Teasing Apart the Many Effects of Lighting Environment on Opsin Expression and Foraging Preference in Bluefin Killifish

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abstract: Coloration and color vision covary with lighting in many taxa. Determining the mechanisms underlying these patterns is difficult because lighting environments can have multiple effects on signaling that occur at multiple timescales. Lighting environments can (1) immediately affect signal propagation and transmission, which determine the radiance spectrum reaching the receiver; (2) induce variation in visual systems via developmental plasticity; and (3) lead to genetic differences in visual systems due to a history of selection in different habitats. We tease apart these effects on pecking preference and examine the relationship between pecking preference and opsin expression. Using killifish from two visually distinct populations (clear vs. tea-stained water), we performed crosses (genetics), raised animals under different lighting conditions (developmental plasticity), and assayed the preference to peck at different-colored dots under different lighting conditions (immediate effects). Pecks are interpreted as foraging preference. Developmental plasticity affected both pecking preference and opsin expression. Lighting environments also had immediate effects on pecking preference, but these depended on the lighting conditions animals experienced during development. Genetic effects were detected in opsin expression, but there were no corresponding effects on pecking preference. Overall, only 3.36% of the variation in pecking preference was accounted for by opsin expression.

Keywords: color vision, developmental plasticity, phenotypic plasticity, sensory bias, sensory drive, visual ecology.

Introduction

The vast diversity in animal color patterns has long attracted the attention of ecologists, evolutionary biologists, and physiologists who have tried to understand the relationship between environmental lighting habitats, color patterns, and color vision (Endler 1992, 1993*b*; Partridge and Cummings 1999; Boughman 2002). Comparative studies indicate that environmental lighting habitats are important in the evolution of color patterns (Marchetti 1993; Seehausen et al. 1997, 2008; Boughman 2001; Scott 2001; Fuller 2002; Leal and Fleishman 2002; Gomez and Théry 2004, 2007; Stuart-Fox et al. 2007) and color vision (Lythgoe et al. 1994; Boughman 2001; Cummings and Partridge 2001; Fuller et al. 2003, 2004; Terai et al. 2006; Seehausen et al. 2008). However, comparative patterns alone cannot determine the mechanisms underlying these correlations (Reznick and Travis 1996; Travis and Reznick 1998). The problem is that lighting environments have myriad effects on visual signaling. Figure 1 shows a simple diagram of the signaling process, wherein signals are given, travel through the environment in which signaling occurs, are detected by the visual system, and are processed by the brain of the receiver, which determines animal behavior (Endler 1992, 1993*a*). This process can be altered by environmental lighting conditions in three distinct ways.

First, the lighting environment affects signaling through its immediate effects on signal propagation, signal transmission, and background. Signal propagation (i.e., the amount of light reflected off the color pattern at each wavelength), signal transmission (i.e., the degree to which signals travel from the signaler to the receiver without degradation), and visual background are easily altered by variation in lighting environment and, therefore, alter the light spectrum reaching the receiver (Endler 1990, 1991; Osorio and Vorobyev 1996; Stevens et al. 2007). Aquatic habitats are particularly variable because depth, dissolved organic matter, and suspended sediments vary and affect the distribution of wavelengths, their transmission, and the background against which color patterns are viewed (Partridge and Cummings 1999).

Second, the visual properties of animals in different lighting habitats can vary due to developmental plasticity (fig. 1). Ontogenetic shifts in visual properties occur in a number of taxa (Carleton et al. 2008), and some of these

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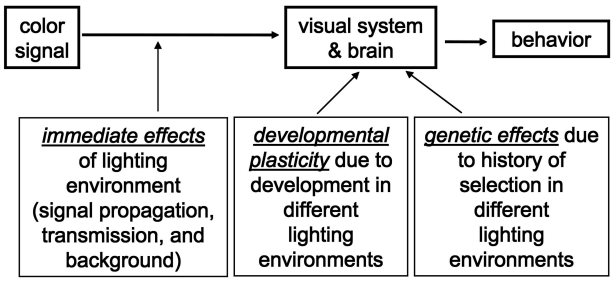

Figure 1: Effects of lighting environment on visual signaling.

changes coincide with shifts in habitat (Shand 1993, 1997; Allison et al. 2006; Hoke et al. 2006). Animals frequently vary the properties of visual filters (oil droplets, lens-filtering pigments, intrarhabdomal filters) when they are raised under different experimental lighting habitats (Cronin et al. 2001; Kroger et al. 2001*a*; Cronin and Caldwell 2002; Cheroske et al. 2003, 2006; Hart et al. 2006; Schartau et al. 2009). Similarly, the relative expression of cone cells and the opsin genes that determine the spectral absorption of photopigment can vary as a direct function of lighting environment (Kroger et al. 1999; Fuller et al. 2005*a*; Shand et al. 2008). In at least one species of cichlid, the wavelength and threshold for depolarization in at least one class of horizontal cells is plastic with respect to lighting environment experienced during development, suggesting plasticity in neural wiring (Kroger et al. 2001*b*; Wagner and Kroger 2005).

Third, different lighting environments can lead to genetic differences in sensory-system properties among populations, due to a history of selection in different habitats (fig. 1). Few studies have systematically teased apart the roles of genetic and environmental variation at the among-population/among-species level in vision using classic quantitative genetic approaches (although see Endler et al. 2001 and Fuller et al. 2005*a* for genetic variation within populations). A variety of studies have examined sequence variation in opsins, which are critical to the spectral sensitivity of photopigments. The extent to which sequence differences in opsins arise as a result of variable lighting environments is less clear (Osorio and Vorobyev 2008; but see Seehausen et al. 2008).

In addition to the three main effects, four types of interactions can also occur. An interaction between developmental plasticity and genetics indicates genetic variation in plasticity between animals adapted to different lighting habitats. An interaction between developmental plasticity and immediate environment indicates that the immediate effects of lighting environment on color-pattern perception varies between animals whose visual-system properties vary as a function of rearing habitat. An interaction between immediate environment and genetics indicates that the effects of the immediate environment vary as a function of genetic differences between animals adapted to different lighting habitats. A three-way interaction between immediate environment, rearing environment, and genetics indicates that the effects of genetic variation in visual system plasticity on color pattern perception vary as a function of the immediate lighting environment. There are a number of studies of each individual component but no published studies that systematically examine all three sources of variation.

In this study, we determine the effects of immediate lighting habitat, developmental plasticity, genetics, and their interactions on foraging preferences. Our ultimate aim is to understand the evolution of male color patterns and female mating preferences. The sensory-bias hypothesis states that female mating preferences evolve as a correlated response to natural selection on nonmating behaviors (Kirkpatrick and Ryan 1991; Fuller et al. 2005*b*). Guppy populations differ in the strength of female mating preferences for orange males, and these differences are also manifested as preferences for pecking at orange disks, which is interpreted as a foraging preference (Rodd et al. 2002; Grether et al. 2005). In this study, we used a similar pecking assay to examine the effects of lighting habitat on visual systems and visually based behaviors in a killifish.

To assess variation in the visual system, we quantified the expression of opsin genes. There has been a recent explosion of interest in opsins (Yokoyama and Yokoyama 1996; Hunt et al. 2004; Horth 2007; Bowmaker 2008; Hofmann and Carleton 2009). Opsins play a critical role in determining the spectral sensitivity of photopigment. 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 and Radlwimmer 1999, 2001; Yokoyama 2000; Hunt et al. 2001). One can make inferences about visual sensitivity (and, presumably, behavior) from sequence data. The opsins are unique in this respect. There are currently no other physiological steps in the detection and processing of visual information where one can make a direct inference between DNA sequence and inferred visual sensitivity.

A variety of claims have been made regarding opsins and behavior. A large number of long wavelength–sensitive (LWS) opsins have been found in guppies, leading to the hypothesis that the vast diversity in guppy color patterns is attributable to the large number of LWS opsins (Hoffmann et al. 2007; Weadick and Chang 2007; Ward et al. 2008). In cichlids, different variants of an LWS allele are present in different sister species that occur in different lighting habitats, suggesting that variation in the LWS op-

sin is critical to speciation and female mating preferences (Seehausen et al. 2008). In bluefin killifish, development in clear versus tannin-stained water has strong effects on the relative expression of opsins (Fuller et al. 2005*a*). The relative abundance of red, yellow, and blue male color patterns also varies between clear and tannin-stained water (Fuller 2002; Fuller and Travis 2004), and the implication is that variation in opsin expression has important effects on male coloration. Despite these intriguing patterns, there are few studies that demonstrate a tight relationship between opsin variation and behavior. In this study we test the hypothesis that opsin expression is correlated with foraging preferences, as measured through a pecking assay.

Study System

The bluefin killifish, *Lucania goodei*, is a compelling system within which to examine the relative effects of genetics and environment on opsin expression and resulting behavior. *Lucania goodei* is a small freshwater fundulid that occurs under a wide range of lighting environments ranging from tea-stained swamps, which have reduced transmission of ultraviolet (UV)/blue wavelengths, to crystal-clear springs, which have high transmission of UV/blue wavelengths (Fuller 2002). Both male coloration and visual properties vary across populations in relation to lighting conditions (Fuller 2002; Fuller et al. 2003, 2004). Males with blue anal fins are more abundant in tea-stained swamps, whereas males with red anal fins (and, to a lesser extent, males with yellow anal fins) are more abundant in clear springs (Fuller 2002). There is genetic and environmental variation as well as an interaction between genetic and environmental variation in male color-pattern expression.

Visual properties of animals also differ between spring and swamp habitats. Swamp animals are less sensitive to UV/blue wavelengths and possess fewer UV and violet cones than do animals from spring populations (Fuller et al. 2003). These differences in cone frequency match differences in expression of opsins (Fuller et al. 2004). *Lucania goodei* expresses five major classes of opsins: short wavelength–sensitive (SWS) 1, SWS2A, SWS2B, rhodopsin (RH) 2, and LWS. In combination with 11-cis retinal, the genes produce the following pigments: SWS1 produces a UV photopigment (maximum absorbance [λ_{max}] = 359 nm), SWS2B produces a violet photopigment (λ_{max} = 405 nm), SWS2A produces a blue photopigment (λ_{max} = 455 nm), RH2 produces a yellow photopigment (λ_{max} = 539 nm), and LWS produces a red photopigment (λ_{max} = 573 nm). *Lucania goodei* has at least two different LWS loci (Genbank accession numbers AY296741 and AY296740). Preliminary evidence indicates no difference in their spectral properties (N. 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 and Kocher 2001; Fuller et al. 2004, 2005*a*).

Both genetic and environmental variation in opsin expression are present at the within-population level (Fuller et al. 2005*a*). Animals raised in clear-water conditions have higher SWS1 and SWS2B expression (which corresponds to UV and violet photopigments), and animals raised in tea-stained water have higher RH2 and LWS expression (which corresponds to yellow and red photopigments; Fuller et al. 2005*a*). The same study found narrow-sense heritability in RH2 expression and broad-sense heritability in LWS and SWS2B expression. In terms of opsin data, this study differs from that of Fuller et al. (2005*a*) in that it assesses (1) the effects of genetics, environment, and their interaction at the among-population level, and (2) the extent to which variation in opsin expression is correlated with foraging preferences. In nature, *L. goodei* feed by pecking at submerged aquatic plants where presumably there are aquatic invertebrates. They eat a variety of different-colored food items, including red chironomid larvae, yellow fish eggs, beetle larvae, gastropods, cladocerans, and limited green algal material (Loftus 2000; Taylor et al. 2001).

Methods

We employed breeding treatments and rearing environments that were expected to maximize variation in the visual system, and then we examined the effects of that variation on behavior under multiple lighting environments. Figure 2 shows our approach. We focused on foraging preferences exhibited as "pecks" at inanimate objects. To induce variation in the visual system, we first performed crosses within and between a spring population and a swamp population, which are known to vary in their visual properties (fig. 2, Step 1). Next, we divided the offspring and reared half in clear-water conditions (which mimics springs) and the other half in tea-stained conditions (which mimics swamps; fig. 2, Step 2). We raised these animals to adulthood and then assayed their visual properties by measuring relative opsin expression (fig. 2, Step 3). Then we measured the preferences of their siblings to peck at various colored dots in both clear and tea-stained water. We addressed three questions. First, what are the effects of genetics, rearing environment, and their interaction on opsin expression? Second, what are the effects of genetics, rearing environment, and testing environment (as well as their interactions) on pecking preferences? Third, what is the relationship between opsin expression and behavior, and does this vary as a function

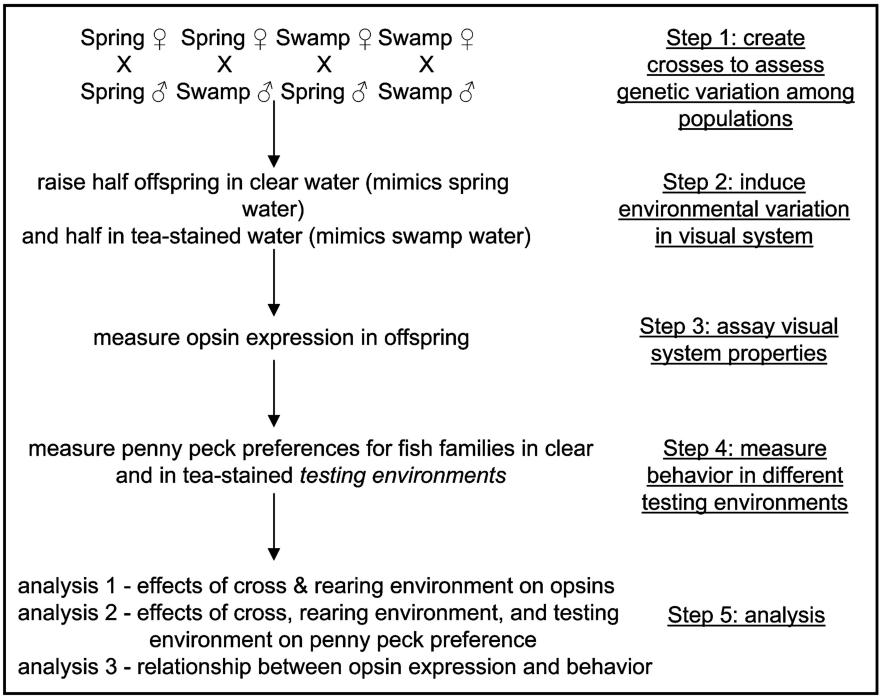

Figure 2: Schematic approach to this experiment.

of the environment? Below we discuss each of these steps in detail.

Breeding and Rearing

We crossed animals within and between a spring population (Upper Bridge, Wakulla River drainage, Wakulla County, FL) and a swamp population (26-Mile Bend, Everglades drainage, Broward County, FL). Animals were collected May–June 2005 with dipnets and minnow seines and were transported back to the Mission Road Greenhouse at Florida State University (FSU), Tallahassee. We established crosses between swamp animals (swamp female × swamp male) and between spring animals (spring female × spring male), and hybrid crosses in both directions (swamp female × spring male; spring female × swamp male). For each type of cross, we used eight different males with specific color patterns. We used two males with red coloration on the rear portion of the dorsal fin and red on the anal fin (R/R), two males with yellow coloration on the rear portion of the dorsal fin and yellow on the anal fin (Y/Y), two males with red on the rear portion of the dorsal fin and blue on the anal fin (R/B), and two males with yellow on the rear portion of the dorsal fin and blue on the anal fin (Y/B). Hence, there was a total of 32 males across the four cross types. Each male was spawned with two females. Males were placed with each female on alternate days, which gave females a day to spawn and a day to recover. We collected eggs until we had a maximum of 100 eggs; however, some individual crosses produced fewer than 100 eggs.

Eggs were raised in tubs until they hatched. The resulting juvenile fish were transferred to 110-L tanks. Juvenile fish were fed live *Artemia* nauplii and frozen *Daphnia*. Adult fish were fed frozen adult *Artemia* and were occasionally supplemented with frozen *Daphnia* and frozen chironomids. Half the offspring were raised under clear-water conditions and the other half was raised under tea-stained conditions. Each tank was given a unique blind code so that experimentation was blind with respect to cross. P-Clear (Nutrafin) was used to remove suspended algae from the water column. Tea-stained water was created by adding Nestea instant tea (decaffeinated, no sugar, no lemon) to the water column. This technique has been used in previous experiments and mimics swamp water conditions quite well (Fuller and Travis 2004; Fuller et al. 2005*a*). Tanks were housed in a greenhouse and exposed to natural sunlight.

The fish were moved to the University of Illinois (UI) in August 2006. Each family was placed in its own bucket and transferred to a greenhouse at UI, where they were exposed to natural sunlight. The greenhouse at UI is made

of polycarbonate and allows the transmission of wavelengths from 385 to over 800 nm. In addition, the greenhouse has several xenon overhanging lamps, which supplement the UV light spectrum. Rearing treatments (clear water vs. tea-stained water) were maintained throughout. The FSU greenhouse was constructed of glass, which allowed for transmission of light into the UV spectrum (Fuller and Travis 2004). Again, fish were housed in 110-L tanks that were equipped with power filters. At UI, we used dechlorinated city water in our tanks. We used UV water filters instead of P-Clear to eliminate algae from the water column. All other husbandry conditions were identical to those at FSU. We ascertained the effects of our lighting environments by measuring the percent transmission of light from 340 to 700 nm through 1-cm cuvettes relative to the transmission of deionized water. The relative transmission varied between clear and tea-stained water, with tea-stained water having reduced transmission between 340 and 498 nm (fig. 3*A*).

We considered the family to be complete if we obtained 50 fry (i.e., hatched eggs). There was variation in the rate at which the females laid eggs. For crosses that produced large numbers of eggs quickly, we divided the broods immediately and reared them in the two alternate lighting environments. For crosses that had low egg production (and that took a long time to complete), we collected the first 50 fry and placed them in a single rearing environment and then started collecting the second 50 fry for the alternate environment. We did this to reduce the variation in size and age within each tank. Occasionally, a dam or a sire died after having produced enough eggs for one of the treatments. We retained the animals from these incomplete crosses (i.e., <50 offspring) and raised them to adulthood. We also replaced the original sire (or dam). If a sire died, we restarted the entire cross with the two dams. If a dam died, we obtained a replacement female, which we crossed with the original sire. Offspring were never pooled between different sires or dams. Table A1, available in Dryad (http://hdl.handle.net/10255/dryad.1247), presents a full description of the crosses and sample sizes, and it explains which crosses were incomplete. The unbalanced design had little practical effect on the analyses presented here. Preliminary analyses indicated little effect of sires or dams, so we made "tanks of fish" the unit of observation and concentrated our analysis on the effects of rearing environment, testing environment, and genetic effects at the among-population level (hereafter referred to as "cross").

Family size (i.e., number of fish in a given tank) varied for multiple reasons. First, there was slight mortality among fry when they were initially transferred to their large rearing tanks. Second, there was most likely slight cannibalism within tanks due to larger fish consuming

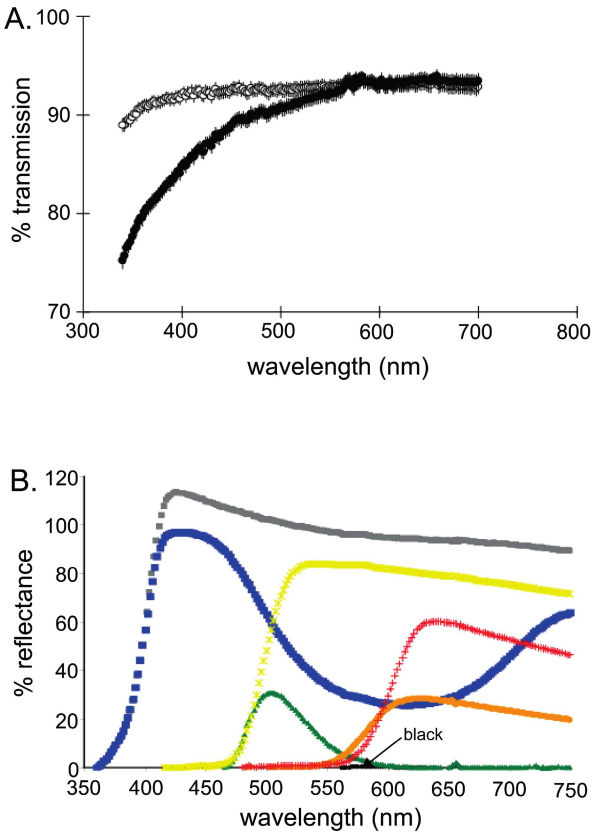

Figure 3: *A*, Relative transmission of wavelengths from 340 to 700 nm across a 1-cm cuvette. Means and standard errors are shown. Open circles denote the clear-water treatment; dark circles denote the tea-stained water treatment. *B*, Reflectance of red, orange, yellow, green, blue, white (in gray), and black dots from 350 to 750 nm.

smaller fish. Third, there was some mortality when we transferred fish from FSU to UI: a few families perished, a few families had significant mortality but a fair number of survivors, and other families had no mortality whatsoever. Fourth, some families were the result of "incomplete" crosses and had a smaller number of initial fry. The family sizes listed in table A1 in Dryad (http://hdl.handle.net/10255/dryad.1247) represent the number of fish present when the pecking assays were performed and after individuals had been removed for opsin measurements (see below).

Opsin Expression

We measured opsin expression for four to six adult animals (two to three males and two to three females) from each treatment combination. For each individual, we obtained cDNA by reverse transcribing RNA isolated from eye tis-

sue. RNA was isolated using Trizol extractions. To control for the effects of circadian rhythms, we euthanized individuals between 1200 and 1400 hours. The collection of animals from a given tank was staggered such that animals were removed and euthanized and RNA was isolated on different days. RNA extractions occurred in January–March 2007. We created primers and probes that were unique to each opsin gene (see Fuller et al. 2004 for details). *Lucania goodei* has at least two different LWS loci, but our primers and probes were common to both alleles. We placed 2 μL of cDNA mixture in a 10-μL reaction with the appropriate primers, probes, and TaqMan mix. We performed three replicate reactions for each of the five opsins for each individual. Fluorescence was monitored over 40 cycles (94°C for 15 s/55C° for 30 s/65°C for 1 min) 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 calculated the average of the three replicate reactions and examined any individual reaction that differed from this average by more than 1.5 cycles. The SWS2A opsin had high critical cycle numbers (meaning it contributed very little to the total pool of opsin cDNA) and ran poorly on some plates, so we excluded it from this analysis. We determined the average critical cycle number for each individual on each plate. Relative opsin expression was calculated as a fraction of total opsin genes for an individual according to the following equation:

$$\frac{T_i}{T_{\text{all}}} = \frac{1/(1 + E_i)^{C_{ti}}}{\sum \{1/[(1 + E_i)^{C_{ti}}]\}},$$

where T_i/T_{all} is the proportional gene expression for a given gene i, E_i is the polymerase chain reaction (PCR) efficiency for each primer/probe set, and C_{ti} is the average critical cycle number for each gene. PCR efficiencies were quantified previously with a multigene construct (Fuller et al. 2004).

We used a general linear model to examine the effect of cross, rearing environment, their interaction, and PCR plate for each of the four main opsins (SWS1, SWS2B, RH2, and LWS). Analyses were performed using Proc Mixed in SAS, which employed an interactive, restricted maximum likelihood method to estimate variance components. The effect of tank nested within the interaction between cross and rearing environment (tank(rearing environment × cross)) was treated as a random effect, ensuring that the effects of rearing environment, cross, and their interaction were tested at the level of tank. The effect of plate was tested at the level of individual. To allow comparisons with our previous work (Fuller et al. 2005*a*), we graphed the least square means and standard errors for the interaction between rearing environment and cross.

Peck Assay

From May to June 2007 we measured the preferences of animals to peck at red, orange, yellow, green, blue, black, and white dots. For each color, we brushed acrylic paint onto overhead transparencies and allowed them to dry. We then used a hole-punch to create colored disks (6 mm diameter). These disks were then attached color-side-up to a petri dish with silicone. On each petri dish, the seven different colored dots were arranged in a circle. We used several petri dishes and varied the order of the disks. The reflectance spectrum of the dots was measured using a reflectance probe positioned at a 45° angle. The probe was coupled with an spectrophotometer and a deuterium-tungsten light source (Ocean Optics). The reflectance of the orange dot was somewhat similar to that of the red dot (fig. 3*B*); however, to us the orange dot looked different from the red dot, and the fish could clearly differentiate the two as well (see "Results" below).

The behavioral assay involved dropping the petri dish in the tank, letting it sink to the bottom, and counting the number of pecks at the red, orange, yellow, green, blue, black, and white dots over a 2-min period. The fish approached the petri dish and pecked at the dots in a manner similar to the way in which they approach and eat food. We performed the behavioral assays in the stock tanks, which contained multiple animals. Ideally, we would have tested single individuals in testing aquaria. In a preliminary study, however, isolated individuals were too timid to peck at the petri dishes. Also, small groups of fish placed in testing aquaria required 2–3 weeks of acclimation before they would peck. The pecking rate was much higher for animals tested in their stock tanks. We therefore performed the assays in the stock tanks. The drawback is that the number of individuals in the tanks varied. We dealt with this by including family size in the statistical model and analyzing the distribution of pecks across the various colors.

Each tank of fish was tested in both clear and tea-stained water. We refer to this as the testing environment. To change the testing environment from clear to tea stained, we simply added instant tea to the water. To change the testing environment from tea stained to clear, we emptied the tank and filled it with clear water. We measured pecking preferences in both testing environments. After assays were completed, we returned the water conditions to their original state (i.e., tea stained or clear). We attempted to measure peck preferences on two separate occasions under each testing environment for each tank of fish (four mea-

surements total for each rearing group between the two testing treatments). However, the fish did not perform pecks in all trials. The data on willingness to peck will be presented in another article. If the fish did not peck, then we tested them again 2–3 days later. If the fish did not peck after four separate attempts on four separate days, then we abandoned the particular testing environment and tested the fish in the alternate testing environment. Some families performed pecks only in a single trial (in a given testing environment), whereas others performed pecks in two trials. For each tank in each testing environment, we calculated the total number of pecks at the red, orange, yellow, green, blue, white, and black dots. For the analysis, we considered a tank in a given testing environment only if the fish performed at least five total pecks. All trials were performed before noon.

We used ordinal multinomial logistic regression to test whether the distribution of accumulated pecks varied as a function of rearing environment, testing environment, or their cross or interactions. We also included the covariate of family size (i.e., total number of fish in the tank) in the model. Preliminary analysis indicated no significant interactions between family size and the other terms in the model, so they were removed from the final model. The analysis was performed using SAS Proc Genmod and assumed a multinomial distribution to the data and a cumulative logit link function. The data were ordered spectrally (red-orange-yellow-green-blue-white-black). Hence, the model analyzes the probability of pecking at colors lower in the spectrum as a function of the treatments and their interactions. We used the repeated statement with tank × testing environment as the designated subject effect to ensure that the analyses took account of our data structure. These data are available in Dryad (http://hdl.handle.net/10255/dryad.1248).

To determine the relationship between opsin expression and pecking preference, we used canonical correlation analysis. Canonical correlation creates linear combinations for each of the two data sets (opsin expression and pecking preference) that maximize the correlations between the two sets of canonical variables. The pecking data included the proportion of pecks at red, orange, yellow, green, and blue dots for each tank of fish measured in each testing environment. We excluded the proportion of pecks at white and black dots because of the large number of zeros in the data set. For the opsin data, we used SAS Proc Mixed to calculate the least square means for each tank from a model that considered the effects of tank and plate. These data are also available in Dryad (http://hdl.handle.net/10255/dryad.1249). We used canonical correlation analysis to (1) test the number of potential dimensions between the two data sets, (2) examine loading patterns to determine whether unique patterns emerged, and (3) determine the total amount of standardized variance in pecking behavior that could be accounted for by the four opsin canonical variates.

Results

Opsin Expression

There were differences in overall expression levels, with LWS and RH2 being expressed at the highest levels, followed by SWS1 and SWS2B (LWS = 0.501 + 0.0072; RH2 = 0.363 + 0.0067; SWS1 = 0.093 + 0.0023; SWS2B = 0.043 + 0.00099). Rearing environment altered opsin expression for three of the four opsin genes: SWS1 and SWS2B were upregulated in clear water, whereas LWS was upregulated in tea-stained water (fig. 4, table 1). Genetic variation at the among-population level was indicated by the significant effect of cross on SWS1. Offspring of spring sires had higher expression levels of SWS1 than did offspring of swamp sires. None of the other opsins varied as a function of cross or a function of cross and rearing environment.

Peck Preferences

The fish displayed clear preferences to peck at some dots over others (red > orange > yellow and green > blue and white > black; fig. 5). The lighting environments under which animals were reared had strong effects on the distribution of pecks across colors (table 2). Animals pecked at red more when they were raised in tea-stained water, and the testing environment had little influence on this behavior (fig. 6*A*). Similarly, animals pecked more at yellow when they were reared under clear-water conditions, regardless of the testing environment (fig. 6*C*).

The distribution of pecks across the colors was also affected by an interaction between the rearing and the testing environments (table 2). The interaction arose because animals that were raised and tested in clear water were less likely to peck at orange (and more likely to peck at green) than were animals raised in clear water and tested in tea-stained water (figs. 5, 6*B*, 6*D*). There were few differences due to testing environment for animals reared in tea-stained water. Most of the pecks at blue dots were made by animals that were reared and tested under tea-stained conditions (fig. 6*E*), but this result must be interpreted with caution, as there were few pecks at blue dots overall.

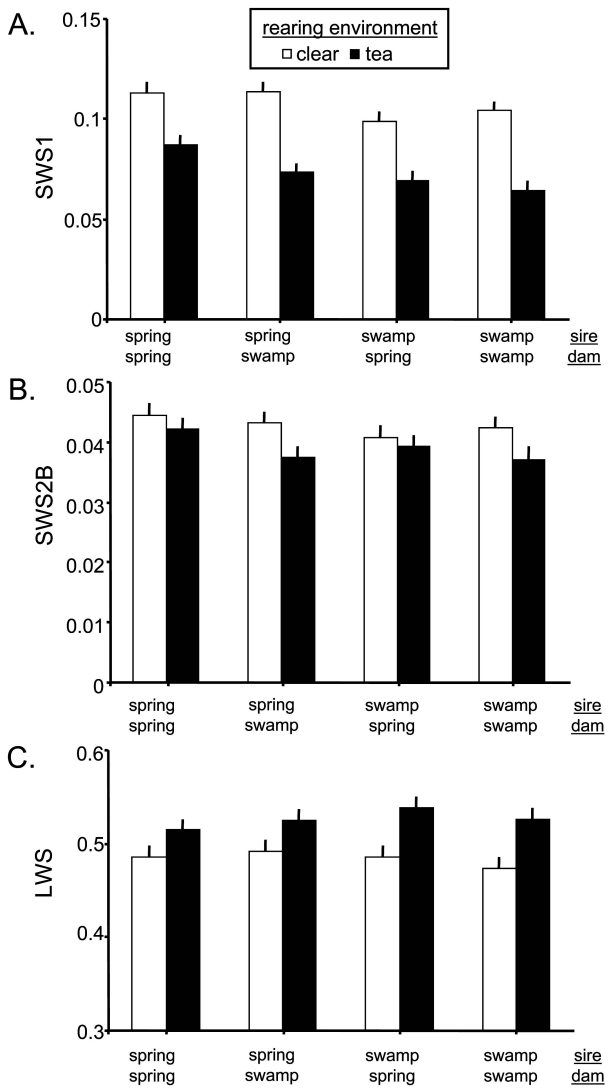

Figure 4: Effects of rearing environment and cross on the relative expression of *A*, short wavelength–sensitive (SWS) 1, *B*, SWS2B, and *C*, long wavelength–sensitive (LWS) opsins. Least squares means and standard errors are shown. Sample sizes for each treatment in each cross are as follows: spring male × spring female: clear water = 14, tea-stained water = 17; spring male × swamp female: clear water = 16, tea-stained water = 18; swamp male × spring female: clear water = 15, tea-stained water = 14; swamp male × swamp female: clear water = 15, tea-stained water = 18.

The Relationship between Opsin Expression and Pecking Preference

The relationship between opsin expression and pecking preference was weak. The first canonical correlation was 0.3455. The hypothesis that all canonical correlations were 0 was rejected (approximate $F_{20,667} = 2.04$, $P < .0048$), but the hypothesis that the remaining canonical variates differed from 0 could not be rejected. The first canonical variate for opsin expression reflected the inverse relationship between SWS1 expression and LWS expression that was mediated by rearing environment (table 3). The first canonical variate for pecking reflected the inverse relationship between pecks at red versus yellow and green dots, which was also mediated by rearing environment (table 3). However, only 2.2% of the standardized variance at pecks could be accounted for by the first opsin canonical variate. Furthermore, only 3.36% of the standardized variance at pecks was accounted for by the four opsin canonical variates together.

Discussion

Our study showed that phenotypic plasticity as a function of differences in lighting environment has large effects on (1) the development of the retina and (2) visually based pecking behavior. Furthermore, lighting environments can have multiple plastic effects that operate at different timescales. Variation in rearing environment altered the distribution of pecks across the various color disks, suggesting that there are long-term effects of lighting habitat on the visual properties and behavior of the animals. The interaction between rearing and testing environments—where the proportion of pecks at some colors differed between testing environments among animals reared in one lighting

Table 1: Effects of rearing environment (RE), cross, RE × cross, and plate on the relative expression of short wavelength–sensitive (SWS) 1, SWS2B, rhodopsin (RH) 2, and long wavelength–sensitive (LWS) opsins, after correcting for effects of plate

Opsin, parameter	df	*F*	*P*
SWS1:			
Rearing environment	1, 118	100.3	<.0001
Cross	3, 118	5.11	.0023
RE × cross	3, 118	1.22	.3072
Plate	23, 248	15.73	<.0001
SWS2B:			
RE	1, 118	7.17	.0085
Cross	3, 118	1.45	.2322
RE × cross	3, 118	.59	.6236
Plate	23, 248	21.91	<.0001
RH2:			
RE	1, 118	.38	.5371
Cross	3, 118	1.17	.3256
RE × cross	3, 118	.81	.4921
Plate	23, 248	38.05	<.0001
LWS:			
RE	1, 118	25.32	<.0001
Cross	3, 118	.53	.6597
RE × cross	3, 118	.54	.6526
Plate	23, 248	36.72	<.0001

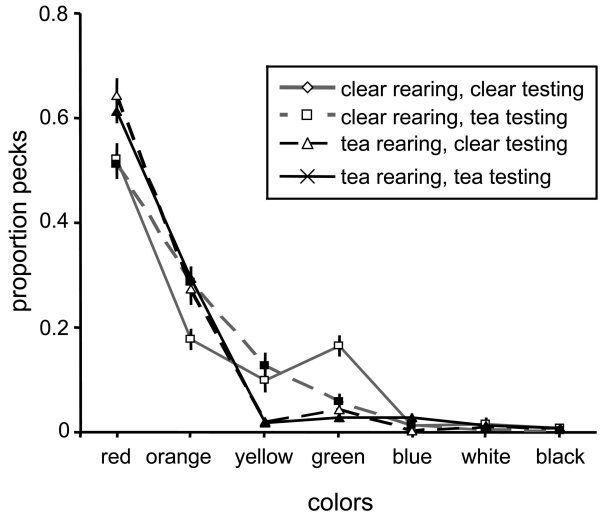

Figure 5: Average proportion of pecks at red, orange, yellow, green, blue, white, and black dots. Means and standard errors are shown.

environment but not the other—is striking, because it suggests that the immediate effects of lighting environment depend on the properties of the visual system induced by rearing environment. By themselves, the effects of rearing environment (i.e., developmental plasticity) and testing environment (i.e., immediate effect of lighting environment) are important but not completely novel. A growing body of literature suggests that the development of visual systems is plastic with respect to light (Kroger et al. 1999; Cronin et al. 2001; Fuller et al. 2005*a*; Wagner and Kroger 2005; Shand et al. 2008; Schartau et al. 2009). Manipulations of lighting environment frequently alter animal behavior (Long and Houde 1989; Milinski and Bakker 1990; Evans and Norris 1996; Cummings et al. 2003).

The unique finding in this study is the interaction between rearing environment and testing environment, where pecking preferences varied as a function of the immediate testing environment, but only for animals raised in one of the two environments (fig. 6). The interpretation is that the immediate effects of lighting environment depend on the properties of the retina. Previous studies have shown that color patterns can differ in appearance to different species (e.g., predators and prey perceive the color pattern differently; Siddiqi et al. 2004), but this is the first study to show that the effects of lighting environment on color-pattern perception can vary among individuals within a species and that this variation is attributable to phenotypic plasticity in the retina.

The interaction is biologically relevant for two reasons. First, the interaction has sobering implications for measuring behavior. Among-site variation in lighting conditions may lead to appreciable differences in vision physiology, but whether one detects meaningful effects on behavior depends on the lighting environment used in the behavioral assay. Second, the interaction between developmental plasticity and immediate effects of lighting environment suggests that variation in the lighting environment under which animals develop (due to either spatial or temporal variation) can have complex effects on foraging preference. In *Lucania goodei*, animals that develop in a tea-stained habitat and subsequently disperse into a clear-water habitat will experience little change in foraging preference, particularly for orange items. Animals that develop in clear water and subsequently disperse into tea-stained water will have shifts in their foraging preferences. To be sure, dispersal between different lighting habitats will generate variation in preference due to developmental plasticity, but there are effects that go beyond those attributable to rearing environment. These effects depend on the interplay between visual system and current lighting conditions. Obviously, foraging behavior will vary given the complex effects of lighting environment. If lighting environments have similar effects on mating preferences, then the direction of sexual selection will also vary due to the phenotypic plasticity of the retina and its interactions with current lighting conditions.

The timing of phenotypic plasticity in visual traits is critical to predicting the levels of variation in color perception. If retinas can reorganize over a short period of time (i.e., quickly track visual habitats), then variation in color perception within populations may be low. If retinas require longer periods to reorganize or if there are critical periods wherein retinas are most responsive to lighting environments, then variation in lighting environment during development may cause appreciable variation in color perception. The extent of spatial and temporal heterogeneity in lighting environments is also critical. The two populations used for the breeding stock came from drainages with low levels of spatial heterogeneity in lighting

Table 2: Ordinal multinomial logistic regression on the accumulated proportion of pecks at red, orange, yellow, green, blue, white, and black dots

Source	df	χ^2	*P*
Rearing environment (RE)	**1**	**29.65**	**<.0001**
Testing environment (TE)	1	3.41	.0647
RE × TE	**1**	**8.65**	**.0033**
Cross	3	1.08	.7817
RE × cross	3	2.06	.56
TE × cross	3	1.07	.7851
RE × TE × cross	3	.02	.9992
Family size	1	.00	.9853

Note: Data are from a Type III analysis. Statistically significant effects are listed in bold.

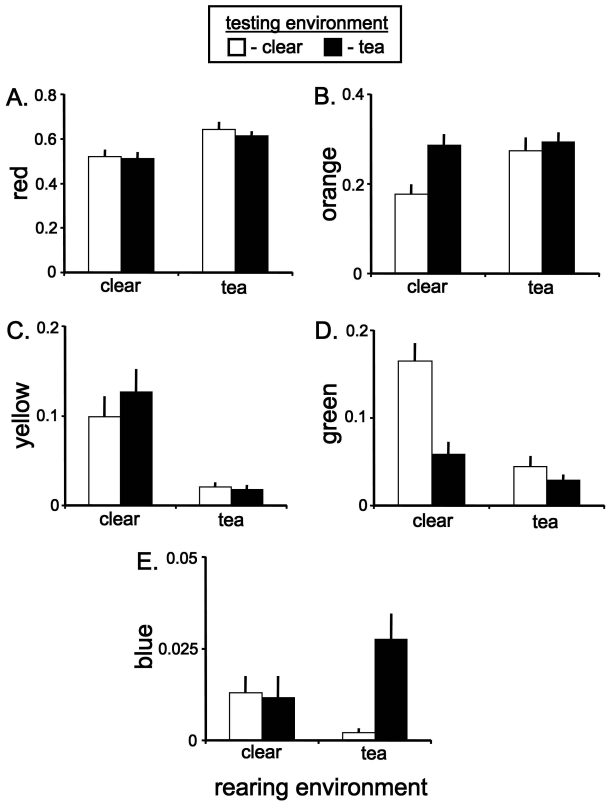

Figure 6: The proportion of pecks at *A*, red, *B*, orange, *C*, green, *D*, yellow, and *E*, blue dots as a function of rearing and testing environments. Note the change of scale on the *Y*-axis. Means and standard errors are shown.

environment (Wakulla River—spring; Everglades—swamp). However, spatial variation can be high along some drainages where the headwaters emerge from swamps but where clear springs connect along the river (e.g., St. John's, Suwanee/Santa Fe). Temporal variation also occurs with wet years leading to higher levels of tannins and dry years leading to clearer water (R. C. Fuller, unpublished data; B. Fugate, personal communication).

The critical question is whether pecking preference has implications for sexual selection and sensory bias. Here, animals pecked most at the red dots. Does red pecking preference correspond to female mating preference for red males? Female *L. goodei* do not exhibit measurable preferences for red males in dichotomous choice tests (McGhee et al. 2007). However, in a test of negative frequency-dependent mating success, red males had slightly higher mating success than did yellow males, regardless of frequency (Fuller and Johnson 2009). Whether this was due to female choice or male-male competition was unclear. Still, the preference to peck at red coupled with the mating advantage of red males is consistent with sensory bias.

The pattern of opsin expression matches that which was shown previously (Fuller et al. 2005*a*), where clear-water habitats resulted in increased expression of SWS1 and SWS2B and decreased expression of LWS. The previous study also showed phenotypic plasticity in RH2 expression as well as narrow-sense heritability in RH2 expression and broad-sense heritability in LWS and SWS2B expression. This study focused on population-level effects. Genetic effects of cross were detected only for SWS1. Animals from spring parents had higher levels of SWS1 expression than did animals from swamp parents, with hybrid offspring being intermediate between the two. The genetic effects appeared to be particularly strong for animals raised in tea, but there was no support of an interaction between cross and rearing environment.

Surprisingly, opsin expression accounted for a small amount of variation in pecking behavior, despite the fact that rearing environment affected both pecking behavior and opsin expression. The loose relationship between opsin expression and pecking preference highlights the fact that opsin expression cannot be used as a surrogate for pecking preference. Whether the same holds for mating preference remains to be seen. The strong effects of rearing environment on pecking preference suggest that portions of the visual system and/or nervous system are plastic with respect to lighting environment. Shifts in chromophore usage (Allison et al. 2004; Flamarique 2005) or filter usage (Flamarique and Harosi 2000) or connections between photoreceptor cells and downstream neurons (Wagner and Kroger 2005) could cause changes in color perception. The central nervous system also allows animals to compare and assign value to the salient signals they encounter. Knowing the spectral properties of photopigment is critical to determine the types of signals animals can detect (Bennett et al. 1994), but the idea that opsin expression alone explains visually based behavior is premature.

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Table 3: Loading factors for the first pair of canonical variates

Opsin	Loading	Pecks	Loading
SWS1	.7473	Red	−.5735
SWS2B	−.0852	Orange	.0859
RH2	.3014	Yellow	.3451
LWS	−.6518	Green	.6196
		Blue	.2886

Note: SWS = short wavelength sensitive; RH = rhodopsin; LWS = long wavelength sensitive.

with fish maintenance. We thank J. Travis for advice at multiple stages of the project. We thank T. Newman and G. Robinson for their assistance using the ABI Prism 7700 Sequence Detection System in the Robinson lab. This work was approved by the University of Illinois Institutional Animal Care and Use Committee (0515). This work was funded by the University of Illinois and a National Science Foundation grant (IOB-0445127).

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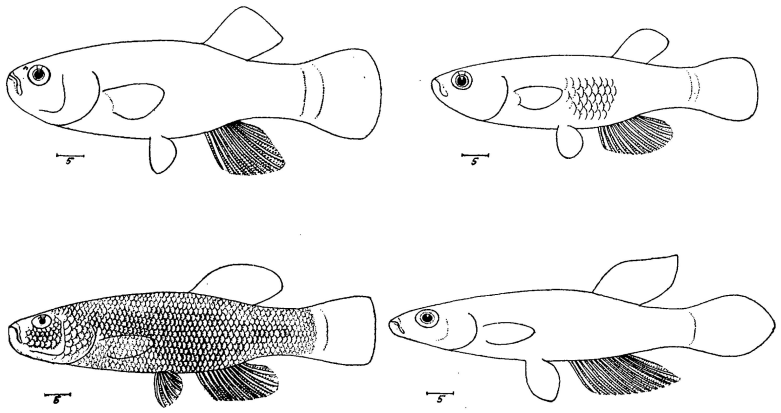

PLATE 1

Fundulus nisorius Cope.
Fundulus zebrinus Jordan and Gilbert.
Fundulus floripinnis (Cope).
Fundulus stellifer (Jordan).

"The killifishes, so named by the early Dutch settlers about New York from their habit of living in the channels or kills, embrace an interesting family of fishes." From "Some Features of Ornamentation in the Killifishes or Toothed Minnows" by Henry W. Fowler (*American Naturalist*, 1916, 50:743–750).