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Title: Rapid adaptive evolution of colour vision in the threespine stickleback radiation.

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Abstract

Vision is a sensory modality of fundamental importance for many animals, aiding in foraging, detection of predators, and mate choice. Adaptation to local ambient light conditions is thought to be commonplace, and a match between spectral sensitivity and light spectrum is predicted. We use opsin gene expression to test for local adaptation and matching of spectral sensitivity in multiple independent lake populations of threespine stickleback populations derived since the last ice age from an ancestral marine form. We show that sensitivity across the visual spectrum is shifted repeatedly towards longer wavelengths in freshwater compared with the ancestral marine form. Laboratory rearing suggests this shift is largely genetically based. Using a new metric, we found that the magnitude of shift in spectral sensitivity in each population corresponds strongly to the transition in the availability of different wavelengths of light between the marine and lake environment. We also found evidence of local adaptation by sympatric benthic and limnetic ecotypes to different light environments within lakes. Our findings indicate rapid parallel evolution of the visual system to altered light conditions. The changes have not, however, yielded a close matching of spectrum-wide sensitivity to wavelength availability, for reasons we discuss.

Keywords: Visual Ecology, Local Adaptation, Evolution, Opsin, Gene Expression, *Gasterosteus aculeatus*.

Background

Sensory systems are often thought to be under strong natural selection [1], and are predicted to evolve to better correspond to signals in the local environment [2]. For example, sensitivity of the visual system to different wavelengths of light is expected to evolve to match roughly the availability of wavelengths [3,4], increasing ability to catch photons and detect contrast between objects and background [5,6,7]. However, few studies have tested the adaptive significance of spectral sensitivity across the whole visual spectrum. The degree of matching between spectral sensitivity of organisms and their light environment across the spectrum has not been quantified.

Aquatic organisms provide excellent opportunities to test for local adaptation and quantify matching [8]. This is because differential attenuation of wavelengths of light with water depth and by suspended particulates result in dramatic and predictable changes in local light spectra [9]. For example, the transition from marine to fresh waters is usually accompanied by a large reduction in the availability of ultraviolet (UV) wavelengths, largely because of an increase in the amount of dissolved organics [9,10].

We used threespine stickleback (*Gasterosteus aculeatus*), which inhabit both marine and freshwater habitats, to investigate predicted evolutionary changes in visual adaptations of populations to the different ambient light environments. Marine stickleback invaded and adapted to numerous lakes and streams at the end of the last ice age (~12,000 years ago) [11]. First, we tested for parallel evolution of opsin gene expression and spectral sensitivity over the visual light spectrum among these derived freshwater populations, which would represent strong evidence of natural selection [12].

Second, utilizing the extant marine form as a proxy for the ancestral state, we evaluated the extent to which shifts in the spectral sensitivity of freshwater populations

78 are correlated with shifts in the ambient light environment, and whether the outcome
79 improves the match to local ambient light spectra. Finally, we tested for parallel
80 divergence of spectral sensitivity of multiple pairs of sympatric limnetic and benthic
81 stickleback ecotypes (or “species pairs”) to finer scale heterogeneity in the light
82 environment within lakes. In each of the three species pairs analyzed here, the benthic
83 stickleback forage in the vegetated littoral regions of the lake and deeper sediments,
84 whereas limnetics are pelagic, found in the open water and near rocky cliffs [13]. The
85 benthic environment contains relatively more long wavelengths than the open water
86 [14].

87 We focused on expression of opsin genes, which encode the light sensitive G-
88 protein coupled receptors that are expressed in retinal rod and cone cells. Opsins
89 conjugate to vitamin A derived chromophores and play an important role in colour vision
90 by mediating the conversion of photons into electrochemical signals, which initiate a
91 neuronal response that is perceived by the brain [15]. The clear and well-characterized
92 link between opsin genotype (coding sequence) and spectral phenotype (wavelength of
93 maximal absorption, λ_{max}) make opsins particularly useful for studying sensory
94 adaptation [16]. Opsin mediated shifts in spectral sensitivity can be achieved by
95 changes in opsin protein coding sequence (*e.g.* [17]) and by changes in levels of gene
96 expression (*e.g.* [18]). We studied gene expression because analysis of whole genomes
97 of marine and freshwater stickleback has not found consistent differences in opsin gene
98 coding sequence between marine and freshwater populations [19]. Compared to other
99 fish, stickleback have relatively few (four) opsins, with a single functional opsin gene in
100 each of the four cone opsin subfamilies: short-wavelength sensitive 1 (*SWS1*), short-

wavelength sensitive 2 (*SWS2*), middle-wavelength sensitive (*RH2*), and long-wavelength sensitive (*LWS*) [20]. We measured expression levels of each of the four unique cone opsin genes in 11 stickleback populations. We also measured expression in fish from two populations raised in a common laboratory environment to test the extent to which it is genetically determined.

We used opsin gene expression levels to estimate spectrum-wide spectral sensitivity to evaluate two general expectations. First, the advantages of photon capture and contrast should result in spectral sensitivity evolving roughly to correspond with wavelength availability [3,4]. We measure this correspondence ("matching") with the correlation across wavelengths between spectral sensitivity and two measures of light availability: irradiance (photons of each wavelength available at a specific water depth) and transmission (indicating the absorption of specific wavelengths by water). Large discrepancies between spectral sensitivity and light availability in specific regions of the visual spectrum might suggest specialized visual functions. Second, changes in wavelength availability from marine to fresh water should lead to similar shifts in spectral sensitivity ("local adaptation"). For example, as some wavelengths become scarce in the new environment and others common, relative to the ancestral environment, we expect spectral sensitivity to shift to correspond [2]. Throughout, we use the whole light spectrum to study association, rather than studying associations between summary measures such as the median. We introduce a new metric to quantify the correlation between shift in spectral sensitivity and the transition between light environments.

123 Shifts in spectral sensitivity can additionally be achieved by differential use of
124 vitamin A derived chromophores [21, 22]. Conjugation of an opsin to an A_1
125 chromophore (11-*cis* retinal) leads to a shorter wavelength of maximal absorption (λ_{max})
126 than conjugation to an A_2 chromophore (3-dehydro 11-*cis* retinal) [21]. Switches in
127 chromophore use have been shown to occur in fishes over ontogeny [23] and between
128 habitats via phenotypic plasticity [22]. Fish in the ocean generally use A_1 chromophores,
129 while freshwater fish have a mixture of A_1 and A_2 chromophores (varying from
130 completely A_1 to completely A_2) [22]. Complete use of A_2 is generally found in lakes
131 whose waters are strongly stained with tannins [*e.g.* 24], and such lakes are not
132 included in our study. To account for possible variation in chromophore use, we model
133 the effects of changes in chromophore and describe how this affects our measures of
134 local adaptation and spectral matching of opsin expression in stickleback.
135
136 **Materials and Methods**
137 **Sampling**
138 Six gravid females were collected from each of 11 populations inhabiting different
139 breeding environments in the Strait of Georgia region of British Columbia, Canada.
140 Collections were made under the Species At Risk Act collection permit 236 and British
141 Columbia Fish Collection permit NA-SU12-76311. The samples came from two marine
142 locations, three lakes containing just a single species of stickleback, and three lakes
143 containing stickleback species pairs (see Supp. Mat. Section 1 and Table S1 for site
144 details). Fish were euthanized at the collection site and eye tissue was immediately

preserved in RNAlater® (Qiagen, Netherlands) and then kept at –20 °C for up to a month before RNA was extracted.

Opsin Expression and Spectral Sensitivity

The expression of each of the stickleback's four unique cone opsin genes (*SWS1*, *SWS2A, RH2-1*, and *LWS* [20]) was measured using a standard reverse-transcriptase quantitative polymerase chain reaction protocol (details in the Supp. Mat. Section 2). We normalized the absolute number of transcripts for each gene from each individual by dividing the expression of a given opsin by the sum of the expression of all four opsins to get relative opsin expression. We also measured gene expression of a reference gene, *Beta actin*, and calculated the expression of each opsin gene relative to it.

All statistical analyses in the paper were conducted in R 3.0.2 [25]. To test for differences in mean expression of each opsin gene between marine and freshwater populations, we used a linear mixed-effects model (using the nlme package, [26]) with water type (marine or fresh) as fixed effect and location as a random effect. For this comparison, individuals from the benthic and limnetic species in a given location were combined and treated as a single population. Results were the same when only the benthics, or only the limnetics, were used instead. In separate analyses we tested for differences in gene expression between the sympatric benthic and limnetic species in three lakes, with lake as a random effect and species as a fixed effect in the model. We treat lake populations as independent replicates that require no phylogenetic correction. This is justified by the geological origins of lakes, which are in separate drainages and were accessible via the sea for a limited period of time. Previous studies show that

168 phylogenies of freshwater stickleback populations in British Columbia based on
169 putatively neutral markers are well approximated by a star phylogeny (*e.g*. [27]).
170 We bred three families of one marine population (Oyster Lagoon) and three
171 families of one benthic population (Priest Lake) by *in vitro* fertilization and reared them
172 under laboratory conditions in stand-alone 100 L tanks with fluorescent lights. Animals
173 were treated in accordance with University of British Columbia Animal Care protocols
174 (Animal Care Permit A11-0402). A gravid adult female from each family was euthanized
175 and her opsin expression was quantified as described above. We used linear models to
176 test differences between lab-reared marine and freshwater fish and between lab- and
177 wild-reared fish from the same populations.
178 Upon finding differences in mean opsin expression between marine and
179 freshwater stickleback, and between sympatric benthic and limnetic stickleback, we
180 estimated how they translated into differences in spectral sensitivity. We calculated a
181 spectral sensitivity curve S_i (350 – 700 nm) for each individual *i* based on its relative
182 expression of the four opsin genes, and using the absorbance templates from
183 Govardovskii *et al*. [28] and estimates by Flamarique *et al.* [24] of the wavelength of
184 maximum absorbance ($\lambda_{\max}$) of each opsin gene (details in Section 3 of the Supp. Mat.).
185 This model assumes that opsin expression contributes additively to spectral sensitivity;
186 at this point in time it is a necessary simplification as we still lack empirical informed
187 models that describe and generalize any potential inhibitory interactions among opsins
188 during signal integration and interpretation.
189 Chromophore (A_1 and A_2) ratios in the surveyed freshwater populations are not
190 known. Based on empirical observations [24] we assumed that marine stickleback used

100% A_1 in the ocean. We estimated spectral sensitivity of stickleback in fresh water using three different chromophore ratios representing the extremes: 100% A_1; 50% A_1 and 50% A_2; and 100% A_2. We assumed that benthic and limnetic stickleback have the same A_1 : A_2 ratio.

Association between Spectral Sensitivity and Ambient Light

We measured the spectral conditions of each location, with the exception of Cranby Lake and Little Quarry Lake. We used two measures to quantify the ambient light environment: irradiance and transmission. Irradiance measures the abundance of photons at each wavelength in the environment at a given point in time. Irradiance measurements of side-welling light (I_s) were taken at 10 cm, 20 cm, 50 cm, 100 cm and 200 cm depth at 10 or more sites within each sampling location using a cosine corrector attached to a spectrophotometer (Ocean Optics, USA). In subsequent analyses we used the irradiance at 50 cm. A limitation of using irradiance to quantify available light is that it varies with depth and with the weather and the angle of the sun. Transmission is the relative rate of loss of photons of a given wavelength per unit distance traveled through water. Transmission is a property of the body of water and may be less variable than irradiance, at least on short time scales. Transmission was measured as the light extinction coefficient with depth (K_S) (method for calculation outlined in Supp. Mat. Section 5).

To test for local adaptation, we developed a statistic to quantify the association between the shift in spectral sensitivity and the transition in light environment, from marine to fresh water, across all wavelengths for each lake population. First, we chose

a marine population (Oyster Lagoon) to represent the ancestral phenotype and breeding environment. Next, we constructed transmission (K_s) and irradiance (I_S) curves by calculating at each wavelength (λ) the median from all samples within a location. At each wavelength we then subtracted the median value of the reference marine location from the median value in each freshwater location. This yielded change in transmission (ΔK_s) and change in irradiance ($\Delta \mathrm{I}_s$) values at every wavelength (λ) at each freshwater location. A positive value of $\Delta \mathrm{I}_s$ at a given wavelength indicates that there are more photons of that wavelength (λ) present at the freshwater location relative to the marine environment. A positive value of ΔK_s at a given wavelength (λ) indicates greater light transmission (fewer photons lost as light travels through water) at the freshwater location than at the reference marine location.

Change in spectral sensitivity ΔS was calculated similarly, as follows. We calculated the median sensitivity at each wavelength (λ) of the sample of individuals from the reference marine population. Change in sensitivity was calculated for each freshwater individual as the difference between its sensitivity curve and the median marine curve. Finally, for each freshwater individual, we calculated the correlation coefficient (*r*) of the change in sensitivity (ΔS) against the change in light environment (ΔK_s or $\Delta \mathrm{I}_s$), with each wavelength yielding a data point for each freshwater individual. A positive *r* indicates that regions of the spectrum with increased irradiance (or transmission) are correlated with increased spectral sensitivity, and regions of the spectrum with a decrease in irradiance (or transmission) are correlated with decreased spectral sensitivity. We used a mixed-effects model (with population as a random effect) to test whether mean correlation coefficients (*r)* differed significantly from zero.

We carried out a similar analysis of local adaptation of spectral sensitivity between the sympatric species in relation to differences in their local light environments. For each lake, we used the limnetic population and the pelagic environment as the reference. Other calculations were the same as described above for the marine and freshwater comparison (see Supp. Mat. Sections 5 and 6, Tables S3 and S4, and Supp. Figs. 1 and 2, for further details and justification of our reference populations).

To quantify the degree to which populations are matched to their native light environments we estimated the correlation, wavelength by wavelength, between each population's mean spectral sensitivity and the transmission and irradiance measured in its local environment. The significance of the mean correlation was tested separately for marine and freshwater populations using linear models.

Because analyses of local adaptation and matching involved a suite of tests that incorporated different measures of light environment and three chromophore scenarios, we adjusted the p-values for multiple testing in each table of results using the "BH" false discovery rate method [29] and the *p.adjust* function in R (Tables S3, S4, and S5). Raw p-values are reported in the main paper and adjusted p-values are reported in the statistics tables in the supplement. In all cases significant p-values remained significant after the correction for multiple testing.

Results

Opsin Expression and Spectral Sensitivity

Freshwater stickleback populations had significantly lower expression of the *SWS1* (UV) opsin gene than the marine populations (difference = -0.20 ± 0.02 SE, $F_{1,6}$

260 = 145.2, p < 0.001) and higher expression of the *RH2* (green) opsin gene (difference =
261 0.21 ± 0.06 SE, $F_{1,6}$ = 18.1, p = 0.005). We did not detect a significant difference in the
262 other two opsin genes, LWS (red) (difference = 0.02 ± 0.04 SE, $F_{1,6}$ = 0.2, p = 0.68) and
263 *SWS2* (blue) (difference = -0.009 ± 0.008 SE, $F_{1,6}$ = 1.2, p = 0.31) (Figure 1).
264 Differences in *SWS1* and *RH2* remained significant if expression was calculated relative
265 to the reference gene *Beta actin* (*SWS1* difference = 2.1 ± 0.3 SE, $F_{1,6}$ = 49.2, p <
266 0.001; *RH2* difference = 2.97 ± 0.9 SE, $F_{1,6}$ = 10.7, p = 0.017). Thus we proceeded
267 using cone opsin proportion as our metric of gene expression when modeling spectral
268 sensitivity, as this has been shown to be best for making inferences about overall colour
269 vision capacities [30].
270 These differences in overall expression translated into large differences in
271 estimated spectral sensitivity in two portions of the spectrum (Figure 2). Freshwater fish
272 had reduced sensitivity in the 350-375 nm (UV and violet) region of the spectrum, and
273 they had greater sensitivity in the 450-600 nm (blue and green) region relative to both
274 marine populations.
275 Within lakes we found that the limnetic stickleback populations had significantly
276 greater *RH2* (green) expression than the benthics (difference = 0.05 ± 0.02 SE, $F_{1,31}$ =
277 7, p = 0.01), and benthics had greater *LWS* (red) expression (0.04 ± 0.02 SE, $F_{1,31}$ =
278 4.3, p = 0.05). However, the magnitudes of the differences were small (Figure 3). The
279 expression of *SWS1 and SWS2* opsins did not differ significantly (p > 0.29) between the
280 two species (Figure 3). The difference in *RH2* expression between the species was still
281 significant when expression was calculated relative to *Beta actin* gene expression
282 (difference = 1.3 ± 0.6 SE, $F_{1,31}$ = 4.4, p = 0.04), but the difference in LWS was not

283 (difference = 0.3 ± 0.84 SE, $F_{1,31}$ = 0.12, p = 0.70). These differences in expression
284 translate to reduced sensitivity in the 525-575 nm (green) region of the spectrum and
285 increased sensitivity in the portion of the spectrum above 600 nm (red) in benthics
286 compared to limnetics (Supp. Figure 3).
287
288 **Laboratory Rearing**
289 In the lab, Oyster Lagoon (marine) and Priest benthic fish (freshwater) had
290 similar expression differences as in the wild (Figure 4). *SWS1* gene expression
291 remained different between the marine and freshwater populations in the lab (difference
292 0.11 ± 0.02 SE, df = 1,4, F = 27.1, p = 0.01) as did *RH2* (difference 0.18 ± 0.03 SE, df =
293 1,4, F = 40.1, p = 0.003). The difference in *SWS1* was, however, greater in the wild
294 samples, as indicated by an interaction between rearing condition (wild or lab) and
295 population of origin (effect size = 0.096 ± 0.039 SE, $t_{1,4}$ = 2.486, p = 0.03). No other
296 interactions were significant (all p > 0.17). Finally, we also detected a small difference in
297 *LWS* expression between the two populations in the lab only (Figure 4; 0.06 ± 0.02 SE,
298 $F_{1,4}$ = 11.5, p = 0.03). Additional tests examining changes in the gene expression of lab-
299 reared fish from each population compared to their wild counterparts are outlined in the
300 Supp. Mat. Section 4.
301
302 **Association between Shifts in Spectral Sensitivity and Ambient Light**
303 The shift in spectral sensitivity from marine to freshwater environments was
304 positively correlated with the change in ambient light spectrum, when sensitivity was
305 estimated assuming that both populations used only the A_1 chromophore. On average,

306 the correlation measured using transmission (mean r = 0.39, ± 0.12 SE, $t_{1,31}$ = 3.3, p =
307 0.002; Figure 5A) was of similar magnitude when using irradiance (mean r = 0.32, ±
308 0.06 SE, $t_{1,31}$ = 4.95, p < 0.0001; Figure 5B). **T**hese correlations arose primarily from
309 shifts in the short- (UV-blue) and middle-wavelength (green) regions (Supp. Figure 4).
310 Decreased transmission of UV (350-400 nm) and violet (380-450 nm) in the freshwater
311 environment (indicated by values below the dashed line in Supp. Figure 4) correspond
312 with decreased sensitivity to these wavelengths in freshwater populations. Increased
313 transmission of blue (450-495 nm) and green (495-570 nm) wavelengths in fresh water
314 is correlated with increased sensitivity to these wavelengths. Freshwater populations
315 varied considerably in the strength of the correlation (Figure 5).
316 These results isolate the effects of shifts in spectral sensitivity caused by
317 changes in opsin gene expression in freshwater, when controlling for chromophore. We
318 also measured the effects of these expression changes if combined with a hypothetical
319 increase in the use of the A_2 chromophore in these freshwater populations. The
320 correlation between shifts in spectral sensitivity and transmission weakens slightly when
321 a 50:50 mix of A_1 and A_2 chromophores is projected (mean r = 0.22, ± 0.12 SE, $t_{1,31}$ =
322 1.85, p = 0.07). When 100% A_2 chromophore is used, the correlation between shifts in
323 sensitivity and transmission weaken further (mean r = 0.14, ± 0.09 SE, $t_{1,31}$ = 1.53, p =
324 0.14) and the correlation between shifts in sensitivity and irradiance becomes negative
325 (mean r = -0.48, ± 0.05 SE, $t_{1,31}$ = -9.3, p = <0.0001) (see Table S3 for details, including
326 adjusted p-values).
327 Within species pair lakes, there was a moderate, although not quite significant,
328 correlation between divergence in spectral sensitivity and the difference in transmission

(modeled using the A_1 chromophore; Figure 5C; mean $r = 0.27 \pm 0.13$ SE, $t_{1,10} = 1.97$, $p = 0.077$). This correlation was not significant for the difference in irradiance (Figure 5D; mean $r = 0.18 \pm 0.18$ SE, $t_{1,10} = 1.00$, $p = 0.339$). The results were similar when other chromophore ratios were used to estimate spectral sensitivity, assuming that ratios were the same in both sympatric forms (see Table S4 for details, including adjusted p-values).

Match of Spectral Sensitivity to Ambient Light

Despite strong correlations between shifts in spectral sensitivity and changes in the distribution of available wavelengths, spectral sensitivity is not closely matched to wavelength availability in either marine or freshwater environments. The mean correlation between spectral sensitivity of freshwater fish and ambient light in lakes, while statistically significant, was small (0.07 ± 0.03 for transmission and 0.12 ± 0.02 for irradiance). This low level of matching has arisen multiple times in parallel in lake stickleback, which suggests that natural selection favors it. Substituting the chromophore did little to alter the mean correlation for transmission (although it became statistically insignificant) and slightly changed the strength for irradiance (See Supp. Mat. Section 7 and Table S5 for details, including adjusted p-values). In the marine environment the mean correlations between marine spectral sensitivity and transmission or irradiance are negative ($r = -0.66 \pm 0.16$ SE and $r = -0.11 \pm 0.07$ SE, respectively). The main cause of the strong negative correlation in marine waters is the excessive UV sensitivity compared with UV light availability. Nevertheless, UV expression declines in fresh water, where these wavelengths are even more scarce,

contributing to the observed correlation between shifts in sensitivity and the change in wavelength distribution.

Discussion

Our findings indicate that there has been rapid parallel evolution of opsin gene expression and spectral sensitivity across the light spectrum in freshwater stickleback populations. All surveyed freshwater populations have their spectral sensitivity shifted towards blue and green wavelengths, and away from ultraviolet and violet, relative to the marine populations. This has been accomplished entirely by shifts in opsin gene expression rather than protein sequence changes. We provide evidence that this difference has a genetic basis, as the main differences in expression were maintained in two lab-reared populations. Our analyses also reveal a strong association between shifts in spectral sensitivity and changes in light transmission from marine to fresh water environments, suggesting that these shifts are in an adaptive direction. On a smaller scale, we also find support for parallel adaptive divergence of gene expression and spectral sensitivity within lakes, between sympatric limnetic and benthic species. The evolution of the visual system in stickleback has been rapid, as these freshwater populations have evolved within the last 12,000 after the last glacial maxima [11].

The degree of phenotypic parallelism in opsin expression and spectral sensitivity that we describe is unprecedented over such a short time span. Nine independently derived populations exhibit the same direction of shift in opsin expression following the colonization of freshwater. In East African cichlids, parallel evolutionary divergence of opsin expression has been detected between species within two of the three major lake

cichlid radiations [31], but these radiations are much older than the freshwater stickleback populations studied here. Our findings are in line with previous work in stickleback, which has found extensive parallel evolution of morphological traits and patterns of genomic divergence among freshwater populations [19,32,33]. Some but not all of this morphological parallelism involves changes at the same underlying genes, which frequently represents adaptation from a common ancestral pool of standing genetic variation [32]. Possibly, the parallelism we observe in spectral sensitivity also represents adaptation from a common pool of standing genetic variation, which would help to explain the speed of evolution in this trait in stickleback. Further genetics work is required to test this idea.

The result from our lab rearing experiment suggests a substantial genetic component to the population differences in opsin expression. This contrasts with many other systems in which differential opsin gene expression and/or spectral sensitivity is largely phenotypically plastic [*e.g.* 34]. For example, wild Bluefin Killifish (*Lucania goodie*) living in clear springs and tannin stained waters exhibit large differences in their opsin gene expression [34]; however, light treatment and rearing experiments in the lab have shown that most of these differences are due to environmental effects [34].

Smaller but detectable differences in opsin expression and sensitivity between limnetic and benthic stickleback inhabiting the same lake were repeated in multiple lakes, suggesting a role for natural selection in divergence of visual systems on a small, within-lake scale. Benthics had slightly higher estimated sensitivity to red wavelengths than did limnetics, in accord with a more red-shifted local light environment. Previous work using optomotor behavioural response assays indicated that limnetic stickleback in

Enos Lake have higher red wavelength sensitivity than the benthic population from the same lake, and similar red wavelength sensitivity to the benthic in Paxton Lake [14]. In contrast, we found higher expression of long wavelength opsins in benthics compared to limnetics. Future work is required to determine how these differences in opsin expression affect foraging and mate choice in stickleback, as has been suggested in Lake Victoria cichlids [35].

Early work in the field of visual ecology focused on the hypothesis that spectral sensitivity should evolve to maximize an individual's photon catch [5,6,7]. Tests of this hypothesis have examined the relationship between the λ_{max} of visual pigments (opsins) and the wavelengths most prevalent in ambient environment and have often found a strong relationship (*e.g*. [36,37]). However, detection of contrast and colour discrimination also likely shapes the evolution of spectral sensitivity. With multiple functions, it may be difficult to predict *a priori* the evolved degree of spectrum-wide matching of spectral sensitivity to the available light spectrum. We did not find a close match in freshwater populations, and indeed, the correlation was negative in marine populations. The low match in marines is driven by their high estimated sensitivity to short wavelengths such as UV, despite the relative rarity of these light wavelengths in the marine environment compared to mid-wavelengths. The low degree of matching suggests that increasing photon capture alone is unlikely to explain the evolution of spectral sensitivity. Predicting a shift in sensitivity with change in light spectrum may be more straightforward: reduced investment in capturing specific wavelengths that are increasingly rare is expected. For example in the deep sea, long-wavelength light is rare, and some deep-sea fish have lost long-wave sensitive opsins and shifted their

421 sensitivity towards shorter wavelengths [37]. Similarly, we found that freshwater
422 stickleback have reduced expression of short wavelengths, which are even scarcer in
423 freshwater than in the sea. Nevertheless, freshwater fish retain relatively high sensitivity
424 to UV light compared to background irradiance.

425 One possible explanation for the low match between sensitivity and ambient
426 wavelengths is that high expression of pigments whose sensitivity is offset from the
427 dominant wavelengths of the environment could play an important role in contrast
428 detection under low light conditions [36]. For example, in stickleback UV wavelengths
429 are important for detection of zooplankton prey against the background light [38]. This
430 idea is consistent with the observed trend toward reduced UV opsin expression in
431 freshwater stickleback populations, since most are less zooplanktivorous than marine
432 stickleback [39]. Experimental work in other fish species has also shown that reduced
433 UV sensitivity coincides with reduced zooplanktivory and zooplankton foraging ability
434 [40,41] A second possible explanation for the low match between spectral sensitivity
435 and ambient light is that detection of specific wavelengths might be important for mate
436 choice and intraspecific signaling. Short (UV-blue) and long wavelengths (yellow-red)
437 are important signals for mate choice in stickleback [42], as male nuptial colouration
438 often involves blue and red pigmentation [43], as well as reflection in the UV [44].
439 Tuning of perception towards these nuptial signals and detection of contrast among
440 them could also contribute to the mismatch of sensitivity to available light. It is also
441 conceivable that our estimates of sensitivity, which do not account for non-additive
442 signal integration during neuronal processing, underestimate the environmental
443 correlation.

444 A_2 opsin chromophore complexes do not necessarily act synergistically with
445 changes in opsin expression to produce adaptive shifts in spectral sensitivity. In the
446 populations surveyed substitution of A_1 chromophores with A_2 chromophores weakens
447 the relationship between shifts in spectral sensitivity and shifts in ambient light. While
448 the empirical ratios of A_1 and A_2 in the wild are unknown for these freshwater
449 populations, our analyses suggest that A_2 domination would be unlikely. A_2 dominated
450 retinas result in shifts in spectral sensitivity that do not correlate to shifts in these
451 environments, and thus are unlikely to be in an adaptive direction. This was a somewhat
452 surprising result as A_2 chromophores are commonly used by many species of fish found
453 in freshwater lakes or streams [22]. The potentially maladaptive shifts seen when
454 substituting to A_2 are a result of overshooting long-wavelength sensitivity relative to the
455 prevalence of these wavelengths in the surveyed freshwater lakes. This finding is
456 consistent with work suggesting A_2 dominated retinas are common for threespine
457 stickleback from dystrophic lakes that are strongly red-shifted relative to the marine
458 environment, as A_2 use in such an environment would likely result in shifts in an
459 adaptive direction [24].
460 In this study we provide three lines of evidence to suggest that observed shifts in
461 spectral sensitivity are adaptive: we show that they have evolved repeatedly, are
462 genetically based and that regions of the spectrum that differ between marine and
463 freshwater locations are largely the same regions that exhibit differences in spectral
464 sensitivity between populations. The methods used in this study help to understand the
465 direction of evolution of spectral sensitivity, and its relationship with ambient light.
466 However, our approach does not allow us to disentangle the relative contribution of

selection on colour discrimination, contrast detection and photon capture to shifts in spectral sensitivity. Future experimental and theoretical work will be required to determine the importance of selection on each of these functions.

Author's Contributions: D.J.R, D.S & T.V conceived of and designed the study; D.J.R, G.L.O, & T.V collected the data; D.J.R, T.V & N.H analyzed the data; T.V and N.H developed the statistical method; D.J.R wrote the manuscript with help from T.V and D.S. All authors contributed to the editing.

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Data Accessibility

The raw data and accompanying meta data are archived in the dryad database, doi: 10.5061/dryad.1mc01

Competing Interests

The authors have no competing interests.

Figure Captions

Figure 1. Normalized cone opsin gene expression of marine and freshwater populations. Marine populations are indicated in black, freshwater populations in grey. Horizontal lines indicate the mean of all populations; circles indicate individual fish. Location abbreviations: Oyster Lagoon (O), Little Campbell River (LC), Priest Lake (Pr), Paxton Lake (Pa), Little Quarry Lake (LQ), Trout Lake (T), Kirk Lake (K), and Cranby Lake (C).

Figure 2. Estimated spectral sensitivity of marine and freshwater populations assuming both only use the A_1 chromophore. Marine populations are indicated in black, freshwater in grey. The thin lines are the fitted values of spectral sensitivity from the mixed-effects model. The shaded regions are one standard error above and below the fitted values, with standard errors also derived from the mixed-effects model.

Figure 3. Normalized cone opsin gene expression of benthic and limnetic populations. The benthic populations are in black, limnetic populations in grey. Horizontal lines indicate the mean of all populations; triangles indicate individual fish. Location names abbreviated as: Priest Lake (Pr), Paxton Lake (Pa), Little Quarry Lake (LQ).

Figure 4. Opsin expression in wild and lab reared fish from a marine (Oyster Lagoon (O)) and freshwater location (Priest Lake (Pr)). Wild fish are indicated in black, lab reared fish in grey. Horizontal lines indicate the mean of the population, and points indicate individual fish.

Figure 5. (A) Correlations between shifts in spectral sensitivity of individuals from freshwater populations and differences in local light transmission relative to the reference marine population, Oyster Bay. (B) As in (A) but using irradiance to measure light environment shift. (C) Correlations between shifts in spectral sensitivity between sympatric benthic and limnetic stickleback species and differences in local light transmission. (D) As in (C) but using irradiance to compare light environments.

References

1. Endler JA. 1991 Variation in the appearance of guppy colour patterns to guppies and their predators under different visual conditions. *Vision Res.* **31**, 587 – 608.

2. Endler JA. 1992 Signals, signal conditions, and the direction of evolution. *Am. Nat.* **139**, S125 – S153.

3. Munz FW, McFarland WN. 1977 Evolutionary adaptations of fishes to the photic environment. In: *The Visual System in Vertebrates*. (ed Crescitelli F). Springer, New York. pp. 194 – 274.

4. Bowmaker JK, Govardovskii VI, Shukolyukov SA, Zueva LV, Hunt DM, Sideleva VG Smirnova OG. 1994 Visual pigments and the photic environment: the cottoid fish of Lake Baikal. *Vision Res.* **34**, 591 – 605.

5. Clarke GL. 1936 On the depth at which fish can see. *Ecology* **17**, 452 – 456.

6. Denton EJ, Warren FJ. 1957 The photosensitive pigments in the retinae of deep-sea fish. *J. Mar. Biol. Assoc. UK* **36**, 651 – 662.

7. Munz FW. 1958 The photosensitive retinal pigments of fishes from relatively turbid coastal waters. J. Gen. Physiol. **42**, 445 – 459.

8. Lythgoe JN. 1979 *Ecology of Vision*. Oxford University Press, Oxford.

9. Kirk JTO. 1994 *Light and Photosynthesis in Aquatic Ecosystems*. Cambridge University Press, Cambridge.

10. Kirk JTO. 1977 Use of a quanta meter to measure attenuation and underwater reflectance of photosynthetically active radiation in some inland and coastal south-eastern Australian waters. *Aust. J. Mar. Freshwater Res.* **28**, 9 – 21.

11. Bell MA, Foster SA. 1994 *The Evolutionary Biology of the Threespine Stickleback*. Oxford University Press, Oxford.

12. Schluter D, Nagel LM. 1995 Parallel speciation by natural selection. *Am. Nat.* **146**, 292 – 301.

13. McPhail JD. 1992 Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): evidence for a species-pair in Paxton Lake, Texada Island, British Columbia. *Can. J. Zool*. **70**, 361 – 369.

14. Boughman JW. 2001 Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* **411**, 944 – 948.

15. Shichida Y, Imai H. 1998 Visual pigment: G-protein-coupled receptor for light signals. *Cell Mol. Life Sci.* **54**, 1299 – 1315.

16. Yokoyama S. 1995 Amino acid replacements and wavelength absorption of visual pigments in vertebrates. *Mol. Biol. Evol.* **12**, 53 – 61.

17. Yokoyama S, Radlwimmer FB, Blow NS. 2000 Ultraviolet pigments in birds evolved from violet pigments by a single amino acid change. *Proc. Nat. Acad. Sci.* **97**, 7366 – 7371.

18. Carleton KL, Kocher TD. 2001 Cone opsin genes of African cichlid fishes: tuning spectral sensitivity by differential gene expression. *Mol. Biol. Evol.* **18**, 1540 – 1550.

19. Jones FC, Grabherr MG, Chan YF, Russell P, Mauceli E, Johnson J, Swofford R, Pirun M, Zody MC, White S, *et al*. 2012 The genomic basis of adaptive evolution in threespine sticklebacks. *Nature* **484**, 55 – 61.

20. Rennison DJ, Owens GL, Taylor JS. 2012 Opsin gene duplication and divergence in ray-finned fish. *Mol. Phylogenet. Evol.* **62**, 986 – 1008.

21. Hárosi FI. 1994 An analysis of two spectral properties of vertebrate visual pigments. *Vision Res.* ***34***, 1359 – 1367.

22. Toyama M, Hironaka M, Yamahama Y, Horiguchi H, Tsukada O, Uto N, Ueno Y, Tokunaga F, Seno K, Hariyama T. 2008 Presence of Rhodopsin and Porphyropsin in the Eyes of 164 Fishes, Representing Marine, Diadromous, Coastal and Freshwater Species—A Qualitative and Comparative Study. *Photochem. Photobiol.* ***84***, 996 – 1002.

23. Flamarique IN. 2005 Temporal shifts in visual pigment absorbance in the retina of Pacific salmon. *J. Comp. Phys. A* ***191***, 37 – 49.

24. Flamarique IN, Cheng CL, Bergstrom C, Reimchen TE. 2013 Pronounced heritable variation and limited phenotypic plasticity in visual pigments and opsin expression of threespine stickleback photoreceptors. *J. Exp. Biol.* **216**, 656 – 667.

25. R Development Core Team. 2015 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

26. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2015 nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-119, http://CRAN.R-project.org/package=nlme.

27. Orti G, Bell MA, Reimchen TE, Meyer A. 1994 Global survey of mitochondrial DNA sequences in the threespine stickleback: evidence for recent migrations. *Evolution* **48**, 608 – 622.

28. Govardovskii VI, Fyhrquist N, Reuter TOM, Kuzmin DG, Donner K. 2000 In search of the visual pigment template. *Visual Neurosci.* **17**, 509 – 528.

29. Benjamini Y, Yekutieli D. 2001 The control of the false discovery rate in multiple testing under dependency. *Ann. Stat.* **29**, 1165–1188.

30. Fuller RC, Claricoates KM. 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.

31. O'Quin KE, Hofmann CM, Hofmann HA, Carleton KL. 2010 Parallel evolution of opsin gene expression in African cichlid fishes. *Mol. Biol. Evol.* **27**, 2839 – 2854.

32. Colosimo PF, Hosemann KE, Balabhadra S, Villarreal G, Dickson M, Grimwood J, Schmutz J, Myers RM, Schluter D, Kingsley DM. 2005 Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science* **307**, 1928 – 1933.

600 33. Hohenlohe PA, Bassham S, Etter PD, Stiffler N, Johnson EA, Cresko WA. 2010 Population
601 genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. *PLoS*
602 *Genetics* **6**, e1000862.
603 34. Fuller RC, Carleton KL, Fadool JM, Spady TC, Travis J. 2005 Genetic and environmental
604 variation in the visual properties of bluefin killifish, *Lucania goodei*. *J. Evol. Biol.* **18**, 516 –
605 523.
606 35. Seehausen O, Terai Y, Magalhaes IS, Carleton KL, Mrosso HD, Miyagi R, van der Sluijs I,
607 Schneider MV, Maan ME, Tachida H, Imai H, Okada N. 2008 Speciation through sensory
608 drive in cichlid fish. *Nature* **455**, 620 – 626.
609 36. Loew ER, Lythgoe JN. 1978 The ecology of cone pigments in teleost fishes. *Vision Res.*
610 **18**, 715 – 722.
611 37. Hunt DM, Dulai KS, Partridge JC, Cottrill P, Bowmaker JK. 2001 The molecular basis for
612 spectral tuning of rod visual pigments in deep-sea fish. *J. Exp. Biol.* **204**, 3333-3344.
613 38. Rick IP, Bloemker D, Bakker T. 2012 Spectral composition and visual foraging in the
614 three-spined stickleback (Gasterosteidae: *Gasterosteus aculeatus* L.): elucidating the role of
615 ultraviolet wavelengths. *Biol. J. Linn. Soc.* **105**, 359 – 368.
616 39. Hagen DW. 1967 Isolating mechanisms in threespine sticklebacks (*Gasterosteus*). *J. Fish*
617 *Board Can.* **24**, 1637 – 1692.
618 40. Bowmaker JK, Kunz YW. 1987 Ultraviolet receptors, tetrachromatic colour vision and
619 retinal mosaics in the brown trout *Salmo trutta*: Age-dependent changes. *Vision Res.* **27**,
620 2101 – 2108.
621 41. Flamarique IN. 2013 Opsin switch reveals function of the ultraviolet cone in fish
622 foraging. *Proc. R. Soc. Lond. B. Biol. Sci*. **280**, 20122490.

623 42. Rick IP, Bakker TC. 2008 Colour signalling in conspicuous red sticklebacks: do
624 ultraviolet signals surpass others? *BMC Evol. Biol.* **8**, 189.
625 43. Rowland WJ. 1994 Proximate determinants of stickleback behaviour: an evolutionary
626 perspective. In *The Evolutionary Biology of the Threespine Stickleback* (eds Bell MA, Foster
627 SA). Oxford University Press, Oxford. pp. 297 – 344.
628 44. Rick IP, Modarressie R, Bakker TC. 2004 Male three-spined sticklebacks reflect in
629 ultraviolet light. *Behaviour* **141**, 1531 – 1541.