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Population variation in opsin expression in the bluefin killifish, *Lucania goodei*: a real-time PCR study

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Abstract Quantitative genetics have not been used in vision studies because of the difficulty of objectively measuring large numbers of individuals. Here, we examine the effectiveness of a molecular technique, real-time PCR, as an inference of visual components in the bluefin killifish, *Lucania goodei,* to determine whether there is population variation in opsin expression. Previous work has shown that spring animals possess a higher frequency of UV and violet cones and a lower frequency of yellow and red cones than swamp animals. Here, we found a good qualitative match between the population differences in opsin expression and those found previously in cone frequency. Spring animals expressed higher amounts of SWS1 and SWS2B opsins (which correspond to UV and violet photopigments) and lower amounts of RH2 and LWS opsins (which correspond to yellow and red photopigments) than swamp animals. The counter-intuitive pattern between color pattern, lighting environment, and vision remains. Males with blue anal fins are more abundant in swamps where animals express fewer SWS1 and SWS2B opsins and where transmission of UV/blue wavelengths is low. Understanding this system requires quantitative genetic studies. Real-time PCR is an effective tool for studies requiring inferences of visual physiology in large numbers of individuals.

Keywords Color · Cones · Fundulidae · Photopigments · Vision

Abbreviations *ERG* electroretinogram · *MSP* microspectrophotometry

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Introduction

Understanding how natural selection acts on visual physiology is an important endeavor not only for understanding the evolution of vision itself (Hisatomi et al. 1994; Yokoyama and Yokoyama 1996; Yokoyama 1997; Hunt et al. 2001), but also for understanding the evolution of mating preferences (Bennett et al.1994; Macias Garcia and de Perera 2002; Rodd et al. 2002), sensory biases (Endler 1992, 1993; Endler and Basolo 1998; Ryan and Keddy-Hector 1992), foraging ecology (Regan et al. 2001; Garamszegi et al. 2002; Martin and Prince 2001), and even speciation (Endler and Houde 1995; Seehausen et al. 1997). However, studies of the evolution of vision typically rely on comparisons across species (Lythgoe et al. 1994; van der Meer and Bowmaker 1995; Yokoyama and Yokoyama 1996; Fleishman et al. 1997; Cummings and Partridge 2001). While comparative studies are immensely important in describing broad patterns, a thorough understanding of adaptation also requires measurements of selection gradients (correlates between trait values and fitness) as well as a determination that the variation in traits has a genetic basis (Reznick and Travis 1996; Travis and Reznick 1998).

Microevolutionary studies of visual physiology—examinations of selection gradients or estimates of quantitative genetic parameters—have been hindered by the difficulty of measuring key characters in large numbers of individuals (Falconer and MacKay 1996; Lynch and Walsh 1998). Three main methods have been used to make inferences about visual physiology and/or visual sensitivity. First, investigators have used the optomotor response, an innate response where animals follow the movement of a visual pattern. Visual sensitivity can be measured by testing animals exposed to various wavelengths and intensities. Boughmann (2001) measured optomotor response for sticklebacks from multiple populations and found a negative correlation between sensitivity to red light and the degree to which

water color is red shifted. Endler et al. (2001) used optomotor responses to select for increased sensitivity to blue and red wavelengths. The limitation of using the optomotor response is that it primarily measures the sensitivity to long wavelengths (Schaerer and Neumeyer 1996). In addition, as a behavioral measure of sensitivity, the optomotor response provides little information on the source of the underlying variation in visual physiology.

More direct measures have been used. Microspectrophotometry (MSP), a cell recording technique that measures the absorbance properties of individual photoreceptor cells, has effectively been used to determine which types of cones animals possess in their retinas. This has been an effective tool in species comparisons (Lythgoe et al. 1994; van der Meer and Bowmaker 1995; Cummings and Partridge 2001) and also in studies of plasticity (Cronin et al. 2001). The drawback of MSP is that it mainly provides data on the presence/absence of cone types (and hence is less effective in measuring the relative abundance of various cone types). Another method for measuring parameters of vision is the electroretinogram (ERG) where the sensitivity of animals to various wavelengths of light is measured across the entire spectrum. This method compares an individual's response to a wavelength of interest with its response to a control. Hence, the control stimulus can affect the interpretation of results (Jacobs et al. 1996). Researchers have also used nitro-blue tetrazolium staining to infer the abundance of various cone classes (Reckel and Melzer 2003). This method relies on dissecting out the retina and then exposing it to a short bandwidth of stimulus light that corresponds to a known cone sensitivity. Treatment with nitro-blue tetrazolium allows one to discern which cones in the retina absorbed the stimulus light. The disadvantage to this method is that multiple animals must be used to infer the relative cone frequencies across the entire retina. Hence, cone frequencies cannot be determined at the individual level, although they can be determined for families, populations, and species. In addition, this method assumes that some wavelengths stimulate a single cone-type. Hence, overlapping cone sensitivities can cause a non-specific response.

Three sets of methods appear amenable to use in microevolutionary studies. The first of these examines the filtering properties of the lens, cornea, oil droplets, and ellipsosomes (Thorpe and Douglas 1993; Flamarique and Harosi 2000; Cronin and Caldwell 2002). The second method involves labeling cones with opsin-specific antibody probes (Vihtelic et al. 1999). Antibody probes can be generated based on the amino acid sequence inferred from the opsin sequences. Retinas are then isolated, cryosectioned, and treated with the antibody probes. By creating probes that fluoresce under different filters, multiple cone/opsin types can be labeled for each section and the relative frequency of each cone type for a single individual can be determined. The third method is real-time PCR which measures the relative expression of opsin genes in the retina. This approach exploits the fact that the spectral properties of cones depend on the photopigments that they contain. Photopigments consist of pairing a vitamin A molecule (either A1 or A2) with an opsin protein. Shifts between A1 and A2 molecules induce relatively small shifts in maximum cone absorbance for short-wavelength-sensitive cones, but can induce larger shifts (30–60 nm) for medium- and long-wavelength-sensitive cones (Bridges 1972; Loew and Dartnall 1976; Munz and McFarland 1977). Opsin properties can induce much larger shifts in maximum cone absorbance (>200 nm) and are the main determinants of cone spectral properties (Yokoyama 1997; Yokoyama and Yokoyama 1996; Yokoyama et al. 1999; Partridge and Cummings 1999). Each opsin is a product of a single gene, and therefore, the relative expression of opsin genes might be used to make inferences concerning the relative abundance of cone types. Carleton and Kocher (2001) have used real-time PCR to measure relative opsin expression in four cichlid species and found that different cichlids use different subsets of genes. They suggested that gene regulation (as opposed to opsin differentiation) may create different spectral sensitivity in these species.

The objective of this study was to determine whether there is variation between populations in the relative expression of opsin mRNAs (measured with real-time PCR) that matches the qualitative and quantitative pattern of relative cone frequencies found in bluefin killifish, *Lucania goodei*, from a spring and a swamp population. We used real-time PCR to ask if differences in expression of opsins between individuals from these different populations match known differences in visual sensitivity ascertained from MSP and ERG methods.

Study system

The bluefin killifish, *Lucania goodei*, is an excellent system to study the evolution of vision. Animals live in a variety of lighting conditions ranging from springs where the water is crystal clear with high transmission of UV and blue wavelengths to tea-stained swamps where the transmission of UV and blue wavelengths is greatly reduced (Fuller 2002). Visual communication appears to be important in this species. The sexes are dimorphic in coloration where males are conspicuously colored and females are cryptic (Foster 1967; Page and Burr 1991; Fuller 2001). In addition, males are polymorphic in dorsal, anal, and pelvic fin coloration (Fuller 2001, 2002). The relative abundance of male color morphs varies across populations in relation to the lighting environment (Fuller 2002). Males with blue anal fins are much more abundant in tea-stained swamps where UV and blue wavelengths do not transmit well. In contrast, males with red anal fins (and to a lesser extent, males with yellow anal fins) are more abundant in populations where there is high transmission of UV and blue wavelengths.

Lucania goodei has five main cone types: UV (λ_{max} 359 nm), violet (λ_{max} 405 nm), blue (λ_{max} 455 nm), yellow (λ_{max} 539 nm), and red (λ_{max} 573) (Fuller et al. 2003). The relative abundance of cones in the retina varies between populations with different lighting environments. In a previous microspectra photometry study, Fuller et al. (2003) compared the relative cone frequencies between a spring and a swamp population. In that study, 10 animals from the swamp population and 11 animals from the spring population were sampled. Furthermore, a minimum of 25 and a maximum of 69 cone cells per individual were measured (average = 49 cones/individual, total number of cones measured = 1,024). Spring animals were much more likely to possess UV cones than swamp animals (Fuller et al. 2003). In addition, spring animals had a higher frequency of UV and violet cones and a lower frequency of yellow and red cones than swamp animals.

Materials and methods

Lucania goodei were collected from a spring population (Wakulla River, Upper Bridge, Wakulla, Fla., USA) and a swamp population (26-Mile Bend, Broward, Fla., USA) on 5–9 September 2002.The animals were transported to the University of New Hampshire. Twelve animals from each population were euthanized and their eyes were removed for RNA isolation. All individuals were sampled within a 2-h period between 10 a.m. and noon to minimize any diurnal variation in opsin expression (Chiu et al. 1995). In addition, the sex and standard length (hereafter referred to as size) was recorded for each individual. Two animals from each population were used for sequencing, and 10 animals were used for real-time PCR.

For each individual, both eyes were homogenized in 1 ml of Trizol solution. The RNA was extracted with chloroform, precipitated with isopropanol, and resuspended in 50 µl of DNAse/RNAse-free water. The RNA was later stored at −80°C. The cDNA was synthesized by reverse transcription using 1 µg of total RNA in the presence of superscript II and an oligo (dT) primer.

Real-time PCR

Data on opsin sequences were obtained from Blow and Yokoyama (Genbank accession numbers AY296735–AY296741). Six distinct opsin genes were sequenced. These genes and their assumed correspondence with the MSP peaks are: SWS1 (365 nm), SWS2B (405 nm), SWS2A (455 nm), RH2 (539 nm) and two distinct LWS (A and B: 560–580 nm). Because Blow and Yokoyama obtained sequences from animals from only the spring population, sequences were also obtained from a small portion of the opsins for two animals from both the swamp and spring population to ascertain that the two populations did not differ in sequence at the primer and probe sites.

Primer express software (ABI version 1.5) was used to design the primers and probes (Table 1). Each probe covered an exon-exon boundary so that only cDNA could be amplified in RT-PCR. The two LWS loci are identical at one exon-exon boundary and, a single set of primers and probes was designed that was common to these two loci. Unique primers and probes were also designed to distinguish the other opsin genes: SWS1, SWS2A, SWS2B, and RH2. Forward and reverse primers were located so that the product length was relatively short (87–109 bp). Finally, Sequencher was used to verify that our primers and probes were unique to each opsin.

The experimental protocol followed Carleton and Kocher (2001). For each real-time PCR (RT-PCR), 0.2 µl of cDNA was placed in a 30-µl reaction with the appropriate primers, probes, and taqman mix. Total RNA that was not reverse transcribed was used as a control. Probes were 5′-labeled with 6′ Fam and 3′-labeled with TAMRA. During PCR, primer extension disrupted the probe and released the 3′ TAMRA allowing it to fluoresce. The amount of fluorescence was monitored over 40 cycles (94°C, 15 s/55°C, 30 s/65°C, 1 min). RT-PCR was performed using the ABI Prism 7700 sequence detection system at Florida State University.

The relative abundance of each opsin in an individual cDNA mixture was based on its critical cycle number following the methodology of Carleton and Kocher (2001). Critical cycle number was determined when the fluorescence exceeded a threshold set close to the background fluorescence. Genes having high expression have smaller critical cycle numbers than genes with low expression. The relative opsin expression was calculated as a fraction of total opsin gene expression for an individual according to the following:

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

where $\frac{T_i}{T_{all}}$ is the proportional gene expression for a given gene i, E_i is the absolute PCR efficiency for each primer/probe set, and C_{ti} is the critical cycle number for each gene.

The absolute PCR efficiency (E_i) of each primer/probe set was measured using a construct that contained fragments of the amplified regions for each opsin gene (Table 1). The construct was generated by first PCR amplifying separate gene fragments for each of the opsin genes (35 cycles) using gene specific primers. These primers contained unique restriction sites for ligating the fragments together. LWSA was used as the template for the three LWS opsins. Products were run on a gel, cut out, and column purified (Qiagen Gel Extraction kit). All fragments were then cut with appropriate restriction enzymes. Pairs of fragments were ligated together (SWS1 to SWS2A and SWS2B to RH2), PCR amplified, and gel purified. SWS1-SWS2A was then ligated to LWS and these then joined with SWS2B-RH2. The full-length construct was TA cloned into pGEM T (Promega TA Cloning kit). The construct was transformed into electrocompetent DH10B cells and sequenced.

Table 1 Primer and probe sequences

Opsin (λ_{max})	Forward primer	Probe	Reverse primer
SWS1 (359 nm)	TTACACCTTGTGTGCCTT GGAA	CCGTAGCAGGCCTGGT GACGTCCT	GGGTTTGCAGATGAC CAGGTAC
SWS2B (405 nm)	GCTGCAAGATTGAAGGA TTTACTG	GGTGTTGGTGGCATGG TCAGCCTTTG	CCAACCATCTTTCGA ATGCAA
SWS2A (455 nm)	CATGCAAGATTGAAGGTTT CATTG	ACACTAGGGGGTATGGT AAGCCTGTGGTCTCT	CCAGCCATCGTTCA AAAGCT
RH2 (539 nm)	CTTCTGCGGTATTGAGGG ATTC	AACACTCGGAGGTGAG GTTGCTCTCTGGT	AACAATATATCTCTCAATAG CCAGAACAA
LWS (573 nm)	TGGTGTGCTCCTCCCA TCTT	TGGAGCAGGTATTGG CCCCATGGAC	TCTTCACTTCCACTGA ACACATCAG

The benefit of the construct is that it allows precise measurement of the relative efficiencies of each primer/probe set. The construct contains equal ratios of all the opsin genes and ensures that the same number of gene copies for each opsin is present for RT-PCR calibration. To determine the relative efficiency, real-time PCR was performed on the construct using each of the 5 primer/probe sets. Six replicate sets (5 primer/probe combinations per set—30 reactions total) were run using 1 µl of construct at a concentration of 0.001 ng μl^{-1}. Efficiencies were also measured across a dilution series of construct concentrations (ng μl^{-1}): 4×10^{-4}, 1×10^{-4}, 4×10^{-5}, 1×10^{-5}, 4×10^{-6}, and 1×10^{-6}. For each replicate set, the opsin with the lowest critical threshold was assigned a relative efficiency of 1. All other relative efficiencies were calculated from

$$\frac{(1+1)^{C_{\text{thigh}}}}{(1+relative\ E_{\text{i}})^{C_{\text{ti}}}}=1$$

where C_{thigh} represents the critical threshold for the opsin with the highest expression level. The relative efficiencies were pooled between all of the replicates and means and standard errors were determined.

To determine the absolute efficiencies, the absolute efficiency of the RH2 opsin was determined via a series of serial dilutions of cDNA (µl): 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002, and 0.001. RT-PCR was performed on five replicates from each of the nine concentrations. The slope of ln(concentration) on critical cycle number allows absolute E to be calculated as [(exp(−slope))−1]. One of the five replicates produced nonsensical data and was excluded from the analysis. In addition, absolute E was also calculated for the RH2 opsin using the dilution series performed using the construct (see above). The mean and standard error of absolute E for the RH2 primer/probe set was determined. The absolute E for the other primer/probe sets was calculated as

$$\text{absolute } E_{\text{i}}=(\text{relative } E_{\text{i}}\times\text{absolute } E_{\text{RH2}})/\text{relative } E_{\text{RH2}}.$$

For the population comparisons of *L. goodei,* measurement error was assessed by calculating the repeatability of the measurements. Three replicate RT-PCR were performed using 0.2 µl cDNA for each opsin for three individuals from each population. In addition, all RT-PCR reactions that were deemed likely to be outliers were flagged and re-run. Because the samples had generic codes, this analysis was performed blind to population of origin, sex, and size. Repeatability was measured as the proportion of variation accounted for by individual effects. The coefficient of variation (CV) across individuals and across all pooled data was also measured, as well as the average coefficient of variation among measures within each individual.

The effects of population of origin, size, and sex on relative gene expression were examined for each of the five opsins using analysis of covariance. All interactions were included in the initial analysis. Provided that they were not statistically significant, the interactions were removed from the model. For individuals used in the repeatability analysis, the mean of the three replicate measures was used. Finally, the relative opsin expression was compared with relative cone frequency as measured by Fuller et al. (2003). Results were considered significant at $P<0.05$. All analyses were performed using SAS V.8.

Results

Table 2 shows both the relative and absolute efficiency for each of the five opsin primer/probe sets. The relative efficiency of the SWS2B opsin was significantly lower than that of the SWS1, SWS2A, RH2, and LWS efficiencies (Table 2, $F_{4,55}=36.43$, $P<0.0001$). Relative efficiencies were used to calculate the absolute efficiencies for each primer/probe set based on the value calculated for the RH2 opsin from a dilution series.

Table 2 Absolute and relative efficiencies

Opsin ($\lambda_{\max}$)	Relative PCR efficiency	Absolute PCR efficiency
SWS1 (359 nm)	0.984 ± 0.009^{a}	0.814
SWS2B (405 nm)	0.857 ± 0.010^{b}	0.712
SWS2A (455 nm)	0.957 ± 0.009^{a}	0.792
RH2 (539 nm)	0.984 ± 0.005^{a}	0.813 ± 0.023
LWS (573 nm)	0.969 ± 0.010^{a}	0.801

[a,b]Different letters refer to statistically significant differences
Data are mean ± SE; $n=3$

The repeatability analysis indicated the presence of an extreme outlier. In the repeatability analysis of the SWS2A opsin, the studentized residual was 814. A value greater than 2 indicates a potential outlier (Sokal and Rohlf 1995). This data point was omitted from subsequent analyses except as noted.

Table 3 shows the repeatability of our measurements both with and without inclusion of the far-outlier. When the outlier is removed, measures of opsin expression were significantly repeatable for all opsins except RH2. The non-significance of RH2 appears attributable to the fact that, despite small experimental error, there is very little variation among individuals, hence little latitude for repeatability. The RH2 opsin had the lowest coefficient of variation across individual means and was two to three times lower than those for the other opsins.

Population had a large effect on relative gene expression for all opsins except SWS2A (Fig. 1a). In general, the spring population was shifted towards greater relative gene expression of opsins that absorbed in the UV and violet range (350–410), whereas the swamp population was biased towards greater relative gene expression of opsins that absorbed in the yellow and red range (530–580) (Fig. 1a). The initial analyses of covariance found no significant interactions between size×population, size×sex, population×sex, or size×population×sex for relative gene expression of any of the five opsins. Furthermore, neither sex nor size accounted for significant amounts of variation in any of the analyses. Both the SWS1 and SWS2B opsins had higher expression in fish from the spring population than those from the swamp population (SWS1: $F_{1,16}=32.03$, $P<0.001$; SWS2B: $F_{1,16}=9.00$, $P=0.0085$). For the SWS2A opsin, there was no effect of population [$F_{1,16}=0.04$, $P=0.835$, spring average = 0.00294 ± 0.000398 (mean ± SE), swamp average = 0.00287 ± 0.000435]. In contrast, both the RH2 and LWS opsins had higher expression in fish from the swamp population (RH2: $F_{1,16}=5.41$, $P=0.033$; LWS: $F_{1,16}=10.68$, $P=0.0048$).

Figure 1a, b compares the relative opsin expression with relative cone frequencies based on data presented in an MSP study (Fuller et al. 2003). The qualitative differences in opsin expression between the two populations match the direction of differences in relative cone frequency. The spring population had a higher frequency of UV and violet cones and also had higher expression of SWS1 and SWS2B opsins. In contrast, the swamp

Table 3 Repeatabilities and coefficients of variation (CV)

Opsin (λ_{max})	Repeatability with far outlier	Repeatability without far outlier	CV (across individual means)	CV (all measures)	Average CV (within samples)
SWS1 (359 nm)	77.7**	82.1**	33.6	34.9	13.8
SWS2B (405 nm)	69.4**	72.6**	21.2	23.5	11.4
SWS2A (455 nm)	20.6	57.2*	40.4	50.2	26.9
RH2 (539 nm)	31.7	43.0	12.0	17.2	13.3
LWS (573 nm)	36.5	96.9**	27.1	25.8	4.9

*$P < 0.05$, **$P < 0.01$

Coefficients of variation are calculated without the far outlier. $n = 6$ for CV (across individual means); $n = 18$ for CV (all measures). Average CV (within samples) is the average CV across the six individuals. Average CV (within samples) represents experimental error, whereas CV (individual means) represents the true coefficient of variation among individuals

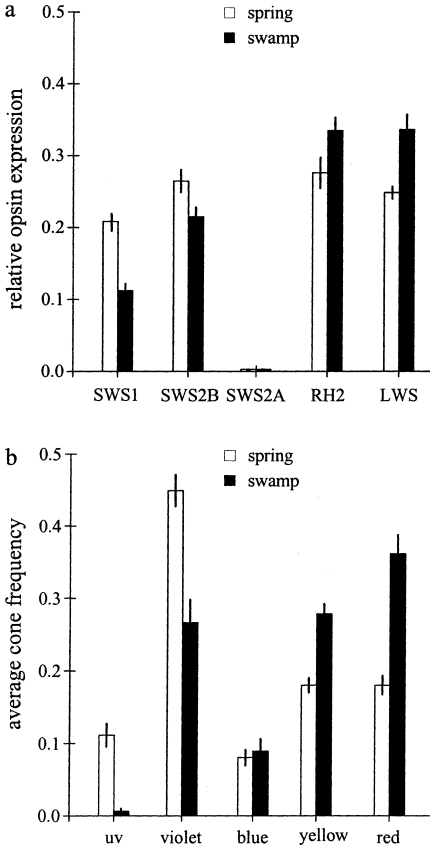

Fig. 1 a Average frequencies of cDNAs coding for *SWS1*, *SWS2B*, *SWS2A*, *RH2*, and *LWS* opsins for the spring and the swamp population. $n = 10$ for each population. **b** Average cone frequencies for UV, violet, blue, yellow, and red cones from data presented in Fuller et al. (2003). $n = 11$ for the spring population; $n = 10$ for the swamp population. A minimum of 25 cone cells were measured for each individual. Data are mean ± SE

population had a higher frequency of yellow and red cones and higher expression of RH2 and LWS opsins.

The quantitative match between cone frequency and opsin expression is relatively good, with the notable exception of the blue opsin/cone. The three most abundant cone types were the same as the three most highly expressed opsins (red, yellow, violet). Some minor discrepancies were present. The frequency of the violet cone in the spring population (0.45) was higher than the relative opsin expression (0.26). In addition, the differences between the populations in average cone frequency are greater than the differences found in relative opsin expression. However, the largest discrepancy is that found between SWS2A opsin expression and blue cone frequency. According to the real-time data, SWS2A opsin represents 0.29% of all the opsins expressed. In the MSP study, blue cones were 8.35% of the measured cones. This is a 28-fold difference.

Discussion

Population differences

In this study, we found higher SWS1 and SWS2B opsin expression in the spring population where there is higher transmission of UV and violet wavelengths. In contrast, animals from the swamp population (where there is lower transmission of UV/blue light) had higher expression of RH2 and LWS opsins. These results further support the pattern seen in male color pattern versus lighting environment and visual physiology. Males with blue anal fins are more abundant in populations where animals express fewer SWS1 and SWS2B opsins and where transmission of UV/blue wavelengths is low (Fuller 2002). How can we explain such a counterintuitive pattern? Clearly, males are not optimizing brightness by possessing blue anal fins in swamps where animals express few SWS1 and SWS2B opsins. One option is that males are maximizing contrast while sacrificing brightness. Contrast is achieved when a color pattern and background stimulate different cone types (Endler 1990). If the retina is set to detect objects against a tea-stained background, blue anal fins may be stimulating the violet and UV cones. In spring populations, where the retina may be set to detect objects against a high-UV/blue background, red and yellow anal fins may be stimulating the yellow and red cones. However, another possibility is that blue males do achieve high conspicuousness in spring populations due to high, perceived brightness, but are selected against due to high predation. We are currently testing these hypotheses by quantifying visual environments and color patterns using the methods of Endler (1990).

The pattern of opsin expression in this study also matches the predominant pattern seen in visual ecology.

In general, the types of cones and filters animals possess in their retinas generally match the available spectrum of light (Lythgoe 1984; Lythgoe et al. 1994; McDonald and Hawryshyn 1995; Hunt et al. 1996; Partridge and Cummings 1999; Yokoyama et al. 1999; Cronin and Caldwell 2002; Cronin et al. 2002). For example, there is evidence that the intraocular filters animals use vary among habitats so that visual sensitivity is maximized for the available wavelengths of light (Cronin et al. 2001; Cronin and Caldwell 2002). In some cases there is evidence of differences in overall spectral sensitivity that correlate with habitat light conditions (McDonald and Hawryshyn 1995; Leal and Fleishman 2002).

However, in some instances, a compensatory pattern is seen where animals adjust the number of photoreceptors and/or length of photoreceptor tips so as to maintain a constant photon catch (Penn and Williams 1986; Kröger et al. 1999). In this case, a decrease in a particular wavelength of light is accompanied by an increase in the number of photoreceptors (or length of photopigment tips) sensitive to that wavelength. For example, Kröger et al. (1999) showed that *Aequidens pulcher* (Cichlidae) raised in monochromatic blue light had a lower proportion of blue sensitive cones in their retina than animals raised in red or green monochromatic light, suggesting decreased sensitivity to blue when reared in a predominantly blue environment. It is unclear why wavelength matching is observed in some circumstances, while wavelength compensation is observed in others.

Are differences in opsin expression in *L. goodei* due to differences in the environment or genetic differences between the populations? We are currently conducting an experiment to investigate the role of genetics and environment on opsin expression. Studies in other organisms indicate that the environment can have a large role in determining visual physiology and visual sensitivity. An experiment in mantis shrimp demonstrated that individuals placed at different depths with different lighting environments also differed in the intraocular filters they used in such a manner as to maximize sensitivity to predominant wavelengths in the environment (Cronin et al. 2001). Experiments on blue acra (*A. pulcher*) have also demonstrated effects of rearing environment on photoreceptors, connectivity between neurons, and behavior (Kröger et al. 1999, 2001, 2003). To date, no quantitative genetic studies have been performed to examine the genetic basis of variation in visual physiology. Such tests are critical given that differences among species (which provide the backbone of studies of adaptive visual physiology) are assumed to have a genetic basis. This study lays the ground work and provides the necessary proof of principle that opsin expression can be used to test for environmental effects on visual sensitivity.

Opsin expression as an inference of visual physiology

Do measurements of relative opsin expression with real-time PCR reflect relative cone abundances in the retina? With respect to qualitative patterns, the answer is yes. Spring animals have higher frequencies of UV and violet cones and lower frequencies of yellow and red cones than animals from the swamp (Fuller et al. 2003). These patterns match those found in this study. We found that spring animals have higher SWS1 and SWS2B opsin expression and lower RH2 and LWS opsin expression than animals from the swamp. There were no differences among the populations in the frequency of blue cones nor in SWS2A opsin expression. The qualitative match in the direction of differences between the populations appears robust.

The quantitative match between relative opsin expression and cone frequency is relatively good with the exception of the SWS2A opsin and blue cone data. The minor discrepancies found in the abundance of UV, violet, yellow, and red opsins/cones could arise from measurement error in either RT-PCR or in MSP. MSP is not an optimal tool for measuring the relative abundance of cone types in the retina. The cones one measures with MSP may come from a small area of retina. Whether this area is representative of the entire retina is unknown. In addition, the amount of photopigment packed into individual cone cells may easily vary among cone types (Flamarique and Harosi 2000) which would create discrepancies between relative cone frequency and relative opsin expression. Such phenomena may easily account for the small discrepancies between cone frequency and opsin expression for SWS1, SWS2B, RH2, and LWS. However, these sources of variation cannot account for the discrepancy in the SWS2A cone/opsin results. If blue cones were as rare as suggested by the real-time data, then we should not have even detected them.

In theory, this difference could be caused by a faulty primer/probe set. However, this is not likely because the SWS2A primer/probe set worked well on the RT construct and did not differ from SWS1, RH2, or LWS primer/probe sets in relative efficiency. Another possibility is that there are multiple SWS2A loci that contribute to blue sensitivity, similar to the LWS loci. Under this scenario, the actual locus creating blue photopigment might not be amplified by our SWS2A primer/probe set. While we are unable to rule out this possibility, we find it doubtful. Blow and Yokoyama sequenced over 250 clones amplified using degenerate primers and have found no evidence of an additional SWS2A opsin (N. Blow and S. Yokoyama, unpublished data).

Assuming the SWS2A opsin expression results are valid, what could cause the disparity between opsin expression and relative cone abundance? One possibility is differential opsin expression throughout the day (Chen et al. 1992). We controlled for variation in diurnal rhythms in opsin expression by euthanizing the animals over a relatively short time (10–12 a.m.). Another possibility is that there is differential reverse transcription from RNA to cDNA among the opsins. This could occur if the SWS2A gene has a secondary structure making

reverse transcription difficult. SWS2A opsins could be more likely to degrade or may anneal less well to the polyT primer. Finally, differences in translation from RNA to protein could create differences between relative opsin expression and cone frequency. Under this scenario, SWS2A opsin transcripts would be translated into SWS2A opsin protein more rapidly than other opsin transcripts. This would create a low abundance of SWS2A opsin transcripts relative to the amount of SWS2A opsin proteins, and presumably, blue cones.

Finally, real-time PCR provides a tool that allows one to infer something about the visual physiology for large numbers of animals in a relatively short amount of time. Hence, this tool is amenable to microevolutionary studies. In particular, real-time PCR can be used in experiments designed to partition the effects of genetics, environment (i.e., plasticity), and the interaction between genetics and environment (i.e. heritable plasticity) on opsin expression (R.C. Fuller et al., unpublished observations). This approach to visual physiology is necessary in order to determine whether population differences in vision are due to genetics or environment. This is a critical endeavor. Many researchers have suggested that population differentiation in sensory systems can easily lead to speciation (Endler 1992; Endler and Houde 1995; Seehausen et al. 1997). Currently, differences in visual physiology across populations are assumed to be due to genetics (Boughmann 2001). Whether or not this assumption is valid needs to be determined.

Real-time PCR may also be useful in selection experiments. First, the build-up of disequilibrium between female mating preferences and male traits is a critical component in sexual selection (Fisher 1930; Lande 1981; Kirkpatrick 1982; Kokko et al. 2002). Hence, selecting for a particular male color morph and looking for correlated responses in vision is a logical step in experimentation. This involves examining the visual physiology of many animals from many generations. Real-time PCR is a feasible tool for such an endeavor. Second, one could use real-time PCR to select for particular opsin profiles and look for correlated responses in behavior. Again, such an experiment would entail measuring opsin expression in many animals. However, because real-time PCR is a destructive method, selection would have to occur at the family level (Falconer and Macay 1996).

In conclusion, we found that measuring relative opsin expression with real-time PCR is a valid method for inferring qualitative differences in cone frequencies. Across populations, greater expression of a given opsin corresponds with a higher frequency of the matching cone type. Spring animals expressed higher levels of SWS1 and SWS2B opsins and lower levels of RH2 and LWS opsins than swamp animals. These results further support the counterintuitive pattern seen between male coloration and visual physiology. Males with blue anal fins are more common in populations with low transmission of UV/blue wavelengths and where the expression of SWS1 and SWS2B opsins is low. Understanding this complex signaling system requires quantitative genetic studies of both the color pattern and the visual system. Using real-time PCR to measure visual physiology for quantitative genetic studies (e.g., heritabilities, selection gradients) will allow us to quantify genetic variation and examine the manner in which selection acts on opsin expression.

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References

Bennett ATD, Cuthill IC, Norris KJ (1994) Sexual selection and the mismeasure of color. Am Nat 144:848–860

Bridges CDB (1972) The rhodopsin-porphyrops in visual system. In: Dartnall HJA (ed) Handbook of sensory physiology, VII/I. Springer, Berlin Heidelberg New York, pp 417–480

Boughman JW (2001) Divergent sexual selection enhances reproductive isolation in sticklebacks. Nature 411:944–947

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

Chen D-M, Christianson JS, Sapp RJ, Stark WS (1992) Visual receptor cycle in normal and *period* mutant *Drosophila*: microspectrophotometry, electrophysiology, and ultrastructural morphometry. Vis Neurosci 9:125–135

Chiu JF, Mack AF, Fernald RD (1995) Daily rhythm of cell-proliferation in the teleost retina. Brain Res 673:119–125

Cronin TW, Caldwell RL (2002) Tuning of photoreceptor function in three mantis shrimp species that inhabit a range of depths. II. Filter pigments. J Comp Physiol A 188:187–197

Cronin TW, Caldwell RL, Marshall J (2001) Sensory adaptation—tunable colour vision in a mantis shrimp. Nature 411:547–548

Cronin TW, Caldwell RL, Erdmann MV (2002) Tuning of photoreceptor function in mantis shrimp species occupying a range of depths. I. Visual pigments. J Comp Physiol A 188:179–186

Cummings ME, Partridge JC (2001) Visual pigments and optical habitats of surfperch (Embiotocidae) in the California kelp forest. J Comp Physiol A 187:875–889

Endler JA (1990) On the measurement of classification of colour in studies of animal colour patterns. Biol J Linn Soc 41:505–523

Endler JA (1992) Signals, signal conditions, and the direction of evolution. Am Nat 139: S125–S153

Endler JA (1993) Some general-comments on the evolution and design of animal communication systems. Philos Trans R Soc London Ser B 340:215–225

Endler JA, Basolo Al (1998) Sensory ecology, receiver biases and sexual selection. TREE 13:415–420

Endler JA, Houde AE (1995) Geographic variation in female preferences for male traits in *Poecilia reticulata*. Evolution 49:356–368

Endler JA, Basolo A, Glowacki S, Zerr J (2001) Variation in response to artificial selection for light sensitivity in guppies (*Poecilia reticulata*). Am Nat 158:36–48

Falconer DS, MacKay TFC (1996) Introduction to quantitative genetics, 4th edn. Longman, Harlow, UK
Fisher RA (1930) The genetical theory of natural selection. Clarendon Press, Oxford
Flamarique IN, Harosi FI (2000) Photoreceptors, visual pigments, and ellipsosomes in the killifish, *Fundulus heteroclitus*: a microspectrophotometric and histological study. Vis Neurosci 17:403–420
Fleishman LJ, Bowman M, Saunders D, Millwer WE, Rury MJ, Loew ER (1997) The visual ecology of Puerto Rican anoline lizards: habitat light and spectral sensitivity. J Comp Physiol A 181:446–460
Foster NR (1967) Comparative studies on the biology of killifishes (Pisces: Cyprinodontidae). PhD Dissertation, Cornell University, Ithaca
Fuller RC (2001) Patterns in male breeding behaviors in the bluefin killifish, *Lucania goodei*: a field study (Cyprinodontiformes: Fundulidae). Copeia 2001:823–828
Fuller RC (2002) Lighting environment predicts relative abundance of male colour morphs in bluefin killifish (*Lucania goodei*) populations. Proc R Soc London Ser B 269:1457–1465
Fuller RC, Fleishman LJ, Leal M, Travis J, Loew E (2003) Intraspecific variation in ultraviolet cone production and visual sensitivity in the bluefin killifish, *Lucania goodei*. J Comp Physiol A 189:609–616
Garamszegi LZ, Møller AP, Erritzøe J (2002) Coevolving avian eye size and brain size in relation to prey capture and nocturnality. Proc R Soc London Ser B 269:961–967
Hisatomi O, Kayada S, Aoki Y, Iwasa Y, Tokunaga F (1994) Phylogenetic relationships among vertebrate visual pigments. Vision Res 34:3097–3102
Hunt DM, Fitzgibbon J, Slobodyanyuk SJ, Bowmaker JK (1996) Spectral tuning and molecular evolution of rod visual pigments in the species flock of cottoid fish in Lake Baikal. Vision Res 36:1217–1224
Hunt DM, Wilkle SE, Bowmaker JK, Poopalasundaram S (2001) Vision in the ultraviolet. Cell Mol Life Sci 58:1583–1598
Jacobs GH, Neitz J, Krough K (1996) Electroretinogram flicker photometry and its applications. J Opt Soc Am A 13:641–648
Kirkpatrick M (1982) Sexual selection and the evolution of female choice. Evolution 36:1–12
Kokko H, Brooks R, McNamara JM, Houston AI (2002) The sexual selection continuum. Proc R Soc London Ser B 269:1331–1340
Kröger RHH, Bowmaker JK, Wagner HJ (1999) Morphological changes in the retina of *Aequidens pulcher* (Cichlidae) after rearing in monochromatic light. Vision Res 39:2441–2448
Kröger RHH, Braun SC, Wagner HJ (2001) Rearing in different photic environments and chromatic environments modifies spectral responses of cone horizontal cells in adult fish retina. Vis Neurosci 18:857–864
Kröger RHH, Knoblauch B, Wagner HJ (2003) Rearing in different photic and spectral environments changes the optomotor response to chromatic stimuli in the cichlid fish *Aequidens pulcher*. J Exp Biol 206 1643–1648
Lande R (1981) Models of speciation by sexual selection on polygenic traits. Proc Natl Acad Sci USA 78:3721–3725
Leal M, Fleishman LJ (2002) Evidence for habitat partitioning based on adaptation to environmental light in a pair of sympatric lizard species. Proc R Soc London Ser B 269: 351–359
Loew ER, Dartnall HJA (1976) Vitamin A1/A2-based visual pigment mixtures in the cones of the rudd. Vision Res 16:891–896
Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer, Sunderland, MA
Lythgoe JN (1984) Visual pigments and environmental light. Vision Res 24:1539–1550
Lythgoe JN, Muntz WRA, Partridge JC, Shand J, Williams D.McB (1994) The ecology of the visual pigments of snappers (Lutjanidae) on the Great Barrier Reef. J Comp Physiol A 174:461–467
Macias Garcia C, Perera TF de (2002) Ultraviolet-based female preferences in a viviparous fish. Behav Ecol Sociobiol 52:1–6
Martin GR, Prince PA (2001) Visual fields and foraging in procellariiform seabirds: sensory aspects of dietary segregation. Brain Behav Evol 57:33–38
McDonald CG, Hawryshyn CW (1995) Intraspecific variation of spectral sensitivity in threespine stickleback (*Gasterosteus aculeatus*) from different photic regimes. J Comp Physiol A 176:255–260
Meer HJ van der, Bowmaker JK (1995) Interspecific variation of photoreceptors in four co-existing haplochromine cichlid fishes. Brain Behav Evol 45:232–240
Munz FW, McFarland WN (1977) Evolutionary adaptations of fishes to the photic environment. In: Crescitelli F (ed) The visual system in vertebrates. Springer, New York, pp 193–274
Page LM, Burr BM (1991) Freshwater fishes: North America North of Mexico. Houghton Mifflin, Boston
Partridge JC, Cummings ME (1999) Adaptations of visual pigments to the aquatic environment. In: Archer SN, Djamgoz MBA, Loew ER, Vallerga S (eds) Adaptive mechanisms in the ecology of vision. Kluwer, Dordrecht, pp 251–283
Penn JS, Williams TP (1986) Photostasis: regulation of daily photon-catch by rat retinas in response to various cyclic illuminances. Exp Eye Res 43:915–928
Reckel F, Melzer RR (2003) Regional variations in the outer retina of Atherinomorpha (Beloniformes, Atheriniformes, Cyprinodontiformes: Teleostei): photoreceptors, cone patterns, and cone densities. J Morphol 257:270–288
Regan BC, Julliot C, Simmen B, Vienot F, Charles-Dominique P, Mollon JD (2001) Fruits, foliage, and the evolution of primate colour vision. Phil Trans R Soc London Ser B 356:229–283
Reznick DA, Travis J (1996) The empirical study of adaptation in natural populations. In: Rose MR, Lauder GV (eds) Adaptation. Academic Press, San Diego, CA, pp 243–289
Rodd HF, Hughes GF Grether, Baril CT (2002) A possible non-sexual origin of mate preference: are male guppies mimicking fruit? Proc R Soc London Ser B 269:475–481
Ryan MJ, Keddy-Hector A (1992) Directional patterns of female mate choice and the role of sensory biases. Am Nat 139: s4–s35
Schaerer S, Neumeyer C (1996) Motion detection in goldfish investigated with the optomotor response is "color blind". Vision Res 36:4025–4034
Seehausen O, Alphen JJM van, Witte F (1997) Cichlid fish diversity threatened by eutrophication that curbs sexual selection. Science 277:1808–1811
Sokal RR, Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research. Freeman, New York
Thorpe A, Douglas RH (1993) Spectral transmission and short-wave absorbing pigments in the fish lens. 2. Effects of age. Vision Res 33:301–307
Travis J, Reznick DN (1998) Experimental approaches to the study of evolution. In: Resitarits WJ, Bernardo J (eds) Issues and perspectives in experimental ecology. Oxford University Press, New York, pp 310–352
Vihtelic TS, Doro CJ, Hyde DR (1999) Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. Vis Neurosci 16:571–585
Yokoyama S (1997) Molecular genetic basis of adaptive selection: examples from color vision in vertebrates. Annu Rev Genet 31:315–336
Yokoyama S, Yokoyama R (1996) Adaptive evolution of photoreceptors and visual pigments in vertebrates. Annu Rev Ecol Syst 27:543–567
Yokoyama S, Zhang H, Radlwimmer FB, Blow NS (1999) Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). Proc Natl Acad Sci USA 96:6279–6284