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RESEARCH ARTICLE

Intra-retinal variation of opsin gene expression in the guppy (*Poecilia reticulata*)

Diana J. Rennison[1,2], Gregory L. Owens[2,3], W. Ted Allison[4] and John S. Taylor[2,*]

[1]Department of Zoology and Biodiversity Research Centre, University of British Columbia, 2370-6270 University Boulevard, Vancouver, BC, Canada, V6T 1Z4, [2]Department of Biology, University of Victoria, PO Box 3020, Station CSC, Victoria, BC, Canada, V8W 3N5, [3]Department of Botany and Biodiversity Research Centre, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC, Canada, V6T 1Z4 and [4]Department of Biological Sciences, University of Alberta, CW 405, Biological Sciences Building, Edmonton, AB, Canada, T6G 2E9

*Author for correspondence (taylorjs@uvic.ca)

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SUMMARY

Although behavioural experiments demonstrate that colouration influences mate choice in many species, a complete understanding of this form of signalling requires information about colour vision in the species under investigation. The guppy (*Poecilia reticulata*) has become a model species for the study of colour-based sexual selection. To investigate the role of opsin gene duplication and divergence in the evolution of colour-based mate choice, we used *in situ* hybridization to determine where the guppy's nine cone opsins are expressed in the retina. Long wavelength-sensitive (*LWS*) opsins were more abundant in the dorsal retina than in the ventral retina. One of the middle wavelength-sensitive opsins (*RH2-1*) exhibited the opposite pattern, while the other middle wavelength-sensitive opsin (*RH2-2*) and the short wavelength-sensitive opsins (*SWS1*, *SWS2A* and *SWS2B*) were expressed throughout the retina. We also found variation in *LWS* opsin expression among individuals. These observations suggest that regions of the guppy retina are specialized with respect to wavelength discrimination and/or sensitivity. Intra-retinal variability in opsin expression, which has been observed in several fish species, might be an adaptation to variation in the strength and spectral composition of light entering the eye from above and below. The discovery that opsin expression varies in the guppy retina may motivate new behavioural experiments designed to study its role in mate choice.

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Key words: gene expression, vision, visual pigments, intra-retinal differences.

INTRODUCTION

In the guppy (*Poecilia reticulata*, Peters 1859), a model system for the study of sexual selection, colouration is an especially important component of female mate choice (Endler, 1980; Endler, 1983; Houde, 1988; Houde and Endler, 1990; Smith et al., 2002; Blows and Brooks, 2003). Little, however, is known about guppy colour vision. Here, we studied the expression patterns of guppy opsin genes. Opsins are the protein component of visual pigments, which absorb light in retinal photoreceptor cells (rods and cones). Opsin proteins are bound to vitamin A-derived chromophores (Wald et al., 1953) and when exposed to light, visual pigments initiate the phototransduction cascade. The sensitivity of a given photoreceptor cell is determined by the opsin gene (or genes) that it expresses and the chromophore, A1 (11-*cis* retinal) or A2 (11-*cis*-3,4-dehydroretinal), that is associated with the opsin. To evaluate colour it is necessary for the retinal circuitry to compare the output from multiple photoreceptor cells with distinct wavelength sensitivities. Thus, opsin expression patterns affect wavelength sensitivity and wavelength discrimination. By determining opsin expression patterns in the guppy we attempted to gain further insight into the visual capacity of the guppy and the role of opsin gene duplication and divergence in the evolution of colour-based mate choice.

Several studies have used microspectrophotometry (MSP) to measure the spectral absorbance of rod and cone cells in the guppy retina and have provided the first evidence that guppies may have more opsins than many other vertebrates (Levine and MacNichol, 1979; Archer et al., 1987; Archer and Lythgoe, 1990). MSP data also suggest that the distribution of cells expressing particular visual pigments differs spatially (Levine et al., 1979). More recently, sequence data from PCR using cDNA and genomic DNA templates (Hoffmann et al., 2007; Weadick and Chang, 2007; Ward et al., 2008; Owens et al., 2009) and from opsin-positive BAC clones (Watson et al., 2010) have shown that guppies possess nine cone opsins: four long wavelength-sensitive (*LWS*) genes, *A180*, *S180*, *S180r* and *P180*; two middle wavelength-sensitive (*RH2*) genes, *RH2-1* and *RH2-2*; two short wavelength-sensitive (*SWS2*) genes, *SWS2A* and *SWS2B*; and one UV-sensitive (*SWS1*) gene. Among these genes there are a large number of amino acid substitutions at positions that influence wavelength sensitivity, the so-called 'key sites' (Yokoyama and Radlwimmer, 1998). All but two of the nine opsins (*LWS S180* and *LWS S180r*) have substitutions generating unique key site haplotypes, and are, therefore, predicted to have distinct wavelengths of maximal absorption. This is an intriguing observation because opsin diversity is a prerequisite for broad spectral sensitivity and wavelength discrimination. However, data on the expression patterns of these opsins are essential for understanding the role of this large opsin repertoire in guppy colour vision.

Opsin expression can vary temporally or spatially (e.g. Takechi and Kawamura, 2005; Veldhoen et al., 2006). Spatial variation (i.e. intra-retinal variation) could be advantageous for fish, given that the light hitting the dorsal retina is of different spectral composition from

that hitting the ventral retina, as a result of the filtering properties of water and any dissolved organic solutes or suspended particulates in it that alter the spectral properties of light. Several species of fish have been shown to possess intra-retinal variation in spectral sensitivity, exhibiting different densities and/or distributions of various photoreceptor types, apparently to deal with varied visual tasks (Takechi and Kawamura, 2005; Allison et al., 2006; Veldhoen et al., 2006; Temple et al., 2010; Temple, 2011). This intra-retinal variation in opsin gene expression may also influence the behaviour of species that use colour in mate choice decisions (Temple, 2011). During mate selection, female guppies have been noted to investigate the male from an elevated position in the water column (Baerends et al., 1955), suggesting that illumination and the location of opsin expression in the retina may be important during mate selection in this species. In addition, the colouration that females find most attractive in male guppies is known to vary among populations (Houde, 1988; Houde and Endler, 1990). This population-level variation in male morphology might be influenced by opsin gene expression as appears to be the case in cichlids (Seehausen et al., 2008). To examine the spatial distribution of opsin gene expression in the guppy retina we used *in situ* hybridization. This technique allowed us to use knowledge of the opsin gene repertoire in our characterization of retinal photoreceptors.

MATERIALS AND METHODS

Animal care

The guppies used in this study were sampled from a lab population descended from fish caught in Cumaná, Venezuela. They were kept on a 14 h light, 10 h dark cycle, at 24°C (±1°C) and were fed flaked fish food. Sampling took place 30 min before the end of their 14 h light cycle. Fish were killed with buffered tricaine methanesulfonate (Sigma-Aldrich, St Louis, MO, USA). All protocols were approved by the animal care committee at the University of Victoria (Victoria, BC, Canada).

Probe design and synthesis

Total RNA was isolated from the freshly removed eyes of an adult male guppy using the Aurum™ Total RNA Fatty and Fibrous Tissue Pack (BioRad, Hercules, CA, USA), and cDNA was synthesized from it using the BioRad iScript™ Select cDNA Synthesis Kit. Opsin probe cDNAs were amplified using locus-specific primers and cloned with the pGEM® T-Easy cloning kit (Promega, Madison, WI, USA).

Two sense and eight anti-sense RNA probes were synthesized from these cDNA clones using the Roche DIG RNA labelling kit (Lewes, UK). It was not possible to produce unique probes for two of the *LWS* opsin genes (*LWS A180* and *LWS S180*) because of the high degree of sequence similarity. Therefore, eight anti-sense opsin probes were produced: *RH2-1*, *RH2-2*, *SWS2A*, *SWS2B*, *SWS1*, *LWS S180r*, *LWS P180* and a probe that was equally complementary (100% identical over the region of probe binding) to *LWS A180* and *LWS S180*. The cRNA probes were synthesized in run-off transcription reactions using T7 promoters for antisense probes and SP6 promoters for sense probes from linearized templates. Riboprobes varied in length from 182 to 827 base pairs (see supplementary material Table S1 for exact lengths and sequences). Probe specificity was assessed by dot blot analysis (see Appendix for details).

Tissue preparation for histology

Twelve eyes (from three adult males and three adult females) were prepared for cryosectioning. The eyes were fixed in 4% (w/v) paraformaldehyde with 5% (w/v) sucrose in 1× phosphate-buffered saline (PBS) overnight at 4°C with agitation. Eyes were then washed in 1× PBS with 5% (w/v) sucrose. This was followed by a series of infiltration steps: briefly, in 1× PBS with 10% (w/v) sucrose, 12.5% (w/v) sucrose, and 15% (w/v) sucrose and overnight at 4°C in 1× PBS with 20% (w/v) sucrose with agitation (modified from Barthel and Raymond, 1990). Next, eyes were embedded in OCT medium (Sakura Finetek Europe, Alphen aan den Rijn, Netherlands) [ratio 2:1, 1× PBS/20% sucrose (w/v):OCT] and stored at –80°C until they were sectioned. Sections 8 µm in width were cut nasal–temporally and dorsal–ventrally using a cryostat at –22°C and were placed onto Superfrost glass slides (Thermo Fisher Scientific, Waltham, MA, USA). The slides were then stored at –80°C.

Whole-mount tissue preparation

Sixteen fish were used for whole-mount *in situ* hybridization. Each fish was dark adapted for 20 h prior to eye removal (see above). Dissection was performed in the dark under a red light. After enucleation, the retina was isolated and fixed in 4% (w/v) paraformaldehye (Sigma-Aldrich) with 5% (w/v) sucrose in 1× PBS overnight at 4°C with gentle agitation.

In situ hybridization on cryosections

Slides were air dried and re-hydrated by soaking in four progressively weaker ethanol solutions: 100% (w/v), 95% (w/v), 70% (w/v), 50% (w/v) followed by 2× saline–sodium citrate (SSC). The slides were treated with a 13 µg ml^{-1} proteinase K (Roche) in PBS Tween-20 (PBST) solution for 4 min and rinsed briefly in diethylpyrocarbonate (DEPC) water. Each slide was washed with 0.1 mol l^{-1} triethanolamine (TEA) and acetylated using acetic anhydride in TEA. Next, slides were dehydrated by soaking in the same ethanol solutions used for hydration but in the reverse order, and allowed to air dry. Digoxigenin (DIG)-labelled RNA probes were then applied to the slides and hybridized overnight at 65°C in an HL-2000 HybriLinker hybridization oven (UVP, Upland, CA, USA). Following hybridization, slides were washed in 2× SSC at room temperature, in 50% (w/v) formamide in 2× SSC at 65°C and in 0.2× SSC at 65°C. They were then rinsed in 1× maleate buffer and put into maleate blocking buffer for 2–3 h. The slides were incubated overnight at 4°C with anti-DIG-AP antibody (Roche), diluted (1:1000) in maleate blocking buffer. The slides were then washed with 1× maleate buffer and Genius 3 buffer (1× Tris-NaCl, 1× $MgCl_2$). Signal detection was performed using Nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP) (Roche) prepared in Genius 3 buffer and incubated for 0.5–2 h in the dark. The reaction was stopped using alkaline phosphatase substrate wash. Slides were viewed under a Zeiss (Oberkochen, Germany) universal light microscope and images were taken with a SPOT Flex colour camera (Diagnostic Instruments, Sterling Heights, MI, USA). Serial adjacent sections were used for all probes as an internal control, giving us confidence that lack of *in situ* hybridization signal was not due to technical issues of tissue quality or target mRNA degradation. Additionally, a sense probe was used as a negative control (see supplementary material Fig. S1).

LWS *in situ* hybridization on retinal whole-mounts

After dissection, 32 fixed retinas (from 16 fish) were rinsed with PBST, and subsequently digested using 13 µg ml^{-1} proteinase K for 15 min. Whole retinas were rinsed with PBST and re-fixed in 4% (w/v) paraformaldehye with 5% (w/v) sucrose in 1× PBS for 20 min. The retinas were rinsed with PBST and pre-hybridized in Hauptmann's buffer at 65°C for 2 h in the hybridization oven. From

each fish one retina was then submerged in Hauptmann's solution with the labelled *LWS A/S180* riboprobe and the other retina was submerged in Hauptmann's solution with the labelled *LWS P180* riboprobe. These preparations were then incubated overnight at 65°C with light agitation. Retinas were then washed in 50% (w/v) formamide in 2× SSC, in 2× SSC, and finally in 0.2× SSC at 65°C. Detection was by the same method described above for the cryosections. The presence or absence of *LWS A/S180* and *LWS P180* expression was noted for each individual.

RESULTS

SWS expression

The *SWS1* opsin gene was expressed uniformly along the dorsal–ventral axis of the retina (Fig. 1A). However, in the peripheral region of the nasal retina fewer cells (1.3 cells per 100 μm) expressed *SWS1* compared with the temporal region of the retina where approximately 8 cells per 100 μm expressed *SWS1* (summarized in Fig. 2). *SWS1* expression did not vary noticeably among individuals; all individuals expressed approximately 8–10 cells per 100 μm in all regions of the retina aside from the peripheral nasal retina.

There are two *SWS2* genes in the guppy, *SWS2A* and *SWS2B*. We designed unique probes for each one. *SWS2A* was expressed uniformly along the nasal–temporal and dorsal–ventral axes in those fish expressing the gene (Fig. 1B) and there were no noticeable differences in the densities of cells expressing *SWS2A* (approximately 14 cells per 100 μm mid-retina). Expression varied among individuals: one of the three males surveyed appeared to have no *SWS2A*-positive cone cells in any of the probed sections, although other sections from the same male expressed other opsins. The expression of *SWS2B* was uniform along the dorsal–ventral and nasal–temporal axes (Fig. 1C) and expression did not vary noticeably among individuals (summarized in Fig. 2).

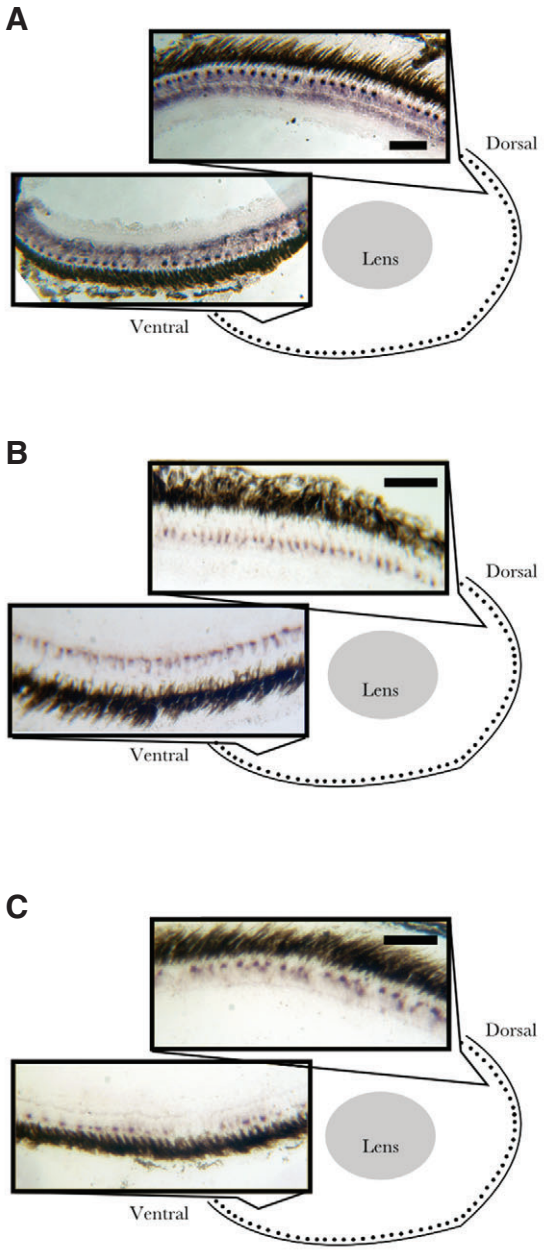

Fig. 1. Expression of short wavelength-sensitive (*SWS*) genes in dorsal–ventral sections of the guppy retina. (A) Section of a female guppy's left eye hybridized with the *SWS1* probe. (B) Section of a male guppy's left eye hybridized with the *SWS2A* probe. (C) Section of a male guppy's left eye hybridized with the *SWS2B* probe. Scale bars, 50 μm.

RH2 expression

There are two *RH2* genes in the guppy. Unique probes were designed for each gene. A uniform pattern of *RH2-1* expression was detected along the nasal–temporal axis (summarized in Fig. 2). We observed more cells expressing *RH2-1* in the ventral region of the retina than in the dorsal region; there were approximately 10.3 *RH2-1*-positive cone cells per 100 μm in the ventral retina and only 5.3 *RH2-1*-positive cells per 100 μm in the dorsal retina (Fig. 3A). This pattern was observed in all six individuals surveyed (three males and three females). *RH2-2*-expressing cells were evenly distributed across both the nasal–temporal and dorsal–ventral axes of the retina (Fig. 3B).

LWS expression

Guppies possess four *LWS* genes; however, only three unique riboprobes were produced. One riboprobe was equally complementary to the *LWS S180* and *LWS A180* opsin transcripts. This probe revealed uniform expression across the nasal–temporal axis of the retina (summarized in Fig. 2). In contrast, there were more cells expressing *LWS S/A180* in the dorsal region of the retina (approximately 7 transcript-positive cells per 100 μm) than in the ventral region (approximately 0.6 transcript positive cells per 100 μm) (Fig. 4A).

LWS P180-expressing cells were detected across the nasal–temporal axis, though fewer *LWS P180*-positive cells were observed at the periphery of the retina (summarized in Fig. 2). Expression along the dorsal–ventral axis was largely localized to the dorsal portion of the retina (4.3 transcript-positive cells per 100 μm) and was absent from the distal portion of the ventral retina (the first 300 μm of the ~1800 μm total width) (Fig. 4B).

Most sections showed no evidence of *LWS S180r* expression. However, in one male (both eyes) and one female (both eyes) there were transcript-positive cells in the peripheral dorsal region of the retina (4.3 transcript-positive cells per 100 μm, and only found in the first 300 μm) (Fig. 4C).

Whole-mount and section *in situ* hybridization data indicated variation in *LWS* expression among individuals. All nine of the females surveyed expressed *LWS S/A180*. However, four of the nine females had few or no *LWS P180* transcript-positive cells. Variation in *LWS* opsin expression was also observed in males. One of the seven fish surveyed expressed only *LWS P180*, two individuals

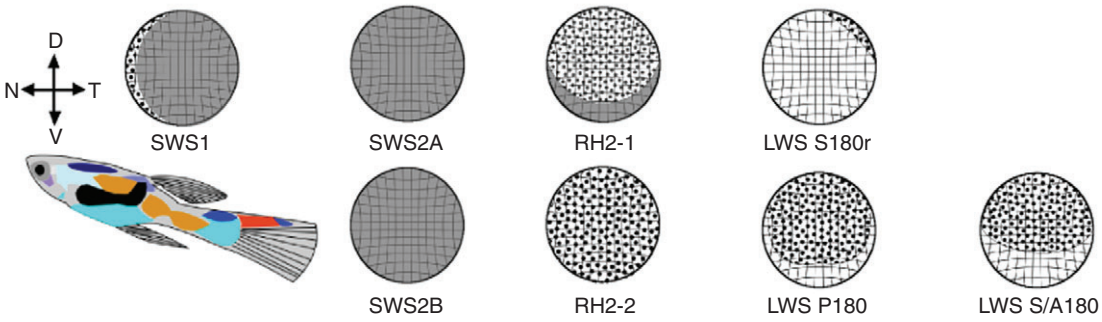

Fig. 2. Summary schematic diagram of spatial expression patterns of eight* cone opsin genes in the guppy retina, as reconstructed from serial sections through the eye. Grey shading represents 8–15 transcript-positive cells per 100 μm; black dots represent 1–7 transcript-positive cells per 100 μm. D, dorsal; V, ventral; N, nasal; T, temporal. *SWS1*, UV-sensitive transcript; *SWS2A* and *SWS2B*, short wavelength-sensitive transcripts; *RH2-1* and *RH2-2*, middle wavelength-sensitive transcripts; *LWS A180*, *S180*, *S180r* and *P180*, long wavelength-sensitive transcripts. *A single probe was used to detect *LWS S180* and *LWS A180* transcripts.

expressed *LWS P180* and *LWS S/A180*, and four fish expressed only *LWS S/A180*.

DISCUSSION

When female mice or Chinook salmon are given the opportunity to mate with 'preferred' males, they produce offspring that score better for a diversity of morphological, behavioural and/or genotypic traits than conspecifics who mate with 'non-preferred' males (Drickamer et al., 2000; Neff et al., 2008). The goal of this study was to improve our understanding of the signals used to evaluate potential mates, by studying the expression patterns of guppy opsin genes. Male colouration plays a key role in this species (Endler, 1980; Endler, 1983; Houde, 1988; Houde and Endler, 1990; Blows and Brooks, 2003), but without characterizing guppy vision, it is difficult to know what it is about a colourful male that attracts females. Guppies have nine cone opsins that span the visual spectrum, including four recently duplicated *LWS* genes (Hoffmann et al., 2007; Ward et al., 2008; Owens et al., 2009). Our results suggest that at least eight, and possibly all nine, of the cone opsin genes are expressed simultaneously in the retinas of many adult male and female Cumaná guppies. As one of the probes was designed to detect two very similar opsins, *LWS A180* and *S180*, our data tell us that either one or both of these genes are expressed.

Our investigation showed that the short wavelength-sensitive opsins, *SWS1*, *SWS2A* and *SWS2B* and one of the middle wavelength-sensitive opsins, *RH2-2*, were expressed uniformly throughout the retina (with the exception of *SWS1* in the nasal retina). In contrast to this, the pattern of expression of *RH2-1* and the long wavelength-sensitive opsins (*LWS*) varied among regions of the retina. Fewer *LWS* opsin-positive cells in the ventral retina may accommodate an increase in *RH2-1* expression (summarized in Fig. 5). We found that *LWS S180r* exhibited the most restricted expression pattern, being found only in the peripheral dorsal–temporal retina. It is possible that *LWS S180r* is expressed in more cone cells or in a different region of the retina at a different life history stage, as only adults were surveyed in this study. Opsin gene expression and regional cone abundance vary during ontogeny in a diversity of fish including cichlids (Carleton et al., 2008), winter flounder (Mader and Cameron, 2004), salmonids (Allison et al., 2006) and zebrafish (Takechi and Kawamura, 2005; Allison et al., 2010).

A number of other *in situ* hybridization and MSP studies in fish have reported intra-retinal variability in opsin expression and spectral absorption [for a recent review see Temple (Temple, 2011)]. For example, Takechi and Kawamura showed that longer wavelength opsins were expressed in ventral and peripheral regions of the retina in zebrafish (Takechi and Kawamura, 2005). Recent work in two members of the family Anablepidae (*Anableps anableps* and *Jenynsia onca*) has also found intra-retinal differences in *RH2* and *LWS* expression (Owens et al., 2011). Additionally, work by Levine and colleagues (Levine et al., 1979) using MSP and Nitro-blue tetrazolium chloride reduction found that a variety of fish, including cichlids (*Cichlasoma longimanus* and *Heterotilapia multispinosa*) and knife fish (*Notopterus*), exhibit intra-retinal variability in visual pigment distributions. MSP in the archerfish (*Toxotes chatereus*) has also shown variation in the abundance of

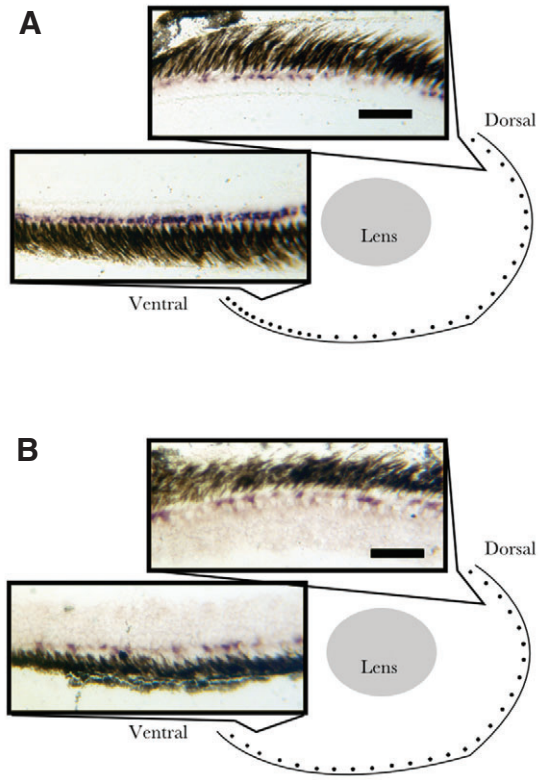

Fig. 3. Expression of *RH2* gene duplicates in dorsal–ventral sections of the guppy retina. (A) Section of a female guppy's left eye hybridized with the *RH2-1* probe. (B) Section of a male guppy's right eye hybridized with the *RH2-2* probe. Scale bars, 50 μm.

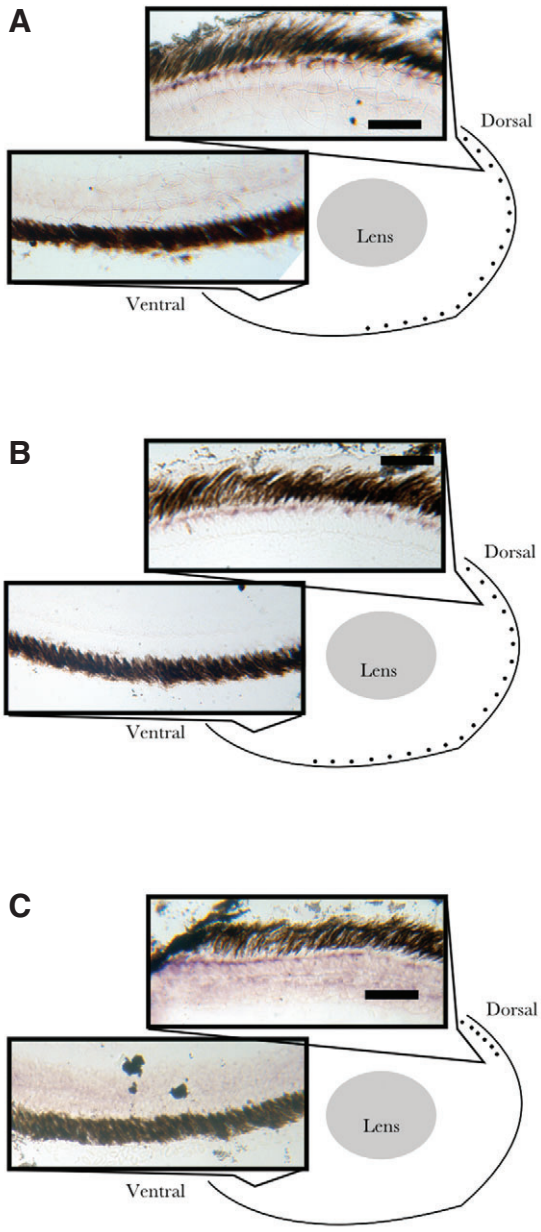

Fig. 4. Expression of *LWS* gene duplicates in dorsal–ventral sections of the guppy retina. (A) Section of a male guppy's left eye hybridized with the *LWS S/A180* probe. (B) Section of a male guppy's left eye hybridized with the *LWS P180* probe. (C) Section of a male guppy's left eye hybridized with the *LWS S180r* probe. Scale bars, 50 μm.

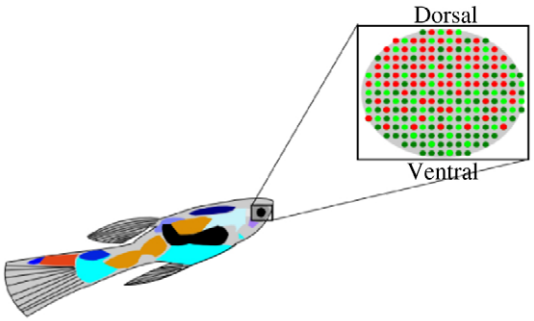

Fig. 5. Schematic representation of *RH2* and *LWS* opsin expression across the retina. Composite expression patterns of *RH2-1* (dark green), *RH2-2* (light green) and *LWS* genes (red) over the retinal surface of male and female guppies.

different photoreceptors within different regions of the retina (Temple et al., 2010). In most aquatic environments the spectral properties of up-welling light differ from those of down-welling light and a non-uniform distribution of opsin pigments might be an adaptation to this environmental heterogeneity (Levine et al., 1979; Takechi and Kawamura, 2005; Temple et al., 2010). Our findings in the guppy lend support to the idea that variable opsin expression (and wavelength sensitivity) along the dorsal–ventral axis of the retina is common in fish, having now been described in a number of distantly related species (see above). Future experiments should examine how water and light properties (e.g. turbidity, depth of water, position in water column) can affect the location and extent of intra-retinal variation. As many ray-finned fish possess large opsin gene repertoires (e.g. Chinen et al., 2003; Parry et al., 2005; Matsumoto et al., 2006; Owens et al., 2009; Windsor and Owens, 2009), perhaps gene duplication has promoted or facilitated this differential intra-retinal gene expression.

Functionally, the differential expression of *RH2-1* and *LWS* genes may play a role in background matching (McFarland and Munz, 1975), luminance detection (Osorio and Vorobyev, 2005) and/or colour vision (Pignatelli et al., 2010). Both *RH2* and *LWS* opsin subfamilies are expressed in double cones and there is evidence that double cones are involved in all of these tasks (McFarland and Munz, 1975; Osorio and Vorobyev, 2005; Pignatelli et al., 2010). As *RH2-1* is a middle wavelength-sensitive opsin, with a maximal absorption of around 519–528 nm (based upon λ_{max} values from *in vitro* reconstitution in *Oryzias latipes* and *Oreochromis niloticus*, which share the same key site haplotype as the guppy), its prevalence in the ventral retina (used for upward viewing) is consistent with the observation that many guppy environments are streams with thick rainforest cover, where the predominant wavelengths are 500–600 nm (Endler, 1993). Thus, the *SWS2* pigments expressed in the ventral retina could act as offset pigments (McFarland and Munz, 1975) and the middle wavelength (*RH2-1*) pigments would be matched to the background light. This combination of pigments in the ventral retina would maximize the contrast of bright and dark objects against the background (McFarland and Munz, 1975). Therefore, a reduction in the number of *LWS*-expressing cones in the ventral retina coinciding with an increase in *RH2-1* expression appears to be a mechanism for tuning to the background light, thereby increasing the contrast of objects viewed against the background. A similar scenario has been suggested in the archerfish (*T. chatareus*) (Temple et al., 2010).

Intra-retinal variation in opsin expression may affect wavelength discrimination in the guppy. Wavelength discrimination requires the integration of signals from multiple cone cells with different spectral sensitivities. The guppy's dorsal retina, which expresses up to nine cone opsins (*versus* only five for the ventral retina), has the potential for increased wavelength discrimination relative to the ventral retina. However, to test this hypothesis it will be necessary to determine whether these opsins are being expressed in different cell types or whether they are co-expressed (e.g. using *in situ* hybridization with dual labelling or single-cell quantitative PCR, qPCR). The co-expression of more than one opsin in a single cone cell has been suggested in another fish species (Temple et al., 2010) and demonstrated in other vertebrates [e.g. the Siberian hamster

(Lukáts et al., 2002)]. Additionally, in the ventral retina, middle wavelength hue discrimination may be increased as a result of the reduction of *LWS* gene expression, allowing for more heterogeneous pairings of *RH2-1* and *RH2-2* in double cones. Intra-retinal variation in opsin expression may also affect wavelength sensitivity in the guppy. An increase in *LWS* opsin expression in the dorsal retina is likely to improve sensitivity to long wavelength light (~550–700 nm) relative to the ventral retina. These intra-retinal patterns may play a role in female mate evaluation during courtship displays, given their potential effects on wavelength discrimination and/or sensitivity to long wavelength light. During mate selection, male guppies have been noted to display in front of and below females (Baerends et al., 1955). Females appear to evaluate males with their dorsal retina, which expresses at least three and possibly all four *LWS* opsins; thus, it is tempting to speculate that this area is specialized to assess male quality, and/or that male courtship displays have evolved to take advantage of this photoreceptor distribution. Behavioural studies tailored to opsin distribution and *in vitro* reconstruction of these opsins will be the next step in understanding how the present findings affect the visual capacity of the guppy.

In our survey we also found that *LWS* gene expression varies among individuals. This finding is consistent with guppy MSP studies that have shown among-individual variation in the cone cell absorbance of longer wavelength-sensitive cones (529–579 nm). However, it is important to note that this MSP work did not account for location and could reflect the dorsal–ventral variation in *RH2-1* and *LWS* opsin expression (Archer et al., 1987; Archer and Lythgoe, 1990). Individual variation in retinal spectral sensitivity may have implications for female mate choice, as perceived brightness (e.g. of orange spots) is dependent upon the *LWS* opsins present. *LWS P180* has three key site mutations, each known to shift the sensitivity to shorter wavelengths (Yokoyama and Radlwimmer, 1998; Davies et al., 2009). Thus, one male with spots that reflect light at the exact wavelength of the *LWS P180* opsin may be perceived as being bright to a female expressing the *LWS P180*, but as slightly less bright or as having less contrast relative to the background to a female only expressing *LWS S180*. As orange brightness, or chroma, is a major determinant in female mate choice (Endler, 1980; Endler, 1983; Houde, 1988; Houde and Endler, 1990; Blows and Brooks, 2003), this inter-individual variation in *LWS* opsin expression may affect mate choice. Furthermore, the polymorphic expression levels of *LWS* opsins may function to maintain or select for variation in male colouration found in guppies (Houde, 1988; Houde and Endler, 1990). The effect of this inter-individual variation on *LWS* opsin expression in males is unclear. Mate choice tests should be designed to investigate the functional implications of inter-individual variability in *LWS* expression.

The opsin expression patterns characterized in this study reveal a complicated and more complete picture of guppy vision at the molecular level. The intra-retinal variation of opsin expression revealed is likely to be a general trend in fish vision, which has often been overlooked by opsin expression analysis, because of the limitations of whole-eye qPCR. Individual level variation reminds us that beauty may truly be in the eye of the beholder, or at least in some portions thereof.

APPENDIX

Supplementary methods

Probe specificity

The level of probe cross-hybridization was assessed by dot blot analysis. Digoxigenin-labelled anti-sense riboprobe aliquots were allowed to hybridize to sense riboprobes from each locus, blotted and cross-linked to a nitrocellulose membrane. Pre-hybridization took place for 30 min at 68°C in DIG Easy Hyb Solution (Roche). Probes diluted in DIG Easy Hyb were then applied to the membrane and hybridized overnight at 68°C in a hybridization oven (UVP). The membrane was blocked for 2 h and an anti-DIG-AP antibody was used. The antibody was detected using CSPD chemi-luminescent substrate (Roche) and the film exposure time for all blots was 1 h.

Results of dot blot analysis

The dot blot data indicated that the *LWS A/S180* probe could bind to a concentrated *LWS P180* template and that the *LWS P180* probe could bind to a concentrated *LWS A180* template. All other probes bound only to their intended targets in our dot blot experiments. Additionally, the *in situ* experiments (sections and whole-mounts) showed that the *LWS A/S180* probe labelled many cells that the *LWS P180* probe did not, suggesting that cross-hybridization among *LWS* probes was rare or non-existent under the experimental conditions of *in situ* hybridization.

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