The Journal of Experimental Biology 216, 656-667

doi:10.1242/jeb.078840

## RESEARCH ARTICLE

# Pronounced heritable variation and limited phenotypic plasticity in visual pigments and opsin expression of threespine stickleback photoreceptors

Iñigo Novales Flamarique[1,*], Christiana L. Cheng[1,†], Carolyn Bergstrom[1,‡] and Thomas E. Reimchen[2]

[1]Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada V3J 4M5 and [2]Department of Biology, University of Victoria, Victoria, BC, Canada V8W 3N5

*Author for correspondence (inigo@sfu.ca)

†Present address: Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

‡Present address: Department of Biology, University of Alaska Southeast, Juneau, AK 99801, USA

## SUMMARY

**Vertebrate colour vision is mediated by the differential expression of visual pigment proteins (opsins) in retinal cone photoreceptors. Many species alter opsin expression during life, either as part of development or as a result of changes in habitat. The latter, a result of phenotypic plasticity, appears common among fishes, but its cellular origin and ecological significance are unknown. Here, we used adult threespine stickleback fish from different photic regimes to investigate heritable variability and phenotypic plasticity in opsin expression. Fish from clear waters had double cones that expressed long (LWS) and middle (RH2) wavelength opsins, one per double cone member. In contrast, fish from red light-shifted lakes had double cones that were >95% LWS/LWS pairs. All fish had single cones that predominantly expressed a short wavelength (SWS2) opsin but ultraviolet cones, expressing a SWS1 opsin, were present throughout the retina. Fish from red light-shifted lakes, when transferred to clear waters, had a ~2% increase in RH2/LWS double cones, though double cone density remained constant. Comparison of visual pigment absorbance and light transmission in the environment indicated that the opsin complements of double cones maximized sensitivity to the background light, whereas single cones had visual pigments that were spectrally offset from the dominant background wavelengths. Our results indicate that phenotypic plasticity in opsin expression is minor in sticklebacks and of questionable functional significance.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/4/656/DC1

Key words: light environment, spectral cone type, retina, colour vision, *Gasterosteus aculeatus*.

Received 2 August 2012; Accepted 11 October 2012

## INTRODUCTION

Cone photoreceptors are specialized cells of the retina whose visual pigments capture light to begin the process of photopic vision (Fein and Szuts, 1982). Each visual pigment is composed of a protein (opsin) and a chromophore (a vitamin A derivative). There are four major classes of cone opsins (SWS1, SWS2, RH2 and MWS/LWS) and one rod opsin (RH1) (Yokoyama, 2000). When combined with the chromophore, these opsins form visual pigments with absorbance maxima ($\lambda_{max}$) in the ultraviolet (SWS1), short (SWS2), middle (RH2, MWS, RH1) or long (LWS) wavelength regions of the spectrum (Yokoyama, 2000; Isayama and Makino, 2012). Besides opsin type, visual pigment absorbance can be modulated by the choice of chromophore: coupling of an opsin to the vitamin A1 chromophore, 11-*cis* retinal, leads to a lower $\lambda_{max}$ than combination with the vitamin A2 chromophore, 3-dehydro, 11-*cis* retinal (Isayama and Makino, 2012). In most vertebrates, the different visual pigments are distributed in morphologically distinct cone types, and comparison of neural outputs from these cells forms the basis for the perception of colour (Fein and Szuts, 1982). Thus, the molecular constituents of visual pigments are primary substrates for the genetic and environmental modulation of colour vision.

Many non-mammalian vertebrates modulate chromophore usage during life, and this often results from migrations to new photic habitats, as with fishes (Beatty, 1984; Novales Flamarique, 2005), or following metamorphic transitions, as with amphibians (Isayama and Makino, 2012). Modulation of opsin expression has also been documented during development (Wood and Partridge, 1993; Szél et al., 1994; Xiao and Hendrickson, 2000; Cheng and Novales Flamarique, 2004; Takechi and Kawamura, 2005; Spady et al., 2006; Hoke et al., 2006; Cheng et al., 2007; Applebury et al., 2007; Shand et al., 2008; Cottrill et al., 2009) and, to a lesser extent, following changes in habitat (Fuller et al., 2005; Shand et al., 2008; Cottrill et al., 2009; Hofmann et al., 2010; Fuller and Claricoates, 2011). Studies using fishes and rodents have demonstrated that opsin switching within individual cones is a major mechanism that re-structures the chromatic organization of the retina and the colour sensitivity of these animals during development and at early life stages (Szél et al., 1994; Cheng et al., 2006; Cheng et al., 2007; Cheng and Novales Flamarique, 2007; Novales Flamarique, 2013). By contrast, the mechanism(s) underlying, and functional significance of, environmentally induced changes in opsin expression are unknown. Despite the lack of this crucial information, such phenotypic plasticity is commonly assumed to be ecologically relevant (Fuller and Claricoates, 2011).

Studies that report phenotypic plasticity in opsin expression have relied on quantification of opsin transcripts from whole retinas using real-time PCR (Fuller et al., 2005; Shand et al., 2008; Cottrill et al., 2009; Hofmann et al., 2010; Fuller and Claricoates, 2011), a

procedure that does not reveal the cellular patterns underlying opsin changes. Microspectrophotometry, the other technique employed in these studies, can only be used to support correlative trends between cone types and opsin expression as sample size is extremely small, in comparison with photoreceptor numbers in the retina (Fein and Szuts, 1982), and most studied animals have topographical variation in both opsin expression and photoreceptor types across the retina (see Hárosi and Novales Flamarique, 2012). In the above studies (Fuller et al., 2005; Shand et al., 2008; Hofmann et al., 2010; Fuller and Claricoates, 2011), differences in opsin expression are assumed to reflect changes in the abundance of the various cone types or differences in the size and/or visual pigment density of cone outer segments (Fuller and Claricoates, 2011), the ciliary structures whose lamellae house the visual pigments (Fein and Szuts, 1982). These studies further assume that the observed changes in opsin expression reflect fundamental alterations to the colour vision of the animal (Fuller and Claricoates, 2011).

The physiological literature, however, casts doubts on many of these assumptions. First, photoreceptor visual pigment density is likely to be at an optimum for proper lamellar packing and functionality of the phototransduction cascade, with overexpression of opsin resulting in expansion of lamellar volume and disrupted kinetics of the light response (Wen et al., 2009). It is therefore unlikely that visual pigment density would increase as a result of experiencing new photic environments in nature. Second, changes in outer segment morphology have only been reported following long-term exposure to artificial light environments (e.g. monochromatic, very bright light) (Kröger et al., 1999; Williams et al., 1999) that have no equivalent in nature. In contrast, migrations to new environments do not induce changes in cone outer segment morphology (e.g. size, taper) in salmonid fishes, and we are not aware of reports to the contrary for other animals featured in the literature (Hárosi and Novales Flamarique, 2012). It thus seems that the reported phenotypic plasticity in opsin expression of fishes is probably based on changes in the relative numbers of spectral cone types. Such alterations in cell numbers can arise *via* the production and/or pruning of photoreceptors, as occurs in several species (Cornish et al., 2004; Cheng et al., 2006; Hoke et al., 2006; Chen et al., 2008; Hu et al., 2011), or as a switch in the opsin expressed within individual photoreceptors, as has been demonstrated in the rods of the European eel (Wood and Partridge, 1993) and in the cones of salmonid fishes and rodents (Szél et al., 1994; Cheng et al., 2006, Cheng and Novales Flamarique, 2007; Glaschke et al., 2011). The importance of opsin phenotypic plasticity in colour sensitivity, and visual function in general, would then depend on the number and types of cones affected and their retinal distributions. It is the topography of these cells, and their relative outputs, that are primary determinants of visual acuity and the colour contrast detection capabilities of the retina (Fein and Szuts, 1982).

We have chosen the threespine stickleback, *Gasterosteus acuelatus*, to study genetic (heritable variability) and environmental (phenotypic plasticity) changes in opsin expression and their ecological significance. Literature on stickleback visually guided behaviour dates back to the 1830s (Rowland, 1994) and this fish has long been a model of ecology and evolutionary research to explore the role of colour in sexual selection and speciation (Rowland, 1994; Boughman, 2001; Smith et al., 2004; Rowe et al., 2004; Albert et al., 2007; Boulcott and Braithwaite, 2007; Rick and Bakker, 2008). In addition to the well-established role of colour in stickleback behaviour, this species is found in waters that expand the range of photic regimes, from dystrophic lakes dominated by long (red) wavelength light (Reimchen, 1989; McDonald and Hawryshyn, 1995; McDonald et al., 1995) to mesotrophic lakes and coastal waters characterized by a full visual spectrum (ultraviolet to red) in surface waters and peak transmission in the middle (green) wavelengths (Novales Flamarique et al., 1992; Novales Flamarique and Hawryshyn, 1993; Novales Flamarique and Hawryshyn, 1997; McDonald and Hawryshyn, 1995; McDonald et al., 1995). Thus, comparison of endemic and displaced sticklebacks from various photic regimes can be used to assess heritable variation and phenotypic plasticity in opsin expression, both components assumed important to the visual ecology of this animal and to the evolutionary divergence between populations (Boughman, 2001; Smith et al., 2004).

In this study, we examined the topographical distribution of opsins, visual pigments and photoreceptor types in adult retinas of seven populations of sticklebacks living in photic habitats ranging from red-stained to clear waters. We then compared the retinas of individuals from dystrophic populations after 14 years and within 6 months of transfer to clear waters to evaluate phenotypic plasticity in opsin expression and its photoreceptor origin.

## MATERIALS AND METHODS

### Animals and sampling locations

Wild adult threespine stickleback (*G. acuelatus* L.) were obtained from seven locations in British Columbia (Canada): Doogan's Lake, Swan Lake, Mayer Lake, Mayer Pond, Drizzle Lake, Drizzle Pond and the Broughton Archipelago (coastal ocean) (Fig. 1). All specimens were in reproductive condition except for those originating from the Broughton Archipelago. Mayer Lake and Drizzle Lake are dystrophic lakes, while Mayer Pond and Drizzle Pond are small mesotrophic ponds. No sticklebacks existed in these ponds until 1993 and 1997, when two of us (C.B. and T.E.R.) transferred sticklebacks from Mayer Lake and Drizzle Lake to Mayer Pond and Drizzle Pond, respectively (Leaver and Reimchen, 2012). Swan Lake is a high productivity, eutrophic, system with large mats of green algae present at various times of the year, but especially during the spring and summer. Doogan's Lake and the oceanic location are mesotrophic systems, characterized by low to medium productivity and clear waters. The dystrophic lakes in this study have no transmission below 500 nm at 1 m depth and no light at 4 m depth (McDonald et al., 1995). The mesotrophic lakes and ocean are characterized by a full light spectrum in surface waters (320–750 nm) and peak transmission in the range 560–565 nm with depth [see comparable systems in previous publications (Novales Flamarique et al., 1992; Novales Flamarique and Hawryshyn, 1993; McDonald and Hawryshyn, 1995; McDonald et al., 1995)]. We measured downwelling and sidewelling light at all locations at 40 cm depth (Fig. 1) using a USB-2000 spectroradiometer equipped with a 600 μm diameter input, 0.22 NA, liquid light guide and a cosine collector (Ocean Optics, Dunedin, FL, USA). The sidewelling light was the average of two scans in the sun and anti-sun directions. Scans were acquired between 11:00 h and 12:30 h in June, with the sun's elevation at ~60 deg. The 40 cm depth is close to the middle of the range (0.1–1 m) at which stickleback nests are found during the breeding season in these water bodies.

Sticklebacks caught in the various locations were either killed on-site, under light adaptation, by quick spinal bisection and decerebration and the retinas fixed for histochemical analyses, or transported live to the aquatic facility of Simon Fraser University (SFU) where they were maintained for up to 6 months. Fish transported to SFU were housed in large aquaria containing water from their locations of origin, or placed in clear water of the same temperature and pH as the source water. Illumination was a 12 h:12 h light:dark cycle provided by tungsten–halogen fluorescent tubes.

Fish were killed upon arrival to SFU for visual pigment measurements and at 3 weeks and 6 months after arrival for histochemical analyses. Supplementary material Table S1 shows the numbers of fish used for the different types of experiments. All experimentation was approved by the Animal Care Committee of SFU, in compliance with guidelines set by the Canadian Council for Animal Care.

### Generation of riboprobes and sequencing of opsins

Opsin partial cDNAs were generated by RT-PCR amplification from total RNA isolated from homogenized retina of adult sticklebacks (RNAqueous-Midi; Ambion, Austin, TX, USA). The cDNAs were synthesized (Ready-To-Go RT-PCR beads, Amersham Biosciences, GE Healthcare, Saskatoon, SK, Canada) using the following primers: SWS1 opsin partial cDNA forward 5′-ATG GGG AAA CAC TTC CAC TT-3′, reverse 5′-TGG AAC AAC AGA TTC CCA TA-3′; SWS2 opsin partial cDNA forward 5′-TCA GCT GAC AAT CAC ATA AA-3′, reverse 5′-AGC CAT CTT TCA AAA GCT AT-3′; RH2 opsin partial cDNA forward 5′-TGG ACA CTT CAC CAC TAT CT-3′, reverse 5′-TGA GTA CCA GAG AAC TTG AA-3′; and LWS opsin partial cDNA forward 5′-TTG CAA GTG ACG AGA AAG GT-3′, reverse 5′-TCC AGG AGA ACA CTA TTC CA-3′. Reverse transcription was carried out at 42°C for 15 min. Cycling parameters for the subsequent PCR were: 95°C × 5 min, 32 cycles of 95°C × 30 s, 56°C × 30 s, and 72°C × 1 min, and 1 cycle of 72°C × 10 min. Each cDNA was gel purified and cloned into a pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced by AmpliTaq Dye terminator cycle sequencing (UBC Sequencing Laboratory). The identities of the sequences were confirmed by comparing them to nucleotide sequence databases using the BLASTn program (Altschul et al., 1997). To make each cRNA probe, a PCR fragment containing the insert and an RNA promoter amplified from the pCRII-TOPO vector were used to generate digoxigenin- and fluorescein-labelled RNA sense and antisense riboprobes by *in vitro* transcription (RNA labelling kit, Roche Diagnostics, Laval, QC, Canada).

To compare RH2 and LWS opsin sequences between stickleback populations, full-length opsin cDNAs were generated as described above using the following primers: RH2 opsin partial cDNA forward 5′-TGG ACA CTT CAC CAC TAT CT-3′, reverse 5′-TTA AGA CAC AGA GGA CAC TT-3′; LWS opsin partial cDNA forward 5′-TTG CAA GTG ACG AGA AAG GT-3′, reverse 5′-TTA TGC AGG AGC CAC AGA-3′. The deduced amino acid sequences were compared using ClustalW2, a multiple sequence alignment program (Larkin et al., 2007).

### *In situ* hybridization

Fish were killed in the light-adapted state. The left eyeball was removed, the iris and lens discarded, and the remaining eyecup, which had been marked on the ventral and nasal retina, immersed in cryo-fixative (4% paraformaldehyde in 0.08 mol l$^{-1}$ phosphate buffer, pH 7.4). After a minimum 24 h fixation at 4°C, the retina was extracted and flattened on a grid while maintaining its natural orientation. This procedure ensured the analysis of similar pieces of retina between individuals. Each retina was then cut into eight pieces, and each piece embedded in OCT frozen blocks for *in situ* hybridization as per previous studies (Cheng et al., 2006; Cheng and Novales Flamarique, 2007). Sections (8 μm thick) obtained from the blocks were collected serially and deposited on alternating slides for simultaneous double-labelling experiments with riboprobes against the SWS1 and SWS2, and the RH2 and LWS opsin transcripts, respectively. Details of the *in situ* hybridization protocol have been published previously (Cheng et al., 2006). Digital images of sections were acquired with an E-600 Nikon fluorescence microscope equipped with DIC optics and a DXM-100 digital camera. The images were used to characterize cone types and to compute the percentage of cones that expressed each opsin mRNA. The densities of double and single cones were counted over a 17,926 μm$^2$ area using a grid system on the computer monitor, and the ratio of double to single cones was computed for each sector of the retina.

### Histology

The eyecup from the right eyeball of the same fish used for *in situ* hybridization was immersed in primary fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.08 mol l$^{-1}$ phosphate buffer, pH 7.4). After 24 h fixation at 4°C, the retina was extracted from the eyecup, rinsed in 0.08 mol l$^{-1}$ phosphate buffer, and pieces cut using the same procedure as for *in situ* hybridization. These pieces were processed for embedding in EPON resin blocks, which were cut radially in 75 nm steps, as detailed previously (Novales Flamarique, 2011), to reveal the overall morphology and the ultrastructure of cones. Electron micrographs were used to obtain average dimensions of cone outer segments and lamellae, and lamellar spacing.

### Microspectrophotometry

Individual fish were dark adapted overnight. Following this adaptation period, the fish was killed, one eye was enucleated, and the retina was removed under infrared illumination. Small pieces of retina were teased apart and prepared for viewing with the dichroic microspectrophotometer (DMSP) as per previous studies (Novales Flamarique and Hárosi, 2000; Cheng et al., 2006; Cheng et al., 2007). The DMSP is a computer-controlled, wavelength-scanning, single-beam photometer that simultaneously records average and polarized transmitted light fluxes through microscopic samples (Novales Flamarique and Hárosi, 2000). The DMSP was equipped with ultrafluar (Zeiss) objectives: 32/0.4 for the condenser and 100/1.20 for the objective. With the aid of reference measurements recorded through cell-free areas, individual photoreceptor outer segments were illuminated sideways with a measuring beam of rectangular cross-section ~2×0.6 μm. Absolute absorbance and linear dichroism spectra were computed in 2 nm increments from the obtained transmittance (each spectrum consisted of an average of eight scans). The solid spectra (fits) were derived from experimental data by Fourier filtering (Hárosi, 1987).

## RESULTS

### Light environments

Spectral irradiance measurements from the various water bodies revealed pronounced differences in light transmission (Fig. 1). The mesotrophic systems had broad spectrum downwelling (300–750 nm) and sidewelling (350–750 nm) light, with the downwelling light being ~4–10 times as intense as the sidewelling light (Fig. 1E,F). Mayer Pond, also a mesotrophic system, had similar broad spectra but the shape of the curve was reduced in the middle to long wavelengths (Fig. 1B). Mayer Lake had a downwelling spectrum shifted toward longer wavelengths, with little downwelling to no sidewelling light below 400 nm, and a ratio of downwelling to sidewelling light of ~33 (Fig. 1A). This light environment is characteristic of dystrophic lakes and is due to the high quantities of dissolved organic matter present in the water. Swan Lake had the most distorted spectra (Fig. 1G), and the ratio of downwelling to sidewelling light was ~4, similar to that of the ocean location (Fig. 1E). Despite the large quantities of algae present in Swan Lake,

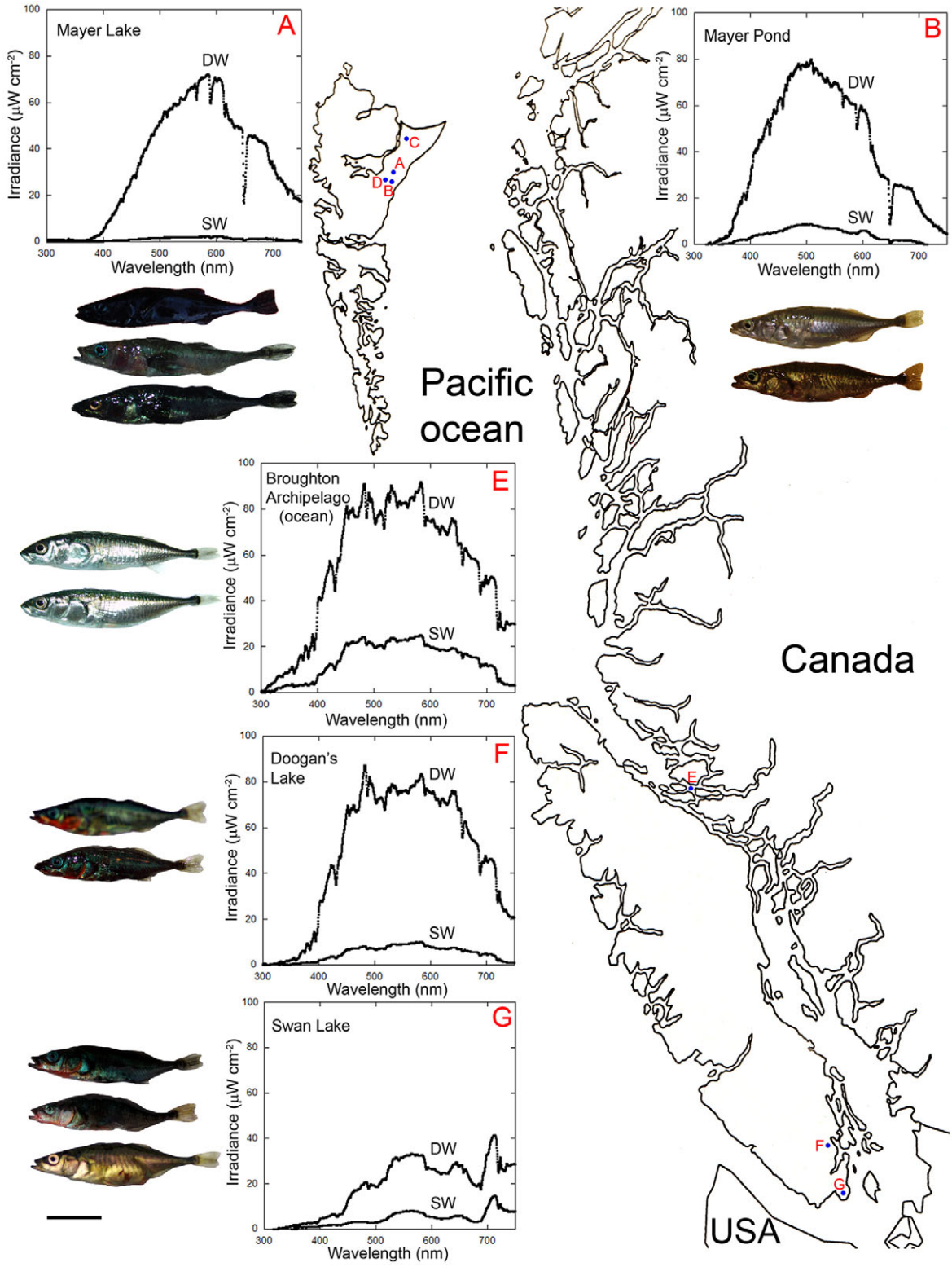


Fig. 1. Map of the stickleback locations studied, including images of representative sticklebacks and light transmission spectra. Locations are designated by red letters and blue spots on the map, corresponding to: (A) Mayer Lake; (B) Mayer Pond; (C) Drizzle Lake; (D) Drizzle Pond; (E) Broughton Archipelago (coastal ocean); (F) Doogan's Lake; and (G) Swan Lake. Spectral irradiance scans of transmitted light in the downwelling (DW) and sidewelling (SW) directions at 40 cm depth are shown for representative water types (i.e. dystrophic, mesotrophic and eutrophic). The data for Drizzle Lake and Drizzle Pond were similar to those from Mayer Lake and Mayer Pond, respectively. The letters on the scans correspond to those on the map. Next to each scan are images of representative adult sticklebacks collected at the corresponding locations. The bottom stickleback associated with Swan Lake is a reproductive female; the rest of the fish in the figure are males. The magnification bar at the bottom left of the figure represents 2.3 cm for the sticklebacks and 42 km for the map.

a broad spectrum of downwelling light (320–750 nm) penetrated surface waters (Fig. 1G).

### Visual pigments and opsin sequences

Microspectrophotometry revealed four cone visual pigment types located within double and single cones, and one rod visual pigment present in the rods (Fig. 2). Single cones had either an ultraviolet (UV) or a short wavelength (S) visual pigment, with mean $\lambda_{max}$ in the range 365–379 nm and 434–460 nm, respectively (Table 1). Double cones had either a long wavelength (L) visual pigment in both double cone members (mean $\lambda_{max}$ range: 566–618 nm), as was predominantly the case in fish from dystrophic lakes or those that had been transferred into clear waters, or an L visual pigment in one member and a middle wavelength (M) visual pigment in the other (mean $\lambda_{max}$ range: 514–547 nm), as was always the case in fish from the remaining water bodies (Table 1). Rods contained a rhodopsin visual pigment with mean $\lambda_{max}$ in the range 507–531 nm. The mean $\lambda_{max}$ values measured for the Broughton Archipelago (ocean) population are consistent with $\lambda_{max}$ values of vitamin A1-conjugated visual pigments in other fish species (see Hárosi, 1994). None of the outer segments examined showed multiple α-band absorbance peaks, which would indicate multiple visual pigments within a photoreceptor (see Cheng et al., 2006; Cheng et al., 2009).

We used semi-empirical models (Hárosi, 1994) to predict the $\lambda_{max}$ shifts of visual pigments when the opsin remains the same but the chromophore is switched from vitamin A1 to vitamin A2. These calculations revealed the following visual pigment A1–A2 pairs, computed based on the $\lambda_{max}$ of vitamin A1-based visual pigments measured: 365–382 nm (UV), 434–441 nm (S), 514–546 nm (M), 566–638 nm (L) and 507–535 (rod). With the exception of the S pigment, the absorbance range of all other visual pigments could be accounted for by variable use of vitamin A1 and A2 chromophores within individual photoreceptors. The lower and upper extremes in the visual pigment absorbance ranges originated from cones of non-reproductive and reproductive fish, respectively. These denote retinas based primarily on vitamin A1 or A2 chromophore, as occurs in other non-mammalian vertebrates at different life stages or seasonally (Beatty, 1984; Novales Flamarique, 2005; Hárosi, 1994; Isayama and Makino, 2012).

Although the proportions of the two chromophores varied between populations, and between photoreceptors within populations, our results indicate vitamin A2-dominated retinas in dystrophic systems and vitamin A1-dominated retinas in the clear waters of the Broughton Archipelago (ocean) (Table 1). Such variation in chromophore use is in line with that found in a related species, the nine-spined stickleback (*Pungitus pungitus*), where the absorbance of all visual pigments (except S) could be accounted for by chromophore shifts (Saarinen et al., 2012). It therefore appears that, in both stickleback species, chromophore use varies between photoreceptor types and multiple SWS2 opsins may be expressed within the single cone population.

Sequencing of the LWS and RH2 opsins, corresponding to the L and M visual pigments (Fig. 2), which showed the greatest ranges in $\lambda_{max}$ (Table 1), revealed only three amino acid differences in the LWS opsins and nine amino acid differences in the RH2 opsins between populations from dystrophic and mesotrophic systems (Figs 3, 4). The differences among the LWS opsins involved three amino acid substitutions (Fig. 3), two located in the N-terminus and one in the fifth transmembrane helix. None of these changes were within proximity of the chromophore and, as such, should be functionally inconsequential (Smith, 2010). In addition, none of these alterations occurred at any of the five key sites that produce major shifts in visual

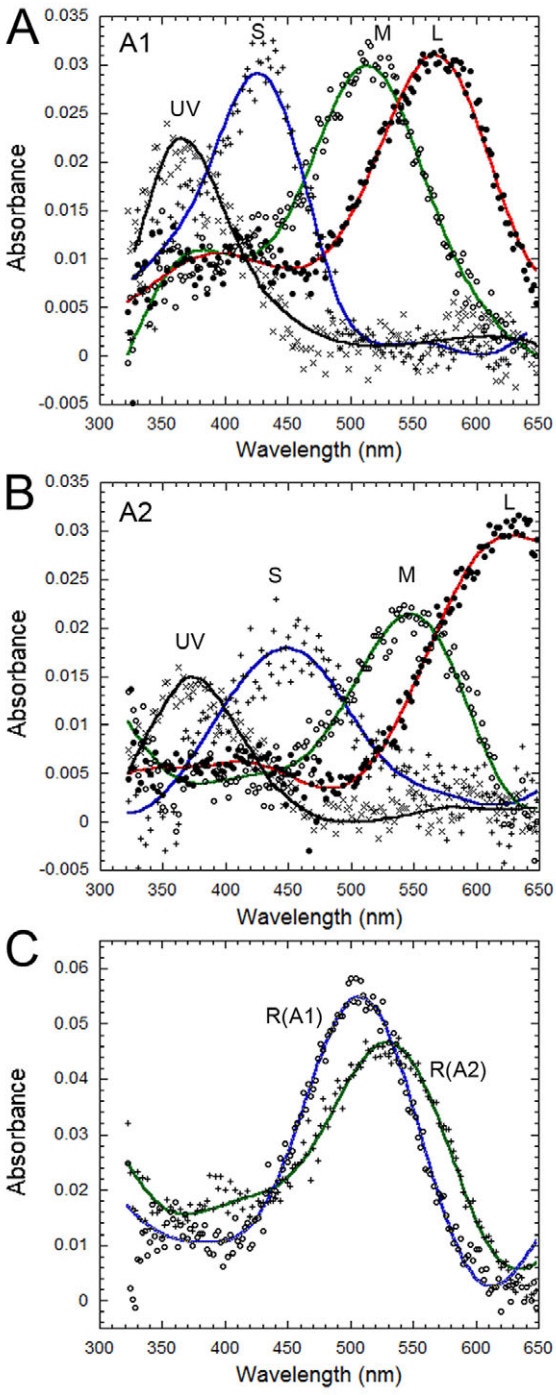


Fig. 2. Representative absorbance spectra of photoreceptors in adult stickleback retina [spectra are means from 3–7 photoreceptors per visual pigment, from 3–5 fish from the Broughton Archipelago (A) and Mayer Lake and Drizzle Lake (B)]. (A,B) Single cones contained a visual pigment maximally sensitive to ultraviolet light (UV) or to short wavelength light (S). Double cones had a visual pigment maximally sensitive to long wavelength light (L) in both double cone members, or one member contained an L visual pigment whereas the other housed a visual pigment maximally sensitive to middle wavelength light (M). Spectra in A correspond to vitamin A1-based visual pigments while those in B correspond to vitamin A2-based visual pigments (see Hárosi, 1994). (C) Rod visual pigments (R), maximally sensitive to middle wavelength light. Visual pigments based on the vitamin A2 chromophore had spectra that were broader and shifted toward longer wavelengths with respect to those from the corresponding vitamin A1-based visual pigments.

Table 1. Stickleback visual pigments

| Photoreceptor parameter | Mayer Lake | Drizzle Lake | Swan Lake | Mayer Pond | Drizzle Pond | Doogan's Lake | Broughton Archipelago |
|---|---|---|---|---|---|---|---|
| UV | | | | | | | |
| $\lambda_{max}$ | 379±6 (*N*=4) | 377 (*N*=2) | 373 (*N*=1) | 372±5 (*N*=3) | 372 (*N*=1) | 369 (*N*=1) | 365±5 (*N*=3) |
| HBW | 4495±612 | 4803 | | 4387±736 | | | 4287±706 |
| S | | | | | | | |
| $\lambda_{max}$ | 460±5 (*N*=11) | 459±5 (*N*=6) | 449±8 (*N*=4) | 447±7 (*N*=8) | 455±6 (*N*=8) | 445±9 (*N*=5) | 434±3 (*N*=14) |
| HBW | 4913±541 | 4795±718 | 4664±518 | 4695±523 | 4680±603 | 4752±702 | 4839±490 |
| M | | | | | | | |
| $\lambda_{max}$ | 547±7 (*N*=6) | | 543±10 (*N*=14) | 543±8 (*N*=5) | | 526±10 (*N*=11) | 514±8 (*N*=26) |
| HBW | 4681±398 | | 4354±455 | 4496±495 | | 4165±372 | 4418±340 |
| L | | | | | | | |
| $\lambda_{max}$ | 616±10 (*N*=32) | 618±8 (*N*=21) | 613±12 (*N*=16) | 603±11 (*N*=27) | 609±12 (*N*=14) | 593±7 (*N*=17) | 566±8 (*N*=22) |
| HBW | 3734±457 | 3655±573 | 3746±298 | 3611±542 | 3497±410 | 3506±525 | 3618±363 |
| Rod | | | | | | | |
| $\lambda_{max}$ | 531±7 (*N*=15) | 530±6 (*N*=18) | 521±7 (*N*=6) | 524±4 (*N*=9) | 527±4 (*N*=13) | 518±8 (*N*=13) | 507±5 (*N*=10) |
| HBW | 4743±369 | 4409±369 | 4543±353 | 4410±403 | 4385±291 | 4385±369 | 4250±276 |

Data are means ± s.d.

The opsins expressed by the various photoreceptors belong to the following gene families: SWS1 (UV), SWS2 (S), RH2 (M), LWS (L) and RH1 (rod). The Broughton Archipelago (ocean) specimens were not in reproductive condition. One reproductive adult collected in another oceanic location near Richmond (BC, Canada) had cone visual pigments with average $\lambda_{max}$ at 366 nm (UV), 445 nm (S), 536 nm (M) and 603 nm (L).

$\lambda_{max}$ is the wavelength of maximum absorbance and HBW is the bandwidth at half-maximum. The number of photoreceptors examined, *N*, is given in parentheses and applies to the corresponding HBW. The missing data are due to a lack of measurements or reliable spectra.

pigment absorbance (Yokoyama et al., 2008). Comparison of RH2 opsin sequences also showed conservation of amino acids at the five key sites between stickleback populations (Fig. 4). Except for position 114, all the differences between sequences involved amino acids belonging to the same, weakly charged, group (Smith, 2010). At position 114, Swan Lake fish had a glutamate (E), a negatively charged amino acid, whereas Mayer Lake and Broughton Archipelago fish had a glycine, a non-polar residue (Fig. 4). Position 114, however, is

```
Mayer Lake          MAEEWGKQAFAARRYN---EDTTRGSMFVYTNSNNTKDPFEGPNYHIAPRWVYNLSTLWM 57
Mayer Pond          MAEEWGKQAFAARRYN---EDTTRGSMFVYTNSNNTKDPFEGPNYHIAPRWVYNLSTLWM 57
Drizzle Lake        MAEEWGKQAFAARRYN---EDTTRGSMFVYTNSNNTKDPFEGPNYHIAPRWVYNLSTLWM 57
Drizzle Pond        MAEEWGKQAFAARRYN---EDTTRGSMFVYTNSNNTKDPFEGPNYHIAPRWVYNLSTLWM 57
Swan Lake           MAEEWGKQAFAARRYN---EDTPRGSMFVYTNSNNTKDPFEGPNYHIAPRWVYNLSTLWM 57
Broughton Ocean     MAEEWGKQAFAARRYN---DDTTRGSMFVYTNSNNTKDPFEGPNYHIAPRWVYNLSTLWM 57
Human LWS           MAQQWSLQRLAGRHPQDSYEDSTQSSIFTYTNSNSTRGPFEGPNYHIAPRWVYHLTSVWM 60

Mayer Lake          FIVVALSVFTNGLVLVATAKFKKLQHPLNWILVNLAIADLGETVFASTISVCNQFFGYFI 117
Mayer Pond          FIVVALSVFTNGLVLVATAKFKKLQHPLNWILVNLAIADLGETVFASTISVCNQFFGYFI 117
Drizzle Lake        FIVVALSVFTNGLVLVATAKFKKLQHPLNWILVNLAIADLGETVFASTISVCNQFFGYFI 117
Drizzle Pond        FIVVALSVFTNGLVLVATAKFKKLQHPLNWILVNLAIADLGETVFASTISVCNQFFGYFI 117
Swan Lake           FIVVALSVFTNGLVLVATAKFKKLQHPLNWILVNLAIADLGETVFASTISVCNQFFGYFI 117
Broughton Ocean     FIVVALSVFTNGLVLVATAKFKKLQHPLNWILVNLAIADLGETVFASTISVCNQFFGYFI 117
Human LWS           IFVVTASVFTNGLVLAATMKFKKLRHPLNWILVNLAVADLAETVIASTISIVNQVSGYFV 120

Mayer Lake          LGHPMCVFEGYVVSVCGITALWSLTIISWERWIVVCKPFGNVKFDAKWATAGIVFSWIWS 177
Mayer Pond          LGHPMCVFEGYVVSVCGITALWSLTIISWERWIVVCKPFGNVKFDAKWATAGIVFSWIWS 177
Drizzle Lake        LGHPMCVFEGYVVSVCGITALWSLTIISWERWIVVCKPFGNVKFDAKWATAGIVFSWIWS 177
Drizzle Pond        LGHPMCVFEGYVVSVCGITALWSLTIISWERWIVVCKPFGNVKFDAKWATAGIVFSWIWS 177
Swan Lake           LGHPMCVFEGYVVSVCGITALWSLTIISWERWIVVCKPFGNVKFDAKWATAGIVFSWIWS 177
Broughton Ocean     LGHPMCVFEGYVVSVCGITALWSLTIISWERWIVVCKPFGNVKFDAKWATAGIVFSWIWS 177
Human LWS           LGHPMCVLEGYTVSLCGITGLWSLAIISWERWLVVCKPFGNVRFDAKLAIVGIAFSWIWS 180

Mayer Lake          AVWCAPPIFGWSRYWPHGLKTSCGPDVFSGSEDPGVQSYMIVLMITCCLIPLAIIILCYL 237
Mayer Pond          AVWCAPPIFGWSRYWPHGLKTSCGPDVFSGSEDPGVQSYMIVLMITCCLIPLTIIILCYL 237
Drizzle Lake        AVWCAPPIFGWSRYWPHGLKTSCGPDVFSGSEDPGVQSYMIVLMITCCLIPLAIIILCYL 237
Drizzle Pond        AVWCAPPIFGWSRYWPHGLKTSCGPDVFSGSEDPGVQSYMIVLMITCCLIPLAIIILCYL 237
Swan Lake           AVWCAPPIFGWSRYWPHGLKTSCGPDVFSGSEDPGVQSYMIVLMITCCLIPLGIIILCYL 237
Broughton Ocean     AVWCAPPIFGWSRYWPHGLKTSCGPDVFSGSEDPGVQSYMIVLMITCCLIPLGIIILCYL 237
Human LWS           AVWTAPPIFGWSRYWPHGLKTSCGPDVFSGSSYPGVQSYMIVLMVTCCIIPLAIIMLCYL 240

Mayer Lake          AVWLAIRAVAMQQKESESTQKAERDVSRMVVVMIVAYIVCWGPYTTFACFAAANPGYAFH 297
Mayer Pond          AVWLAIRAVAMQQKESESTQKAERDVSRMVVVMIVAYIVCWGPYTTFACFAAANPGYAFH 297
Drizzle Lake        AVWLAIRAVAMQQKESESTQKAERDVSRMVVVMIVAYIVCWGPYTTFACFAAANPGYAFH 297
Drizzle Pond        AVWLAIRAVAMQQKESESTQKAERDVSRMVVVMIVAYIVCWGPYTTFACFAAANPGYAFH 297
Swan Lake           AVWLAIRAVAMQQKESESTQKAERDVSRMVVVMIVAYIVCWGPYTTFACFAAANPGYAFH 297
Broughton Ocean     AVWLAIRAVAMQQKESESTQKAERDVSRMVVVMIVAYIVCWGPYTTFACFAAANPGYAFH 297
Human LWS           QVWLAIRAVAKQQKESESTQKAEKEVTRMVVVMIFAYCVCWGPYTFFACFAAANPGYAFH 300

Mayer Lake          PLAAAMPAYFAKSATIYNPVIYVFMNRQFRSCIMQLFGKEVDDGSEVS-TSKTEVS---S 353
Mayer Pond          PLAAAMPAYFAKSATIYNPVIYVFMNRQFRSCIMQLFGKEVDDGSEVS-TSKTEVS---S 353
Drizzle Lake        PLAAAMPAYFAKSATIYNPVIYVFMNRQFRSCIMQLFGKEVDDGSEVS-TSKTEVS---S 353
Drizzle Pond        PLAAAMPAYFAKSATIYNPVIYVFMNRQFRSCIMQLFGKEVDDGSEVS-TSKTEVS---S 353
Swan Lake           PLAAAMPAYFAKSATIYNPVIYVFMNRQFRSCIMQLFGKEVDDGSEVS-TSKTEVS---S 353
Broughton Ocean     PLAAAMPAYFAKSATIYNPVIYVFMNRQFRSCIMQLFGKEVDDGSEVS-TSKTEVS---S 353
Human LWS           PLMAALPAYFAKSATIYNPVIYVFMNRQFRNCILQLFGKKVDDGSELSSASKTEVSSVSS 360

Mayer Lake          VAPA 357
Mayer Pond          VAPA 357
Drizzle Lake        VAPA 357
Drizzle Pond        VAPA 357
Swan Lake           VAPA 357
Broughton Ocean     VAPA 357
Human LWS           VSPA 364
```

Fig. 3. Amino acid sequence comparison between long wavelength-sensitive (LWS) opsins of various stickleback populations and humans. Yellow regions are the predicted transmembrane helical domains, blue letters indicate differences between stickleback sequences, and green letters point to the locations of the five key sites (Yokoyama et al., 2008). The amino acids at the five key sites of middle wavelength-sensitive (MWS) and LWS opsin sequences are solely responsible for the spectral tuning of these opsins (Yokoyama et al., 2008).

```
Mayer Lake      MAWEGGLE-PNGTEGKNFYIPMSN-------RTGVVRSPFEYQQYYLADPIMFKILALYM 52
Swan Lake       MAWEGGLE-PNGTEGKNFYIPMSN-------RTGVVRSPFEYQQYYLADPIMFKILALYM 52
Broughton Ocean MAWEGGLE-PNGTEGKNFYIPMSN-------RTGVVRSPFEYQQYYLADPIMFKILALYM 52
Human MWS       MAQQWSLQRLAGRHPQDSYEDSTQSSIFTYTNSNSTRGPFEGPNYHIAPRWVYHLTSVWM 60

Mayer Lake      FFLICTGTPINGLTLLVTAQNKKLRQPLNYILVNLAVAGLIMCAFGFTITITSAVNGYFI 112
Swan Lake       FFLICTGTPINGLTLLVTAQNKKLRQPLNYILVNLATVAGVIMCAFGFAITITSAVNGYFI 112
Broughton Ocean FFLICTGTPINGLTLLVTAQNKKLRQPLNYILVNLAVAGLIMCAFGFTITITSAVNGYFI 112
Human MWS       IFVVIASVFTNGLVLAATMKFKKLRHPLNWILVNLAVADLAETVIASTISVVNQVYGYFV 120

Mayer Lake      LGATACAVEGFMATLGGEVALWSLVVLAVERYIVVCKPMGSFKFSGTHAGAGVLFTWIMA 172
Swan Lake       LEATACAVEGFMATLGGEVALWSLVVLTVERYIVVCKPMGSFKFSGTHAGAGVLFTWIMA 172
Broughton Ocean LGAFACALEGFMATLGGEVALWSLVVLAVERYIVVCKPMGSFKFSGTHAGAGVLFTWIMA 172
Human MWS       LGHPMCVLEGYTVSLCGITGLWSLAIISWERWMVVCKPFGNVRFDAKLAIVGIAFSWIMA 180

Mayer Lake      MACAAPPLFGWSRYLPEGMQCSCGPDYYTLAPGFNNESYVIYMFVVHFFTPVFIIFFTYG 232
Swan Lake       MACAAPPLFGWSRYLPEGMQCSCGPDYYTLAPGFNNESYVIYMFVVHFFTPVFIIFFTYG 232
Broughton Ocean MACAAPPLFGWSRYIPEGMQCSCGPDYYTLAPGFNNESYVIYMFVVHFFTPVFIIFFTYG 232
Human MWS       AVWTAPPIFGWSRYWPHGLKTSCGPDVFSGSSYPGVQSYMIVLMVTCCITPLSIIVLCYL 240

Mayer Lake      SLVLTVKAAAAQQQESESTQKAEREVTRMCILMVFGFLVAWVPYASFAGWIFLNKGAPFS 292
Swan Lake       SLVLTVKAAAAQQQESESTQKAEREVTRMCILMVFGFLVAWVPYASFAGWIFLNKGAPFS 292
Broughton Ocean SLVLTVKAAAAQQQESESTQKAEREVTRMCVLMVFGFLVAWVPYASFAGWIFLNKGAPFS 292
Human MWS       QVWLAIRAVAKQQKESESTQKAEKEVTRMVVVMVLAFCFCWGPYAFFACFAAANPGYPFH 300

Mayer Lake      ALTAAIPAFFAKSSALYNPVIYVLLNKQFRNCMLTTIGMGGMVEDETSVS-ASKTEVSSV 351
Swan Lake       ALTAAIPAFFAKSSALYNPVIYVLLNKQFRNCMLTTIGMGGMVEDETSVS-ASKTEVSSV 351
Broughton Ocean ALTAAIPAFFAKSSALYNPVIYVLLNKQFRNCMLTTIGMGGMVEDETSVS-ASKTEVSSV 351
Human MWS       PLMAALPAFFAKSATIYNPVIYVFMNRQFRNCILQLFGK--KVDDGSELSSASKTEVSSV 358

                S----- 352
Mayer Lake      S----- 352
Swan Lake       S----- 352
Broughton Ocean SSVSPA 364
Human MWS
```

Fig. 4. Amino acid sequence comparison between RH2 opsins of various stickleback populations and the human MWS opsin. Symbols as in Fig. 3.

located in extracellular loop 1 of the stickleback RH2 opsin, which is not in the vicinity of the retinal chromophore and should therefore not influence the absorbance of the visual pigment (Smith, 2010; Yokoyama et al., 2008). Overall, these results corroborated the conclusion reached from visual pigment data that all stickleback populations studied had the same opsins.

### Cone mosaics and distributions of opsins

In accordance with the correspondence between visual pigments and morphological cone types, single cones contained a SWS1 or SWS2 opsin (Fig. 5) and double cones contained either LWS opsin or RH2 and LWS opsins, one per double cone member (Figs 6, 7). At the ellipsoid level, single and double cones formed square mosaics in the ventral retina (Fig. 5G) and mostly row mosaics in the rest of the retina (Fig. 5D). As is the case for the majority of fish species that have been studied (see Hárosi and Novales Flamarique, 2012), the square mosaic unit of the threespine stickleback consisted of four double cones forming a square, or cruciform, arrangement and a variable number of single cones, from 1 to 5 (Fig. 5A–C,G–I). Single cones were predominantly central in the mosaic unit, at the hypothetical intersection of neighbouring double cone partitions (Novales Flamarique, 2001; Cheng et al., 2006), but a few corner cones could also be found in the central and centro-dorsal areas of the retina (Fig. 5A,B). In contrast to reports on the adults of other fish species (Cheng et al., 2006; Cheng and Novales Flamarique, 2007), corner cones expressed SWS2 opsin, whereas the neighbouring centre cones expressed predominantly SWS1 opsin (Fig. 5A–F). In the ventral retina, where corner cones were absent, the remaining (centre) cones expressed primarily SWS2 opsin, though a minority expressed SWS1 opsin (Fig. 5G–I). UV cones were substantially smaller than S cones (Table 2) and showed proportionately smaller areas of mRNA expression in the cone myoids (Fig. 5J–L). All stickleback populations examined had similar distributions of UV and S cones. The highest densities of UV cones occurred in the centro-dorsal and centro-temporal areas, and S cones were always more numerous than UV cones throughout the retina (Fig. 8).

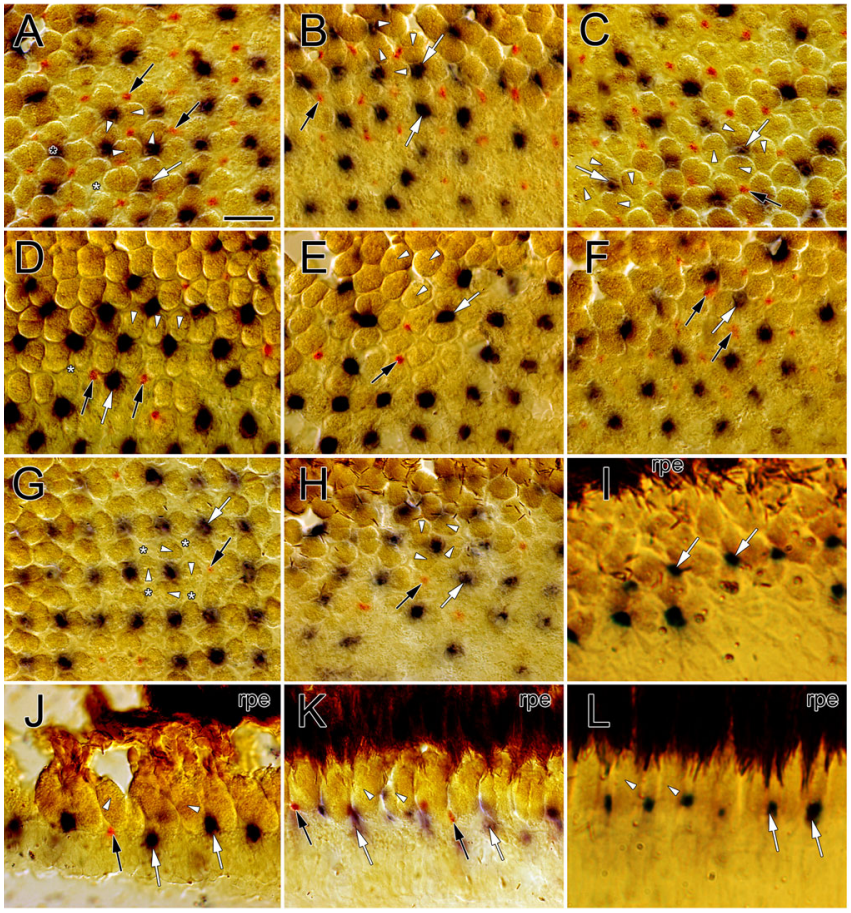


Fig. 5. Micrographs of sections from the retinas of sticklebacks following double label *in situ* hybridization with riboprobes encoding the SWS1 and SWS2 opsin mRNAs. (A–C) Transverse section through the centro-dorsal retina of sticklebacks from Mayer Lake (A), Mayer Pond (B), and Mayer Lake after 3 weeks of transplantation to full-spectrum clear waters in the laboratory (C). All the single cones, and none of the double cones, labelled with either of the two riboprobes. Most corner cones were S cones, labelling with the SWS2 riboprobe (blue colour, white arrow), while most centre cones were UV cones, labelling with the SWS1 riboprobe (red colour, black arrow). The cones formed primarily a square mosaic with double cones making the sides of the unit square (white arrowheads point to the double cone partitions). Corner cones could be present (black asterisk) or absent (white asterisk). (D–F) Transverse section through the centro-temporal retina of sticklebacks from Mayer Lake (D), Mayer Pond (E), and Mayer Lake after 3 weeks of transplantation to full-spectrum clear waters in the laboratory (F). The mosaic in this part of the retina was primarily a row. (G–I) Transverse section through the ventral retina of sticklebacks from Mayer Lake (G), Mayer Pond (H) and Swan Lake (I). Here, the cone mosaic was square and the corner cones had disappeared. The remaining single (centre) cones were predominantly S cones, but some were UV cones. (J–L) Radial section through the centro-dorsal retina of sticklebacks from Mayer Lake (J), Mayer Pond (K) and Swan Lake (L). The UV cones were noticeably smaller than the S cones. rpe, retinal pigment epithelium. Scale bar in A represents 10 μm and holds for all panels except I, where it represents 12.5 μm.

Table 2. Microspectrophotometry-derived parameters for stickleback cones from fish inhabiting the range of photic environments examined

| Cone | $A_{max}$ | $R$ | $d$ (μm) | $S_{\perp}$ (μm$^{-1}$) | $l_{os}$ (μm) |
|---|---|---|---|---|---|
| UV ($N$=7) | 0.018±0.0033 | 1.7±0.13 | 1.3±0.068 | 0.018±0.0031 | 7.1±1.9 |
| S ($N$=15) | 0.025±0.0042 | 1.8±0.14 | 2.1±0.073 | 0.015±0.0024 | 8.4±1.6 |
| M ($N$=21) | 0.025±0.0050 | 1.7±0.12 | 2.9±0.084 | 0.011±0.0025 | 11±2.3 |
| L ($N$=23) | 0.030±0.0015 | 1.6±0.09 | 2.9±0.079 | 0.013±0.0017 | 11±2.5 |

Data are means ± s.d.
$A_{max}$ refers to the absorbance at $\lambda_{max}$, $R$ is the dichroic ratio, $d$ is the mean outer segment diameter, $l_{os}$ is the length of the outer segment, and $S_{\perp}$ is the transverse specific density.

Double cones from dystrophic lake populations were predominantly (>95%) L/L pairs, the rest were M/L pairs (Fig. 6A,D, Fig. 7A–J, Fig. 8A). Double cones from transplanted populations, whether to mesotrophic ponds 15–20 years ago (Fig. 6B,E, Fig. 7B) or to full-spectrum holding tanks and examined at 3 weeks and 6 months post-arrival (Fig. 7C), were also overwhelmingly (>93%) L/L double cones. The ~2% increase in M cones over the entire retina following transplantation was nonetheless statistically significant (ANOVA, $F$=24.93, $P$<0.001), and *post hoc* comparison tests (Student–Newman–Keuls, Tukey, with α=0.05) revealed that M cone numbers were the same between transplanted populations, regardless of holding time, and different from the source (dystrophic lake) population. The highest increases in M cones occurred away from the central retina, primarily in the ventral and temporal regions of the retina (Fig. 6D,E, Fig. 7D,E, Fig. 8B,C), though numbers between sectors were not statistically different. The M cone population did not exhibit any discernible topographical pattern (Fig. 6A,B,D,E, Fig. 7A–J) but appeared randomly distributed throughout the retina, reminiscent of the smaller populations of UV cones, blue cones and 'green' rods in the retinas of amphibians (Chen et al., 2008). In contrast, double cones of fish from the remaining water bodies were M/L pairs, alternating in position within the mosaic unit (Fig. 6C,F, Fig. 7K,L, Fig. 8D). As in salmonid fishes (Beaudet et al., 1997; Cheng and Novales Flamarique, 2007), the highest cone densities occurred in the ventral and nasal periphery, which were the areas characterized by the lowest UV cone presence (Fig. 8).

### Cone morphology and ultrastructure

Cone dimensions of fish from the various populations were statistically the same (Table 2). Ultrastructure investigations revealed that lamellar thickness and inter-lamellar spacing were also invariant between populations, with means of 14.5 nm and 9.1 nm, respectively. In particular, these means ± s.d. for the dystrophic lake populations were 14.4±0.95 nm and 9.3±1.8 nm ($N$=63 cones, 6 fish), and 14.7±0.89 nm and 9.2±1.6 nm ($N$=75 cones, 8 fish) for the transplanted populations. These numbers, which are in the range reported for other fishes (Borwein and Hollenberg, 1973; Collin et al., 1996), indicate no changes in outer segment morphology as a result of transplantation.

## DISCUSSION

### Heritable variation in opsin expression tuned to the spectral background

Genetic variation in opsin expression between populations was restricted to the longer wavelength opsins, RH2 and LWS. Dystrophic lake populations had double cones that were overwhelmingly L/L pairs whereas fish endemic to clear water systems had M/L double cones, each member alternating in position within well-organized mosaics (Figs 6–8). There was a clear correspondence between the mean $\lambda_{max}$ of double cones, weighted by their cone fractions, and the light environments that fish inhabited. Based on our downwelling irradiance measurements and those in the literature (Novales Flamarique et al., 1992; Novales Flamarique and Hawryshyn, 1993; McDonald and Hawryshyn, 1995; McDonald et al., 1995), we computed the depth at which the integrated light power transmission median ($\lambda P_{50}$), a measure of the prevailing ambient quanta (McFarland and Munz, 1975), best approximated the weighted $\lambda_{max}$ of double cones. Surprisingly, this depth (60 cm) was the same for both dystrophic and mesotrophic systems, and was within 5 cm of the mid-range for nest deposition, an activity that marks the time when adult sticklebacks are found close to the water surface. At 60 cm depth, the mean $\lambda P_{50}$ was 610 and 565 nm for dystrophic and mesotrophic systems, respectively. These numbers are almost a match for the weighted $\lambda_{max}$ of double cones from the corresponding fish populations, with a mean of 613 nm for fish originating from dystrophic systems and 562 nm for those from mesotrophic systems. Such close correspondence, based on the vitamin A2-conjugated opsins found in reproductive fish, indicates evolutionary selection for double cone visual pigments with maximum sensitivity to the ambient light spectrum.

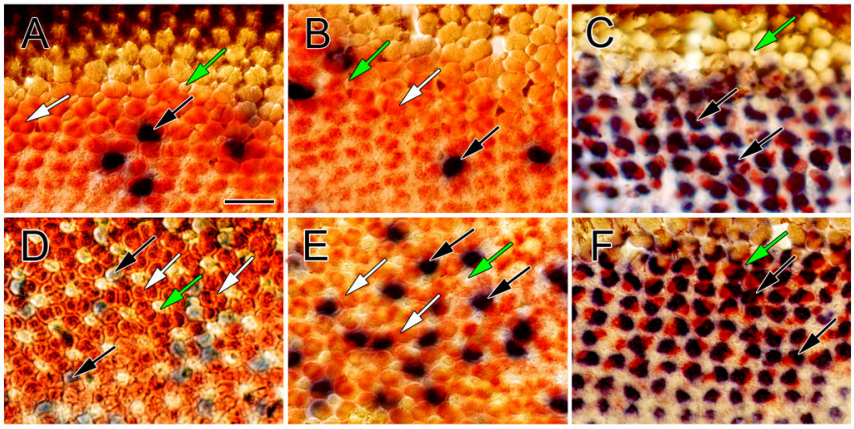


Fig. 6. Micrographs of sections from the retinas of sticklebacks following double label *in situ* hybridization with riboprobes encoding the RH2 and LWS opsin mRNAs. Dorsal (A–C) and ventral (D–F) sections from Mayer Lake (A,D), Mayer Pond (B,E) and Swan Lake (C,F). The Swan Lake population had double cones that were M/L pairs (the M member, blue colour, is designated by a black arrow); the double cones of the Mayer Lake and Mayer Pond populations were overwhelmingly L/L pairs (red colour, white arrows). Green arrows point to single cones, which were not labelled by either riboprobe. Scale bar in A represents 10 μm and holds for all panels.

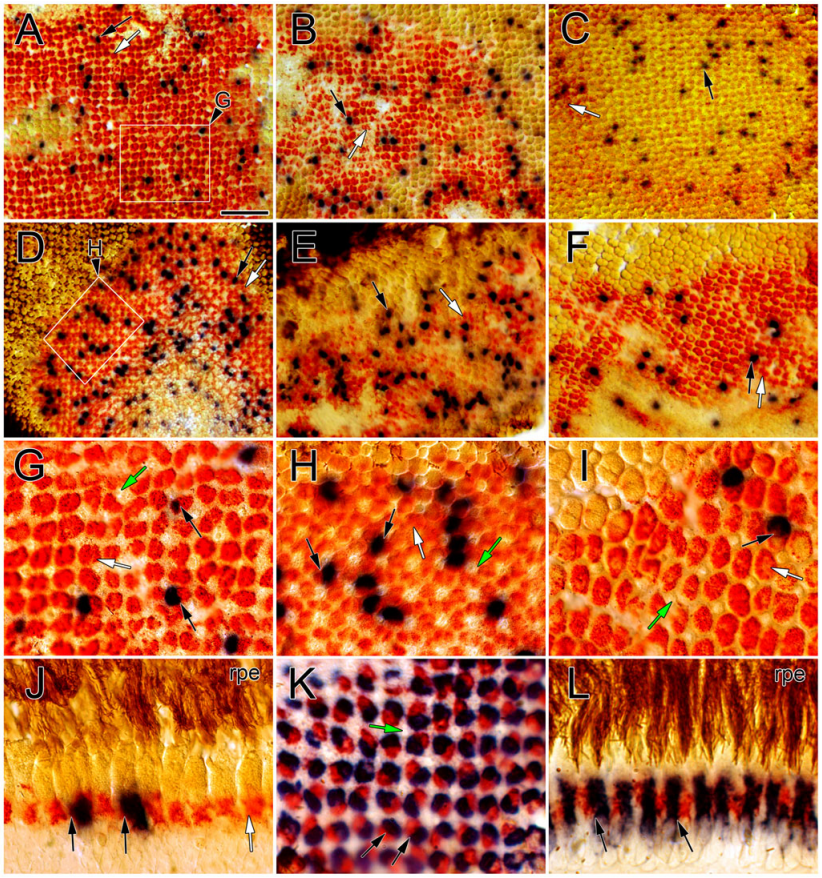


Fig. 7. Micrographs of sections from the retinas of sticklebacks following double label *in situ* hybridization with riboprobes encoding the RH2 and LWS opsin mRNAs. (A–C) Transverse section through the dorsal retina of sticklebacks from Mayer Lake (A), Mayer Pond (B), and Mayer Lake after 3 weeks of transplantation to full-spectrum clear waters in the laboratory (C). All the double cones, and none of the single cones, labelled with either of the two riboprobes. Most double cones were L/L pairs (white arrow), both members labelling with the LWS riboprobe (red colour). A minority of double cones were M/L pairs (black arrow), one member labelling with the RH2 riboprobe (blue colour) and the other with the LWS riboprobe (red colour). (D–F) Transverse section through the ventral (D), nasal (E) and centro-temporal (F) retina of a stickleback from Mayer Pond. RH2 opsin mRNA expression increased toward the naso-ventral retina. (G–I) Higher magnification views of cone mosaics from dorsal (G), ventral (H) and centro-temporal (I) retina. G and H are magnifications of the regions shown in A and D, respectively. Green arrows indicate single cones. (J) Radial section through the centro-dorsal retina of Mayer Lake. (K,L) Transverse (K) and radial (L) sections from the central retina of Swan Lake stickleback. All the double cones were M/L pairs and the M member alternated with the L member around the square mosaic unit. Other symbols and abbreviations as in Fig. 5. Scale bar in A represents 30 μm for A–F and 10 μm for G–L.

There were two cases where the $\lambda P_{50}$ at 60 cm depth and the weighted $\lambda_{max}$ of double cones were distant from each other. These involved fish transplanted to clear water ponds ($\lambda P_{50}$≈540 nm and $\lambda_{max}$≈603 nm) and those from Swan Lake ($\lambda P_{50}$≈613 nm and $\lambda_{max}$≈578 nm). The lack of correspondence in these cases reveals the slow pace of evolution and major differences in population ecology between water bodies. Stickleback populations from the dystrophic and mesotrophic systems examined are genetically very stable, having evolved over thousands of years in relative isolation (Deagle et al., 1996). In contrast, the Swan Lake population undergoes frequent genetic drift as this small water body becomes periodically anoxic, resulting in the death of resident fish, and marine stickleback populations can readily invade as a result of the ocean's proximity. Thus, the Swan Lake population, though living in an environment dominated by long wavelengths, has an opsin complement resembling that found in mesotrophic waters and probably reflects the latest oceanic invasion. Similarly, the relatively recent introduction (15–20 years ago) of fish from dystrophic lakes into mesotrophic ponds has failed to change the inherited pattern of opsin expression from the source populations. As several generations of fish have been produced in the new environments, it appears that maximizing the sensitivity of double cones to the light background of surface waters, though beneficial in the long term, is not crucial for immediate survival.

Intraspecific, heritable, variation in opsin expression has also been reported in killifishes and in African cichlids using real-time PCR and microspectrophotometry. In Lake Victoria, cichlid populations living at greater depths have increased expression of LWS opsin alleles that absorb at longer wavelengths compared with those from surface-dwelling populations (Carleton et al., 2005; Hofmann et al., 2010; Smith et al., 2011). There is also an apparent increase in L/L double cones, as opposed to L/M double cones, in cichlids inhabiting murkier waters (Carleton et al., 2005). In Lake Victoria, the differential expression of LWS alleles with depth is thought to drive speciation according to the premises of the 'sensory bias' hypothesis (Terai et al., 2006; Seehausen et al., 2008), though such a scheme does not hold for Lake Malawi populations (Smith et al., 2011). In killifishes, populations from 'tea-stained' swamps appear to have fewer UV and violet cones than populations from clear water springs (Fuller et al., 2003), and this result correlates with mRNA expression of these opsins (Fuller et al., 2004). The topographical organization of spectral cone types in the retinas of cichlids and killifishes is unknown, however, making functional interpretation difficult.

By comparison, our study shows the chromatic organization of the retina of sticklebacks from a wide range of photic regimes, revealing a difference in double cone opsin expression that is much greater than that estimated for cichlid and killifish cones based on microspectrophotometry data (Fuller et al., 2003; Carleton et al., 2005). This difference in double cone opsin expression is reflected in the spectral sensitivity of ganglion cells as measured by compound action potential recordings from the optic nerve (McDonald and Hawryshyn, 1995). Such extracellular potentials indicate a single sensitivity peak in the long wavelengths (600–620 nm) in sticklebacks from dystrophic lakes and two peaks, in the middle (540–560 nm) and long (600 nm) wavelengths, in fish from a mesotrophic lake (Quamichan Lake, BC, Canada) with similar spectral characteristics to the one studied here (Doogan's Lake). There is therefore a dominant contribution of the L cone mechanism to the spectral sensitivity of sticklebacks from dystrophic lakes, a result that is in line with expectations from our findings.

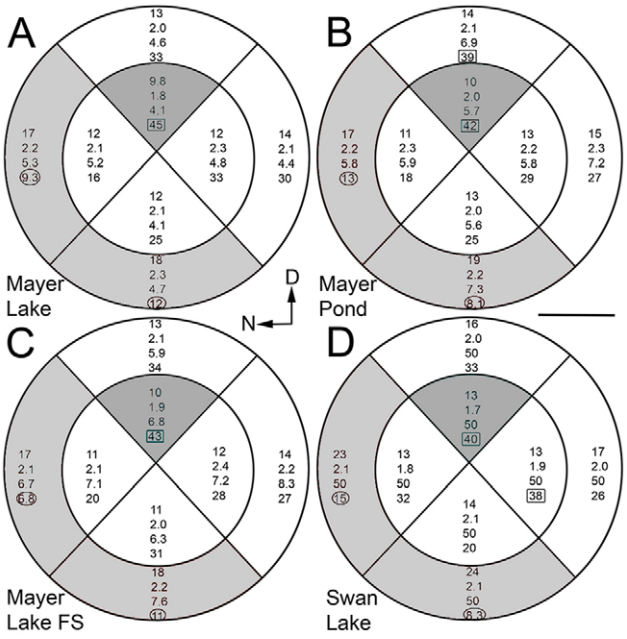


Fig. 8. Retinal maps of cone distributions in the retinas of sticklebacks from different photic regimes. Each retinal sector has four numbers arranged in a column. These are averages of 5–8 replicate observations, depending on retinal sector. From top to bottom, the first is the average number of double cones (in thousands per mm$^2$), the second is the average ratio of double cones to single cones, the third is the average percentage of RH2 mRNA-expressing cones within the double cone population, and the fourth is the average percentage of single cones that labelled with the SWS1 riboprobe. For each retina, light and dark grey sectors indicate double cone densities that are 1 s.d. above or below the retinal average, respectively. SWS1 percentages that are 1 s.d. above or below the retinal average are bounded by a rectangle or ellipse, respectively. Over the entire retina, only the percentage of cones expressing RH2 was significantly different (at α=0.05) between the Swan Lake population and the other three fish groups shown. Mayer Lake FS depicts the average combined retinal statistics from Mayer Lake fish after 3 weeks (*N*=4) and 6 months (*N*=3) following transplantation to full-spectrum clear waters in the laboratory; the retinas were grouped together because the averages were statistically the same. Drizzle Lake and Drizzle Pond fish had analogous photoreceptor distributions to those from Mayer Lake and Mayer Pond, while fish from Doogan's Lake and the Broughton Archipelago had similar distributions to those from Swan Lake. The scale bar represents 1.8 mm. D, dorsal retina; N, nasal retina.

It is intriguing yet enigmatic that the previous electrophysiological study failed to find any spectral sensitivity functions corresponding to either the UV or S cone mechanisms (McDonald and Hawryshyn, 1995). In fact, even the presence of an M cone mechanism in Swan Lake sticklebacks could not be clearly discerned. The electrophysiological technique used relied on extracellular recordings from a subset of optic nerve fibres and this limitation, along with restricted illumination of the ventral retina (McDonald and Hawryshyn, 1995), could easily have resulted in the absence of detected signal from smaller cone populations. Other taxa, like amphibians, also have reduced populations of UV and S cones dispersed throughout the retina (accounting for ~1–6% of all cones) (Chen et al., 2008), and their physiological presence was only revealed following action potential recordings from the entire retina (Koskelainen et al., 1994; Deutschlander and Phillips, 1995). In primates, S cones (expressing a SWS1 opsin) are present in reduced numbers (<10%) compared with other cone types (Roorda et al., 2001), yet play a role in non-foveal vision. In the case of adult sticklebacks, it is important to note that these fishes often feed in surface waters, where a full spectrum of light is present, providing the necessary wavelengths to stimulate UV cones.

### Phenotypic plasticity in opsin expression: cellular origin and functional significance

Fish transplanted to clear waters from dystrophic lakes had the same ~2% increase in M cones regardless of residence time in the mesotrophic ponds or in aquaria exposed to a full light spectrum. Such an increase could have resulted from apoptosis of L cones and their replacement by newly differentiated M cones, or by a switch in opsin expression from LWS to RH2 within individual cones. Although we did not perform experiments to distinguish between these two possibilities, it is worth noting that full differentiation of cones in goldfish takes approximately 3 weeks (Wu et al., 2001). Apoptosis followed by differentiation, two processes that are usually sequential (Candal et al., 2005), would take longer than our first sampling time (3 weeks) to complete. Thus, our data suggest opsin switching as the cellular mechanism behind the double cone phenotypic plasticity observed. In the retinas of salmonid fishes and rodents, cell labelling studies with proliferation and differentiation markers have shown that cone production in the main (non-peripheral) retina is absent after development (Julian et al., 1998; Candal et al., 2005; Cheng et al., 2009; Glaschke et al., 2011). Our histological analyses further indicated that cone density and morphology did not change following transplantations. This result, together with the similarity in absorbance curves obtained for the various visual pigments between populations, strongly suggests that opsin density remained the same following transplantations.

The timing of M cone density increase, occurring within 3 weeks of transfer to clear waters, is in line with reported differences in opsin mRNA expression between adult killifish following 1–3 days of rearing in new light environments (Fuller and Claricoates, 2011). It is also consistent with the timing of the opsin switch in the single cones of salmonid fishes after 3–5 days of exogenous treatment with thyroid hormone (Cheng et al., 2009; Gan and Novales Flamarique, 2010).

In contrast to the opsin switches that occur during development of salmonid fishes and rodents (Cheng et al., 2006; Cheng and Novales Flamarique, 2007; Applebury et al., 2007), the increase in M cone density reported here for sticklebacks does not appear to have any significance for the visual ecology of the animal. This is because the total photon catch of M cones in the retina would be altered by less than 1% (see Novales Flamarique, 2013). Similar changes in cone numbers following metamorphosis of amphibians are considered inconsequential (Chen et al., 2008). Our results are in line with those from cave-dwelling mollies, where differences in LWS opsin mRNA expression between populations living in different light environments show little to no phenotypic plasticity (Tobler et al., 2010). It remains to be determined whether the changes in opsin mRNA expression due to phenotypic plasticity reported for other fishes (Fuller et al., 2005; Shand et al., 2008; Hofmann et al., 2010; Fuller and Claricoates, 2011) are ecologically meaningful.

### ACKNOWLEDGEMENTS

We thank Lisa Grebinsky for laboratory and field assistance, and Dr Simon Jones (Pacific Biological Station, Department of Fisheries and Oceans, Canada) for sticklebacks from the Broughton Archipelago.

### FUNDING

The entirety of this research was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant [no. 238886] to I.N.F. Additional funding for light measurements in Haida Gwaii was provided by NSERC grant no. NRC 2354 to T.E.R.

## REFERENCES


**Albert, A. Y. K., Millar, N. P. and Schluter, D.** (2007). Character displacement of male nuptial colour in threespine sticklebacks (*Gasterosteus acuelatus*). *Biol. J. Linn. Soc. Lond.* **91**, 37-48.

**Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J.** (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

**Applebury, M. L., Farhangfar, F., Glösmann, M., Hashimoto, K., Kage, K., Robbins, J. T., Shibusawa, N., Wondisford, F. E. and Zhang, H.** (2007). Transient expression of thyroid hormone nuclear receptor TRbeta2 sets S opsin patterning during cone photoreceptor genesis. *Dev. Dyn.* **236**, 1203-1212.

**Beatty, D. D.** (1984). Visual pigments and the labile scotopic visual system of fish. *Vision Res.* **24**, 1563-1573.

**Beaudet, L., Novales Flamarique, I. and Hawryshyn, C. W.** (1997). Cone photoreceptor topography in the retina of sexually mature Pacific salmonid fishes. *J. Comp. Neurol.* **383**, 49-59.

**Borwein, B. and Hollenberg, M. J.** (1973). The photoreceptors of the 'four-eyed' fish, *Anableps anableps* L. *J. Morphol.* **140**, 405-442.

**Boughman, J. W.** (2001). Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* **411**, 944-948.

**Boulcott, P. and Braithwaite, V. A.** (2007). Colour perception in three-spine sticklebacks: sexes are not so different after all. *Evol. Ecol.* **21**, 601-611.

**Candal, E., Anadón, R., DeGrip, W. J. and Rodríguez-Moldes, I.** (2005). Patterns of cell proliferation and cell death in the developing retina and optic tectum of the brown trout. *Brain Res. Dev. Brain Res.* **154**, 101-119.

**Carleton, K. L., Parry, J. W., Bowmaker, J. K., Hunt, D. M. and Seehausen, O.** (2005). Colour vision and speciation in Lake Victoria cichlids of the genus *Pundamilia*. *Mol. Ecol.* **14**, 4341-4353.

**Chen, Y., Znoiko, S., DeGrip, W. J., Crouch, R. K. and Ma, J. X.** (2008). Salamander blue-sensitive cones lost during metamorphosis. *Photochem. Photobiol.* **84**, 855-862.

**Cheng, C. L. and Novales Flamarique, I.** (2004). Opsin expression: new mechanism for modulating colour vision. *Nature* **428**, 279.

**Cheng, C. L. and Novales Flamarique, I.** (2007). Chromatic organization of cone photoreceptors in the retina of rainbow trout: single cones irreversibly switch from UV (SWS1) to blue (SWS2) light sensitive opsin during natural development. *J. Exp. Biol.* **210**, 4123-4135.

**Cheng, C. L., Novales Flamarique, I., Hárosi, F. I., Rickers-Haunerland, J. and Haunerland, N. H.** (2006). Photoreceptor layer of salmonid fishes: transformation and loss of single cones in juvenile fish. *J. Comp. Neurol.* **495**, 213-235.

**Cheng, C. L., Gan, K. J. and Novales Flamarique, I.** (2007). The ultraviolet opsin is the first opsin expressed during retinal development of salmonid fishes. *Invest. Ophthalmol. Vis. Sci.* **48**, 866-873.

**Cheng, C. L., Gan, K. J. and Novales Flamarique, I.** (2009). Thyroid hormone induces a time-dependent opsin switch in the retina of salmonid fishes. *Invest. Ophthalmol. Vis. Sci.* **50**, 3024-3032.

**Collin, S. P., Collin, H. B. and Ali, M. A.** (1996). Ultrastructure and organisation of the retina and pigment epithelium in the cutlips minnow, *Exoglossum maxillingua* (Cyprinidae, Teleostei). *Histol. Histopathol.* **11**, 55-69.

**Cornish, E. E., Xiao, M., Yang, Z., Provis, J. M. and Hendrickson, A. E.** (2004). The role of opsin expression and apoptosis in determination of cone types in human retina. *Exp. Eye Res.* **78**, 1143-1154.

**Cottrill, P. B., Davies, W. L., Semo, M., Bowmaker, J. K., Hunt, D. M. and Jeffery, G.** (2009). Developmental dynamics of cone photoreceptors in the eel. *BMC Dev. Biol.* **9**, 71.

**Deagle, B. E., Reimchen, T. E. and Levin, D. B.** (1996). Origins of endemic stickleback from the Queen Charlotte Islands: mitochondrial and morphological evidence. *Can. J. Zool.* **74**, 1045-1056.

**Deutschlander, M. E. and Phillips, J. B.** (1995). Characterization of an ultraviolet photoreception mechanism in the retina of an amphibian, the axolotl (*Ambystoma mexicanum*). *Neurosci. Lett.* **197**, 93-96.

**Fein, A. and Szuts, E. Z.** (1982). *Photoreceptors: Their Role in Vision*. Cambridge, UK: Cambridge University Press.

**Fuller, R. C. and Claricoates, K. M.** (2011). Rapid light-induced shifts in opsin expression: finding new opsins, discerning mechanisms of change, and implications for visual sensitivity. *Mol. Ecol.* **20**, 3321-3335.

**Fuller, R. C., Fleishman, L. J., Leal, M., Travis, J. and Loew, E.** (2003). Intraspecific variation in retinal cone distribution in the bluefin killifish, *Lucania goodie*. *J. Comp. Physiol. A* **189**, 609-616.

**Fuller, R. C., Carleton, K. L., Fadool, J. M., Spady, T. C. and Travis, J.** (2004). Population variation in opsin expression in the bluefin killifish, *Lucania goodei*: a real-time PCR study. *J. Comp. Physiol. A* **190**, 147-154.

**Fuller, R. C., Carleton, K. L., Fadool, J. M., Spady, T. C. and Travis, J.** (2005). Genetic and environmental variation in the visual properties of bluefin killifish, *Lucania goodei*. *J. Evol. Biol.* **18**, 516-523.

**Gan, K. J. and Novales Flamarique, I.** (2010). Thyroid hormone accelerates opsin expression during early photoreceptor differentiation and induces opsin switching in differentiated TRα-expressing cones of the salmonid retina. *Dev. Dyn.* **239**, 2700-2713.

**Glaschke, A., Weiland, J., Del Turco, D., Steiner, M., Peichl, L. and Glösmann, M.** (2011). Thyroid hormone controls cone opsin expression in the retina of adult rodents. *J. Neurosci.* **31**, 4844-4851.

**Hárosi, F. I.** (1987). Cynomolgus and rhesus monkey visual pigments. Application of Fourier transform smoothing and statistical techniques to the determination of spectral parameters. *J. Gen. Physiol.* **89**, 717-743.

**Hárosi, F. I.** (1994). An analysis of two spectral properties of vertebrate visual pigments. *Vision Res.* **34**, 1359-1367.

**Hárosi, F. I. and Novales Flamarique, I.** (2012). Functional significance of the taper of vertebrate cone photoreceptors. *J. Gen. Physiol.* **139**, 159-187.

**Hofmann, C. M., O'Quin, K. E., Smith, A. R. and Carleton, K. L.** (2010). Plasticity of opsin gene expression in cichlids from Lake Malawi. *Mol. Ecol.* **19**, 2064-2074.

**Hoke, K. L., Evans, B. I. and Fernald, R. D.** (2006). Remodeling of the cone photoreceptor mosaic during metamorphosis of flounder (*Pseudopleuronectes americanus*). *Brain Behav. Evol.* **68**, 241-254.

**Hu, M., Hu, Z., Xue, L., Yang, Z., Zheng, Z., He, D. and Zhang, X.** (2011). Guinea pigs reared in a monochromatic environment exhibit changes in cone density and opsin expression. *Exp. Eye Res.* **93**, 804-809.

**Isayama, T. and Makino, C. L.** (2012). Pigment mixtures and other determinants of spectral sensitivity of vertebrate retinal photoreceptors. In *Photoreceptors: Physiology, Types and Abnormalities* (ed. E. Akutagawa and K. Ozaki), pp. 1-31. New York, NY: Nova Science Publishers.

**Julian, D., Ennis, K. and Korenbrot, J. I.** (1998). Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina. *J. Comp. Neurol.* **394**, 271-282.

**Koskelainen, A., Hemilä, S. and Donner, K.** (1994). Spectral sensitivities of short- and long-wavelength sensitive cone mechanisms in the frog retina. *Acta Physiol. Scand.* **152**, 115-124.

**Kröger, R. H., Bowmaker, J. K. and Wagner, H. J.** (1999). Morphological changes in the retina of *Aequidens pulcher* (Cichlidae) after rearing in monochromatic light. *Vision Res.* **39**, 2441-2448.

**Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R. et al.** (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948.

**Leaver, S. D. and Reimchen, T. E.** (2012). Abrupt changes in defence and trophic morphology of the giant threespine stickleback (*Gasterosteus* sp.) following colonization of a vacant habitat. *Biol. J. Linn. Soc.* **107**, 494-509.

**McDonald, C. G. and Hawryshyn, C. W.** (1995). Intraspecific variation of spectral sensitivity in threespine stickleback (*Gasterosteus acuelatus*) from different photic regimes. *J. Comp. Physiol. A* **176**, 225-260.

**McDonald, C. G., Reimchen, T. E. and Hawryshyn, C. W.** (1995). Nuptial colour loss and signal masking in *Gasterosteus*: an analysis using video imaging. *Behaviour* **132**, 963-977.

**McFarland, W. N. and Munz, F. W.** (1975). Part III: The evolution of photopic visual pigments in fishes. *Vision Res.* **15**, 1071-1080.

**Novales Flamarique, I.** (2001). Gradual and partial loss of corner cone-occupied area in the retina of rainbow trout. *Vision Res.* **41**, 3073-3082.

**Novales Flamarique, I.** (2005). Temporal shifts in visual pigment absorbance in the retina of Pacific salmon. *J. Comp. Physiol. A* **191**, 37-49.

**Novales Flamarique, I.** (2011). Unique photoreceptor arrangements in a fish with polarized light discrimination. *J. Comp. Neurol.* **519**, 714-737.

**Novales Flamarique, I.** (2013). Opsin switch reveals function of the ultrviolet cone in fish foraging. *Proc. R. Soc. Lond. B* **280**, 20122490.

**Novales Flamarique, I. and Hárosi, F. I.** (2000). Photoreceptors, visual pigments, and ellipsosomes in the killifish, *Fundulus heteroclitus*: a microspectrophotometric and histological study. *Vis. Neurosci.* **17**, 403-420.

**Novales Flamarique, I. and Hawryshyn, C. W.** (1993). Spectral characteristics of salmonid migratory routes from southern Vancouver Island (BC, Canada). *Can. J. Fish. Aquat. Sci.* **50**, 1706-1716.

**Novales Flamarique, I. and Hawryshyn, C. W.** (1997). Is the use of underwater polarized light by fish restricted to crepuscular time periods? *Vision Res.* **37**, 975-989.

**Novales Flamarique, I., Hendry, A. and Hawryshyn, C. W.** (1992). The photic environment of a salmonid nursery lake. *J. Exp. Biol.* **169**, 121-141.

**Reimchen, T. E.** (1989). Loss of nuptial color in threespine sticklebacks (*Gasterosteus acuelatus*). *Evolution* **43**, 450-460.

**Rick, I. P. and Bakker, T. C. M.** (2008). Color signaling in conspicuous red sticklebacks: do ultraviolet signals surpass others? *BMC Evol. Biol.* **8**, 189.

**Roorda, A., Metha, A. B., Lennie, P. and Williams, D. R.** (2001). Packing arrangement of the three cone classes in primate retina. *Vision Res.* **41**, 1291-1306.

**Rowe, M. P., Baube, C. L., Loew, E. R. and Phillips, J. B.** (2004). Optimal mechanisms for finding and selecting mates: how threespine stickleback (*Gasterosteus aculeatus*) should encode male throat colors. *J. Comp. Physiol. A* **190**, 241-256.

**Rowland, W. J.** (1994). Proximate determinants of stickleback behaviour: an evolutionary perspective. In *The Evolutionary Biology of the Threespine Stickleback* (ed. M. A. Bell and S. A. Foster), pp. 297-344. Oxford, UK: Oxford University Press.

**Saarinen, P., Pahlberg, J., Herczeg, G., Viljanen, M., Karjalainen, M., Shikano, T., Merilä, J. and Donner, K.** (2012). Spectral tuning by selective chromophore uptake in rods and cones of eight populations of nine-spined stickleback (*Pungitius pungitius*). *J. Exp. Biol.* **215**, 2760-2773.

**Seehausen, O., Terai, Y., Magalhaes, I. S., Carleton, K. L., Mrosso, H. D. J., Miyagi, R., van der Sluijs, I., Schneider, M. V., Maan, M. E., Tachida, H. et al.** (2008). Speciation through sensory drive in cichlid fish. *Nature* **455**, 620-626.

**Shand, J., Davies, W. L., Thomas, N., Balmer, L., Cowing, J. A., Pointer, M., Carvalho, L. S., Trezise, A. E. O., Collin, S. P., Beazley, L. D. et al.** (2008). The influence of ontogeny and light environment on the expression of visual pigment opsins in the retina of the black bream, *Acanthopagrus butcheri*. *J. Exp. Biol.* **211**, 1495-1503.

**Smith, S. O.** (2010). Structure and activation of the visual pigment rhodopsin. *Annu. Rev. Biophys.* **39**, 309-328.

**Smith, C., Barber, I., Wootton, R. J. and Chittka, L.** (2004). A receiver bias in the origin of three-spined stickleback mate choice. *Proc. R. Soc. Lond. B* **271,** 949-955.

**Smith, A. R., D'Annunzio, L., Smith, A. E., Sharma, A., Hofmann, C. M., Marshall, N. J. and Carleton, K. L.** (2011). Intraspecific cone opsin expression variation in the cichlids of Lake Malawi. *Mol. Ecol.* **20**, 299-310.

**Spady, T. C., Parry, J. W., Robinson, P. R., Hunt, D. M., Bowmaker, J. K. and Carleton, K. L.** (2006). Evolution of the cichlid visual palette through ontogenetic subfunctionalization of the opsin gene arrays. *Mol. Biol. Evol.* **23**, 1538-1547.

**Szél, Á., van Veen, T. and Röhlich, P.** (1994). Retinal cone differentiation. *Nature* **370**, 336.

**Takechi, M. and Kawamura, S.** (2005). Temporal and spatial changes in the expression pattern of multiple red and green subtype opsin genes during zebrafish development. *J. Exp. Biol.* **208**, 1337-1345.

**Terai, Y., Seehausen, O., Sasaki, T., Takahashi, K., Mizoiri, S., Sugawara, T., Sato, T., Watanabe, M., Konijnendijk, N., Mrosso, H. D. J. et al.** (2006). Divergent selection of opsin drives incipient speciation in Lake Victoria cichlids. *PLoS Biol.* **4**, e433.

**Tobler, M., Coleman, S. W., Perkins, B. D. and Rosenthal, G. G.** (2010). Reduced opsin gene expression in a cave-dwelling fish. *Biol. Lett.* **6**, 98-101.

**Wen, X. H., Shen, L., Brush, R. S., Michaud, N., Al-Ubaidi, M. R., Gurevich, V. V., Hamm, H. E., Lem, J., Dibenedetto, E., Anderson, R. E. et al.** (2009). Overexpression of rhodopsin alters the structure and photoresponse of rod photoreceptors. *Biophys. J.* **96**, 939-950.

**Williams, T. P., Squitieri, A., Henderson, R. P. and Webbers, J. P. P.** (1999). Reciprocity between light intensity and rhodopsin concentration across the rat retina. *J. Physiol.* **516**, 869-874.

**Wood, P. and Partridge, J. C.** (1993). Opsin substitution induced in retinal rods of the eel (*Anguilla anguilla* (L.)): a model for G-protein-linked receptors. *Proc. R. Soc. Lond. B* **254,** 227-232.

**Wu, D. M., Schneiderman, T., Burgett, J., Gokhale, P., Barthel, L. and Raymond, P. A.** (2001). Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. *Invest. Ophthalmol. Vis. Sci.* **42**, 2115-2124.

**Xiao, M. and Hendrickson, A.** (2000). Spatial and temporal expression of short, long/medium, or both opsins in human fetal cones. *J. Comp. Neurol.* **425**, 545-559.

**Yokoyama, S.** (2000). Molecular evolution of vertebrate visual pigments. *Prog. Retin. Eye Res.* **19**, 385-419.

**Yokoyama, S., Yang, H. and Starmer, W. T.** (2008). Molecular basis of spectral tuning in the red- and green-sensitive (M/LWS) pigments in vertebrates. *Genetics* **179**, 2037-2043.