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EVOLUTION OF MALE COLORATION DURING A POST-PLEISTOCENE RADIATION OF BAHAMAS MOSQUITOFISH (*GAMBUSIA HUBBSI*)

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Sexual signal evolution can be complex because multiple factors influence the production, transmission, and reception of sexual signals, as well as receivers' responses to them. To grasp the relative importance of these factors in generating signal diversity, we must simultaneously investigate multiple selective agents and signaling traits within a natural system. We use the model system of the radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting blue holes to test the effects of resource availability, male body size and other life-history traits, key aspects of the transmission environment, sex ratio, and predation risk on variation in multiple male color traits. Consistent with previous work examining other traits in this system, several color traits have repeatedly diverged between predation regimes, exhibiting greater elaboration in the absence of predators. However, other factors proved influential as well, with variation in resource levels, body size, relative testes size, and background water color being especially important for several color traits. For one prominent signaling trait, orange dorsal fins, we further confirmed a genetic basis underlying population differences using a laboratory common-garden experiment. We illustrate a promising approach for gaining a detailed understanding of the many contributing factors in the evolution of multivariate sexual signals.

KEY WORDS: **Animal communication, condition dependence, indicator traits, predation, sensory drive, sexual selection and ornamentation.**

Secondary sexual signals, such as brightly colored body parts, form a conspicuous feature of biological diversity and can vary dramatically within and between populations and species. Because such signals play an integral role in mate choice and sexual isolation among species, investigating the causes of this diversity is essential to understanding the evolution of mating systems and processes underlying speciation (e.g., Andersson 1994; Panhuis et al. 2001; Boughman 2002).

At the most basic level, a signal originates from an individual, travels through the environment, and is received by one or more individuals that may respond to it (Endler 1992; Fuller 2002). Although a signal's path can seem relatively simple, the production, transmission, reception of, and response to the signal may all be affected by multiple ecological factors, comprising a network of interactions that can influence signal evolution and expression (Fig. 1). Thus, a range of factors—from food and pigment availability to body condition to the transmission environment and the receiver's behavioral response and sensory system—affect signal variation, and can drive divergent signal evolution if these factors differ in degree or kind across space or time (Endler 1992; Ord et al. 2002).

Although the complexity of signal evolution is well appreciated, the majority of studies on signal evolution in the wild focus on the role of a single or a few selective agents and signaling

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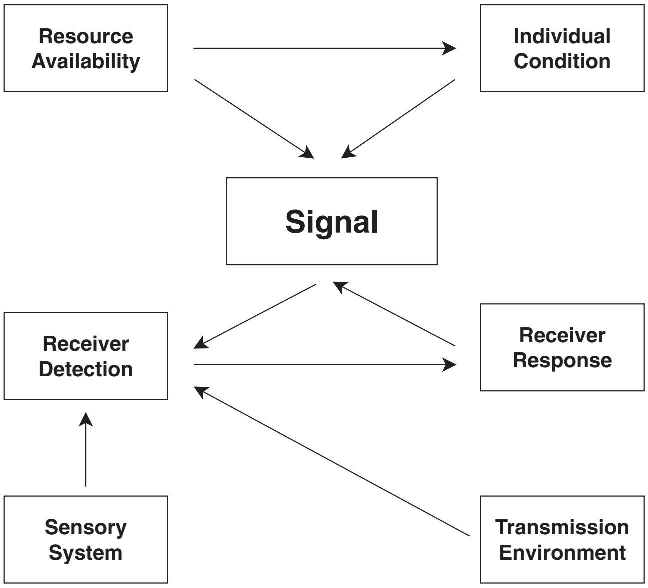

Figure 1. Conceptual illustration of communication, centered on understanding the major underlying causes of signal diversity.

traits. For example, numerous studies across diverse taxa have explored the hypothesized trade-off between sexual selection favoring conspicuous traits, and natural selection from predation or parasitism constraining trait elaboration (sensu Otte 1974; Endler 1980, 1983; McGregor 2005), with varying levels of support (e.g., Langerhans et al. 2005; Zuk et al. 2006; Weese et al. 2010; Chen et al. 2012; reviewed in Zuk and Kolluru 1998). Similarly, variation in sexual signal diversity arising from availability of environmental resources (e.g., Grether et al. 1999; Craig and Foote 2001; but see Schwartz and Hendry 2010) and the transmission environment (e.g., Endler 1992, 1993; Marchetti 1993; Boughman 2002; Fuller 2002; Leal and Fleishman 2002) have all been well studied individually. However, few studies have taken a multivariate approach to study signal evolution in the wild (but see Millar et al. 2006; Gotanda et al. 2013). This discrepancy raises several issues.

First, by focusing on one or a few possible selective agents, researchers may miss the actual causal factor(s) influencing signal evolution. Furthermore, spurious associations between environmental factors and signal diversity may occur when measured environmental factors covary with causal selective agents, leading to incorrect inferences of causality (Mitchell-Olds and Shaw 1987; MacColl 2011). Although no observational study can conclusively identify causation, examining multiple potential causal agents can both increase the probability of identifying the true causal factors and result in well-informed hypotheses for future experimentation. Finally, focusing on only one or a few sexual signals may lead to inaccurate conclusions regarding (1) the target of selection, owing to correlations among traits; and (2) the most important selective agent(s) underlying signal diversity, owing to potentially stronger influence of other agents on unmeasured traits (MacColl 2011). To date, multivariate approaches have generally found evidence suggesting that multifarious selective agents of varying importance influence signal evolution (Millar et al. 2006; Gotanda et al. 2013). To gain a greater understanding of the evolution of sexual signal diversity—identifying and disentangling independent and interactive contributions of various factors (e.g., predation, transmission environment, condition) on multivariate signal variation—we must examine multiple environmental factors and multiple signaling traits in the wild.

STUDY SYSTEM AND AIMS

Here we evaluate the relative importance of six environmental factors and five individual traits in shaping the evolution and expression of six male color traits in a model system for ecologically driven divergence and speciation, the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting inland blue holes across Andros Island, The Bahamas. Blue holes are water-filled vertical caves found in some carbonate banks and islands (Mylroie et al. 1995). On the northern half of Andros Island, *G. hubbsi* colonized and adaptively radiated across inland blue holes during the past ∼15,000 years (Langerhans et al. 2007) as rising sea levels lifted the freshwater lenses of the island, flooding the voids. *Gambusia hubbsi* exhibit adaptive phenotypic differences between blue holes with and without the piscivorous bigmouth sleeper (*Gobiomorus dormitor*; life history, body shape, locomotor performance, genital morphology, behavior: e.g., Langerhans et al. 2005, 2007; Langerhans 2009a, 2010; Riesch et al. 2013; Heinen et al. 2013; Heinen-Kay and Langerhans 2013), and are undergoing ecological speciation, as sexual isolation between divergent populations has resulted as a by-product of divergent natural selection (Langerhans et al. 2007; Langerhans and Makowicz 2013).

Although this radiation has become a textbook example of adaptive diversification (e.g., Freeman and Herron 2007; Reece et al. 2010), no study has yet quantitatively investigated how male color varies among blue holes, or if it has diverged between high- and low-predation environments. Yet, because divergence in mating traits often enhances sexual isolation during ecological speciation, female mate choice based on male coloration might influence reproductive isolation (e.g., Nosil 2012; Langerhans and Riesch 2013). Bahamas mosquitofish are sexually dimorphic in body size, body shape, and coloration (Supplemental Material A). Males exhibit exaggerated color patterns compared to females, which may make them more visible against the background environment of blue holes (i.e., more conspicuous). Specifically, males have bright orange dorsal fins, black spotting on the dorsal and caudal fins, a black "shoulder patch" (situated anterodorsal of the insertion of pectoral fin ray 1), and an iridescent eye patch just posteroventral of the eye, as well as less obvious color patterns,

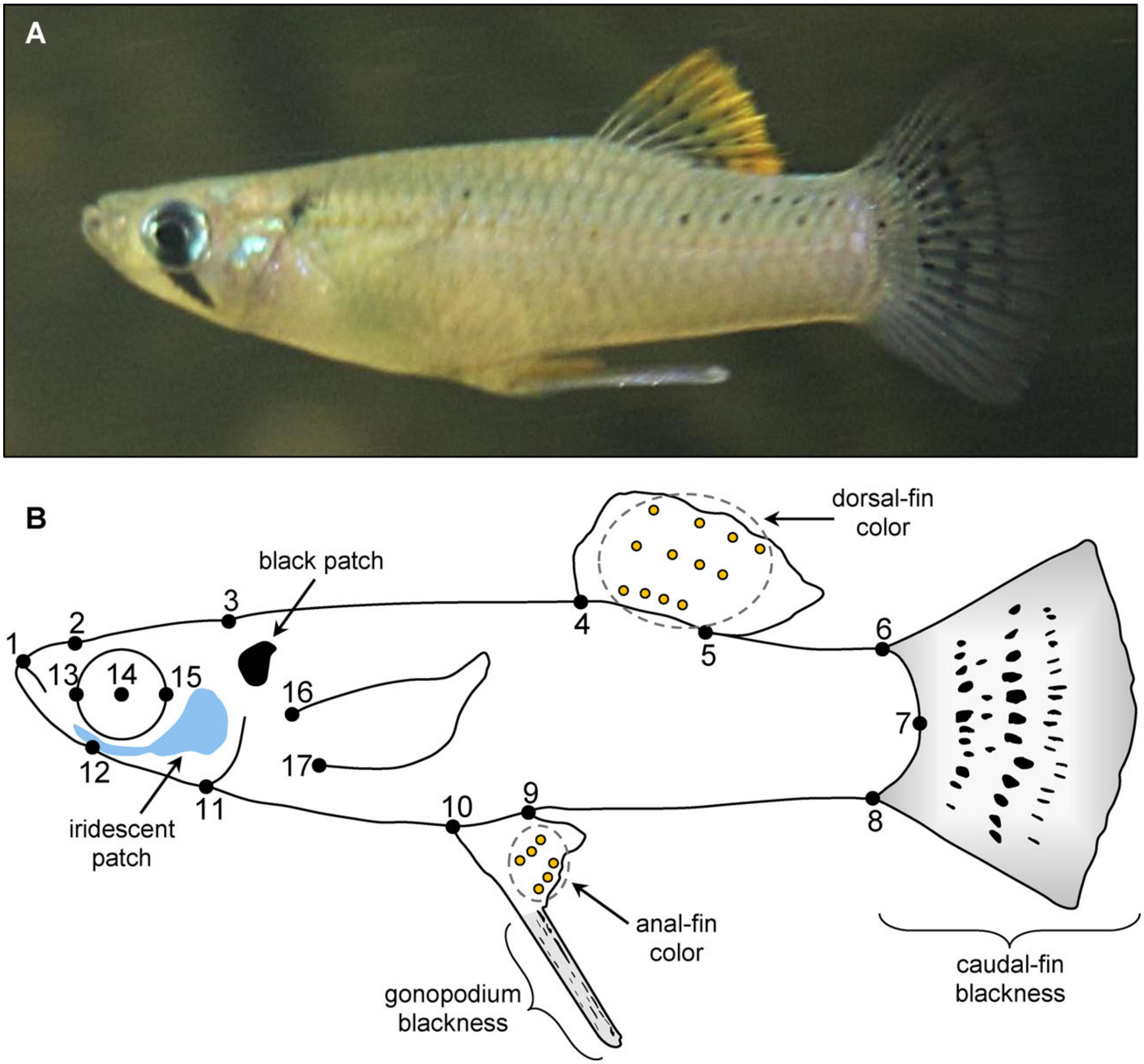

Figure 2. (A) *Gambusia hubbsi* male photographed in Stalactite blue hole. (B) Illustration of locations of color measurements and landmarks used for geometric morphometric analysis of body shape. See Supplemental Material B for landmark descriptions.

including a black-speckled gonopodium (highly modified anal-fin rays 3–5 used as a sperm-transfer organ) and orange coloration at the base of the anal fin (Fig. 2). Males also facultatively express a dark subocular bar, which was not measured in this study.

Bahamas mosquitofish represent an ideal system for exploring the complexity of signal evolution and disentangling effects of predation and other environmental factors in signal divergence for several reasons. First, blue holes comprise temporally stable environments (Heinen et al. 2013). Second, Bahamas mosquitofish have independently diverged between replicated high-predation risk and low-predation risk blue holes (e.g., Langerhans et al. 2007; Langerhans 2009a; Riesch et al. 2013; Supplemental Material B). And finally, blue holes with different predation regimes do not systematically differ in abiotic environmental variables or resource availability (Langerhans et al. 2007; Heinen et al. 2013; Riesch et al. 2013; Supplemental Material B).

To uncover the primary factors driving the evolution of signal diversity in this system, we use a comparative approach to test the effects of resource availability, male condition and other traits, key aspects of the transmission environment, sex ratio, and predation risk on variation in multiple male signal characteristics. This investigation encompasses all factors depicted in Figure 1 except the receiver's sensory system, which we assume here to be capable of detecting the signals (for spectral sensitivity of poeciliid fishes see, e.g., Archer and Lythgoe 1990; Watson et al. 2010; for spectral sensitivity of Gobiodei fishes see, e.g., Jokela et al. 2003; Utne-Palm and Bowmaker 2006).

Our goal in this study was to investigate the factors underlying male color variation, both within and between populations of *G. hubbsi*. We specifically asked: (1) Do males exhibit larger and more-colorful (i.e., exaggerated) sexually dimorphic traits in the absence of the predator *G. dormitor*, consistent with the hypothesized tradeoff between sexual selection and natural selection (e.g., Endler 1980; Millar et al. 2006)? (2) What are the relative contributions of differences in predation risk and other external environmental factors on color divergence between populations? (3) Does male coloration covary with male condition or body shape, and do these relationships differ between predation regimes? (4) Is there a heritable basis to variation in male coloration among populations?

To investigate the first three questions, we measured male coloration, body shape, and condition, as well as environmental factors from a total of 10 blue holes on Andros Island (Supplemental Material B): five lacking *G. dormitor* and five with *G. dormitor* (hereafter referred to as low-predation and high-predation blue holes; sensu Langerhans et al. 2007). To investigate the heritable basis of male coloration, we measured dorsal-fin color of wild-caught males from four populations (two high-predation, two low-predation) after receiving a long-term common diet in the laboratory, as well as their laboratory-reared F1 and F2 male offspring.

Methods

MALE COLORATION, CONDITION, AND BODY SHAPE IN BLUE HOLES

Photography and image analysis

We collected 10–15 mature males from each site using hand-held dip nets and minnow traps during the first 2 weeks of May 2011. Immediately after capture, we took individual lateral photographs of each male inside a portable photo studio (see Supplemental Material B for methodological details). We used Adobe Photoshop CS5.1 to measure the color of four fin regions and the surface areas of two color patches on the body (see Fig. 2B, for locations of all measurements). To estimate color in the images, we employed the Commission Internationale de l'Éclairage (CIE) Laboratory color space—an approach taken in many previous studies to quantify fish coloration (e.g., Craig and Foote 2001; Svensson et al. 2006; Morrongiello et al. 2010). CIE *L*a*b** is a device independent, perceptually uniform color space that consists of three orthogonal parameters: *L** values describe relative lightness ranging from black to white, *a** values describe variation from green to red, and *b** values describe variation from blue to yellow.

We measured male dorsal-fin and anal-fin coloration by sampling the average *a** and *b** color values from 12, 3 × 3 pixel squares on the dorsal fin (four each from distal, middle, and proximal fin regions), and six squares on the anal fin (three each from distal and middle fin regions) using the eyedropper tool. Color values were taken from the fin membrane between dorsal-fin rays 3–4, 4–5, 5–6, and 6–7, and between anal-fin rays 5–6, 6–7, and 7–8. We measured areas of the black shoulder patch and iridescent eye patch with the lasso tool. Finally, to estimate the degree of black spotting and speckling of the caudal fin and gonopodium, we measured the average blackness of these features by tracing their outlines and calculating the average *L** value.

CIE *L*a*b* is based on the human visual system (Stevens et al. 2007), and therefore its measured values do not represent how *G. hubbsi* or *G. dormitor* perceive the *G. hubbsi* color traits, a topic that should be explored in future studies. Furthermore, our methods do not allow us to investigate color traits outside the range of human color sensitivity (e.g., ultraviolet). However, the CIE *L*a*b* color space values, along with measures of color patch area, allow us to evaluate how aspects of *G. hubbsi* color vary in relation to environmental and biological variation within and between populations. Moreover, reflectance spectrometry has failed to uncover any significant UV component to any color traits examined in this study (R. B. Langerhans, unpubl. data). To ensure that our results were robust across particular choices of color space, we additionally analyzed our fin-color data for both wild-caught and lab-reared males (*a** and *b** channel information for dorsal and anal fins) using species-independent measures of color variation (Endler 2012, Supplemental Material C). Poeciliid fishes have well-developed color vision, and while visual sensitivity of *G. hubbsi* has not yet received direct examination—although a congener has—the broad similarity among features of rods and cones across poeciliid genera suggest *G. hubbsi* also has similar visual abilities (Levine and MacNichol 1979; Archer and Lythgoe 1990; Archer and Hirano 1997; Ward et al. 2008; Watson et al. 2010).

Male condition

Because condition can influence expression of sexual signals, we measured two estimates of body size (standard length [SL, mm] and lean weight [g]), testes dry weight (g), and fat content (as dry weight [g]); these life-history traits were reanalyzed from a previous study (Riesch et al. 2013). We log transformed all variables before analysis. SL and lean weight were highly correlated ($r = 0.92$) and we selected lean weight as our body-size estimate because this reflected an overall estimate of body size and muscle mass rather than simply body length. To obtain "size-free" values for testes weight and fat weight, we used an analysis of covariance (ANCOVA) approach to derive residuals from log–log regression of these two traits onto lean weight using pooled within-group slopes across predation regimes after first confirming no significant deviation from homogeneity of slopes (Reist 1986; Berner 2011).

Male body shape

Male body shape may covary with color, as body shape is known to influence fitness via locomotor performance and female mating preference (Langerhans et al. 2007; Langerhans 2009a; Langerhans and Makowicz 2013), and thus may indicate some aspects of male quality. We digitized 17 landmarks (Fig. 2) on all digital images using tpsDig software (Rohlf 2010), and used geometric morphometric methods to examine morphological variation in body shape (Rohlf and Marcus 1993; Marcus et al. 1996; Zelditch et al. 2012). We used the morphological divergence vector (**d**) in analyses described later, capturing the aspects of body shape most influenced by the presence and absence of *G. dormitor*—a multivariate axis ranging from relatively streamlined bodies to relatively deep mid-body/caudal regions and small heads (see Supplemental Material B for details). We additionally measured size of the median fins to include as covariates in the analyses of fin coloration. In each case, we measured two components of fin size (see Supplemental Material B) and used the first principal component of a principal components analysis (PCA; see Supplemental Material D) based on the correlation matrix of the two variables. We used the ANCOVA approach described earlier to derive size-corrected residuals of PC1 for each fin.

ENVIRONMENTAL MEASUREMENTS

Because we were interested in determining the major biotic and abiotic factors driving color variation among populations of *G. hubbsi*, we selected a priori environmental agents other than predation regime that we hypothesized to play important roles in the evolution and expression of male coloration. Specifically, we measured background water color and habitat color of each site using underwater digital photography (estimating the transmission environment), two estimates of resource availability (zooplankton density measured from a plankton tow and algal biomass measured using fluorometry; see Heinen et al. 2013 for original data and methodological details), and the tertiary (adult) sex ratio of *G. hubbsi* using underwater visual surveys (providing an estimate of the strength of sexual selection; see Heinen et al. 2013 for methodological details). All environmental variables were measured at the time of fish sampling. See Supplemental Material B for details.

COMMON-GARDEN EXPERIMENT

To evaluate whether observed divergence in dorsal-fin color between predation regimes has a genetic basis, and did not simply reflect dietary differences between blue holes, we measured dorsal-fin color from four populations (two high-predation, two low-predation) in wild-caught male *G. hubbsi* after being housed 8–13 months under common laboratory conditions (low-predation: $n = 6$; high-predation: $n = 10$), as well as males born and raised in the laboratory (low-predation: $n = 7$; high-predation: $n = 12$; see Supplemental Material B). All fish were housed in 38-L aquaria and fed a varied diet of newly hatched brine shrimp nauplii, frozen daphnia, and frozen bloodworms.

Live males were photographed laterally in front of a black background while immersed in water inside a small glass photo tank. We manually white balanced each photograph using a white egg-crate lighting panel present in each image and then measured dorsal-fin coloration as previously described. Color patterns other than dorsal-fin color were not measured for these fish due to reduced confidence in these aspects of images due to lighting conditions and immersion in water, positioning of the gonopodium of live fish in water, and the black background.

Statistical Analyses

TESTING COLOR DIVERGENCE BETWEEN PREDATION REGIMES

We conducted mixed-model multivariate analysis of covariance (MANCOVA) for our primary test of multivariate color divergence between predation regimes (question 1). Before running the model we first reduced the dimensionality of our dorsal-fin and anal-fin color measurements by performing a PCA separately for a^* and b^* values for each fin. For the dorsal fin, we calculated the average a^* and b^* values for the proximal, middle, and distal measurements. We then conducted a PCA on the correlation matrix separately for a^* and b^* values. We conducted a similar PCA for the middle and distal a^* and b^* values for the anal fin. We retained all PC axes that explained more variation than expected under a broken-stick model (Frontier 1976; Jackson 1993), resulting in one retained PC axis for each trait (Supplemental Material D). Larger values of PC1 for both fins correspond with higher a^* or b^* values (more red and yellow, respectively).

These four PC axes, along with log-transformed black shoulder patch area, log-transformed iridescent eye patch area, caudal-fin blackness, and gonopodium blackness served as response variables in our MANCOVA, with predation regime as our predictor variable, log-transformed lean weight as a covariate to control for body size, and population nested within predation regime included as random effect. Statistical significance was determined using an F test based on Wilks's Λ for lean weight and population nested within predation regime, and an F test employing restricted maximum likelihood (REML) and the Kenward–Roger degrees of freedom adjustment (Kenward and Roger 1997) for predation regime to appropriately test this fixed effect while treating population as a random term (i.e., effectively treat population as the unit of replication for this test). The latter significance test was conducted using the MIXED procedure in SAS (SAS Institute; for sample code, see Riesch et al. 2013). We evaluated the relative importance of model terms using the effect size measurement of Wilks's partial η^2.

To assess which color traits were most important in explaining broad patterns between predation regimes, we examined canonical axis loadings derived from the predation regime term in the MANCOVA, and to provide an intuitive metric regarding the magnitude of trait differences between predation regimes we also present standardized effect sizes (Cohen's *d*, describing the difference between means in standard deviation units; Cohen 1988). In addition, we conducted univariate linear mixed models for each trait (model terms were identical to MANCOVA). We used one-tailed tests for significance because we made a priori predictions that male body-color traits would be more exaggerated (i.e., larger and more colorful) in low-predation blue holes. Random effects were fit using REML and we employed the Kenward–Roger degrees of freedom adjustment. The univariate models were fit in JMP (SAS Institute).

MODEL SELECTION AND MODEL AVERAGING: DETECTING INFLUENCE OF NUMEROUS FACTORS ON MALE COLORATION

We employed model selection and multimodel inference (Burnham and Anderson 2002; Grueber et al. 2011) to evaluate the predictive power and statistical effects of predation regime, other environmental factors, and individual condition and morphology on male color (questions 2 and 3). We constructed two global generalized mixed models for each of the previously described male color traits. Both approaches included predation as a predictor variable, but differed in alternative predictors, with one global model focusing on internal/condition effects and one global model focusing on effects of the external environment. All of the predictors could not be included in a single model largely due to a lack of degrees of freedom, with 10 populations serving as a limiting factor. For both global models, we standardized all variables to a mean of zero and standard deviation of 1, and included biologically relevant interactions with predation regime identified via graphical data exploration.

The first global model for each trait included predation regime, and the following measures of individual condition and morphology: log-transformed lean weight, size-corrected residuals of fat weight, size-corrected residuals of testes weight, and body shape (**d**). For color traits measured on fins, we included an estimate of the respective fin size in the model (size-corrected residuals of PC1 for a given fin). The global model for each trait also included population identity as a random intercept.

The second global model for each trait included predation regime, the environmental variables of mean a^* and b^* water color values, PC1 of habitat color, relative chlorophyll *a* density, log-transformed (+1) zooplankton density, and sex ratio of each population. The global model also included log-transformed lean weight as a covariate to control for effects of body size, and population identity as a random intercept.

We fit each global generalized mixed model using the R package lme4 (Bates et al. 2011) and performed model selection on each global model using the R package MuMIn (Bartoń 2012). Specifically, we employed the dredge function to fit all possible permutations of each global model while including population as a random intercept in every model, fit using maximum likelihood (ML) to compare and rank models by their information criteria. We ranked the model pool for each global model using Bayesian Information Criteria (BIC) because it tended to favor more parsimonious models than Akaike Information Criteria, corrected for small sample size (AIC*c*). See Grueber et al. (2011) for a review of information criteria for use in model selection. With the exception of two cases there was no one best model and so we chose a subset of the most informative models with $\Delta\text{BIC} \leq 4$ (variance inflation factor < 5 for all chosen models).

We next carried out model averaging with each model subset using the function model.avg within the R package MuMIn. Model averaging uses information criteria (i.e., BIC) to assess the predictive power of explanatory variables (i.e., relative importance values: the sum of the Akaike model weights for each model the variable occurs in, across the candidate models of the chosen model subset) and obtain averaged parameter estimates, standard errors, and 95% confidence intervals using the natural averaging method from a set of models when there is no single best-supported model or hypothesis (sensu Burnham and Anderson 2002; reviewed in Grueber et al. 2011).

COMMON-GARDEN EXPERIMENT

As previously described for wild-caught males we calculated the average a^* and b^* values of the proximal, middle, and distal measurements of the dorsal fin for the lab-reared males. We were specifically interested in whether the observed overall divergence in dorsal-fin color between predation regimes reflects genetically based differences (question 4). We first reduced dimensionality of the proximal, middle, and distal a^* and b^* average values by conducting a PCA on the correlation matrix from which we retained the first two PC axes (PC1, PC2). Larger values of PC1 correspond with higher a^* and b^* values (i.e., greater red and yellow coloration), whereas larger values of PC2 correspond with higher a^* and lower b^* values (see Supplemental Material D). In JMP, we then ran separate linear mixed-models with each PC axis as our response variable, predation regime, birth status (i.e., originally wild caught or lab born), and their interaction as main effects, log-transformed SL as a covariate (lean weight was not measured for these individuals), and population as a random intercept fit using REML. For significance testing, we employed the Kenward–Roger degrees of freedom adjustment.

Table 1. Results from mixed-model nested multivariate analysis of covariance evaluating variation in eight color variables of male *Gambusia hubbsi* from 10 blue holes on Andros Island, The Bahamas (treating population as a random effect).

Term	*DF*	*F*	*P*	Partial variance explained (%)
Log lean weight	8,72	8.43	<0.001	48.35
Predation regime	7,315	10.41	<0.001	56.37
Population (predation regime)	64,422	4.24	<0.001	30.10

Table 2. Summary of univariate general linear mixed models examining body color variation, standardized univariate effect sizes (Cohen's *d*), and canonical variate loadings for the predation regime term from the mixed-model multivariate analysis of covariance (bold text emphasizes significant differences between predation regimes).

Trait	Term	df	*F*	P^*	Cohen's *d*	CV Loading
Dorsal fin a^*	Log lean weight	1,99.30	4.56	0.018		
	Predation regime	1,8.05	4.37	0.035	**1.47**	**– 0.50**
Dorsal fin b^*	Log lean weight	1,96.86	14.76	<0.001		
	Predation regime	1,8.14	7.11	0.014	**1.87**	**– 0.86**
Anal fin a^*	Log lean weight	1,87.45	36.44	<0.001		
	Predation regime	1,7.73	3.73	0.045	**1.39**	**– 0.49**
Anal fin b^*	Log lean weight	1,86.31	45.41	<0.001		
	Predation regime	1,7.97	4.41	0.035	**1.49**	**– 0.64**
Iridescence patch	Log lean weight	1,99.42	20.19	<0.001		
	Predation regime	1,8.19	0.10	0.382	0.22	– 0.24
Shoulder patch	Log lean weight	1,99.85	0.02	0.447		
	Predation regime	1,8.13	3.52	0.049	**1.32**	**– 0.28**
Caudal L^*	Log lean weight	1,101.44	8.89	0.002		
	Predation regime	1,7.93	0.19	0.337	0.31	– 0.14
Gonopodium L^*	Log lean weight	1,91.99	1.82	0.090		
	Predation regime	1,8.28	2.16	0.090	1.02	0.41

Note: One-tailed *P* values shown for tests of the predation regime term.

Results

TESTING COLOR DIVERGENCE BETWEEN PREDATION REGIMES

MANCOVA revealed a strong effect of predation regime on multivariate male body coloration (Table 1). Lean weight and population nested within predation regime also had highly significant effects (Table 1). Inspection of Cohen's *d* and the canonical axis derived from the predation regime term indicated that dorsal- and anal-fin coloration diverged the most between predation regimes, whereas univariate mixed models further revealed that five of the eight color variables exhibited significant differences between predation regimes, with males from low-predation populations having greater red (↑ a^*) and yellow (↑ b^*) dorsal and anal-fin coloration, and larger shoulder patches, as predicted (Table 2).

MODEL SELECTION AND MODEL AVERAGING: DETECTING INFLUENCE OF NUMEROUS FACTORS ON MALE COLORATION

Predation was among the strongest predictors of male color for several traits in our model selection framework (Fig. 3 and Table 3). Specifically, our results show that males from low-predation populations tended to have greater red (↑ a^*) and yellow (↑ b^*) dorsal-fin coloration, larger black shoulder patches, and larger iridescent patches than males from high-predation blue holes (see Fig. SB4). In addition, differences between predation regimes in anal-fin yellowness were only evident in sites with low to moderate yellow background water color (i.e., interaction effect: trait increased with water yellowness in high-predation sites only, whereas low-predation populations maintained relatively high levels irrespective of water color), and dorsal-fin redness exhibited stronger differences between predation regimes in cases of moderate yellow background water color (i.e., interaction effect: trait decreased with water yellowness in high-predation sites only, whereas low-predation populations maintained relatively high levels irrespective of water color; Fig. 3). Although predation was included as a predictor of other traits, it either had low predictive value or high variance in effect size such that the 95% confidence intervals of the model averaged effect size bounded zero.

Individual condition and body shape also explained variation in male body coloration, both within and between blue

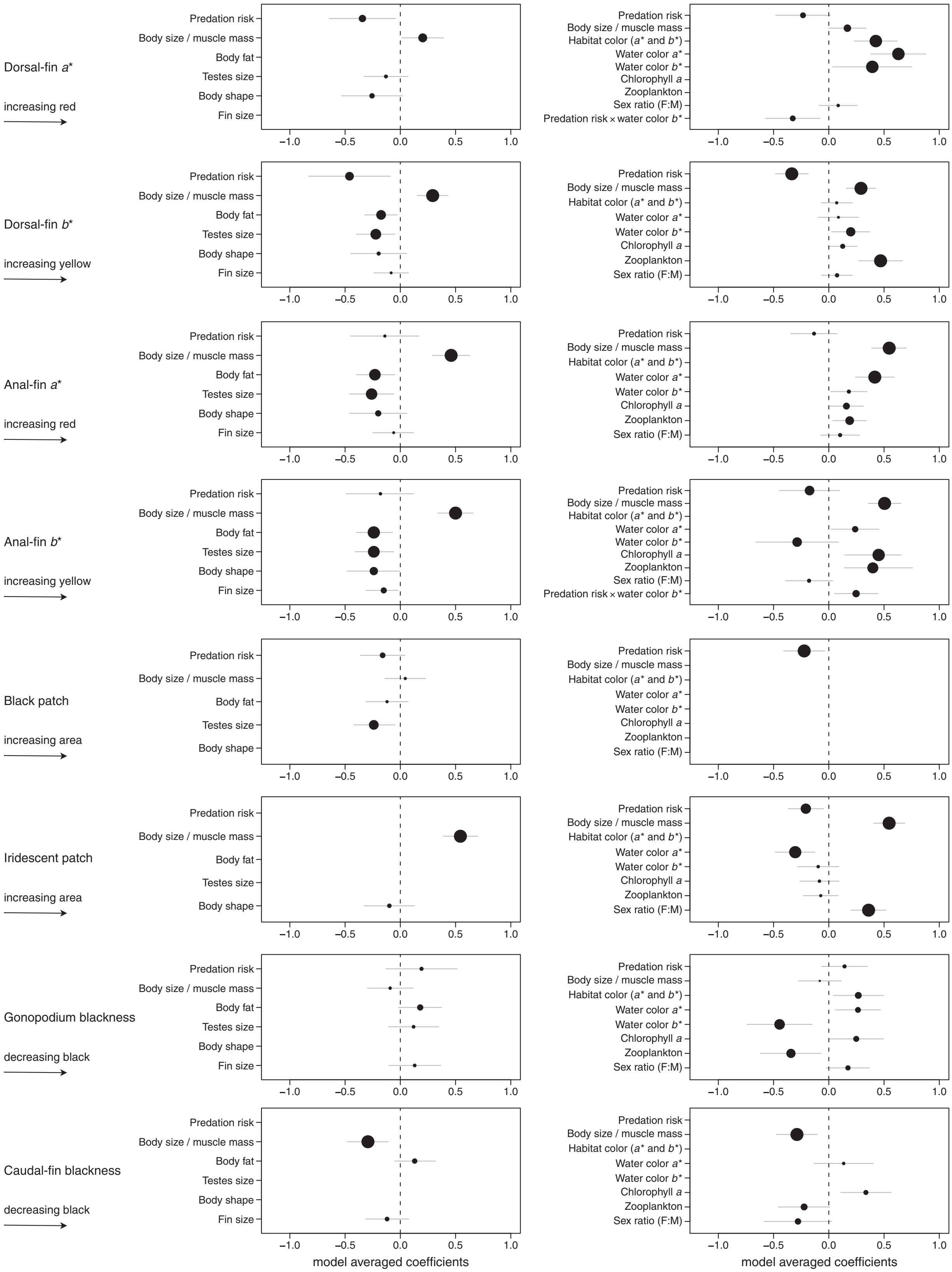

Figure 3. Summary of model averaging results evaluating the relative importance and possible interactions of predation and variation in individual condition and morphology (left column) and predation and environmental variation (right column) on male coloration. Shown are model averaged coefficients with 95% confidence intervals (or ±SE for traits with a single best model), and relative importance values (larger symbols = greater relative importance).

Table 3. Summary of results from our model selection and model averaging analyses. We present the direction of each effect in relation to exaggeration (+) or reduction (−) of the color for each trait. Symbols within parentheses indicate an effect that is not statistically significant from zero, whereas "0" indicates that the variable was not included in the chosen models. "Cumulative effect" is a tally of the number of color variables with a significant average effect for each term.

Explanatory variables	Dorsal-fin *a**	Dorsal-fin *b**	Anal-fin *a**	Anal-fin *b**	Black patch	Iridescent patch	Gonopodium blackness	Caudal-fin blackness	Cumulative effect	Median relative importance	Mean (abs) effect size
Body size/muscle mass (condition)	+	+	+	+	0	+	(+)	+	6	1	0.30
Body size/muscle mass (environment)	(+)	+	+	+	0	+	(+)	+	5	1	0.30
Body fat	0	−	−	−	(−)	0	(−)	(−)	3	0.20	0.13
Testes size	(−)	−	−	−	−	0	(−)	0	4	0.33	0.15
Body shape (deeper mid-body/caudal)	(−)	(−)	(−)	−	0	(−)	0	0	1	0.11	0.12
Fin size covariate	0	(−)	(−)	−	NA	NA	(−)	(+)	1	0.07	0.09
Predation risk (condition)	−	−	(−)	(−)	(−)	0	(−)	0	2	0.09	0.18
Predation risk (environment)	(−)	−	(−)	(−)	−	−	(−)	0	3	0.38	0.18
Habitat color (*a** and *b**)	+	(+)	0	0	0	0	−	0	2	0	0.1
Water color *a**	+	(+)	+	+	0	−	−	(−)	5	0.22	0.26
Water color *b**	+	+	+	(−)	0	(−)	+	0	4	0.31	0.20
Chlorophyll *a*	0	(+)	+	+	0	(−)	−	−	4	0.15	0.18
Zooplankton	0	+	+	+	0	(−)	+	(+)	4	0.37	0.21
Sex ratio (F:M)	(+)	(+)	(+)	(−)	0	+	(−)	(+)	1	0.11	0.16
Predation risk × water color *b**	−	0	0	+	0	0	0	0	2	0.07	0.07

holes (Table 3). None of these associations differed between predation regimes (no evidence for importance of any interaction terms). Lean weight had strong predictive power for all male color traits except black shoulder patch area and gonopodium blackness (Fig. 3). For traits where lean weight was an important predictor, males with greater lean weight possessed larger, darker, or more red/yellow traits (Fig. 3). Relative testes weight also had strong predictive power. Specifically, males with relatively heavier testes tended to have less yellow dorsal fins, less yellow and red anal fins, and smaller black shoulder patch areas (Figs. 3, SB4). Similarly, males with relatively more body fat tended to have less yellow dorsal fins, and less yellow and red anal fins (Fig. 3). Finally, body shape (**d**, the morphological index) was a moderately strong predictor of anal-fin color regardless of predation regime. Within populations, males with more streamlined body shapes tended to have more yellow anal-fins (Fig. 3).

A number of environmental factors other than predation regime were also important in predicting and explaining variation in male body color (Fig. 3 and Table 3). Importantly, these factors did not covary with predation regime (zooplankton density, chlorophyll *a*, and sex ratio: Heinen et al. 2013; background water color and habitat color: Supplemental Material B). Zooplankton density was a strong environmental predictor of dorsal-fin and anal-fin yellowness, and gonopodium blackness (Figs. 3, SB4). Model averaging showed that males from sites with greater zooplankton density tended to have more yellow (↑ *b**) dorsal fins, more yellow and red anal fins (↑ *a** and *b**), and blacker (↓ *L**) gonopodia.

Water color was also an important predictor of male body color (Fig. 3 and Table 3). In addition to the interactive effects of water color and predation risk on dorsal-fin and anal-fin coloration described earlier, males tended to have more red (and yellow) dorsal fins in blue holes with increasingly red (and yellow, respectively) water color, regardless of predation risk (Figs. 3, SB4). Similarly, males tended to have more red anal fins with increasingly red water color across both high- and low-predation sites. Furthermore, iridescent patch size increased with increasing green background water color (↓*a**; Fig. SB4). Finally, males in blue holes with more yellow (↑*b**) and more green (↓*a**) water backgrounds tended to have more pigmented gonopodia.

In contrast, variation in the background habitat color, relative chlorophyll *a* density, and sex ratio were rarely important, each comprising a strong predictor for only a single male color trait. Finally, environmental variation had little effect or predictive power for black shoulder patch area or caudal-fin blackness (Fig. 3).

COMMON-GARDEN EXPERIMENT

Results from our common-garden experiment provide strong evidence for a genetic basis to divergence in male dorsal-fin coloration between predation regimes. Males originally derived from

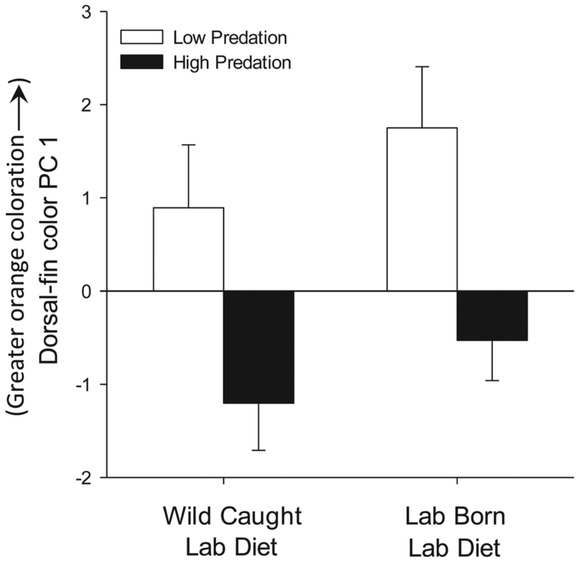

Figure 4. Dorsal-fin coloration of *Gambusia hubbsi* from two high- and two low-predation blue holes when kept or reared under common conditions in the lab. Shown are least-squares means ± SE of dorsal-fin color, controlling for body size.

Table 4. Results from a linear mixed-model (treating population as a random effect) evaluating divergence in dorsal-fin color (PC1) of male *Gambusia hubbsi* from two high- and two low-predation blue holes from Andros Island, The Bahamas, when kept or reared under common conditions in the lab (i.e., birth status).

Term	df	*F*	*P*
SL	1,29.21	7.02	0.013
Birth status	1,27.05	2.98	0.096
Predation regime	1,3.77	29.67	0.007
Predation regime × Birth status	1,22.80	0.001	0.922

low-predation blue holes had, on average, greater PC1 scores indicating more yellow and red dorsal-fin coloration (Fig. 4 and Table 4). Furthermore, the degree of dorsal-fin coloration (PC1) did not significantly differ between males originally captured in the wild (and subsequently raised in the lab under common conditions for 8–13 months) and males born and raised in the lab (F1 or F2 generation; Fig. 4 and Table 4). There were no significant relationships found for PC2 ($P > 0.4$ for all variables).

Discussion

Signal diversity in *G. hubbsi* inhabiting blue holes is associated with multiple ecological and phenotypic factors. Overall, we found that male coloration is generally more exaggerated in the absence of *G. dormitor*; however, the relative importance of predation and other factors—resource availability, background color, body size, condition, and body shape—in explaining variation in male coloration differed between particular traits. Furthermore, divergence between predation regimes appears to largely reflect genetically based trait differentiation in dorsal-fin color. Although many of our findings matched a priori predictions, the results also highlight the complexity of signal evolution, as multiple factors influenced the expression of most traits, and the nature of these associations were not uniform across traits (see also Millar et al. 2006; Ord and Martin 2006).

PREDATION RISK AND MALE COLORATION

Low-predation males possessed more orange dorsal and anal fins, larger black shoulder patches, and larger iridescent patches (when controlling for differences in background water color and sex ratio). These patterns may reflect (1) a trade-off between sexual selection favoring elaboration and natural selection via predation selecting against conspicuousness, (2) a shift in the magnitude or nature of sexual selection across predation regimes due to changes in the riskiness of sexual behaviors, or both.

First, observed trends could arise from the antagonistic interplay of predation and sexual selection (e.g., Endler 1980, 1983; Zuk et al 2006; but see Weese et al 2010; reviewed in Zuk and Kolluru 1998). That is, although intra- and intersexual selection may favor elaborate color traits, these same exaggerated patterns may make males more conspicuous to predators. For at least one of the traits examined here, orange dorsal fins, this seems particularly likely, as males regularly display these fins to females and rival males during sexual/social behaviors.

Second, predation risk can drive signal divergence by altering the context of sexual selection without inducing any natural selection on sexual signals. Predation is known to influence many reproductive traits in diverse taxa, such as courtship displays, mating tactics, and copulation duration (e.g., Lima and Dill 1990; Magnhagen 1991; Sih 1994; Godin 1995; Candolin 2003). Predation can modify the context of sexual selection by changing the magnitude (e.g., higher frequency and duration of courtship behaviors in low risk, but higher frequency of coercive male mating tactics in high risk) or nature of sexual selection (e.g., preference for more complex, multidimensional signal patterns in lower risk, where sexual/social behaviors can occur over longer timescales). The general trend of increased color elaboration in low-predation blue holes observed here could reflect stronger sexual selection in the absence of predators (e.g., Forsgren 1992; Godin and Briggs 1996), instead of, or in combination with, stronger natural selection against elaboration in the presence of predators. For instance, the observed pattern for the black shoulder patch suggests that higher dimensionality of mating cues might occur in low-predation environments. Unique among the traits, we measured in this study, the black shoulder patch is sexually dimorphic with males expressing relatively larger patches than females in low- but not high-predation blue holes (Supplemental Material A). A common way to increase complexity and conspicuousness of a signal is to pair saturated pigments with iridescent colors, producing high contrast (reviewed in Doucet and Meadows 2009), and indeed in *G. hubbsi* males, black shoulder patches are situated adjacent to patches of iridescence, consistent with the idea that the black shoulder patch acts as a secondary signal in low-risk environments but may not be favored in high-risk environments.

INFORMATION CONTENT OF COLOR SIGNALS

What information, if any, might male color traits convey to conspecifics? The association between male coloration and measures of male body size, body shape, and condition suggest that these traits may reflect honest signals of male quality. For example, with the exceptions of black patch area and gonopodium blackness, increased trait values were strongly associated with greater lean weight, a measure of body size and muscle mass. Although only preliminary evidence suggests that female *G. hubbsi* prefer larger males (R. B. Langerhans, unpubl. data), female preference for large males is widespread in poeciliid fishes (Bisazza et al. 2001; Rios-Cardenas and Morris 2011), including some *Gambusia* species, and may also be favored in male–male competition (Bisazza et al. 1996, Rios-Cardenas and Morris 2011).

Somewhat counter-intuitively, black shoulder patch area and dorsal- and anal-fin coloration were negatively associated with body fat (weakly for the black shoulder patch) and testes size (controlling for body size). This relationship may reflect alternative mating strategies employed by colorful versus dull males: in *G. hubbsi*, more colorful males appear to spend more time actively courting females and displaying to other males, whereas duller males appear to spend more time foraging and attempting forced copulations (J. L. Heinen-Kay and R. B. Langerhans, unpubl. data). Although the potential for alternative mating tactics has so far not been thoroughly investigated in this species, it is widespread in poeciliids (Grether and Kolluru 2011; Rios-Cardenas and Morris 2011). Moreover, the negative relationship between testes size and signal expression is concordant with evidence for a general trade-off between traits that increase fertilization success and those that increase mating success (Kvarnem and Simmons 2013).

The relationship between body shape and coloration was relatively straightforward: male body shape was strongly associated with anal-fin color and weakly associated with dorsal-fin color, regardless of predation risk. Males with more streamlined body shapes (shallower mid-body/caudal regions) tended to exhibit more exaggerated fin coloration. This might partially reflect resource-based limitations, as males with more streamlined bodies likely experience greater swimming endurance and higher foraging success (Langerhans 2009b; Langerhans and Reznick 2010). The association between body shape and fin color is an especially interesting finding because female *G. hubbsi* prefer males with more streamlined bodies in low-predation environments and males with deeper mid-body/caudal regions in high-predation environments (Langerhans et al. 2007; Langerhans and Makowicz 2013). Therefore, increased orange fin coloration may be associated with preferred males in low-predation but not in high-predation populations.

RESOURCE ALLOCATION AND INVESTMENT IN TRAITS

Several color traits (i.e., dorsal- and anal-fin color and gonopodium blackness) were positively associated with either zooplankton density or chlorophyll *a* concentration (a measure of algal biomass) across blue holes, suggesting resource-based limitations on male investment in fin coloration. Interestingly, these relationships were similar within each predation regime. For instance, although males from blue holes with *G. dormitor* were less colorful than males from blue holes lacking *G. dormitor*, fin coloration increased with resource availability within each predation regime. Orange pigmentation often derives largely from carotenoids, pigments that must be acquired by animals from the biotic environment (Goodwin 1986). Such carotenoid-based pigments have long been predicted to indicate aspects of male quality (e.g., Endler 1980; reviewed in Olson and Owens 1998), such as foraging ability and immune function. Although preliminary work has found multiple carotenoid pigments in *G. hubbsi* dorsal fins (S. T. Giery et al., unpubl. data), orange coloration in *G. hubbsi* fins may reflect a combination of yellow carotenoids and red drosopterins (similar to orange spots in guppies: Grether et al. 2001; Hudon et al. 2003), with only carotenoids expected to exhibit resource-based limitations. Consistent with this notion, we found that effects of zooplankton density and chlorophyll *a* (potential sources of carotenoids for *G. hubbsi*) were much stronger for yellow than red coloration in the fins.

SIGNAL TRANSMISSION AND RECEPTION

The sensory drive hypothesis posits that the transmission environment of a signal can profoundly affect both how and if a signal is received, and that signals should be locally adapted to their environments (Endler 1992). As predicted by theory, variation in color properties of the transmission environment was important in explaining variation in male color between blue holes. In general, the dorsal and anal fins exhibited greater orange coloration in blue holes with a slightly more orange-shifted ambient water color. Because yellows and reds are less visible against a yellow-red background as compared to a blue-green background (Endler 1992), this suggests a pattern that maintains high conspicuity across all water colors. Similarly, dorsal fins exhibited greater red coloration in blue holes with vertical wall backgrounds that were more red and yellow, and males had larger iridescent patches in greener background waters. Gonopodium black pigmentation increased in more yellow-green waters, perhaps because black pigmentation is conspicuous against a wide range of backgrounds (Endler 1992; Morrongiello et al. 2010). Together these results are consistent with adaptive responses of male color traits for efficient signal transmission in local environments.

Interestingly however, we found evidence that the relationship between fin color and the background water color sometimes differed between high- and low-predation blue holes. First, although red dorsal-fin coloration decreased in more yellow background water colors in high-predation sites, it did not change across the same gradient in low-predation blue holes. Second, yellow anal-fin coloration tended to increase in more yellow background water colors across high-predation blue holes but was again relatively invariable across low-predation sites. Although we can only speculate at this point, these differences may derive from the way these fins are used for communication purposes. Dorsal-fin color in *G. hubbsi* males and other poeciliid fishes serves as a public signal that makes males stand out even when the fin is folded and held close to the body. Anal-fin color, however, is more likely to serve as a private signal (sensu Cummings et al. 2003; Millar and Hendry 2012) as anal-fin coloration is only visible when males display their gonopodium at close ranges to females during courtship or to other males during antagonistic interactions (all authors, pers. obs.). Thus, while increasing anal-fin color based on background water color may enhance detectability by conspecifics, it unlikely makes males stand out more over longer distances to predators. However, decreasing dorsal-fin coloration to better match the background water color will undoubtedly decrease overall conspicuousness.

EVOLUTIONARY DIVERGENCE AND SPECIATION

Does color divergence between environments primarily reflect genetic differentiation or phenotypic plasticity? We effectively ruled out diet-induced phenotypic plasticity as the primary explanation for differences between predation regimes, as dorsal-fin color differences persisted in fish raised under a common diet in the lab. Thus, at least some differences in male coloration between populations reflect genetic divergence. However, these results do not imply that plasticity plays no role in dorsal-fin coloration. Indeed, although the pattern was marginally nonsignificant, offspring born and raised in the lab tended to express greater dorsal-fin coloration than wild-caught males from their parental populations held in the lab for 8–13 months. This suggests that dietary constraints, probably due to resource availability, have at least some additional impact on dorsal-fin coloration in the wild.

Finally, male color divergence between predation regimes may prove important for ongoing ecological speciation in this system (Langerhans et al. 2007; Langerhans 2009a). Previous work has centered on the roles of body shape and locomotor performance in elevating reproductive isolation between populations with different predation regimes through isolation by adaptation (reduced fitness of immigrants) and sexual selection on body shape (Langerhans et al. 2007; Langerhans 2009a; Langerhans and Makowicz 2013). Male coloration might affect reproductive isolation between populations through several routes. First, migrant males from low- to high-predation might experience increased conspicuousness to predators (perhaps suffering increased predation, e.g., Nosil 2004), and migrants from high- to low-predation might experience reduced conspicuousness to conspecifics (perhaps suffering reduced mating success, e.g., Labonne and Hendry 2010; Schwartz et al. 2010). Second, if females prefer certain male color traits, such as more exaggerated dorsal-fin color, and only locally adapted males can produce strong orange coloration (i.e., honest indicators of male fitness), then immigrants between predation regimes would suffer reduced mating success compared to residents (van Doorn et al. 2009). Finally, males with color patterns fine-tuned for their local background color might experience reduced fitness in environments with a different background color, regardless of predation regime. This highlights how the complexity of signal evolution can have important implications for the process of speciation (Boughman 2002; Gray and McKinnon 2007).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure SA1. The relationship between body color traits and standard length (SL) for male (triangle symbols and dashed lines) and female (filled circles and solid lines) *Gambusia hubbsi* in low-predation (gray symbols and lines) and high-predation (black symbols and lines) blue holes.
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Figure SB3. Thin-plate spline transformation grids illustrating morphological divergence between predation regimes in male *G. hubbsi* inhabiting blue holes on Andros Island, The Bahamas, based on analysis of 115 males from 10 blue holes included in this study (observed variation depicted along **d**, lines drawn to aid interpretation).
Figure SB4. Relationships between population means for four body color variables and environmental variation/individual condition for male *Gambusia hubbsi* in low-predation (open symbols and dashed lines) and high-predation (filled symbols and solid lines) blue holes.
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