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Rapid light-induced shifts in opsin expression: finding new opsins, discerning mechanisms of change, and implications for visual sensitivity

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Abstract

Light-induced shifts in cone frequency and opsin expression occur in many aquatic species. Yet little is known about how quickly animals can alter opsin expression and, thereby, track their visual environments. Similarly, little is known about whether adult animals can alter opsin expression or whether shifts in opsin expression are limited to critical developmental windows. We took adult wild-caught bluefin killifish *(Lucania goodei)* from three different lighting environments (spring, swamp and variable), placed them under two different lighting treatments (clear vs. tea-stained water) and monitored opsin expression over 4 weeks. We measured opsin expression for five previously described opsins (SWS1, SWS2B, SWS2A, RH2-1 and LWS) as well as RH2-2 which we discovered via 454 sequencing. We used two different metrics of opsin expression. We measured expression of each opsin relative to a housekeeping gene and the proportional expression of each opsin relative to the total pool of opsins. Population and lighting environment had large effects on opsin expression which were present at the earliest time points indicating rapid shifts in expression. The two measures of expression produced radically different patterns. Proportional measures indicated large effects of light on SWS1 expression, whereas relative measures indicated no such effect. Instead, light had large effects on the relative expression of SWS2B, RH2-2, RH2-1 and LWS. We suggest that proportional measures of opsin expression are best for making inferences about colour vision, but that measures relative to a housekeeping gene are better for making conclusions about which opsins are differentially regulated.

Keywords: 454 sequencing, colour vision, cyprinodontiformes, phenotypic plasticity, RH2-2, rhodopsin, tannins, visual ecology

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Introduction

Visual systems – once heralded as such complex systems as to pose problems for evolution – are highly variable (Partridge & Cummings 1999), and this variation has both genetic and environmental influences (Cronin *et al.* 2000; Lindstrom 2000; Cronin & Caldwell 2002; Hunt *et al.* 2004; Fuller *et al.* 2005; Horth 2007; Hofmann & Carleton 2009; Hofmann *et al.* 2010). Much of the recent work on visual systems has focused on the expression of cone cells in the retinas and their corresponding opsins. Opsins play a critical role in determining the spectral sensitivity of the photopigment in cones (Wald 1968; Yokoyama 1997). Photopigment consists of combining a vitamin A molecule with an opsin protein. Different opsin proteins vary in the way that they bind to vitamin A leading to differences in spectral absorbance (Yokoyama & Radlwimmer 1999, 2001; Yokoyama 2000b; Hunt *et al.* 2001). One can make inferences about visual sensitivity (and presumably behaviour) from both opsin sequence data and opsin expression patterns (Horth 2007; Hofmann & Carleton 2009).

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Populations and/or species can become locally adapted to their environmental conditions via genetic change (Yokoyama 2000a; Spady *et al.* 2005; Larmuseau *et al.* 2009, 2010) and/or adaptive phenotypic plasticity (Kawecki & Ebert 2004). There is an abundance of data suggesting genetic differences among species and, to a lesser extent, among populations and individuals (Fuller *et al.* 2005; Jokela-Maatta *et al.* 2009). However, phenotypic plasticity also plays a critical role in the tuning of visual systems. There are most likely multiple physiological mechanisms via which environmental variation in lighting conditions can alter visual sensitivity (for examples involving oil droplets and carotenoid accumulation, see Goyret *et al.* 2009; Knott *et al.* 2009; Toomey *et al.* 2010; Toomey & McGraw 2009). Shifts in chromophore usage [i.e. 11-*cis*-retinal (vitamin A1) versus 11-*cis*-3-dehydroretinal (vitamin A2)] can induce shifts in the spectral sensitivity of cone cells (Hawryshyn 1997; Partridge & Cummings 1999).

A number of species also undergo ontogenetic changes in opsin expression (Carleton *et al.* 2008), and some of these alterations correspond with shifts in photic environments (Shand *et al.* 2002; Cheng & Flamarique 2004, 2007a; Cottrill *et al.* 2009). Experimental rearing studies in bluefin killifish (*Lucania goodei*), bream (*Acanthopagrus butcheri*), and cichlids show that opsin expression frequently varies in response to the photic environment such that animals maximize photic capture (i.e. there is a correlation between opsin/cone abundance sensitive to a particular wavelength and the abundance of that wavelength in the downwelling spectrum) (Fuller *et al.* 2005, 2010; Shand *et al.* 2008; Hofmann *et al.* 2010).

The question of how quickly animals can alter opsin expression is unknown, yet has important implications for animals experiencing large fluctuations in environmental lighting conditions. Consider a system where animals can readily disperse between different lighting environments. If animals can readily alter their opsin expression (and presumably their retinas), then phenotypic plasticity will decrease variation in colour vision/spectral sensitivity because visual systems will quickly track the lighting environment. On the other hand, if changes in opsin expression are slow, or if opsin expression is only sensitive to lighting environments during critical windows of development, then dispersal between different lighting environments creates populations where there is high variation in colour vision/spectral sensitivity (Fuller & Noa 2010; Fuller *et al.* 2010).

A second issue in regard to opsin expression is how best to measure it. Most studies of gene expression using quantitative PCR (qRT-PCR) measure the expression of a gene relative to a housekeeping gene (e.g. Scott & Schulte 2005; Fangue *et al.* 2006; Scott *et al.* 2006). In contrast, studies of opsin expression frequently measure expression relative to the total pool of opsins (% relative abundance of each opsin) (Fuller *et al.* 2005, 2010; Spady *et al.* 2006; Carleton *et al.* 2008; Shand *et al.* 2008). The rationale for this approach is that colour vision relies on the differential stimulation of cones and photopigment (Endler 1990; Vorobyev *et al.* 1998). Differences in the proportional abundance of each opsin reflect potential differences colour sensitivity (Fuller *et al.* 2003, 2004; Cheng & Flamarique 2004, 2007a; Parry *et al.* 2005; Shand *et al.* 2008), whereas differences in opsin expression relative to a housekeeping gene might not be indicative of differences in colour vision *per se,* but instead reflect high or low overall opsin abundance.

However, measures of opsin abundance relative to the total opsin pool are somewhat problematic because the expression values for each opsin are not independent of one another. This makes inferences about opsin regulation (i.e. which opsins are up-regulated vs. down-regulated) difficult because an increase in the expression of one opsin necessitates a decrease in the relative expression of the others because of an increased total opsin pool. The second issue with measuring opsin expression relative to the total opsin pool is that there is the potential to miss some of the opsins which may affect the inferred patterns of expression. Massive efforts have been made to find all the opsins in a number of fish including cichlids, poeciliids and other cyprinodontiforms. A large number of opsins have been found in some groups (Carleton & Kocher 2001; Spady *et al.* 2005; Hoffmann *et al.* 2007; Weadick & Chang 2007; Ward *et al.* 2008; Windsor & Owens 2009; Watson *et al.* 2010). The implication of potentially missing an opsin that in reality contributes to the total opsin pool is unclear.

The current study had three goals. Our first goal was to examine the tempo and magnitude of changes in opsin expression with respect to lighting environment in adult bluefin killifish, *Lucania goodei*. To do this, we took wild-caught adult animals from three populations (spring, swamp and variable) and placed them in clear and tea-stained conditions and monitored opsin expression over 4 weeks. Our second goal was to compare opsin expression relative to a housekeeping gene (hereafter referred to as '$\text{relative}_{(hk)}$' expression) versus opsin expression relative to the total opsin pool (hereafter referred to as 'proportional' expression). We note that 'proportional' opsin expression is a slight change in terminology from previous studies which have referred to this metric as relative opsin expression (see citations given earlier). Our third goal was to determine the effects of missing one of the

opsins. We performed a 454 sequencing study using *L. goodei* eyes, fins, brains, ovaries and testes. We examined the resulting contigs for additional opsins – in particular Rhodopsin 2-2 (RH2-2). We determined the effects of measuring opsin expression with and without this opsin to determine how its presence/absence affects proportional expression patterns in the other opsins.

The study system

The bluefin killifish, *Lucania goodei*, is a compelling system within which to examine the tempo of light-induced changes in opsin expression. *L. goodei* is a small freshwater fundulid that occurs under a wide range of lighting environments ranging from tea-stained swamps that have reduced transmission of UV/blue wavelengths to crystal clear springs that have high transmission of UV/blue wavelengths (Fuller 2002). Swamp animals are less sensitive to UV/blue wavelengths and possess fewer UV and violet cones than animals from spring populations (Fuller *et al.* 2003). These differences in cone frequency match differences in expression of opsins (Fuller *et al.* 2004). *L. goodei* express five major classes of opsins—SWS1, SWS2A, SWS2B, RH2 and LWS (Fuller *et al.* 2004; Yokoyama *et al.* 2007). In combination with 11-cis retinal, the genes produce the following pigments: SWS1 - UV photopigment (maximum absorbance (λ_{max}) = 359 nm); SWS2B - violet photopigment (λ_{max} = 405 nm); SWS2A - blue photopigment (λ_{max} = 455 nm); RH2-1 - yellow photopigment (λ_{max} = 539 nm); and LWS - red photopigment (λ_{max} = 573 nm, for *L. goodei*). *L. goodei* has at least two different LWS loci (genbank accession numbers AY296741, AY296740). Preliminary evidence indicates no difference in their spectral properties (N.S. Blows and S. Yokoyama, personal communication). Because the map of genotype to phenotype is straightforward for these proteins, we can use differences in opsin expression to infer qualitative differences in cone frequency (Carleton & Kocher 2001; Fuller *et al.* 2004, 2005). Two separate experiments have documented pronounced phenotypic plasticity in opsin expression in *L. goodei* (Fuller *et al.* 2005, 2010). Animals raised in clear water conditions have higher SWS1 and SWS2B expression (corresponds to ultraviolet and violet photopigment). Animals raised in tea-stained water have higher RH2 and LWS expression (corresponds to yellow and red photopigment) (Fuller *et al.* 2005, 2010). However, these animals were raised from the larval stage until adulthood under their respective lighting conditions. Hence, the temporal dynamics over which differences in opsin expression emerge are unknown.

Methods

Finding all the opsin genes—454 sequencing

We created an EST library for *L. goodei* using 454 sequencing. This is part of a larger endeavour to develop more genetic resources for the *Lucania* system which includes research into visual ecology (eyes), colour pattern polymorphism (fins), speciation with respect to intrinsic genetic incompatibilities relative to its sister species (ovaries and testes), adaptation to salinity (gills) and behaviour (brains). Hence, we created a pooled sample of RNA from fin, eye, gill, ovary, testes, and brain. We used animals from five distinct populations across Florida (1- Upper Bridge Wakulla River, Wakulla, Co., FL, USA; 2- St. Mark's National Wildlife Refuge Gambo Bayou, Wakulla, Co., FL, USA; 3- 26-Mile Bend, Everglades, Broward Co., FL, USA; 4- Rum Island Park, Santa Fe River, Columbia Co., FL, USA; 5- Delk's Bluff Bridge, Oklawaha River, Marion Co., FL, USA). RNA was extracted using a standard Trizol protocol with phase-locked tubes, and subsequently purified with sodium acetate/ethanol precipitation. Details on cDNA synthesis/normalization, library preparation, and sequencing can be found in Dassanayake *et al.* (2009). Preparation of barcoded GS FLX libraries from the normalized cDNA samples, emulsion-based clonal amplification and sequencing on the 454 Genome Sequencer FLX system were performed in the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign according to the manufacturer's instructions (454 Life Sciences, Branford, CT, USA). Contig assembly was carried out with SeqmanPro from Lasergene with a match length of 40 bp and minimum match of 90%. Sequence annotation was carried out using a series of blast searches based on inferred amino acid sequences (Altschul *et al.* 1997) that compared *L. goodei* with the non-redundant database from NCBI. A detailed manuscript with the details and results on the entire project is in preparation.

For the purpose of this study, we wanted to determine whether there were additional opsins that were missed in earlier studies (Fuller *et al.* 2004, 2005, 2010; Yokoyama *et al.* 2007; Fuller & Noa 2010). In particular, a number of fish species possess two separate RH2 opsins, but *L. goodei* appeared to only possess one (Gojobori & Innan 2009; Hofmann & Carleton 2009). We first examined our contigs to determine whether the previously described opsins had been recovered and whether additional opsins were present. Indeed, we found a second RH2 opsin. To determine its relationship with other

RH2 opsins, we constructed a neighbour-joining tree. We included RH2 sequence from all atherinomorph species for which two RH2 opsins had been detected (*Xiphophorus helleri*, *Poecilia reticulata*, *Jennynsia onca*, *Anableps anableps and Oryzias latipes*) as well as sequences from a cichlid (*Orechromis niloticus*). The overall genetic distance was low indicating that neighbour joining is appropriate (Hall 2008). We used the RH1 opsin gene from *L. goodei* as an outgroup. Identical tree structure was found using maximum parsimony. We aligned the sequences using ClastalW in Mega (Tamura *et al.* 2007). The phylogeny was based on p-distances, and all sites were considered informative. Identical results were obtained when the analysis was limited to third codon positions. A bootstrap analysis with 1000 replicates was used to determine node confidence.

Opsin expression

We collected bluefin killifish from three populations in Florida, May 22–27, 2010, using seines and dipnets. We chose a swamp population (26-Mile Bend, Everglades Drainage, Broward Co.), a spring population (Upper Bridge, Wakulla River, Wakulla/St.Mark's Drainage, Wakulla Co.), and a population that varies between spring and swamp conditions (Rum Island Park, Columbia Co.). The Rum Island Park site occurs along a stretch of the Santa Fe River where two springs (Rum Island Springs and Blue Springs) connect to the river over a 1-mile stretch. The lighting environment is variable both temporally and spatially. During dry years, the Santa Fe River is exceedingly clear, but during wet years, the river is tannin-stained because of large amounts of incoming organic material. Spatial variation occurs because of clear spring water flowing into a tannin-stained main stem. We collected the fish in tannin-stained water in the Sante Fe River approximately 200 m away from Rum Island Springs. These fish were transported to our laboratory at the University of Illinois.

The goal of this experiment was to determine the effects of population, lighting environment and time on opsin expression. To do this, we housed field-caught fish in either tea-stained or clear water conditions, and measured opsin expression over 4 weeks.

Twelve 76 -litre (20 gallon) fish tanks were established (three populations * two water treatments * two replicate tanks = 12). Tanks were filled with dechlorinated city water, and half contained tea-stained water that mimicked swamp conditions and half contained clear water that mimicked spring conditions. The tea-stained treatment was created by adding Nestea instant decaffeinated tea. Each tank also had a UV filter and a power filter that kept unicellular algae out of the water column. All the fish were measured, and sex was identified before placing twenty fish of a given population into a tank.

We measured opsin expression over a 4-week period. We established our treatments on 'day 0' (June 1, 2010) and subsequently sampled 2 fish from each tank on days 1, 3, 7, 14, 21 and 28. Unfortunately, we lost a few of our RNA samples, so we were forced to combine days 1 and 3 into an 'early' time treatment, days 7 and 14 into a 'middle' time treatment, and days 21 and 28 into a 'late' time treatment in order to achieve sufficient replication across all treatment combinations. The RNA samples were lost when the pellet was accidentally dislodged during precipitation and ethanol rinses. To account for circadian rhythms, all fish samples were collected between 10:00 and 11:30 am. The fish were euthanized with an overdose of MS-222. From each fish, both eyes were removed, placed in cold 95% ethanol, and punctured so that the ethanol could enter the eye. The samples were stored at −80 °C until RNA extraction. Each fish was considered to be a sample (two eyes per sample), and each sample was labelled with a unique identifying code. We used roughly equal numbers of males and females from each treatment combination. Sex had little effect on opsin expression and is not considered further.

We quantified opsin expression via qRT-PCR. Methods are described in detail elsewhere (Fuller *et al.* 2004, 2005, 2010). RNA was extracted using a standard Trizol protocol with phase-locked tubes. RNA was reverse transcribed into cDNA using SuperScript II. We used taqman primers and probes that were specific to each of six opsin genes plus a housekeeping gene, elongation factor 1-alpha (hereafter EF1-α; see Table 1 for primer and probe sequences). The gene-specific primers and probes were made for RH2-2 and EF1-α using Primer Design software (ABI). We used Sequencher (version 4.6) to verify that primers and probes did not hybridize with any other opsin. The ABI Prism 7700 Sequence Detection System was used for our qRT-PCR. We performed three replicate reactions for each of our seven genes. Fluorescence was monitored over 40 cycles (94C-15s/55C-30s/65C-1 minute) using the ABI Prism 7700 Sequence Detection System. We examined the three replicate reactions for each opsin for each individual and discarded any apparent outliers. We then calculated the average critical threshold value for each gene for each individual.

From these data, we calculated both the relative$_{(hk)}$ and the proportional expression of the six opsin genes. The proportional expression of the six opsins with respect to the total opsin pool was calculated as

Table 1 Forward primer, probe and reverse primer sequence and access numbers and efficiencies for each of the six cone opsins plus the housekeeping gene, elongation factor 1-alpha. The data on Dryad are available at doi:10.5061/dryad.k8073

Locus	Forward primer	Probe	Reverse primer	Accession no.	Efficiency
SWS1	TTA CAC CTT GTG TGC CTT GGA A	CCG TAG CAG GCC TGG TGA CGT CCT	GGG TTT GCA GAT GAC CAG GTA C	AY296735.1	1.878
SWS2B	GCT GCA AGA TTG AAG GAT TTA CTG	GGT GTT GGT GGC ATG GTC AGC CTT TG	CCA ACC ATC TTT CGA ATG CAA	AY296736.1	1.935
SWS2A	CAT GCA AGA TTG AAG GTT TCA TTG	ACA CTA GGG GGT ATG GTA AGC CTG TGG TCTCT	CCA GCC ATC GTT CAA AAG CT	AY296737.1	1.877
RH2-2	ACA CCA TCA GGA CGC ACA TG	CAC TTC CTT TTC AGC TTT CTG GGT GGA AG	GCC CAG CAG CAG GAC TCA	Dryad	1.940
RH2-1	CTT CTG CGG TAT TGA GGG ATT C	AAC ACT CGG AGG TGA GGT TGC TCT CTG GT	AAC AAT ATA TCT CTC AAT AGC CAG AAC AA	AY296739.1	1.930
LWS	TGG TGT GCT CCT CCC ATC TT	TGG AGC AGG TAT TGG CCC CAT GGA C	TCT TCA CTT CCA CTG AAC ACA TCA G	AY296740.1AY296741.1	1.933
EF 1-α	ACT CTC TCC ACC CTG GGT CAT	TTC TCT GCT CAA GGA CTG GCT TAT	TTG CGA TGG GTT TTG ATC AG	Dryad	1.937

$$\frac{T_i}{T_{all}} = \frac{\frac{1}{(1+E_i)^{C_{ti}}}}{\sum \frac{1}{(1+E_i)^{C_{ti}}}}.$$

$\frac{T_i}{T_{all}}$ is the proportional gene expression for a given gene *i*. E_i is the PCR efficiency for each primer/probe set, and C_{ti} is the average critical cycle number for each gene. PCR efficiencies were quantified using a 5-fold dilution series for each gene (1×, 0.5×, 0.1×, 0.05×, 0.01×, 0.005×, 0.001×—see Table 1 for values). Proportional opsin expression values represent the proportion of the total opsin pool that is attributable to each of the six opsin genes. For each individual, the six proportional expression values sum to one.

We also calculated the relative$_{(hk)}$ expression of the six opsin genes with respect to our housekeeping gene EF1-α according the following equation:

$$\frac{T_i}{T_{ef}} = \frac{\left(1/(1+E_i)^{Ct_i}\right)}{\left(1/(1+E_{ef})^{Ct_{ef}}\right)}.$$

T_i/T_{ef} is the expression of each individual opsin *i* relative to the expression of EF1-α. Relative$_{(hk)}$ expression values represent the level of expression relative to the housekeeping gene EF1-α. Although all six opsin genes are measured with respect to EF1-α, the expression of each opsin gene is independent of the expression of the other opsins. These data have been submitted to Dryad (doi:10.5061/dryad.k8073). Sequence data for EF1-α and RH2-2 were also submitted to Dryad (doi:10.5061/dryad.k8073). Because we used pooled cDNA from several populations for our 454 sequencing, the data were not acceptable for Genbank. We are currently sequencing these genes from a single population and will submit them to Genbank soon.

To determine the effects of time, population and lighting environment on opsin expression, we performed two different analyses. First, we performed a repeated measures analysis where the relative$_{(hk)}$ expression values of the six opsins were the repeated measures. We used a general linear model to examine the effects of time, population, water and their interactions on opsin expression. This analysis allowed for an examination of treatment effects on overall expression and also for a determination of whether the treatments differentially affected the expression of different opsins. To determine whether the patterns of relative$_{(hk)}$ gene expression could simply be attributable to EF1-α expression, we performed the same analysis on EF1-α critical threshold values. Second, we performed a set of univariate analyses on both the relative$_{(hk)}$ and proportional expression of each of the six opsins. Again, we examined the effects of time, population, water and their

interactions on opsin expression. Relative$_{(hk)}$ opsin expression was natural log-transformed to meet the assumptions of analysis of variance (Sokal & Rohlf 1995). Finally, we examined the effects of 'missing' the RH2-2 on proportional opsin expression. We recalculated proportional expression excluding RH2-2 from the calculations and reanalysed the data. All statistical analyses were performed using SAS v. 9.1 (SAS Institute, Cary, NC, USA).

Results

454 sequencing and opsins

Our 454 sequencing and subsequent assembly resulted in 69 581 contigs and 87 105 singlets in *L. goodei*. We recovered most of the major opsin classes. A blast search of our assembled contigs indicated that six of the seven previously documented opsins were detected. Specifically, we found contigs that represented full-length cDNAs that were >99% identical to SWS1, SWS2B, RH1, RH2, LWSA and LWSB. Small fragments of SWS2A were detected, but there was no single contig that corresponded to the full-length SWS2A cDNA. This finding is consistent with previous studies showing exceedingly low levels of SWS2A expression in *L. goodei*.

RH2-2 was also detected among our contigs. We obtained a long contig whose initial blast result indicated was most similar to RH2-2 found in guppy. To verify this, we performed a small phylogenetic analysis that included all of the species of Atherinomorpha for which two RH2 loci had been detected plus a cichlid species. Figure 1 shows the consensus bootstrap tree for RH2. Two distinct clades with high bootstrap support emerged that correspond to these two gene families (RH2-1 and RH2-2). The newly discovered *L. goodei* opsin is well supported in a clade with the RH2-2 opsin of the other members of Cyprinodontiformes as well as medaka and cichlid. In-situ expression work in cichlids (Spady *et al.* 2006) and medaka (Matsumoto *et al.* 2006) indicates that the other RH2 opsins which belong to this clade are 'blue-green' sensitive (Fig. 1).

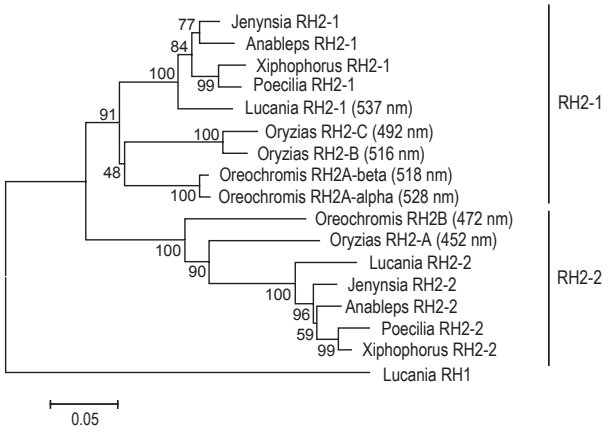

Fig. 1 Phylogenetic analysis of RH2 opsin coding sequences in Atherinomorpha plus a representative cichlid. The neighbour-joining tree was constructed in Mega (Tamura *et al.* 2007) using p-distances. All codon positions were considered informative. The scale bar indicates the number of substitutions per 100 sites. RH1 for *L. goodei* was used as the outgroup. Taxa and accession numbers are as follows: (*Jennynsia onca* RH2-1: GQ221668, RH2-2: GQ221669; *Anableps anableps* RH2-1: FJ11149, RH2-2: FJ11150; *Poecilia reticulata* RH 2-1: DQ234859, RH2-2 DQ234858; *Xiphophorus helleri* RH2-1: GU454732, RH2-2: GU454733; *L. goodei* RH1: AY296738.1, RH2-1: AY296739, RH2-2: dryad: doi:10.5061/dryad.k8073; *Oryzias latipes* RH2a: AB223053, RH2b: AB223054, RH2c: AB223055; *Oreochromis niloticus* RH2A-alpha: DQ235683, RH2A-beta: DQ235682.1, RH2B: DQ235681.1). λ_{max} values are denoted in parentheses where applicable.

Repeated measures—relative$_{(hk)}$ opsin expression

Overall relative$_{(hk)}$ opsin expression varied as a function of time, water and population (Table 2A). Opsin expression was lowest in the first sampling period, but increased over time (Fig. S1A, Supporting information). Opsin expression was highest in the tea-stained lighting treatment compared with the clear treatment (Fig. 2). Opsin expression was also highest in the spring and variable populations compared with the swamp population (Fig. 2). The effects were not attributable to variation in the expression of the reference gene, EF-1α (Table 2C). Neither population nor water affected EF-1α critical threshold values. There was an effect of time on EF-1α, but the pattern could not, by itself, explain the effect of time on opsin expression. Furthermore, including the critical cycle-values of EF-1α as a covariate in the opsin analyses did not alter the treatment effects. Hence, the pattern for relative$_{(hk)}$ opsin expression does not appear to be driven by variation in EF-1α. Repeated measures analysis of relative$_{(hk)}$ expression indicated overall variation in the expression levels of the six opsin classes (Table 2—LWS > RH2-1 > RH2-2 > SWS1 > SWS2B > SWS2A). The within-treatment effects indicated that water, population and time had different effects on different opsins (Table 2B). We discuss these patterns in the following paragraphs.

Comparing relative$_{(hk)}$ and proportional measures of opsin expression

The two measures of opsin expression (relative$_{(hk)}$ and proportional) produced drastically different patterns with respect to the experimental treatments (Supporting information Table S1, Fig. 2A–L). SWS1 shows the

Table 2 Repeated measures of natural log-transformed relative$_{(hk)}$ opsin expression (A and B) and the analysis of variance for the critical threshold value for the housekeeping gene elongation factor 1- alpha (C). df = degrees of freedom, MS = mean-square, *P* = probability value

Source	df	MS	*F*	*P*
(A) Between-subject effects				
Population (P)	**2,97**	**19.16**	**11.31**	**<.0001**
Water (W)	**1,97**	**12.62**	**7.45**	**0.0075**
Time (T)	**2,97**	**11.13**	**6.57**	**0.0021**
T*P	4,97	1.41	0.83	0.5092
T*W	2,97	0.79	0.46	0.6303
P*W	2,97	1.97	1.17	0.316
T*P*W	4,97	2.27	1.34	0.2604
(B) Within-subject effects				
Opsins	**5,485**	**469.35**	**2662.36**	**<.0001**
Opsins*population	**10,485**	**1.41**	**8.01**	**<.0001**
Opsins*water	**5,485**	**1.06**	**6.00**	**<.0001**
Opsins*time	**10,485**	**0.36**	**2.03**	**0.029**
Opsins*T*P	20,485	0.17	0.99	0.4754
Opsins*T*W	10,485	0.09	0.49	0.8987
Opsins*P*W	10,485	0.06	0.31	0.9776
Opsins*T*P*W	20,485	0.26	1.50	0.0765
(C) EF-1a				
Population	2,97	5.62	2.26	0.1099
Water	1,97	0.66	0.27	0.6068
Time	**2,97**	**12.96**	**5.21**	**0.0071**
T*P	4,97	2.82	1.13	0.3458
T*W	2,97	2.11	0.85	0.4308
P*W	2,97	1.48	0.6	0.5531
T*P*W	4,97	1.25	0.5	0.7345

Terms in bold indicate $P < 0.05$.

most drastically different patterns between relative$_{(hk)}$ and proportional expression (Fig. 2 A1-A2). Supporting information Table S1(A1, A2) shows the univariate analyses for relative$_{(hk)}$ and proportional expression of SWS1. Both analyses indicate a large effect of population. However, the analysis of proportional expression shows a large effect because of water treatment ($F_{1,97} = 15.86$, $P = 0.0001$), whereas this effect is completely absent from the relative$_{(hk)}$ expression pattern ($F_{1,97} = 0.03$, $P = 0.8546$). An examination of the graphs shows an even more disturbing pattern. Relative$_{(hk)}$ SWS1 expression shows little difference as a function of water treatment – particularly for the spring and variable populations. However, the proportional expression values show a fairly substantial difference where SWS1 is expressed at higher levels in the clear water condition – particularly for the spring and variable populations.

The exact opposite effect of water treatment can be seen for SWS2B, RH2-1, RH2-2 and LWS (Fig. 2). For these opsins, water had large effects on the relative$_{(hk)}$ expression, but less effect on the proportional measures of opsin expression (Supporting information Table S1G–L and Fig. 2). SWS2B, RH2-2, RH2-1 and LWS all had increased relative$_{(hk)}$ expression in the tea-stained water treatment – particularly for the spring and variable populations. However, proportional measures of SWS2B, RH2-2 and LWS (and to a lesser extent, RH2-1) showed little effect of water treatment.

The effect of population also produced counter-intuitive patterns for some opsins. Relative$_{(hk)}$ expression of RH2-2, RH2-1 and LWS varied as an effect of population where expression was highest in the spring and variable populations. Analysis of proportional expression indicated the exact opposite pattern where proportional expression of RH2-2 and RH2-1 was highest in the swamp population.

Opsin expression over time

Different opsins demonstrated different patterns with respect to time (Table 2B, Supporting information Table S1 and Fig. S1). The SWS opsins tended to be highest at the intermediate time points. The RH2 opsins showed little pattern with respect to time. LWS increased over time. These patterns were somewhat consistent for relative$_{(hk)}$ and proportional measures of expression (see Supporting information Fig. S1).

For most opsins, there was little evidence that plasticity differed between the populations (i.e. no time*population, water*population, nor time*water*population interaction for SWS1, SWS2A, RH2-1 and RH2-2; see Supporting information Figures S2 and S3). However, LWS expression varied as a function of both the time*population and the time*population*water interactions (Fig. 3, Supporting information Table S1). Similarly, relative$_{(hk)}$ SWS2B varied as a function of time*population*water (Supporting information Fig. S3 and Table S1), but there was no such effect for proportional SWS2B expression. The spring population had particularly high LWS expression early in the experiment in the tea-stained treatment, whereas the variable population had high expression in the tea-stained treatment in the later part of the experiment.

Does 'missing' RH2-2 alter the interpretation for proportional opsin expression?

Previously, Fuller *et al.* have calculated opsin expression relative to the total pool of measured opsins (Fuller *et al.* 2004, 2005, 2010). However, these studies did not measure RH2-2 expression, because it was hitherto unknown. To address the question of whether excluding RH2-2 had large effects on our previous analyses, we re-calculated proportional opsin expression excluding the RH2-2 data and re-analysed our data. The effects of our treatments were robust to whether or not RH2-2 was

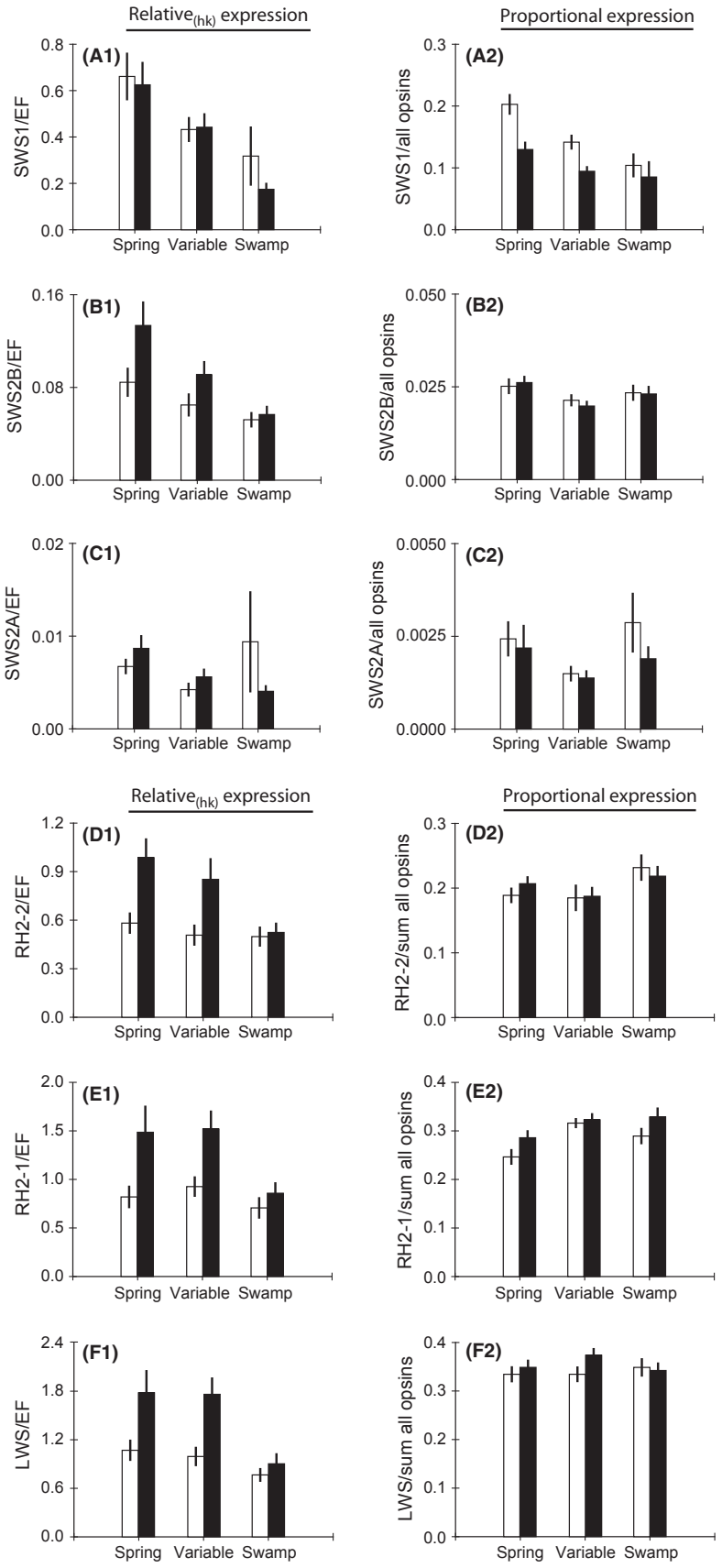

Fig. 2 Relative$_{(hk)}$ (expression relative to EF 1-α) and proportional (expression relative to total opsin pool) expression of the SWS1, SWS2B, SWS2A, RH2-2, RH2-1 and LWS opsins across populations and water treatments. Mean ± SE. Clear water treatments are denoted by open bars, and tea-stained water treatments are denoted by black bars. Sample sizes are as follows: Spring: clear $n = 19$, tea $n = 21$; Variable: clear $n = 21$, tea $n = 18$; Swamp: clear $n = 18$, tea $n = 18$.

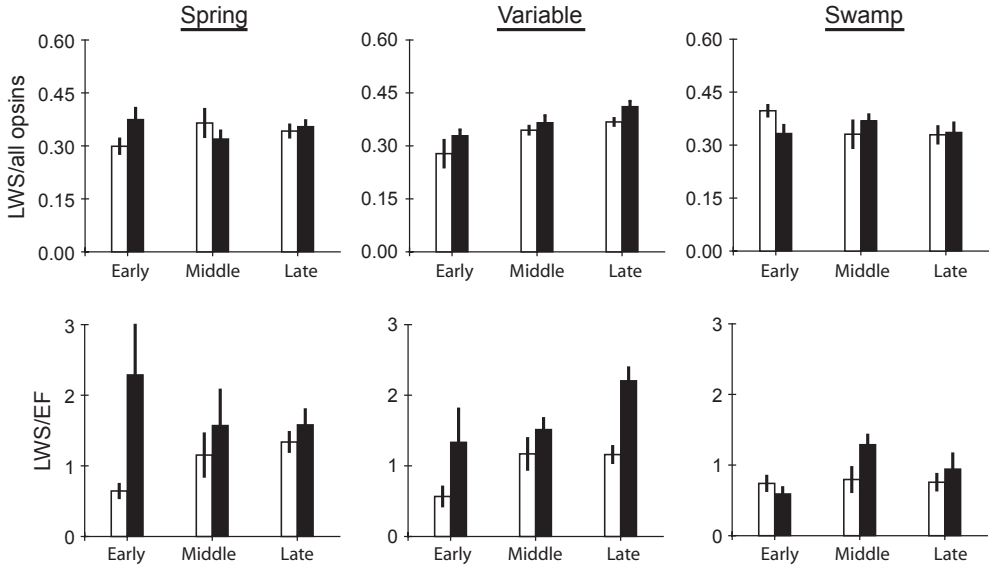

Fig. 3 Proportional and relative$_{(hk)}$ expression of LWS as a function of population, water treatments and time. Mean ± SE. Clear water treatments are denoted by open bars and tea-stained water treatments are denoted by black bars. Opsin expression was measured at early (days 1 and 3), middle (days 7 and 14) and late (days 21 and 28) time periods. Sample sizes are as follows: Spring clear: early $n = 6$, middle $n = 5$, late $n = 8$; Spring tea: early $n = 6$, middle $n = 7$, late n = 8; Variable clear early $n = 7$, middle $n = 7$, late $n = 8$; Variable tea early $n = 6$, middle $n = 4$, late $n = 8$; Swamp clear: early $n = 5$, middle $n = 6$, late $n = 7$; Swamp tea: early $n = 6$, middle $n = 4$, late $n = 8$.

included in the analysis (Supporting information Table 2). Treatments that previously had large effects ($F > 5$) remained statistically significant ($P < 0.01$). A few effects that were marginally significant (or non-significant) changed. In our previous analysis, there was a trend for the interaction between time and population to affect SWS2B expression ($F_{4,97} = 2.27$, $P = 0.0669$), and this effect became statistically significant when RH2-2 was excluded from the analysis ($F_{4,97} = 3.36$, $P = 0.0128$). On the other hand, the previous analysis showed a significant effect of time*population and time*population*water on LWS expression ($F_{4,97} = 3.57$, $P = 0.0092$, $F_{4,97} = 2.62$, $P = 0.0395$, respectively), but removing RH2-2 from the analysis rendered this effect non-significant ($F_{4,97} = 2.07$, $P = 0.0904$, $F_{4,97} = 2.62$, $P = 0.0866$).

Discussion

Four main results emerge from this study. First, *L. goodei* express RH2-2 opsin in their retinas. The RH2-2 is most likely responsible for 'blue' photopigment. Second, the analysis of proportional opsin expression is robust to the inclusion/exclusion of the RH2-2 opsin. Third, relative$_{(hk)}$ and proportional measures of opsin expression differ dramatically with respect to the inferred effects of time and population. Fourth, there was a surprisingly small effect of time on opsin expression. We discuss each of these findings and their implications in turn.

Lucania goodei *and RH2-2*

Prior to this study, five major classes of cone opsins and one rod opsin were known to be expressed in *L. goodei:* SWS1, SWS2A, SWS2B, RH2-1, LWS and RH1 (Fuller *et al.* 2004, 2005, 2010; Yokoyama *et al.* 2007). However, throughout the teleosts, the RH2 locus was known to have undergone multiple duplication events because zebrafish, cichlids, medaka and several cyprinodontiforms (*Xiphophorus helleri*, *Anableps anableps*, *Jenynsia onca* and *Poecilia reticulate*) all have 2–4 different RH2 loci (Hoffmann *et al.* 2007; Gojobori & Innan 2009; Hofmann & Carleton 2009; Owens *et al.* 2009; Watson *et al.* 2010). The current study shows that *L. goodei* has two RH2 opsins as well.

We contend that the RH2-2 opsin is responsible for blue photopigment. Previously, we assumed that the blue photopigment was attributable to the SWS2A opsin because in vitro studies indicated that SWS2A in combination with 11-cis retinal produced a λ_{max} value of 448 nm (Yokoyama *et al.* 2007), which was similar to the λ_{max} value measured for blue cones (455 nm). The idea that blue photopigment is attributable to the RH2-2 stems from three findings. First, *L. goodei* express RH2-2 at much higher levels than SWS2A. Previous work on *L. goodei* has found extremely low levels of SWS2A expression (<1%). The low levels of SWS2A expression were perplexing because microspectrophotometry (MSP)

studies showed that the blue cone was relatively common in *L. goodei* retinas (Fuller *et al.* 2003; see Watson *et al.* 2010 for a similar finding in Xiphophorus). Second, Molstad (2008) performed an in-situ immunloabeling study in *L. goodei* using SWS1, SWS2A, SWS2B, RH2-1, LWSA and LWSB probes. Molstad found that the SWS2B and SWS2A probes hybridized to the same single cone. However, Fuller *et al.* (2003) found that the blue cone was usually a member of a double cone. Molstad also found many unlabelled cone cells (i.e. they failed to hybridize with any of her probes) which were members of a double cone. We propose that the unlabelled cones in Molstad's study express the RH2-2 opsin. Third, three other fishes have an RH2 gene that is maximally sensitive in the blue-green spectrum (zebrafish λmax of 467 nm - Chinen *et al.* 2003; medaka λmax of 452 nm - Matsumoto *et al.* 2006; cichlids λmax of 472 nm - Spady *et al.* 2006). While these opsins have different names, they form a well-resolved clade with the RH2-2 reported here for *L. goodei* (Fig. 1).

The fact that *L. goodei* expresses RH2-2 at relatively high levels raised the question of whether previous quantitative genetic and population studies in this system produced aberrant patterns of gene expression (Fuller *et al.* 2005, 2010). While the proportion of opsins from each class contributing to the total opsin pool undoubtedly differs with the addition of RH2-2, the overall patterns in gene expression were robust to whether or not RH2-2 was included in the calculations.

Proportional vs. relative$_{(hk)}$ measures of opsin expression

Proportional and relative$_{(hk)}$ measures of opsin expression produced drastically different patterns with respect to treatment effects. The most glaring example can be seen in the SWS1. Proportional SWS1 expression was highest in the clear water treatment – particularly for the spring and variable populations. In contrast, the relative$_{(hk)}$ measures found no effect of water treatment – particularly for the spring and variable populations. The phenomenon generating these patterns can be discerned when one considers the relative$_{(hk)}$ expression of the other opsins. SWS2B, RH2-2, RH2-1 and LWS all have higher relative$_{(hk)}$ expression in the tea-stained condition – particularly for the spring and variable populations. Hence, the expression of nearly all the opsins (except SWS1) increases under the tea-stained condition. Because SWS2B, RH2-2, RH2-1 and LWS show the same pattern of relative$_{(hk)}$ expression and numerically dominate the total opsin pool, the effect of the environment on their proportional expression is diminished.

The question of which measure of opsin expression is best depends on the biological question at hand. If one is solely interested in differences in colour vision, then proportional measures of opsin expression may be more relevant. Colour vision relies on the differential stimulation of cones in the retina (Hofmann & Carleton 2009). Differences in proportional opsin expression are thought to reflect differences in either the proportional abundance of cone cells in the retina or differences in size and/or photopigment density of the cone tips (Carleton & Kocher 2001; Fuller *et al.* 2004; Parry *et al.* 2005; Shand *et al.* 2008; Hofmann *et al.* 2010), and, hence to reflect fundamental differences in the colour vision of animals. In *L. goodei,* animals from the spring population kept in clear water conditions had the highest proportional amount of SWS1 and presumably were more sensitive to UV light than were swamp animals kept in tea-stained water.

The problem with proportional measures of opsin is that they have the potential to mislead with regard to the mechanism by which differences in opsin expression arise. In four separate studies, we have found that proportional SWS1 expression is higher in clear water conditions and that the environment effects are largest for the SWS1 opsin (Table 3). This led us to hypothesize that the SWS1 opsin was being 'up-regulated' in clear water conditions and 'down-regulated' in tea-stained conditions. This hypothesis seemed particularly likely, given that UV cone cells in salmon and trout were present in young fish, lost upon migration to the sea, and regained upon return to fresh water streams (Bowmaker & Kunz 1987; Beaudet & Hawryshyn 1999; Allison *et al.* 2003; Hawryshyn *et al.* 2003) suggesting that UV cones and SWS1 expression are particularly plastic (Cheng & Flamarique 2004, 2007a,b). In addition, our previous work shows that UV cones are greatly reduced in swamp animals relative to spring animals and that SWS1 expression mirrors this pattern (i.e. many animals lack detectable UV cones; Fuller *et al.* 2003). However, the current data suggest that – at least when adult animals are placed under tea and clear water conditions – animals increase expression of everything but the SWS1 opsin.

Timing of opsin expression

We found that the effects of water treatment were present across all three sampling periods. This suggests that changes in opsin expression occur rapidly with respect to lighting environment. In essence, we missed the critical time periods when opsin expression diverged between the two lighting environments. Unfortunately, we lacked the power to resolve opsin expression patterns between days 1 and 3. Regardless, these results indicate that changes in opsin expression can occur quite quickly and that these changes can occur in adult animals.

Table 3 Summary of opsin expression studies in *Lucania goodei*. Spring and swamp represent overall phenotypic differences in Fuller *et al.* (2004) and in the current study. Fuller *et al.* (2005) was a quantitative genetic study conducted within a single swamp populations where half-sib families were divided between clear and tea-stained water. Fuller *et al.* (2010) was a quantitative genetic study comparing crosses within and between the spring and swamp populations and raised in clear and tea-stained water. Bold terms show effects that differ between the proportional and relative$_{(hk)}$ measures of opsin expression

	Study comparisons being made (measure of opsin expression)				
Opsin	Fuller *et al.* 2004 population differences (proportional expression)	Fuller *et al.* 2005 V_e and V_g within the swamp population (proportional expression)	Fuller *et al.* 2010 V_e and V_g within and among spring and swamp population (proportional expression)	This Study population differences and subsequent V_e (proportional expression)	This Study population differences and subsequent V_e (relative$_{(hk)}$ expression)
SWS1	spring > swamp	clear > tea	spring > swamp (V_g) clear > tea	spring > variable & swamp **clear > tea (large effect)**	spring & variable > swamp **no water effect**
SWS2B	spring > swamp	V_g due to dams clear > tea	clear > tea	spring > variable & swamp	spring & swamp > variable
SWS2A	no effect	V_g due to dams	no effect	no effect	spring > variable & swamp
RH2-2	not examined	not examined	not examined	**swamp > spring & variable**	**spring & variable > swamp** tea > clear
RH2-1	swamp > spring	V_g due to sires tea > clear	no effect	**swamp & variable > spring** tea > clear	**spring & variable > swamp** tea > clear
LWS	swamp > spring	V_g due to dams tea > clear	tea > clear	no overall effects	spring & variable > swamp tea > clear

Exactly what is changing in the retina is unclear. One possibility is that the distribution of cone cells varies with alterations in lighting environment. However, major alterations in the retinal mosaic would presumably occur over a longer period of time which would lead to an interaction between lighting environment and time. Another possibility is that cone cells remain unchanged, but that the size of the cone tips and/or the density of the photopigment are altered. In-situ expression studies are needed to resolve these issues. Both scenarios suggest alterations in visual sensitivity. The third possibility is that opsin expression reflects the immediate dynamics of photopigment recycling. Cones continuously shed outer segments and generate new photopigment. Hence, one can imagine a scenario where the rate of opsin production matches the rate of photoreceptor stimulation simply because of a need to maintain the current population of various cones. However, this hypothesis predicts that opsin expression should be greatest for the SWS1, SWS2B, SWS2A and RH2-2 in the clear water treatment – which we did not find.

Evolutionary and ecological implications of population and water treatment effects

Table 3 summarizes past opsin expression studies on *L. goodei* as well as the current study. The proportional measures of opsin expression we detected were largely consistent with previous population comparisons where spring animals had higher SWS1 and SWS2 expression and swamp animals had higher RH2-1 expression. Previous work also documented differences in LWS expression which were not observed here. The variable population was somewhat intermediate in expression between the spring and swamp populations. Similarly, proportional opsin expression varied as a function of lighting environment for SWS1 and RH2-1, and the direction of these effects corroborated our previous work (Table 3). However, SWS2B and LWS had previously shown consistent differences in expression as a function of lighting environment which were not observed in this study. Still, the proportional gene expression data paint a picture where spring animals or animals in clear water habitats have a higher relative abundance of SWS1 and SWS2B opsins in their retinas which match their higher relative abundance of UV and violet cone cells (Fuller *et al.* 2003). Swamp animals or animals in tea-stained water have higher relative abundance of RH2-1 and LWS. Presumably, there is important visual information to be acted upon (e.g. zooplankton and fish coloration) that reflect in the ultraviolet range in clear water habitats.

Interpreting the population effects in this current study is difficult because it obviously reflects both genetic differences and potential long-lasting environmental/maternal effects. There is good reason to expect that long-lasting developmental plasticity is high in *L. goodei*. In a previous study, Fuller *et al.* (2010) raised fish from various crosses in either tea-stained or clear water for over a year but then tested their behaviour in both clear and tea-stained water. This allowed them to distinguish the effects of lighting environment on the development of the visual system versus the immediate effects of the lighting environment (because of altering background conditions or differential filtering of the light spectrum) on visually based behaviours. Surprisingly, they found that the lighting environments experienced during development had larger effects on visual behaviour than did the immediate lighting environments in which they were tested. Hence, there was long-lasting developmental plasticity in the visual system in *L. goodei*. The same study also documented genetic differences between populations in proportional SWS1 expression, but no genetic effects of population for the other opsins. However, the genetic effects of population in Fuller *et al.* (2010) may be conservative because that study had a higher level of experimenter induced error (see the plate effect in all models). The current study used wild-caught fish that may differ because of both genetics and the fact that they developed in different lighting environments. Still, the fact that Fuller *et al.* (2010) only found population effects (because of genetics) for proportional SWS1 expression whereas the current study found population effects of SWS1, SWS2B, RH2-2 and RH2-1 suggests that there may be large effects of developmental plasticity on visual systems that subsequent shifts in opsin expression cannot completely overcome.

The other novelty of the current study is the discrepancy between the patterns in proportional and relative$_{(hk)}$ expression and the manner in which these vary among populations and water treatments. Spring and variable populations dramatically increased relative$_{(hk)}$ expression of nearly all the opsin genes (LWS, RH2-1, RH2-2 and SWS2B) except the SWS1 in response to the tea-stained water treatment. However, swamp animals showed little alteration in gene expression. Why should this happen? One possibility is that reflects photostasis – a compensation mechanism that was proposed based on studies of albino rats (Williams *et al.* 1999). Rats exposed to high-light environments were found to have reduced rod outer segments, whereas rats exposed to lower light had larger rod outer segments. The idea is that there is an optimal level of photon capture and that animals alter the amounts of photopigment in an inverse manner to the lighting environment (i.e. high light–low photopigment; low light–high photopigment).

However, this argument requires an explanation as to why this should happen in some populations but not others. The spring and variable populations are more temporally heterogeneous. While swamp populations vary in the degree of tannin staining, they rarely, if ever, approach the clarity typically seen in springs (R.C. Fuller, personal observation). However, during wet years, the variable population is tannin stained. Fuller has also seen at least 1 year (2005) when the Upper Bridge population was slightly tannin stained. Small amounts of tannins greatly reduce the penetrance of UV wavelengths into depths (>0.5 m) where *L. goodei* are found (Fuller unpublished data).

The exciting aspect of opsin expression is that it might be predictive of visually based behaviours. However, the emerging picture in *L. goodei* is somewhat complicated. Spring and swamp animals vary in opsin expression and in some aspects of female mating behaviour (Fuller & Noa 2010). Similarly, the lighting conditions experienced during development alter opsin expression and also alter foraging preferences (Fuller *et al.* 2010). Similarly, among cichlids, there are strong comparative patterns where species that forage on zooplankton have high levels of SWS1 opsin (O'Quin *et al.* 2010). While there are strong patterns at the among species/population and among treatment groups, correlations between opsin expression and behaviours among individuals within these groups are not nearly as strong (Fuller & Noa 2010; Fuller *et al.* 2010). More work is needed to determine the extent to which opsin expression has implications on variation in mating and foraging decisions among individuals.

In conclusion, we found that opsin expression is highly plastic with respect to lighting environment in the adult stages of *L. goodei*. These shifts in opsin expression were quite rapid. Proportional measures of gene expression (which included the newly discovered RH2-2 opsin) were concordant with previous studies indicating high proportional SWS1 expression for animals from spring populations kept in clear water conditions. However, relative$_{(hk)}$ measures of opsin expression indicated the exact opposite pattern with no differences in SWS1 expression as a function of lighting environment. Rather, the vast majority of opsins (SWS2B, RH2-2, RH2-1 and LWS) had pronounced increases in tea-stained water, particularly for the spring and variable populations, which caused the apparent pattern in proportional SWS1 expression. This alteration in relative$_{(hk)}$ may represent a strategy to increase overall visual sensitivity in reduced lighting environments. These findings argue that both measures (proportional and relative$_{(hk)}$) should be employed. Proportional measures are more likely to reflect differences in visual sensitivity, but measures made relative to an independent housekeeping gene may well indicate the mechanisms via which shifts in opsin expression occur.

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R.C.F. has two main research themes in her lab - the evolution of color patterns and color vision in fish and speciation in fish. K.M.C. is interested in veterinary science.

Data accessibility

DNA sequences and expression data: DRYAD entry (doi:10.5061/dryad.k8073).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Univariate analyses of relative$_{(hk)}$ and proportional measures of each of the six opsins.

Table S2 Analysis of variance on proportional opsin expression for each opsin with RH2-2 removed from the calculations.

Fig. S1 Effects of time on relative$_{(hk)}$ and proportional opsin expression.

Fig. S2 Relative$_{(hk)}$ gene expression for all opsins as a function of population, water treatment and time.

Fig. S3 Proportional gene expression for all opsins as a function of population, water treatment and time.

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