizontal spacelight because the proportion of long wavelength light decreases, as is typical for clear waters (figure 4*a*).

Increasing sensitivity to the background spacelight will improve contrast and therefore detectability of dark objects [25], including potential prey, predators and mates. We used a standard model [25] to compare the contrast and detection distance of a dark object viewed horizontally by pure and mixed pigments, and we modified this model to predict detection when the object was directly above the observer. The average RH2B/RH2Aβ mixture extended maximum horizontal detection distance over that of pure RH2B by 0.27 m

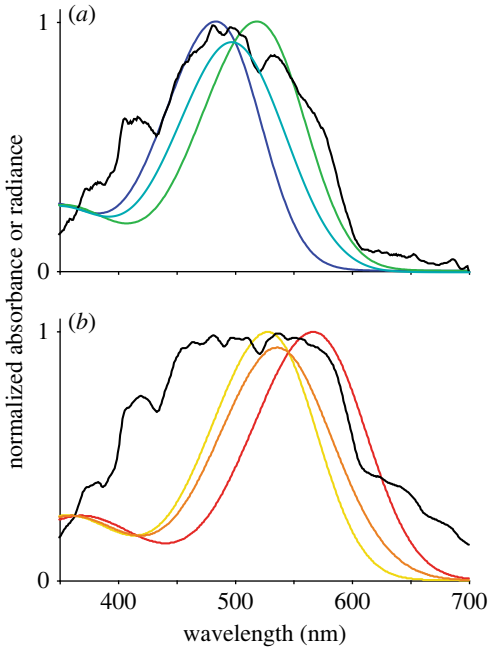

Figure 4. Absorbance templates [21] of *Metriaclima zebra* pure and mixed visual pigments compared to backgrounds that they view in Lake Malawi (depth = 3 m). (*a*) Horizontal spacelight radiance (black line) is viewed by the nasal retina, where the addition of RH2Aβ (green) to RH2B (blue) produces a mixture (teal) that is better than pure RH2B at aligning with the brightest part of the background spectrum. (*b*) Overhead spacelight radiance (black) is viewed by the ventral retina, where the addition of LWS (red) to RH2Aα (yellow) produces a mixture (orange) that has broader sensitivity than pure RH2Aα and absorbs more of this background light.

(from 12.92–13.19 m, or 2.1%), equivalent to approximately four body lengths of an adult *M. zebra*. Overhead detection distance was also improved by pigment mixtures. The average RH2Aα/LWS mixture increased overhead detection by 0.1 m over that of pure RH2Aα (from 17.55–17.65 m, or 0.6%), equivalent to 1.5 body lengths. Similarly, the RH2B/RH2Aβ mixture extended overhead detection from 17.23–17.35 m, or 0.7%. This could be relevant when the viewer's body is angled downward, as when foraging on rocks, causing the RH2B/RH2Aβ mixture in the naso-ventral retina to gaze overhead.

When the contrast of a distant object is near the threshold of sensitivity, increasing its contrast may improve the likelihood that the viewer will notice it. This might be accomplished by mixing visual pigments. At the maximal detection distance of the pigment mixtures, the RH2B/RH2Aβ mixture yields 8.5% greater apparent contrast of the dark object than does pure RH2B, while the RH2Aα/LWS mixture improves apparent contrast by 2.7% over pure RH2Aα.

(d) Effect on colour discrimination

While coexpression of *RH2Aβ* (with *RH2B*) and coexpression of *LWS* (with *RH2Aα*) both increase sensitivity to longer wavelengths, they are likely to affect colour vision in different ways. Combining RH2Aβ with RH2B makes the mixed receptor sensitivity more similar to that of the other double cone containing RH2Aα (figure 4). However, mixing LWS with RH2Aα increases the difference between absorbance spectra of the mixed (RH2Aα/LWS) and the RH2B double cone members. Adding RH2Aβ pigment to RH2B generally decreased the distance in colour space between *M. zebra* colours and five backgrounds determined previously for a depth of 3 m [22]. The distance between colours and the backgrounds decreased by an average of 4.3–7.6% compared with the greatest *M. zebra* colour distance for the corresponding background (electronic supplementary material, table S1). On the other hand, adding LWS to RH2Aα increased a great majority of these distances, with an average increase of 2.9–5.9% relative to the different backgrounds (electronic supplementary material, table S2). Thus, although coexpression of *LWS* confers a smaller advantage than *RH2Aβ* in contrast detection, it also enhances colour discrimination. If both pigment mixtures were present, distances of yellow colours decreased relative to each background, while the distances of other colours increased relative to some backgrounds and decreased relative to others (electronic supplementary material, table S3).

4. Discussion

Metriaclima zebra expressed *RH2B* and *RH2Aα* in opposite members of nearly every double cone. This agrees with previous MSP and protein expression studies of *M. zebra* [9,31]. These double cone members also coexpressed different opsins in different regions of the retina. *RH2Aβ* was coexpressed with *RH2B*, most frequently in the nasal retina, while *LWS* was coexpressed with *RH2Aα* predominantly in the ventral retina. The two gradients of coexpression may reflect the spatial distribution of transcription factors regulating expression of *RH2Aβ* and *LWS*. Photoreceptors may temporarily contain two opsins as they switch expression from one opsin to another during development [32], but this is unlikely to explain the coexpression we observed. Although opsin expression changes during development of the *M. zebra* retina, this stabilizes by adulthood [33] when our measurements were made. Thus, opsin coexpression is likely a persistent feature of cichlid retinas, raising the question of its effect on vision.

Double cones in the nasal and ventral retina of *M. zebra* contain different mixtures of visual pigments, and they view different coloured backgrounds (figure 5). In each retinal region, the calculated absorbance of the background light was increased by the mixing of the two visual pigments. Several published reports of opsin coexpression involve mixing of opsins that differ greatly in spectral sensitivity. The photoreceptors of butterflies [5] and rodents [4] achieve extremely broad sensitivity by mixing two opsins that differ in λ_{max} by up to 150 nm. Salamanders coexpress UV, green and red opsins in a single cone [2,34]. By comparison, the opsins that are coexpressed by *M. zebra* are more similar to each other in spectral absorbance, with λ_{max} differing by approximately 40 nm. In either member of the *M. zebra* double cone (containing RH2B or RH2Aα), expressing the second opsin (RH2A*β* or LWS) adds the pigment with the next longer λ_{max} and increases sensitivity to longer-wavelength light. To our knowledge, this is the first documentation of photoreceptors coexpressing opsin genes in a manner that tunes sensitivity to the environment. Known mechanisms of tuning photoreceptor sensitivity include altering the opsin [35], changing the chromophore [36] and incorporating pre-receptor filtering [37,38]. Thus, opsin coexpression may be a novel mechanism of spectral tuning. Archerfish (*Toxotes chatareus* Hamilton 1822) also may tune retinal regions by mixing chromophores and opsins, though opsin coexpression has yet to be confirmed by molecular techniques [39].

When a spectral class of photoreceptor is tuned so that its absorption is matched to the spectrum of the background

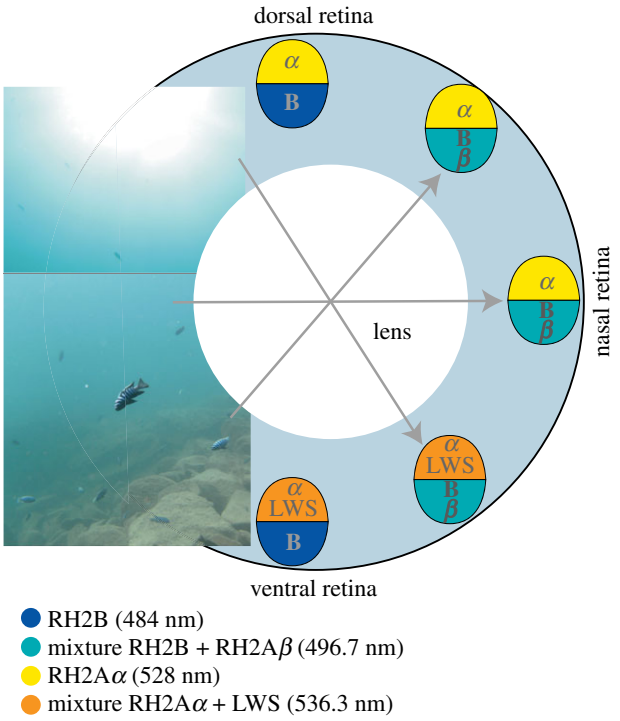

Figure 5. *Metriaclima zebra* tunes photoreceptors by mixing different opsins in retinal regions that view distinct backgrounds. Peak absorbance wavelength is given for pure visual pigments and for average pigment mixtures measured by MSP.

light that it views, the contrast of dark objects such as silhouetted predators increases [25]. In fishes, the sensitivity of double cones, which are pairs of partially fused cone cells (figure 1*a*), often matches the background spectra of their environments [39]. However, in many habitats the background spectrum differs with angle of view. Thus, maximizing contrast across the visual field requires multiple receptors tuned to heterogeneous backgrounds [39,40]. In several fishes sensitivity varies between retinal regions, matching the corresponding backgrounds. These include two cichlid species [40] and archerfish [39]. Our results show that *M. zebra* accomplishes this through regional coexpression of different pairs of opsins. Our calculations indicated that dark objects can be detected at slightly greater distances by double cones containing different visual pigment mixtures in the ventral and nasal retina. In addition, near the maximum detection distance, the apparent contrast of a dark object is increased by the pigment mixtures. For a cichlid engaged in defending territories or foraging on rocks, this increase by contrast may improve the probability that it will notice a potential predator or mate entering the range of detectability. This might include predators specialized in stealing eggs during mating.

Cells containing RH2Aβ also look downward and upward because RH2Aβ expression extends ventrally and dorsally in the nasal retina, and these fish tilt vertically as they forage on rocks. Thus, fish with nasal *RH2B*/*RH2Aβ* coexpression would have increased sensitivity to a vertical swath behind them, even as they forage. As the viewer descends, the advantage of *RH2Aβ* expression should persist because the downwelling spectrum becomes increasingly similar to the horizontal spacelight, which itself changes little with depth. This prediction is supported by the radiance we measured 7 m below the surface.

LWS was rarely expressed outside of the upward-looking ventral retina. If *RH2Aα*/*LWS* coexpression evolved for detection of distant dark objects, the mid retina may have less LWS because the horizontal spacelight is better matched by pure RH2Aα. LWS may be absent from the downward-looking dorsal retina because *M. zebra* typically remain just above the brown rocks. Thus, animals approaching from a distance would tend to be in a more horizontal or overhead part of the visual field and not coming from below. On the other hand, the lack of *RH2Aβ* coexpression outside of the nasal retina may be due to its negative effects on colour vision.

Colour patterns figure prominently in mate choice and male–male competition among cichlids [41–43], indicating that colour discrimination is likely an important visual function for cichlids [8]. Although in most species it is unknown whether members of double cones contribute individually to colour vision, they do so in triggerfish (*Rhinecanthus aculeatus* Linnaeus 1758) [44]. In addition, electrophysiological measurements of *M. zebra* indicate an opponent interaction, which is required for wavelength discrimination, between RH2B and RH2A pigments (*RH2Aα* and *β* gene expression values were averaged) [45]. Coexpressing *RH2Aβ* with *RH2B* reduced colour space distances between *M. zebra* colours and backgrounds, but coexpressing *LWS* with *RH2Aα* increased most of these distances. Thus, in the case of coexpressed *RH2Aβ*/*RH2B*, but not *RH2Aα*/*LWS*, there appears to be a trade-off between colour and luminance vision, as has been found in surfperch (Embiotocidae) [46]. This trade-off may explain the lack of RH2Aβ in the central and temporal retina, regions that are more likely to be used for examining the colours of nearby objects such as prey and mates.

It is noteworthy that the presence of regional opsin coexpression varies among cichlids. We observed significant variation in coexpression levels among fish from the same population (figure 2). This variation is consistent with the variation in opsin expression determined by qPCR in both laboratory-reared and wild populations [13,47]. In addition, the absence of *RH2Aβ* expression in the wild fish we examined is consistent with a previous qPCR study that found *RH2Aβ* comprises less than 0.1% of total cone opsin transcripts in wild *M. zebra* from the same population at Mazinzi Reef, and from the Thumbi West Island population [47]. However, that study did find significant *RH2Aβ* expression in a wild population of *M. zebra* at Zimbawe Rock, where it was 5–7% of total opsin in 2 out of 10 individuals examined. Thus, expression of *RH2Aβ* appears to vary between and within wild populations of *M. zebra*.

The sensory trade-off between colour discrimination and contrast detection of *RH2Aβ* coexpression may partially explain why this gene is expressed by fewer wild fish when compared with *LWS*. Additional sources of variation in coexpression levels likely include both genetic factors and plasticity of development in response to the rearing light environment [48,49]. Investigating behaviours involving different visual field sectors and the temporal variation in light environments would advance our understanding of the ecological significance of opsin coexpression.

5. Conclusion

In summary, double cones across the *M. zebra* retina consistently express *RH2B* and *RH2Aα* in opposite members. In addition,

RH2Aβ was coexpressed with *RH2B*, and *LWS* was coexpressed with *RH2Aα* in particular retinal regions (figure 5). The degree of coexpression formed two separate gradients, with *RH2Aβ* concentrated nasally and *LWS* concentrated ventrally. We confirmed the presence of these visual pigment mixtures by MSP. Our calculations show that both pigment mixtures tune their respective retinal regions to the different lake background spectra that they view, increasing quantum catch of environmental light and potentially aiding detection of dark objects such as silhouetted prey, predators or mates. However, our modelling suggests that *RH2Aβ* coexpression hinders colour discrimination, leading to a trade-off between colour and luminance visual functions.

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