GENETICS, LIGHTING ENVIRONMENT, AND HERITABLE RESPONSES TO LIGHTING ENVIRONMENT AFFECT MALE COLOR MORPH EXPRESSION IN BLUEFIN KILLIFISH, *LUCANIA GOODEI*

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Abstract.—Determining the degree to which variation in traits is controlled by genetics and/or environment is fundamental to understanding adaptation. In this study, we examine the genetic and environmental influences on color pattern expression in male bluefin killifish, *Lucania goodei*. This is a compelling system because both male color patterns and vision physiology are correlated with basic properties of the environment. Across populations, males with blue anal fins are more abundant in waters with low transmission of UV and blue wavelengths. Here, we present results from two paternal half-sib breeding experiments (one in the laboratory, one in the greenhouse) in which offspring were raised under light treatments that mimicked natural variation in the spectral composition of light. In both experiments, we found that red-versus-yellow expression is controlled by an autosomal locus of large effect where yellow (Y) is dominant over red (y). There was little blue expression in the laboratory. In the greenhouse, we found higher expression of blue anal fin morphs when males were raised in tea-stained water (low transmission UV/blue) than when raised in clear water (high transmission UV/blue). We also found genetic effects of sires and an interaction between sire and lighting environment (i.e. heritable plasticity). These results show that a relatively simple, environmentally dependent, epistatic interaction can produce a large amount of variation in male color patterns that presumably function in sexual selection.

Key words.—Color polymorphism, epistasis, Fundulidae, heritable plasticity, ultraviolet light.

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Determining the degree to which sex-limited color patterns are controlled by genetics and/or environment is a critical step in the study of natural and sexual selection (Fisher 1958; Endler 1986; Arnold 1994; Reznick and Travis 1996; Travis and Reznick 1998; Mousseau et al. 2000). Yet, few studies have tried to tease apart the relative roles of genetics and environment for sexually selected color patterns despite many intriguing correlations between color patterns and environmental conditions across populations (Houde 1997; Seehausen et al. 1997; Grether et al. 2001). Although many studies have examined the roles of condition and parasites on color pattern production (Hill and Montgomerie 1994; Thompson et al. 1996; Brawner et al. 2000), few have examined the effects of abiotic conditions, such as lighting environment, while simultaneously examining the influence of genetics. This is striking because a large literature suggests that environmental lighting conditions should have large effects on the perception of male color patterns (Endler 1992, 1993).

In this study, we examine the genetic and environmental influences on color pattern expression in male bluefin killifish, *Lucania goodei*. We specifically focus on anal fin coloration, which is used in sexual selection (Foster 1967; Fuller 2001). *Lucania goodei* is a compelling system because both male color patterns (Fuller 2002) and vision physiology (Fuller et al. 2003) are correlated with basic properties of the environment indicating either some adaptive variation or some very striking environmental influence on phenotypes. In a comparative study across 30 Florida populations, Fuller (2002) found large effects of lighting environment on the relative abundance of male color morphs. Populations vary in the transmission of UV/blue wavelengths (360–478 nm) through the water column where high UV/blue transmission is associated with clear springs and low UV/blue transmission is associated with tea-stained swamps and lakes. Males with

blue anal fins were more abundant in waters with low transmission of UV and blue wavelengths (i.e. tea-stained swamps and lakes). In contrast, males with red anal fins (and to a lesser extent, males with yellow anal fins) were more abundant in populations with high transmission of UV and blue wavelengths (i.e. clear springs). These differences in color morph frequencies are correlated with differences in vision physiology: animals in a clear spring population have proportionately more UV and violet cones and fewer yellow and red cones than do animals from a tea-stained swamp population (Fuller et al. 2003). This pattern of spectral matching follows that typically found across species (Levine and MacNichol 1979; Lythgoe 1984; Lythgoe et al. 1994). Are these differences in male color morph frequencies due to genetic differentiation as a result of differential selection in different lighting environments, or does phenotypic plasticity also contribute to the pattern? To answer these questions, we performed a paternal half-sib breeding experiment to determine whether genetics, environment, and/or the interaction between genetics and environment affect male color pattern expression.

There is good reason to expect large genetic effects. Inheritance studies of color pattern have frequently found large genetic effects. In poeciliids, color patterns of many morphs are controlled by sex-linked genes (Winge 1922; Kallman 1975; Angus 1989; Houde 1992, 1997; Brooks and Endler 2001). In the side-blotched lizard, *Uta stansburiana*, variation in color pattern expression has a large genetic component (Sinervo and Lively 1996; Sinervo et al. 2000; Zamudio and Sinervo 2000) and is hypothesized to be under the control of a single locus. There is also good evidence that environmental conditions can have strong effects on color patterns. The production of both carotenoid-based and structural colors has been linked to foraging ability and general health (Ko-
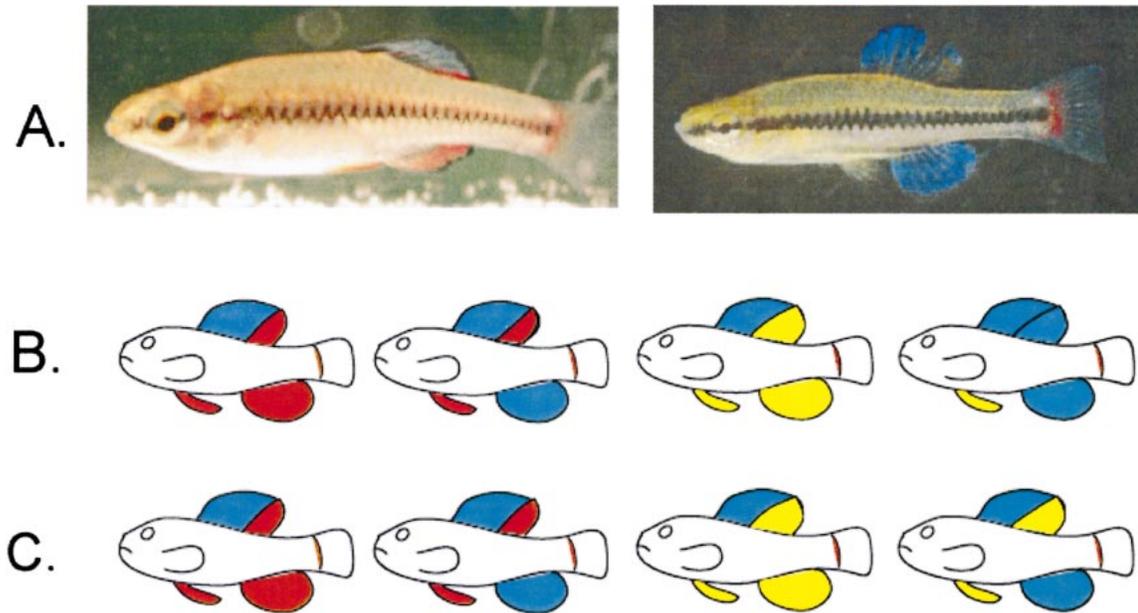
FIG. 1. (A) Two male color morphs. One male is red on the posterior region of the dorsal fin, red on the anal fin, and red on the pelvic fins. The other is yellow on the posterior region of the dorsal fin, blue on the anal fin, and yellow on the pelvic fins. (B) The four color morphs used in the laboratory experiment: R/R-red pelvics; R/B-red pelvics; Y/Y-yellow pelvics; B/B-yellow pelvics. (C) The four color morphs used in the greenhouse experiment: R/R-red pelvics; R/B-red pelvics; Y/Y-yellow pelvics; Y/B-yellow pelvics.

dric-Brown 1989; Houde and Torio 1992; Evans and Norris 1996; Grether 2000; Keyser and Hill 2000; Doucet 2002; Siitari and Huhta 2002). Brakefield and Larsen (1984) have shown that many butterflies develop distinctly different color patterns in different seasons (wet vs. dry). Similarly, Horth (2001) has shown that temperature can affect the expression of a polymorphic, melanistic color pattern in eastern mosquito fish, *Gambusia holbrooki*.

In this paper, we present the results of two experiments that investigated the sources of variation among males in color expression. In each experiment, we crossed males of different colors with several females and raised the offspring in different lighting environments. Our goal was to determine the relative effects of genetics, environment and the interaction between genetics and environment on male color pattern expression.

MATERIALS AND METHODS

Study System

The bluefin killifish, *Lucania goodei*, is a freshwater fundulid found throughout peninsular Florida with a few populations inhabiting southeastern Georgia and South Carolina (Page and Burr 1991). The breeding system is promiscuous. Males guard patches of vegetation that serve as substrates for females to attach eggs (Fuller 2001). There is no evidence for male parental care (Fuller and Travis 2001). Males use their dorsal and anal fins when fighting other males and also when courting females. In fights, males flare their dorsal and anal fins and engage in circle fights (Foster 1967; Fuller 2001). Males also use their fins in the initial stages of courting females. Males swim circle loops in front and around the female while flashing their anal and dorsal fins.

The color pattern is dimorphic between the sexes. Males have a red dot at the base of the caudal fin (Fig. 1A). The dorsal, anal, and pelvic fins are colored in males but lack color in females. The anterior three-fourths of the dorsal fin is blue on all males. The posterior one-fourth of the dorsal fin, the pelvic fins, and the anal fin are polymorphic among males. The polymorphism on the pelvic fins and posterior dorsal fin is relatively simple. The posterior one-fourth of the dorsal fin can be blue, red, or yellow. The pelvic fins are either red or yellow (Fig. 1A).

The polymorphism on the anal fin is much more complex. There are five main categories of anal fin color patterns: solid red, solid yellow, solid blue, combination of red and blue, and combination of yellow and blue. In a statewide Florida census, over 99% of males expressing coloration could be placed into these categories. However, in one population, males with orange anal fins were found in addition to the yellow and red anal fin morphs (Fuller 2002). In this study, we do not consider the single population with the orange color morph. In addition, the red-blue combination and yellow-blue combination anal fin color patterns can take a variety of forms. In our study, we use only males with solid-colored anal fins as sires and score male offspring as belonging to one of the five categories.

We collected *L. goodei* at the 26-Mile Bend boat ramp in the Everglades (Broward County, FL) using dip nets and seines in January 2000 and August 2001. This is a high-density population with good representation of most color patterns (Fuller 2002). We transported the animals to Florida State University and housed them in 76-L aquaria until the experiments began. Animals were fed daily with frozen chironomids and adult *Artemia*.

Laboratory Experiment

We chose four sires with distinctly different color patterns to maximize our ability to detect a genetic effect (Fig. 1B). One male was blue on the posterior region of the dorsal fin, blue on the anal fin, and yellow on the pelvic fins, and is referred to as the ''B/B-yellow pelvics'' sire hereafter. The second male was yellow on the posterior region of the dorsal fin, yellow on the anal fin, and yellow on the pelvic fins, and is referred to as the ''Y/Y-yellow pelvics'' sire hereafter. The third male was red on the posterior region of the dorsal fin, red on the anal fin, and red on the pelvic fins, and is referred to as the ''R/R-red pelvics'' sire hereafter. The fourth male was red on the posterior region of the dorsal fin, blue on the anal fin, and red on the pelvic fins, and is referred to as the ''R/B-red pelvics'' sire hereafter.

We crossed each of the four sires with nine randomly chosen females resulting in a total of 36 females (hereafter referred to as dams). For each sire, the nine dams were divided among three environmental lighting treatments (UV filter, UV/blue filter, gray filter). For each pairing, we placed a sire with a dam in a 19-L (5-gallon) aquarium filled with clear well water and covered by one of the light filters. Each aquarium contained one sponge filter. Dams were not virgins. Because *L. goodei* are external fertilizers, whose sperm and egg combine in the water, we assume that there is no sperm storage. Each aquarium contained yarn mops that served as spawning substrate. We checked the mops daily for the presence of eggs. The sire and dam remained in the aquarium until a minimum of 20 healthy eggs was obtained, at which time the two fish were removed. We began crossing sires and dams in January 2000 and finished in July 2000.

In this experiment, offspring were raised under the lighting treatments from conception. We carefully removed the eggs from the mops and placed them in small plastic tubs that we floated in the aquarium. Upon hatching, fry were fed *Artemia* nauplii. Once all of the fry were eating and the parents had been removed, we released the fry into the aquarium. At approximately two to three months of age, offspring were switched to a diet of frozen brine shrimp and frozen bloodworms. Throughout the experiment, room temperature was kept at 22°C.

The environmental lighting treatments were created by attaching plastic theater gels with known spectral properties to wooden frames that sat on top of the aquaria. Light from fluorescent bulbs (that mimic sunlight) had to pass through the filters before entering the aquaria. The no-UV treatment was created by attaching two layers of a plastic UV filter to the frame. This treatment reduced all wavelengths below 400 nm (Fig. 2A). The no-UV/blue treatment combined one sheet of plastic UV filter layered with one sheet of blue filter that together reduced wavelengths below 500 nm. The gray filter treatment consisted of two layers of clear filter that removed roughly 20% of all the wavelengths and controlled for the presence of a filter. Lights were maintained on a 14L:10D cycle. There were no windows in the room. Hence, there was no access to sunlight.

To ascertain the effects of our treatments, we measured down-welling irradiance using an Ocean Optics S2000 spectrophotometer equipped with a cosine corrector (Ocean Optics, Dunedin, FL). Specifically, we measured down-welling irradiance in four aquaria for each lighting treatment (12 aquaria total; Fig. 2A). Measurements were taken 7.62 cm (3 inches) below the surface of the water. We chose 3 inches below the water surface for ease of measurements. Due to the depth of the aquaria (25 cm) and the dimensions of the cosine corrector, it was difficult to measure the irradiance at greater depths. In the field, *L. goodei* are typically found at depths of 5–100 cm (R. Fuller, pers. obs.). Hence, we measured the irradiance within the range at which they are found (although admittedly on the shallow side). We calibrated the spectrophotometer using an LS-1 calibrated lighting source (Ocean Optics). This apparatus gives accurate measurements of irradiance ($\mu W/cm^2$) under water provided that the cosine corrector is filled with water. These measurements were then converted into units of photon flux ($\mu M \ m^{-2} \ s^{-1}$). We performed ANOVA among lighting treatments at each wavelength (361–701 nm) to determine the effects of the filters. We could not measure irradiance below 360 nm because our calibrated light source does not cover these wavelengths.

We censused the aquaria once every two to three months from August 2000 to August 2001. During each census, we recorded the number of males, females, and juveniles, the standard length of males and females, and the color pattern of males. Densities were not held constant across tanks. To increase the number of males expressing coloration, we occasionally removed the largest, most brightly colored males. This results in more males expressing coloration because large, dominant males can frequently suppress smaller males from expressing coloration. At the end of the experiment, we calculated the proportions of males bearing each color pattern. In addition to males present at the last census, we included males that had been removed and males that had apparently died. There was no evidence that the date of census affects male color pattern expression. *Lucania goodei* can reach sexual maturity in three months (R. Fuller, pers. obs.). The largest males expressed coloration at this time. However, the time until smaller males expressed color was longer due to inhibition from larger males. There was no evidence that differential time until sexual maturation affected male color morph expression.

After obtaining offspring, we treated dams with androgens to induce them to express the male color pattern. Our objective was to determine whether dams also carried genes for the male color pattern, and, if so, to determine the phenotype each dam expressed. After obtaining adequate eggs for the breeding experiment, we placed each dam in a 1.9-L glass jar containing well water aerated with a small air stone. Each day, we added 20 μl of a 17α-methyltestosterone solution (concentration: 3 mg/ml ethanol). To prevent a build-up of ethanol (which is toxic to fish), we replaced the well water twice weekly and recorded the female color pattern.

These data were analyzed using a general linear model to determine whether sires, lighting environment, or the interaction between sires and lighting environment accounted for a significant amount of variation in the relative abundances of males with solid red, solid yellow, and solid blue anal fins. We treated sires and lighting environment as fixed effects. In this experiment, there was no replication within individual dams. Hence, the effect of sire was tested over the
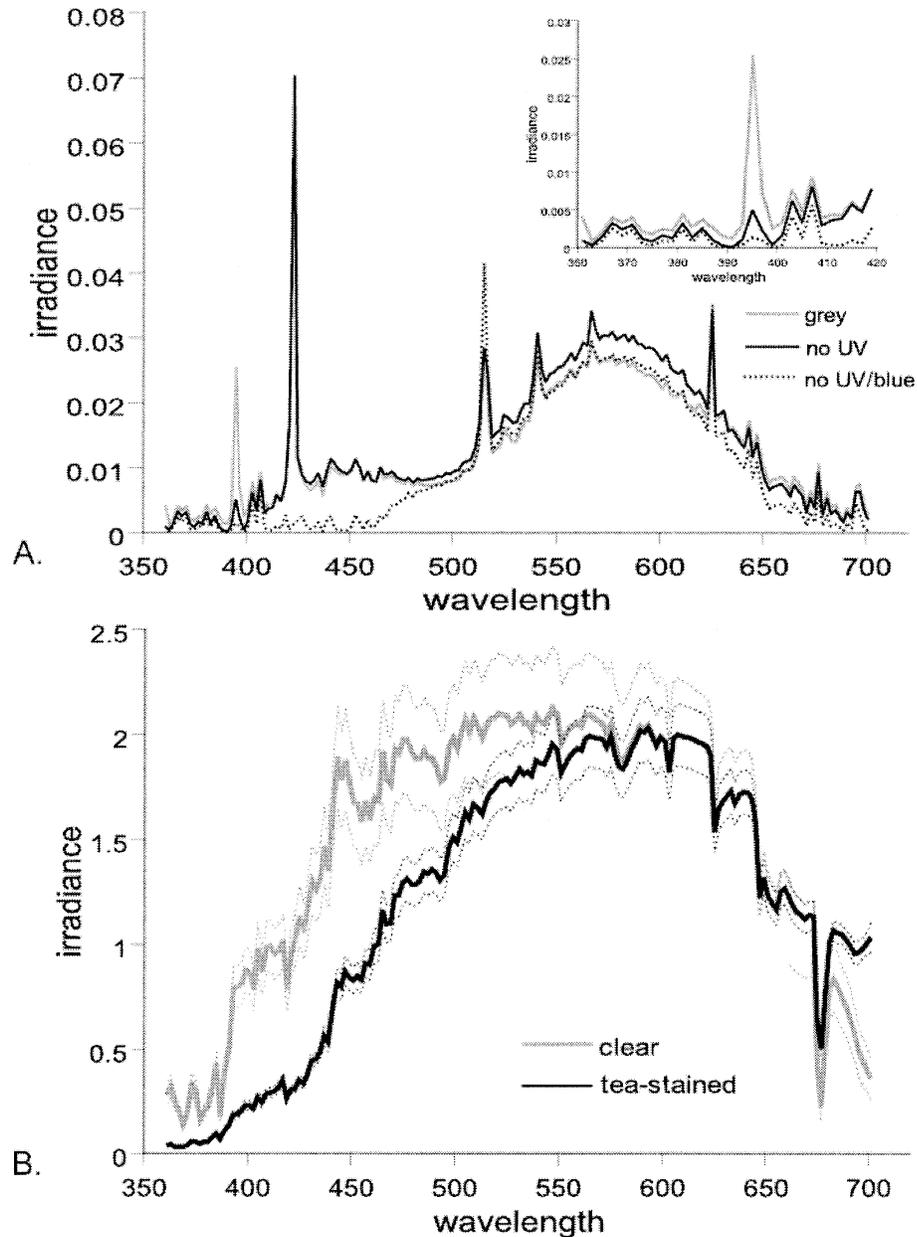
FIG. 2. (A) Down-welling irradiance ($\mu M\ m^{-2}\ s^{-1}$) across lighting treatments in the laboratory experiment. The inset graph illustrates the pattern in irradiance in the shorter wavelengths. (B) Down-welling irradiance ($\mu M\ m^{-2}\ s^{-1}$) in clear versus tea-stained water in the greenhouse experiment. The black dotted lines denote $\pm$ 1 SE for the tea-stained treatment. The gray lines denote $\pm$ 1 SE for the clear-water treatment.

mean-square error term. We also included the effect of density in the model to control for the fact that densities were not held constant across aquaria. We used the arcsine transformation of the square root of the proportion of males in a clutch bearing a given color pattern as our dependent variable. In the text, we report means and standard errors calculated on the untransformed proportions. We visually examined the residuals from all models to determine whether the assumption of normality and homogeneity of variances was upheld. Finally, we calculated a chi-square statistic to test whether the hidden phenotype of females met with the predictions of a Mendelian model. Results were considered significant at

$P < 0.05$. All P-values are based on two-tailed tests. All analyses were performed using SAS Version 8 (SAS Institute, Cary, NC).

Greenhouse Experiment

We chose four sires with distinctly different color patterns (Fig. 1C). One male was yellow on the posterior region of the dorsal fin, blue on the anal fin, and yellow on the pelvic fins, and is referred to as the ''Y/B-yellow pelvics'' sire hereafter. The second male was yellow on the posterior region of the dorsal fin, yellow on the anal fin with a slight tinge

TABLE 1. Number of animals at various life stages for each family in the greenhouse experiment.

Sire	Dam	Eggs	Fry	Fry eating	Fry in clear water	Fry in tea-stained water	Males w/ color (clear)	Males w/ color (tea)	Males w/o color (clear)	Males w/o color (tea)	Females (clear)	Females (tea)
R/B	1	236	198	105	53	52	7	10	0	0	12	8
R/B	2	271	251	135	63	62	9	6	0	0	6	5
R/B	3	191	186	130	66	64	15	10	2	0	17	10
R/R	1	218	182	69	34	35	5	12	0	0	11	10
R/R	2	222	181	127	59	59	8	9	9	0	12	8
R/R	3	310	233	168	83	85	0	15	1	0	0	15
Y/B	1	261	222	119	58	58	9	7	0	0	8	6
Y/B	2	101	74	46	17	17	4	3	0	0	2	2
Y/B	3	295	249	167	45	67	13	18	4	0	13	13
Y/B	4	152	128	66	27	27	8	7	0	2	7	3
Y/Y	3	286	234	177	85	85	17	14	6	0	19	9
Y/Y	4	54	26	20	8	10	6	4	0	0	3	6
Y/Y	5	369	304	210	104	106	10	13	1	0	9	17

of blue at the base, and yellow on the pelvic fins, and is referred to as the ''Y/Y-yellow pelvics'' sire hereafter. The third male was red on the posterior region of the dorsal fin, red on the anal fin, and red on the pelvic fins, and is referred to as the ''R/R-red pelvics'' sire hereafter. The fourth male was red on the posterior region of the dorsal fin, blue on the anal fin, and red on the pelvic fins, and is referred to as the ''R/B-red pelvics'' sire hereafter.

We crossed each sire with three to four randomly chosen dams in the laboratory. We then divided each clutch between two environmental lighting treatments (clear vs. tea-stained water). Crosses were made using a similar protocol as in the laboratory experiment. Upon hatching (approximately 10–14 days postfertilization), we fed animals *Artemia* nauplii in the laboratory for approximately two to four weeks. We then transported them to the greenhouse, divided each clutch so that there were equal numbers of similarly aged fry, and placed them in 114-L tanks containing either clear or tea-stained water. We began crosses in August 2001 and finished March 2002. Again, we determined the hidden phenotype for a fraction of the dams by treating them with 20 μl of a 17α-methyltestosterone solution (concentration: 3 mg/ml ethanol).

For each combination of dam and sire, we set up two 114-L (30 gallon) tanks containing either clear or tea-stained water (26 tanks total). We filled tanks with well water and stocked them with mops and plastic plants. All water was treated with a buffer to keep the pH above 7. In addition, we set up external filters (Whisper Power 30, Tetra, Blacksburg, VA) to remove excess wastes and algae from the tanks via filtration through mesh bags containing activated carbon. For the tea-stained water treatment, we added a small amount of instant, decaffeinated tea to the water two to three times each week. Whenever algae began to grow in the water column, we drained the water, added new water, re-established the treatment, and added a slow-release algicide (Green Water Control, Aquatronics, Oxford, CA) to the tanks to keep the water column clear. All aquaria were treated with algicide. Filamentous algae were allowed to grow on aquarium walls. Daylight was the only light source. This experiment ran from August 2001 to December 2002.

To quantify treatment differences, we measured down-welling irradiance (μM m^{-2} s^{-1}) using an Ocean Optics spec-

trophotometer and a cosine-corrector (Fig. 2B). All measurements were taken between 1000h and 1400h on clear, sunny days. Measurements were taken 7.62 cm below the surface of the water in all aquaria following the methods described in the laboratory experiment. We performed AN-OVA among lighting treatments at each wavelength (361–701 nm) to determine the effects of the treatments.

We planned a balanced breeding design in which each sire was crossed with three dams. However, unforeseen complications resulted in an unbalanced design. Because one dam paired with the Y/B-yellow pelvics sire died after producing a small clutch, we paired this sire with another dam resulting in 4 dams for the Y/B-yellow pelvics sire (Table 1). In addition, we accidentally lost all but one female offspring from one clutch during one census (Table 1, R/R, dam 3, clear water). Finally, we inadvertently contaminated a clutch with unrelated animals. Luckily, we still had both the dam and sire, and could spawn another clutch (Table 1, Y/B, dam 3, clear water, 45 fry). These complications resulted in an unbalanced experimental design.

We analyzed this experiment using a general linear model to determine whether sires, dams nested within sires, lighting environment, or the interaction between sires and lighting environment accounted for a significant amount of variation in the proportion of male offspring with solid red, solid yellow, solid blue, or any blue on the anal fins. We treated sire and lighting environment as fixed effects and dams nested within sires as a random effect. In addition, we included density in the model to control for unequal densities of fish across aquaria. Because the design was unbalanced, SAS used the Satterthwaite approximation to determine the error and degrees of freedom in the denominator of the sire *F*-test (Sokal and Rohlf 1995). This resulted in an error term equal to 0.9608 × mean square of dam (sire) + 0.0392 × mean-square error. All other *F*-tests were calculated using the mean-square error in the denominator. Again, we visually examined the residuals from all models to determine whether the assumption of homogeneity of variances was met. The assumption was met in all but one case. Here, we employed a log-linear model (that makes no assumption concerning homogeneity of variances) to assess the effects of sires, lighting environment, and the interaction between sires and lighting environment on the frequency of males expressing solid blue anal
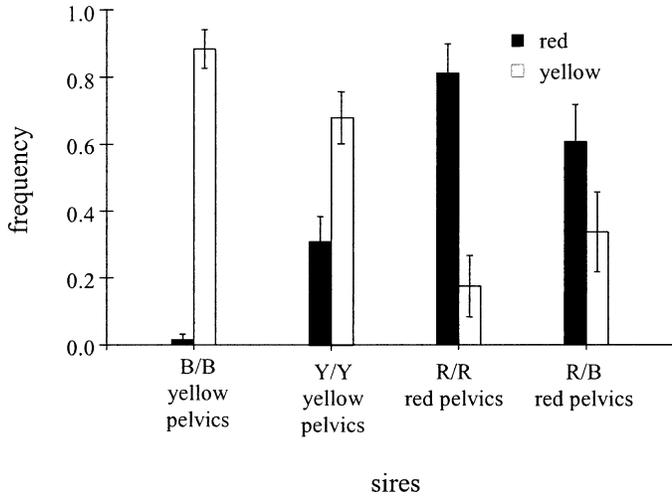
FIG. 3. Average frequency of F_1 males expressing a red or yellow anal fin in laboratory crosses. Bars are standard errors. B/B-yellow pelvics sire ($n = 8$), Y/Y-yellow pelvics sire ($n = 8$), R/R-red pelvics sire ($n = 8$), R/B-red pelvics sire ($n = 9$).

fins. All other details of the analysis followed the procedures used for the laboratory experiment.

RESULTS

Laboratory Experiment

Analysis of variance at each wavelength indicated that irradiance differed significantly among the treatments in both the shorter wavelengths (361–469 nm) and in the longer wavelengths (667–701 nm) ($P < 0.05$ in all ANOVAs; Fig. 2A). Irradiance was significantly higher under the gray filter than under the no UV/blue filters (361–467 nm, 667–737 nm, Tukey post-hoc tests at each wavelength, $P < 0.05$). Similarly, irradiance was higher under the no-UV filter than it was under the no UV/blue filter, particularly in the blue wavelengths (403 nm, 407 nm, 411–469 nm), but also in the longer, red wavelengths (677 nm, 681–701 nm). Irradiance was also higher under the gray filter than under the no-UV filter in the shorter wavelengths (361–363 nm, 373–375 nm, 379 nm, 383–399 nm; Tukey post-hoc tests of means). Finally, average irradiance over all wavelengths did not differ between the treatments ($F_{2,9} = 0.52$, $P = 0.6115$).

Few males expressed the blue anal fin coloration in the lab. Across all 36 families, 324 males expressed coloration on the anal fin (346 males total—22 died before expressing color). Most males expressed either a red anal fin (142 males; 44%) or a yellow anal fin (163 males; 50%). Only fourteen males expressed a solid blue anal fin (4%). Five males (2%) expressed either a yellow-blue or red-blue combination anal fin. On average, there were 9.00 males expressing color (0.747 SE, $n = 36$) in each clutch. Two clutches had only two males expressing color and were excluded from the analysis.

The proportion of males expressing red anal fins varied significantly among sires (Fig. 3; $F_{3,21} = 16.54$, $P < 0.0001$). Males with red anal fins were most abundant in the clutches of the R/R-red pelvics and R/B-red pelvics sires. In addition, lighting treatments also accounted for a significant amount

of variation ($F_{2,21} = 4.69$, $P = 0.021$). Males with red anal fins were more abundant in the gray filter treatment (mean $= 0.573 \pm 0.125$ SE, $n = 12$) than in the no-UV filter treatment (mean $= 0.347 \pm 0.101$ SE, $n = 11$). There was no effect of the interaction between sire and environment ($F_{6,21} = 0.92$, $P < 0.502$) nor of fish density ($F_{1,21} = 0.17$, $P < 0.681$).

The proportion of males with yellow anal fins was similarly affected by sire (Fig. 3; $F_{3,21} = 10.08$, $P = 0.0003$). Males with yellow anal fins were most abundant in the clutches sired by the B/B-yellow pelvics and Y/Y-yellow pelvics sires. In contrast to the pattern with red males, yellow males tended to be more abundant in the no-UV treatment (mean $= 0.638 \pm 0.102$ SE, $n = 12$) than in the gray filter treatment (mean $= 0.399 \pm 0.117$, $n = 12$) (lighting treatment: $F_{2,21} = 3.50$, $P = 0.0487$). The interaction between sire and environment did not account for a significant amount of variation ($F_{6,21} = 1.11$, $P < 0.3878$) nor did density ($F_{1,21} = 1.27$, $P < 0.273$).

Neither sire, lighting environment, nor the interaction between sire and environment accounted for a significant amount of variation in the relative abundance of males with blue anal fins (sire $F_{3,21} = 1.04$; lighting environment $F_{2,21} = 0.26$; sire $\times$ environment $F_{6,21} = 0.94$). Density did account for a significant amount of variation ($F_{1,21} = 4.74$, $P < 0.041$); blue males were more likely to be found in aquaria with high fish density (slope $= 0.007 \pm 0.005$ SE). However, given that the entire model does not account for a significant amount of variation ($F_{11,21} = 0.86$, $P < 0.592$), this result is somewhat tenuous.

Similar results were found when analyzing only clutches with five or more males expressing color pattern. The proportion of males expressing red fins varied with sires ($F_{3,18} = 15.57$, $P < 0.0001$) and lighting environment ($F_{2,18} = 5.35$, $P = 0.015$), but not with the interaction between sires and lighting environment ($F_{6,18} = 1.54$, $P = 0.223$) nor with density ($F_{1,18} = 1.84$, $P = 0.192$). The proportion of males expressing yellow fins also varied with sires ($F_{3,18} = 7.96$, $P = 0.0014$) and lighting environment ($F_{2,18} = 4.40$, $P = 0.028$), and tended to vary with density ($F_{1,18} = 4.41$, $P = 0.0501$), but not with the interaction between sires and lighting environment ($F_{6,18} = 1.48$, $P = 0.242$). The proportion of males with blue anal fins was significantly affected by density ($F_{1,18} = 6.96$, $P = 0.017$), but not by sires ($F_{3,18} = 2.11$, $P = 0.134$), lighting environment ($F_{2,18} = 1.18$, $P = 0.330$), nor the interaction between sires and lighting environment ($F_{6,18} = 1.07$, $P = 0.417$).

A Test for Mendelian Ratios

Almost all males expressed some component of either yellow or red. Of the 14 males with solid blue anal fins, four had either red pelvic fins or a red posterior region on the dorsal fin. Seven males with solid blue anal fins had either yellow pelvic fins or a yellow posterior region of the dorsal fin. Three males were not diagnosable for red or yellow because they were removed from aquaria prematurely or died. Of the five males with combination anal fins, one expressed as yellow and blue, and four expressed as red and blue. This
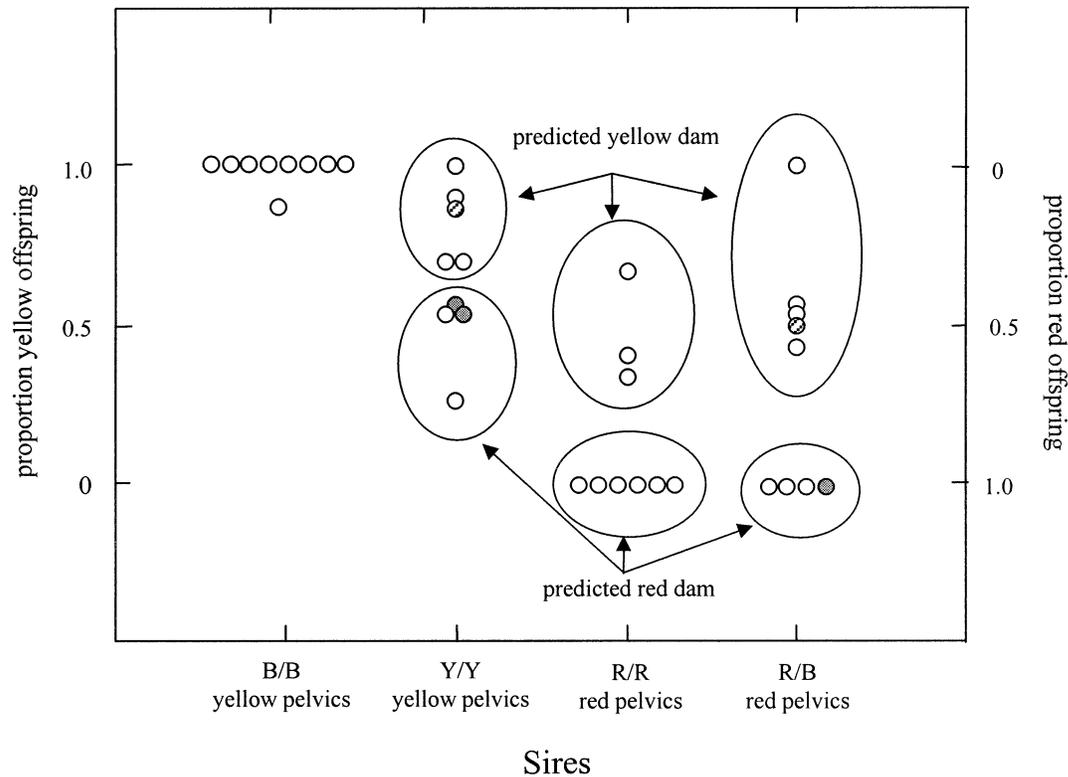
FIG. 4. Distribution of clutches expressing some element of red or yellow in laboratory crosses. Each dot is a clutch. The predicted female hidden phenotype is indicated. Gray dots indicate females that were predicted to carry the red phenotype but actually expressed as yellow. Hashed dots indicate females that were predicted to carry the yellow phenotype but actually expressed as red.

phenomenon allows us to categorize 99% of the males as expressing some component of either yellow or red.

An examination of the relative abundance in each clutch of males with any yellow versus males with any red shows a pattern of expression that is consistent with Mendelian inheritance in which there is one autosomal locus controlling the expression of yellow and red with the yellow allele (Y) being dominant over the red allele (y) (Fig. 4). The B/B-yellow pelvics sire had 100% yellow offspring in all of his clutches with the exception of one clutch in which seven male offspring were yellow and one was red. This pattern is consistent with this male being homozygous dominant (YY). The Y/Y-yellow pelvics sire had clutches that varied between 50% and 100% yellow male offspring with the exception of one clutch in which one male offspring was yellow, and three were red. This is consistent with this male being heterozygous (Yy). Mating with a female carrying the homozygous dominant genotype (YY) should produce 100% yellow male offspring. A mating with a female carrying the heterozygous genotype (Yy) should produce 75% yellow and 25% red male offspring. A mating with a female carrying the homozygous recessive phenotype (yy) should produce 50% yellow and 50% red offspring.

The red sires also produced offspring in patterns consistent with the Mendelian hypothesis (Fig. 4). The R/R-red pelvics sire produced clutches with 100% red offspring and clutches with roughly 50% red and 50% yellow offspring. The R/B-red pelvics sire produced a similar distribution of clutches (100% red, 50% red) with the notable exception of one clutch

with 100% yellow offspring (8/8 males expressed as yellow). These ratios are consistent with these sires being homozygous recessive (yy). Matings with homozygous recessive females (yy) should produce 100% red male offspring. Matings with heterozygous females (Yy) should produce 50% red and 50% yellow male offspring. Matings with homozygous dominant females (YY) should produce 100% yellow male offspring.

We determined the hidden phenotype for 32 dams to test the Mendelian hypothesis. Dams expressed the same range of color patterns as males (i.e. red, yellow, and blue color elements) on their dorsal and anal fins and appeared to express color patterns as strongly as sires. All 32 of the dams expressed some element of either red or yellow on either their pelvic, rear-dorsal, or anal fins allowing us to discern which phenotype (yellow or red) they possessed. Based on the relative abundance of F1 male phenotypes and the assumption of Mendelian inheritance, we predicted the phenotype of the dam (Fig. 4). Given that we had no knowledge of dam phenotype when pairing dams and sires, we assumed mating between male and female genotypes was random. We could make no predictions for the B/B-yellow pelvics sire because we posited that he was a homozygous dominant male. Hence, the dam genotype should have no effect on the F1 male phenotypes. For the Y/Y-yellow pelvics sire, we predicted clutches with roughly 50% red and 50% yellow male offspring would have dams carrying the red phenotype (yy) whereas clutches with 75–100% yellow offspring would have dams carrying the yellow phenotype (YY or Yy). For both the R/R-red pelvics and R/B-red pelvics sires, we predicted

clutches with 100% red offspring would have dams carrying the red phenotype. For clutches with roughly 50–100% yellow male offspring, we predicted dams would carry the hidden yellow phenotype (YY or Yy).

The predictions were met in 18/23 cases (Fig. 4; $\chi^2 = 7.34$, df = 1, $P = 0.0067$). Four of the five cases in which the predictions were not upheld can be attributed to sampling error. For the Y/Y-yellow pelvics sire, three of our predictions were not upheld, but in all of these clutches the proportion of males with yellow anal fins was between 50% and 75%. In one clutch, 9/16 (56.25%) male offspring were yellow, leading us to predict a red dam. In the second clutch, 11/20 (55%) male offspring were yellow, leading us to predict a red dam. In the third clutch, 5/7 (71%) male offspring were yellow, leading us to predict a yellow dam. The R/B-red pelvics sire had one clutch in which 4/4 (100%) male offspring were red, leading to the prediction of a red dam. In all of these cases, the addition of one or two male offspring with the appropriate phenotype changes the frequencies to the point at which the predicted dam phenotype matches the expressed dam phenotype. However, in one case the deviation from the predicted dam phenotype cannot be attributed to sampling error. The R/B-red pelvics sire had one clutch in which 5/10 (50%) offspring were red, leading to the prediction of a yellow dam. The expressed phenotype was red. We cannot explain this, as only red offspring should result from the pairing of two animals carrying the homozygous recessive (yy) phenotype.

Greenhouse Experiment

In the greenhouse experiment, irradiance was significantly higher in the clear-water treatment than in the tea-stained treatment for UV and blue wavelengths 361–487 nm ($P < 0.05$ in all ANOVAs; Fig. 2B). In contrast, irradiance was higher in the tea-stained treatment than in the clear-water treatment for the longer wavelengths; 675–679 nm and 689–701 nm, respectively ($P < 0.05$). There were no statistically significant differences in average irradiance over all wavelengths (361–737 nm, $F_{1,18} = 1.37$, $P = 0.2578$).

Blue expression appeared to be higher in male offspring raised in the greenhouse across all treatments. Of 239 male offspring expressing coloration, 28 had solid blue anal fins (12%), and 16 had either a yellow-blue (8 males; 3%) or red-blue (8 males; 3%) combination anal fin. Still, the vast majority of males had either solid red (95 males; 40%) or solid yellow (100 males; 42%) anal fins. There were an average of 11.0 (1.13 SE) males expressing color across the remaining 25 clutches (91% of all males). In addition, there was no evidence for differential survival across sires, dams, or lighting environments (see Table 1 for raw data).

The relative abundance of males with solid blue anal fins was significantly affected by sire ($F_{3,10.6} = 6.45$, $P = 0.00495$), lighting environment ($F_{1,7} = 21.03$, $P = 0.0025$), and the interaction between sire and lighting environment ($F_{3,7} = 4.56$, $P = 0.0451$; Fig. 5a). There was no statistically significant effect of dam ($F_{9,7} = 1.00$, $P = 0.513$) nor of density ($F_{1,7} = 1.02$, $P = 0.346$). An examination of Figure 4a shows that male offspring with solid blue anal fins were most abundant in the clutches of the Y/B-yellow pelvics sire
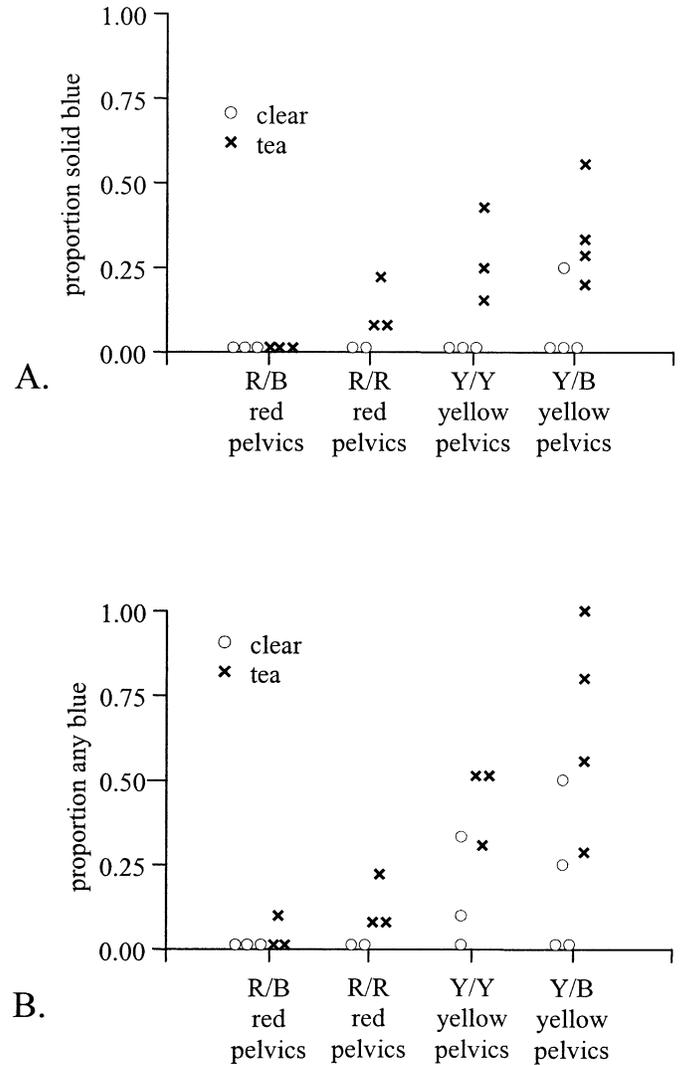

A.

B.

FIG. 5. The proportion of males expressing (A) solid blue anal fins and (B) any element of blue on the anal fin in greenhouse crosses. Open circles, clutches raised in clear water; x, clutches raised in tea-stained water.

(mean = 0.203 ± 0.070 SE, $n = 8$) followed by the Y/Y-yellow pelvics sire (mean = 0.139 ± 0.072 SE, $n = 6$) followed by the R/R-red pelvics sire (mean = 0.074 ± 0.041 SE, $n = 5$) followed by the R/B-red pelvics sire (mean = 0 ± 0 SE, $n = 6$). Across lighting environments, male offspring with solid blue anal fins were much more common in the tea-stained water treatment (mean = 0.198 ± 0.048 SE, $n = 13$) than in the clear-water treatment (mean = 0.021 ± 0.021 SE, $n = 12$). The significant interaction between lighting environment and sire is attributable to the fact that male offspring from the R/B-red pelvics sire did not express solid blue anal fins when raised in tea-stained water (Fig. 5A).

These data may not be suited for ANOVA because of the small numbers of individuals with solid blue anal fins in some groups and the heterogeneity of replicate variance among groups (Fig. 5A). We employed an alternate analysis, which was to use a log-linear model to examine the effects of sire, lighting environment, and the interaction between sire and

lighting environment on the numbers of individuals with and without any blue coloration. We found a significant effect of both lighting environment ($\chi^2 = 6.00$, df = 1, $P = 0.0143$) and of the interaction between sire and lighting environment ($\chi^2 = 17.23$, df = 3, $P = 0.0006$), and a marginally significant effect of sire ($\chi^2 = 7.60$, df = 3, $P = 0.0551$) on the frequency of males expressing any blue on their anal fins.

Sire, lighting environment, and the interaction between sire and lighting environment had similar effects on the proportion of males expressing any blue on their anal fins. A significant effect of sire (Fig. 5B; $F_{3,9.3} = 4.74$, $P = 0.029$) was driven by both the Y/B-yellow pelvics sire and the Y/Y-yellow pelvics sire having a higher proportion of male offspring with some component of blue on their anal fins than the R/R-red pelvics sire and the R/B-red pelvics sire (Y/B-yellow pelvics sire mean = 0.424 ± 0.127 SE, $n = 8$; Y/Y-yellow pelvics sire mean = 0.290 ± 0.084 SE, $n = 6$; R/R-red pelvics sire mean = 0.074 ± 0.041, $n = 5$; R/B-red pelvics sire mean = 0.017 ± 0.017 SE, $n = 6$). Again, males raised in tea-stained water were more likely to have some element of blue on their anal fins than were males raised in clear water (Fig. 5B, $F_{1,7} = 37.95$, $P = 0.0005$; tea-stained water mean = 0.340 ± 0.087 SE, $n = 13$; clear water mean = 0.099 ± 0.049 SE, $n = 12$). The significant interaction between sire and lighting environment ($F_{3,7} = 9.49$, $P = 0.0073$) was driven by the fact that male offspring of the Y/B-yellow pelvics, Y/Y-yellow pelvics, and R/R-red pelvics sires were more likely to express some element of blue on their anal fins when raised in tea-stained water than were offspring from the R/B-red pelvics sire (Fig. 5B). There was also a significant effect of dam nested within sire ($F_{9,7} = 6.93$, $P = 0.0091$). Finally, density significantly affected the proportion of males with any blue on their anal fins ($F_{1,7} = 7.07$, $P = 0.032$). Males were more likely to have some element of blue on their anal fins when raised at a low density (slope = -0.018 ± 0.008).

Neither sire, lighting environment, the interaction between sire and lighting environment, nor density accounted for significant amounts of variation in the relative abundance of males with solid yellow anal fins nor in the relative abundance of males with solid red anal fins (yellow morphs: sire $F_{3,9.5} = 0.66$, lighting environment $F_{1,7} = 0.52$, sire × lighting environment $F_{3,7} = 0.29$, density $F_{1,7} = 0.14$; red morphs: sire $F_{3,9.3} = 1.90$, $P = 0.197$, lighting environment $F_{3,7} = 2.69$, $P = 0.145$, sire × lighting environment $F_{3,7} = 1.60$, $P = 0.273$, density $F_{1,7} = 1.41$, $P = 0.274$). Dams had a significant effect upon the relative abundance of males with solid red anal fins ($F_{9,7} = 5.16$, $P = 0.021$) and tended to affect the relative abundance of males with solid yellow fins ($F_{9,7} = 3.06$, $P = 0.077$).

The results were largely unchanged when the analysis was performed on only those clutches with five or more males expressing color pattern. Again, the proportion of males with solid blue anal fins was significantly affected by lighting environment ($F_{1,5} = 48.50$, $P = 0.0009$), sires ($F_{3,8.62} = 9.11$, $P = 0.005$), and the interaction between lighting environment and sires ($F_{3,5} = 11.48$, $P = 0.011$). There was no significant effect of dams ($F_{8,5} = 1.24$, $P = 0.425$) nor of density ($F_{1,5} = 1.96$, $P = 0.221$). Similar results were found for the analysis of the proportion of males with any blue on their anal
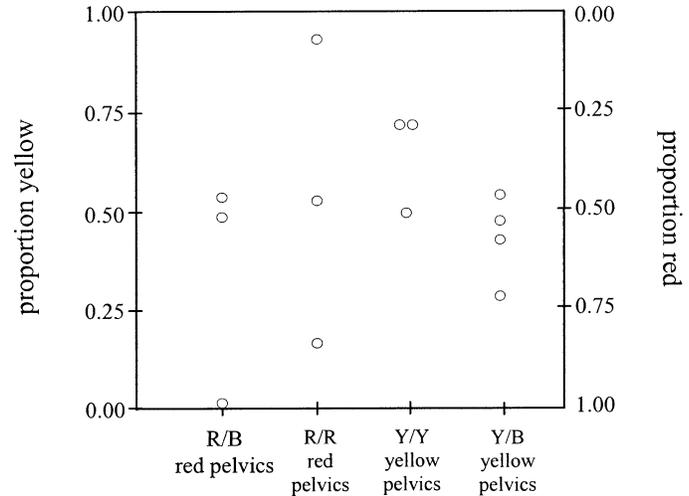

FIG. 6. The proportion of males expressing some element of yellow or red in greenhouse crosses. The data are pooled across lighting treatments for each combination of sire and dam.

fins. There was a statistically significant effect of lighting environment ($F_{1,5} = 52.35$, $P = 0.0008$), sires ($F_{3,8.13} = 5.91$, $P = 0.020$), density ($F_{1,5} = 9.65$, $P = 0.027$), dams ($F_{8,5} = 6.17$, $P = 0.030$) and of the interaction between lighting and sires ($F_{3,5} = 11.22$, $P = 0.012$). No treatments accounted for a significant amount of variation in the proportion of males with solid yellow anal fins (sires: $F_{3,8.3} = 0.37$, $P = 0.776$; dams $F_{8,5} = 3.04$, $P = 0.118$; lighting environment: $F_{1,5} = 0.27$, $P = 0.628$; density: $F_{1,5} = 0.12$, $P = 0.7392$; sire × lighting environment: $F_{3,5} = 0.26$, $P = 0.8528$). The proportion of males with solid red anal fins tended to be significantly affected by dams nested within sires ($F_{8,5} = 3.62$, $P = 0.086$), but not by sires ($F_{3,8.21} = 1.55$, $P = 0.275$), lighting environment ($F_{1,5} = 1.21$, $P = 0.322$), density ($F_{1,5} = 1.18$, $P = 0.327$), nor by the interaction between sires and lighting environment ($F_{3,5} = 0.96$, $P = 0.478$).

Again, over 99% of the animals could be diagnosed as expressing some element of either red or yellow (Fig. 6). Examination of the data shows ratios roughly consistent with Mendelian inheritance in which there is an autosomal locus of large effect controlling yellow versus red with the yellow allele (Y) being dominant over the red allele (y). There were no clutches in which either the Y/B-yellow pelvics sire or the Y/Y-yellow pelvics sire had 100% red male offspring. In contrast, both the R/R-red pelvics and R/B-red pelvics males had clutches distributed at approximately 100% red and 50% red. In addition, the R/R-red pelvics male had one clutch with close to 100% yellow offspring (14/15 yellow).

We discerned the hidden female phenotype for six dams. Five of the six females met the predicted phenotype based on Mendelian ratios. The one deviation came from the R/R-red pelvics sire in which 82% of the males were red (18% yellow). Yellow offspring should only result from matings between a red phenotype (yy) and a yellow phenotype (Yy). We therefore predicted the dam would carry the yellow phenotype, but she expressed as red.

Both the Y/Y-yellow pelvics and the Y/B-yellow pelvics sires appear to be heterozygotes since both produced clutches

with both yellow and red offspring. Hence, the fact that we did not find a strong sire effect on red versus yellow expression in male offspring is not surprising. Dams did have significant effects on the production of yellow/red offspring as we would expect when the four sires are either heterozygous for yellow (Yy) or homozygous for red (yy).

DISCUSSION

In this study, we showed that a relatively simple, environmentally dependent epistatic interaction can produce a large amount of phenotypic variation in male color patterns that presumably function in sexual selection. The effects of some genes can be masked by others depending upon the environmental conditions under which animals were raised. In the laboratory experiment, we found strong evidence for an autosomal locus that has a large effect on whether males can develop yellow or red anal fins. There were strong effects of sire, and the expression of the hidden phenotype in dams met with the predictions of Mendelian inheritance. Mendelian inheritance was indicated when we determined the underlying yellow versus red phenotype and disregarded blue expression. This indicated that blue expression was not governed by the same gene that governed yellow versus red expression. This meets with our understanding of animal color patterns in which yellow and red are thought to be carotenoid- or pteridine-based pigments, whereas blue is most likely structural; hence, colors are controlled via different metabolic pathways (Fox and Vevers 1960). In the greenhouse experiment, we detected an orthogonal genetic effect on whether males develop blue anal fins essentially covering the yellow/red phenotype. Furthermore, we detected a heritable, plastic effect in which some males are more likely to develop blue anal fins when raised in tea-stained water.

Hence, a single locus largely determines whether males can express red or yellow, but this can be suppressed by expression of blue, which depends on orthogonal sets of genes that determine a male's propensity to express blue as well as his response to the environment. These results are similar to those found in an African satyrine butterfly, *Bicyclus anynana*, in which several single gene mutants have large effects on eyespot color patterns, but overall expression of eyespots is dependent on temperature, and genotypes differ in their response to temperature (reviewed in Brakefield et al. 1996; Brakefield 1998; but see Wijngaarden and Brakefield 2001). In both systems (*B. anynana* and *L. goodei*), there are genes with large effects on color pattern that act orthogonally to the expression of phenotypic plasticity. Variation in each of these axes can create a large number of color patterns.

The existence of multiple color morphs is also consistent with these traits being quantitative threshold traits in which the value of an underlying continuous variable determines the morph an animal adopts (Falconer and Mackay 1996; Roff 1996). Blue coloration is a potential quantitative threshold trait, but yellow/red is unlikely to be determined by a threshold. Under very restrictive conditions, quantitative threshold traits can in theory appear as Mendelian traits. However, Roff (1996) suggests that quantitative threshold models be considered when single locus models do not fit

the distribution of morphs. Further genetic studies will resolve this issue.

The fact that both alleles (Y and y) are maintained in nearly all *L. goodei* populations suggests the presence of a balanced polymorphism. In a statewide Florida census, Fuller (2002) found males with red in all populations and males with yellow in 29 of 30 populations. Heterozygote advantage could maintain this variation if yellow heterozygous males (Yy) have higher relative fitness than either homozygous yellow males (YY) or red males (yy). This scenario seems unlikely, because we cannot distinguish yellow homozygotes from yellow heterozygotes. However, such an effect could occur if there are differences in reflectance spectra between the two genotypes that individual *L. goodei* can detect but that are undetectable to humans (Endler 1990; Bennett et al. 1994; Andersson et al. 1998; Hunt et al. 1998; Cuthill et al. 1999). We have not yet analyzed the reflectance spectra for these genotypes. Although our results are dependent upon human perception of color, this does not present a problem because our analysis was restricted to inherited differences among phenotypes that we could recognize. Admittedly, there could be more variation in these color patterns that we can not detect.

Negative frequency dependence could also maintain both alleles (as well as genes for blue morphs) within populations. Negative frequency dependence could occur if males compete more intensely with males bearing the same phenotype (Gross and Charnov 1980; Partridge 1983; Sinervo and Lively 1996) or if there are female mating preferences for rare males (Farr 1977; Partridge 1983; Hughes et al. 1999). Finally, both alleles (as well as genes for blue morphs) could be maintained within populations by variation in environmental conditions. Light is filtered as it passes through the water column resulting in different lighting environments at different depths (Endler 1990; Loew and McFarland 1990; Johnsen 2002). Variation in color morphs could be maintained within populations if each phenotype had the highest mating success under a fraction of the lighting conditions (see Endler 1991; Endler and Théry 1996). Similarly, different lighting niches could occur at different times of the day (Endler 1991, 1993). ''Early/late'' (low sun angle) conditions of early morning and late afternoon should create lighting environments relatively rich in both short shorter wavelengths (<500 nm) and very long wavelengths (>650 nm), which could favor blue morphs. Conversely, ''daylight'' conditions are richer in the intermediate wavelengths (500–650 nm) and could favor yellow morphs.

Plasticity and Its Heritable Variation

The overall pattern in plasticity matches the pattern of color pattern variation across populations. Males were more likely to express blue anal fins when raised in tea-stained water where there was lower transmission of UV and blue wavelengths. In a statewide Florida census, Fuller (2002) found more males with blue anal fins in populations with low transmission of UV and blue wavelengths. The congruence between these two studies demonstrates the robustness of this counterintuitive pattern. Males are obviously not maximizing perceived brightness (total number of photons reflected off the color pattern and detected by a receiver) by

expressing blue anal fins in conditions where blue does not transmit well and where animals possess fewer UV and violet retinal cones (Fuller et al. 2003). The most likely scenario is that blue males create high contrast with the water column or with other color elements on the body. If no photons are being detected from the anal fin, then it will appear black, which will produce high contrast with the body and visual background. On the other hand, clear water has high transmission of UV/blue wavelengths causing the water column to have a bluish tint. This environment should create high contrast for yellow and red color morphs, but lower contrast for blue morphs. Another possibility is that blue males really are more conspicuous in clear water due to high, perceived brightness but that they suffer high mortality costs due to predation. We acknowledge that these speculations would benefit from a proper analysis of reflectance spectra (Endler 1990; Bennett et al. 1994). We are currently analyzing reflectance spectra to compute actual brightness and contrast of blue anal fins in tea-stained and in clear water using the methods of Endler (1991) and Marshall et al. (2003).

The existence of heritable plasticity in blue morph expression raises several intriguing issues. Why don't all males respond to the environment in the same manner? Variation in plasticity may be maintained if the reliability of the cue used for plasticity varies (Lively 1986; Weinig 2000a,b). In other words, the degree to which cues predict future conditions must vary. High variation in environmental conditions due to dry versus rainy seasons, variation in managed hydrology through canals, or variation in storms (i.e. hurricanes) could conceivably result in variation in the predictability of future conditions (Trexler et al. 2001). A second possibility is that there are costs to plasticity resulting in the maintenance of variation in plasticity (Dorn et al. 2000; Relyea 2002). Intuitively, the cost/benefit argument relies on a balance between fitness components maintaining variation within a population. A third possibility is that negative frequency dependence maintains variation in plasticity. If a rare-male mating advantage exists (due either to female choice or male-male competition), then this could result in the maintenance of variation in plasticity. In tea-stained environments, rare-male mating advantage would select for intermediate values of plasticity, which results in the maximum number of male color morphs. This explanation relies on blue, yellow, and red morphs being acceptable alternatives in tea-stained water, but only yellow and red morphs being acceptable alternatives in clear water.

Finally, what do plastic male signals imply about sexual selection? Obviously, the handicap and direct benefits models of sexual selection rely on plasticity between male trait and condition (see Andersson 1994 for a review). However, the situation in *L. goodei* is entirely different. The signals males use in communication vary depending on the environment—not on individual male condition. This raises a host of questions. Does the information provided by the signal also vary with environmental conditions? Do receivers ''want'' to detect different information in different environments? Does receiver perception of the signal vary with lighting conditions or are males simply tracking the environment? Clearly, the signal perception of some males must vary because males vary in their response to the environment. If the relationship between males and signal content/signal perception varies with environment, then does the direction of sexual selection also vary with the environment? These are questions that we hope to address in the future.

In conclusion, we found that genetics, environment, and an interaction between genetics and environment affect color pattern expression in male *L. goodei*. Specifically, we found evidence for an autosomal locus of large effect that controls the expression of yellow versus red in which yellow is dominant over red. Lighting conditions also played a large role in the expression of blue anal fins. More males expressed blue anal fins when raised in tea-stained water than when raised in clear water. In addition, sires had strong effects on whether male offspring expressed blue anal fins and also on the expression of plasticity. These results raise two questions. First, how is all of this variation maintained (red vs. yellow, genes for blue expression, genes for plasticity)? Frequency dependence, heterozygote advantage, variation in the environment, and/or a balance in fitness components are all possibilities. Second, given that the direction of plasticity is in accordance with across-population patterns, how can we explain this counterintuitive pattern (i.e., more blue males in low UV/ blue-transmittance environments)? Males with blue anal fins are most likely maximizing contrast with background lighting conditions and sacrificing perceived brightness. The next step in this research program is to determine the fitness correlates of these color morphs and the degree to which sexual selection varies under different lighting conditions.

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