doi:10.1111/j.1558-5646.2007.00171.x

ECOLOGICAL SPECIATION IN *GAMBUSIA* FISHES

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Received September 1, 2006

Accepted April 30, 2007

Although theory indicates that natural selection can facilitate speciation as a by-product, demonstrating ongoing speciation via this by-product mechanism in nature has proven difficult. We examined morphological, molecular, and behavioral data to investigate ecology's role in incipient speciation for a post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting blue holes. We show that adaptation to divergent predator regimes is driving ecological speciation as a by-product. Divergence in body shape, coupled with assortative mating for body shape, produces reproductive isolation that is twice as strong between populations inhabiting different predator regimes than between populations that evolved in similar ecological environments. Gathering analogous data on reproductive isolation at the interspecific level in the genus, we find that this mechanism of speciation may have been historically prevalent in *Gambusia*. These results suggest that speciation in nature can result as a by-product of divergence in ecologically important traits, producing interspecific patterns that persist long after speciation events have completed.

KEY WORDS: **Adaptive radiation, divergent natural selection, ecological speciation, geometric morphometrics, mate choice, parallel evolution, predation, premating isolation.**

Since the inception of evolutionary biology, understanding the mechanisms leading to speciation has been of fundamental importance (e.g., Muller 1942; Dobzhansky 1951; Simpson 1953; Mayr 1963). Although long neglected, the importance of natural selection in the evolution of reproductive isolation is now receiving much focused attention (Hendry et al. 2000; Schluter 2001; Funk et al. 2002, 2006; Coyne and Orr 2004; Dieckmann et al. 2004; Gavrilets 2004; Rundle and Nosil 2005). The simplest model of ecological speciation—the evolution of barriers to gene flow resulting from ecologically based divergent selection (Schluter 2001; Rundle and Nosil 2005)—describes adaptation to divergent selective regimes, which incidentally results in reproductive isolation as a by-product. This by-product mechanism can occur in any geographical context, and does not require selection to directly favor reproductive isolation (i.e., reinforcement). Theory suggests that divergent natural selection between environments might often result in speciation as a by-product; however, only a handful of promising examples from nature have so far been revealed (e.g., Funk 1998; Schluter 2001; Nosil et al. 2003; Vines and Schluter 2006).

Among the most convincing evidence of the by-product mechanism is ecological speciation among allopatric populations. In the scenario in which populations are allopatric, selection cannot act directly on reproductive isolation itself (interbreeding opportunities do not exist, or are extremely rare), but rather selection's role in speciation must be incidental. Evidence for ecological speciation in the wild has now been uncovered in several cases (e.g., Funk 1998; McPeek and Wellborn 1998; Rundle et al. 2000; Jiggins et al. 2001; Nosil et al. 2002; McKinnon et al. 2004; Boughman et al. 2005), and a general role of natural selection in promoting speciation has been uncovered across diverse taxa (Funk et al. 2006). However, many of the populations

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examined to date have been sympatric or parapatric—thus, the exclusive role of the by-product mechanism has rarely been investigated (see Funk 1998; Nosil et al. 2003; Vines and Schluter 2006). Although laboratory experiments confirm the plausibility of this model (Rice and Hostert 1993; Rundle et al. 2005), we still have limited knowledge regarding the general importance of the by-product mechanism in nature (Schluter 2001; Coyne and Orr 2004; Rundle and Nosil 2005). Here we test the hypothesis of ecological speciation via the by-product mechanism using allopatric populations of a live-bearing fish, and in doing so, further address two important gaps in our understanding of ecological speciation (Rundle and Nosil 2005; Vamosi 2005; Langerhans 2006; Nosil and Crespi 2006): the importance of predation as a selective agent facilitating speciation, and the particular phenotypes actually influencing reproductive isolation among ecologically divergent populations.

Blue holes are water-filled voids in carbonate banks and islands, often possessing now-submerged cave passages (Mylroie et al. 1995) (Fig. 1). The Bahamas mosquitofish (*Gambusia hubbsi*; Family Poeciliidae) colonized inland blue hole environments during the past ~15,000 years (Fairbanks 1989) as rising sea levels lifted the freshwater lenses of Bahamian islands (freshwater aquifers floating atop marine groundwater, common to many small islands), flooding the voids. Inland blue holes are analogous to aquatic islands in a sea of land, as mosquitofish populations in these isolated habitats seem to exhibit little gene flow with outside populations, showing some of the highest F_{ST} values reported for fish populations (Schug et al. 1998). Probably because dispersal and colonization abilities are greater for mosquitofish (smaller-bodied, shorter generation time, live bearing) than for larger predatory fish, mosquitofish currently inhabit many blue holes, whereas larger piscivorous fish only inhabit a subset. Thus, in some blue holes mosquitofish experience a relatively predator-free environment devoid of any piscivorous fish, and in others they face a strong predation threat from the bigmouth sleeper (*Gobiomorus dormitor*), a major predator of mosquitofish (McKaye et al. 1979; Winemiller and Ponwith 1998; Bacheler et al. 2004; R. B. Langerhans, unpubl. data). Because blue holes with divergent predator regimes do not systematically differ in abiotic environmental variables (Appendix), the system provides a "natural experiment" to test the effects of predation-mediated natural selection on evolutionary diversification in mosquitofish (Downhower et al. 2000; Langerhans et al. 2005).

Especially strong confirmation of the hypothesis of ecological speciation via the by-product mechanism is provided when each of three kinds of evidence is available: divergent natural selection among environments, replicated trait evolution in independent populations, and greater reproductive isolation between ecologically divergent pairs of populations than ecologically similar ones resulting as a by-product of divergent traits. In this study, we use morphological data to test for divergent natural selection, use molecular data to test for evolutionary independence among populations exhibiting similar phenotypes, and conduct mate-choice trials to test for ecologically associated premating isolation.

Regardless of whether *G. hubbsi* exhibits ongoing ecological speciation driven by differences in predator regime, an intriguing

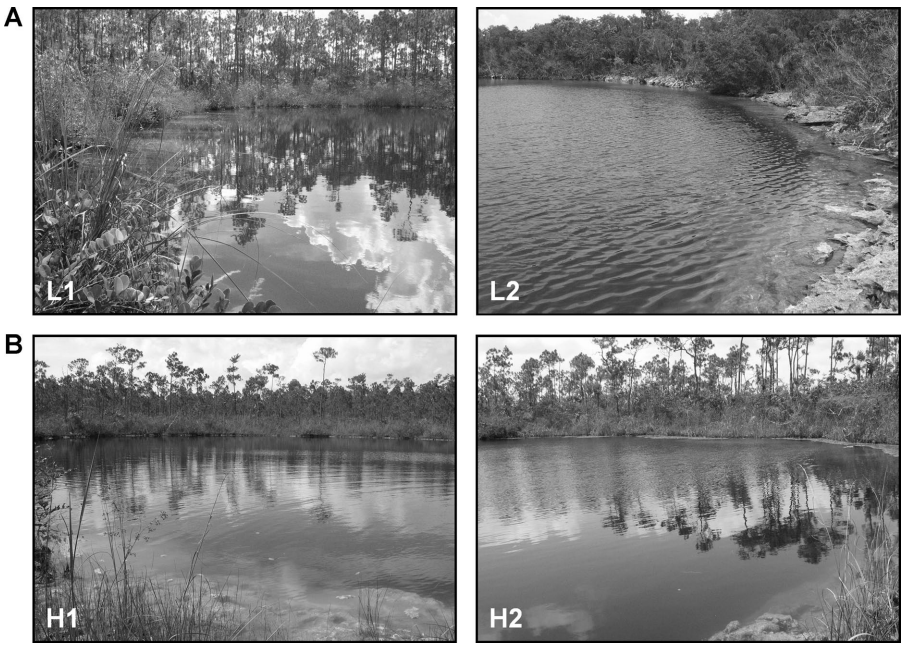

Figure 1. Photographs of four blue holes used in this study. (A) Blue holes without any piscivorous fish. (B) Blue holes with the predatory fish *Gobiomorus dormitor*. Population names follow Figure 2.

question is whether this mechanism of speciation might reflect a common scenario in *Gambusia* speciation. If that is the case, extant species might exhibit the signature of ecological speciation. Because variation in predator regime exists among as well as within *Gambusia* species, we can address this question using available data on premating isolation between species. We examine published measures of sexual isolation between *Gambusia* species to determine whether predation-driven ecological speciation might have produced interspecific patterns in the genus.

Materials and Methods

COLLECTIONS

We examined over 600 *G. hubbsi* individuals from a total of 12 blue holes (six with predators, six without) on Andros Island, the Bahamas (Fig. 2; see online Supplementary Material Table S1 for details regarding sample sizes). We collected fish from all 12 blue holes in August 2004, and additionally collected fish from four of these blue holes in August 2005—those used in the mate-choice experiment. Populations were classified "low-predation" or "high-predation" based on the absence or presence of the bigmouth sleeper, a piscivorous fish. Because of high water clarity and the fish's active behavior, detection of this predatory fish was easily accomplished using underwater visual observations (e.g., detection of the bigmouth sleeper always occurred within the first 30 sec of observations, despite $\geq$ 6 h of observations within each blue hole). Fish communities are very simple in most blue holes (see Appendix), with *G. hubbsi* typically coexisting with only one to three other fish species. In the one exception in this study (six sympatric fish species in one blue hole), a piscivorous fish, redfin needlefish (*Strongylura notata*), likely provides an important source of predation in addition to the bigmouth sleeper.

While piscivorous fish serve as major predators of mosquitofish, avian predators pose an additional potential threat (Kushlan 1973; Britton and Moser 1982). Because blue holes are steep-sided and deep, wading birds (e.g., egrets, herons) are virtually excluded from these sites (Downhower et al. 2000). However, it is possible that diving birds (e.g., kingfishers, grebes) may sometimes visit blue holes. Thus, predation on *G. hubbsi* from birds may occur, but is not expected to differ among blue holes with and without predatory fish (i.e., should not confound effects of piscivorous fish).

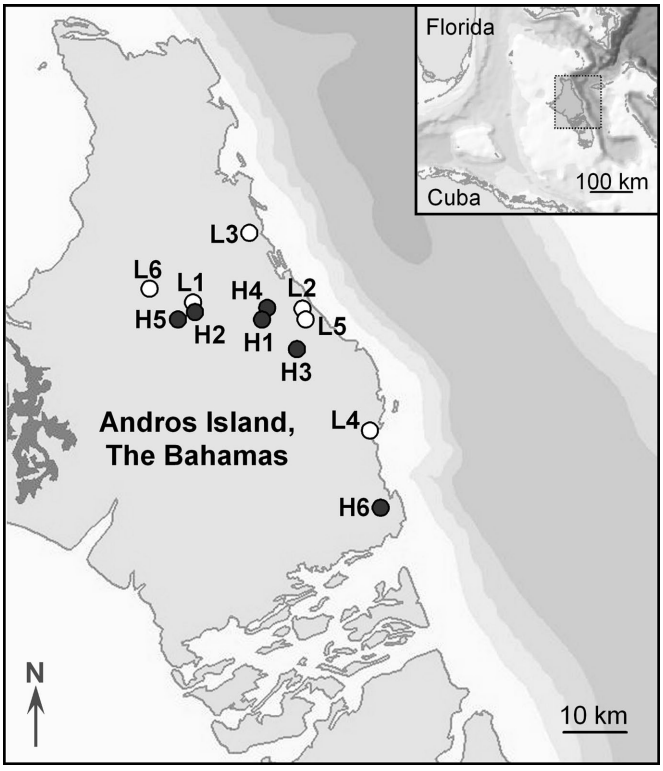

Figure 2. **Map of study sites. Low-predation (open circles; labels beginning with "L") and high-predation (filled circles; labels beginning with "H") blue holes where *G. hubbsi* were collected. Geographic distance between populations does not differ between blue holes with similar (mean $\pm$ 1 standard error, 19.15 $\pm$ 2.19 km) or different predator regimes (17.79 $\pm$ 2.03 km) (Mantel test, $r = -0.06$, $P = 0.68$).**

MORPHOLOGICAL ANALYSES

Based on both theoretical and empirical work, clear a priori predictions exist regarding divergent selection on mosquitofish morphology between predatory environments. Mosquitofish use body and caudal fin propulsion for both prolonged and fast-start swimming modes (see Webb 1984; Blake 2004). Because this locomotor system is mechanically coupled (i.e., same propulsor for different swimming activities), and because prolonged and fast-start swimming are optimized with different propulsor arrangements (see below), optimizing one swimming mode necessarily compromises the other. Morphology is strongly linked to swimming performance in mosquitofishes, and biomechanical research demonstrates that prolonged swimming performance is optimized with a relatively shallow caudal peduncle (body region between the dorsal and anal fins and the base of the caudal fin; see posterior shaded region in Fig. 3) and a deep anterior body/head region (fusiform body shape), while fast-start swimming is optimized with the opposite trait values (deep caudal peduncle, shallow anterior body/head) (e.g., Blake 1983; Webb 1984, 1986; Walker 1997; Plaut 2002; Blake 2004; Langerhans et al. 2004, 2005; R. B. Langerhans, unpubl. data). Due to this performance trade-off, environments favoring alternative swimming modes should generate divergent selection on morphology. This scenario is predicted for low- and high-predation environments: resource competition generates selection favoring enhanced prolonged swimming in low-predation environments (important for finding and consuming food, acquiring mates, reserving energy supplies for reproduction), whereas predation creates selection favoring enhanced

fast-start swimming in high-predation environments (important for evading predator strikes) (e.g., Vogel 1994; Domenici 2003; Blake 2004; Langerhans et al. 2004; Walker et al. 2005). We examined divergent selection on body shape in Bahamas mosquitofish by testing for the predicted differences in morphology between blue holes with divergent predator regimes.

We examined three morphological datasets: (1) lateral x-ray radiographs of preserved male *G. hubbsi* specimens from 12 blue hole populations collected in 2004, (2) lateral x-ray radiographs of preserved male and female *G. hubbsi* from the four focal blue holes used in the mate-choice experiment collected in 2004 and 2005, and (3) lateral images of live male and female *G. hubbsi* from these four focal blue holes collected in 2005. Radiographs were taken with a Hewlett-Packard (Palo Alto, CA) Faxitron cabinet X-ray system, and images of live fish were captured with a Canon (Tokyo, Japan) PowerShot A95 using a Tiffen (Hauppauge, NY) 37 mm + 7 macro filter following methods in Langerhans et al. (2004). We digitized 10 landmarks on each image (see Fig. 3) using the software program tpsDig (Rohlf 2004a). Landmarks were selected to provide adequate coverage of the lateral body profile, and followed the methods of previous studies in *Gambusia* (e.g., Langerhans and DeWitt 2004; Langerhans et al. 2004). We digitized the following landmarks: most anterodorsal point of premaxilla (tip of snout), most posterodorsal point of skull (or indentation at the posterodorsal end of head for external photographs), anterior insertion of dorsal fin, posterior insertion of dorsal fin, dorsal insertion of caudal fin, ventral insertion of caudal fin, posterior insertion of anal fin, anterior insertion of anal fin, most posteroventral point of skull (or intersection of the operculum and body profile for external photographs), center of eye. The eye was included as a landmark because of its importance in previous studies of predator-associated morphological divergence in live-bearing fishes (Langerhans and DeWitt 2004; Langerhans et al. 2004). We conducted geometric morphometric analyses (e.g., Rohlf and Marcus 1993; Adams et al. 2004; Zelditch et al. 2004) using the digitized landmarks. We used tpsRegr software (Rohlf 2004b) to align landmark coordinates via generalized Procrustes analysis (i.e., rotating, translating, and scaling coordinates to remove positioning effects and isometric size effects; Bookstein 1991; Marcus et al. 1996). Superimposed landmark configurations were used to calculate geometric shape variables—uniform components and partial warps—describing affine and nonaffine shape variation. Affine components describe uniform spatial covariation of landmarks in the x–y plane, while nonaffine components describe heterogeneous changes (local deformations). We examined body shape variation among predator regimes using nested MANCOVA; geometric shape variables (uniform components and partial warps) served as dependent variables, centroid size served as the covariate (controlling for multivariate allometry), and predator regime and population nested within predator regime served as independent variables. Shape variation along canonical variate axes was visualized using thin-plate spline transformation grids (for details, see Bookstein 1991; Rohlf et al. 1996; Klingenberg et al. 2003; Klingenberg and Monteiro 2005).

In all MANCOVAs, we tested for heterogeneity of slopes, and included the interaction term when significant. The interaction term had minimal influence on the effect of predator regime within each dataset as revealed by the high correlation among canonical axes derived from the predator regime term with and without the inclusion of the interaction term (all $r > 0.99$, all $P < 0.0001$). Because the effect of predator regime on morphology was highly similar for males and females (correlation between canonical axes derived separately for males and females in each morphological dataset: all $r > 0.87$, all $P < 0.0001$), and because we wished to place both sexes on the same morphological axis to examine effects of morphological similarity on mating preference, we only present results from analyses pooling sexes for datasets 2 and 3 (Table 1). Further, because results were highly similar among years (correlation among canonical axes: $r = 0.94$, $P < 0.0001$), we only present results from analyses pooling data across years for dataset 3. Because the use of either centroid size or the natural logarithm of centroid size as the covariate in these analyses produced nearly identical results (correlation among canonical axes: all $r > 0.99$, all $P < 0.0001$), we only present results from analyses using untransformed centroid size.

We conducted a discriminant function analysis (DFA) for each dataset to provide an intuitive metric regarding the magnitude of morphological divergence (i.e., percentage of fish correctly classified according to predator regime). Each DFA used the geometric shape variables as the dependent variables and predator regime as the independent variable. DFAs were conducted using jackknife sampling as a cross-validation technique (i.e., each individual was sequentially removed from the dataset and classified according to a discriminant function derived with the remaining data).

We assessed body size differences between predator regimes using nested analysis of variance (ANOVA). The natural logarithm of standard length served as the dependent variable, while predator regime and population nested within predator regime served as independent variables. These analyses were conducted separately for males and females for all datasets.

Because we had a priori hypotheses concerning differences in caudal peduncle and head size, we calculated caudal peduncle area and head area using the convex polygon area of interconnected landmarks in those regions (see Fig. 3). We examined differences in caudal peduncle area and head area between predator regimes, controlling for body size, by conducting nested ANCOVA using standard length as the covariate, and predator regime and population nested within predator regime as independent variables. All morphological traits were natural log-transformed prior to

analysis. These analyses were conducted for all datasets, and percent differences in least-squares means between predator regimes were calculated to provide quantitative metrics of the magnitude of divergence for particular traits. The predator regime term was significant in all cases (all $P < 0.002$).

To confirm that morphological variables were repeatable, we digitized the 10 landmarks for two separate images of 10 live individuals. We used live fish to provide a conservative estimate, as repeatability is expected to be lower for photographs of live fish compared to radiographs of preserved specimens. Repeatability was calculated using the intraclass correlation coefficient from a model II ANOVA (Lessells and Boag 1987; Sokal and Rohlf 1995). Estimates of body size were highly repeatable (centroid size: intraclass correlation coefficient, $r = 0.998$, $P < 0.0001$; standard length: $r = 0.998$, $P < 0.0001$). All geometric shape variables (uniform components and partial warps) also exhibited high repeatability (mean $r = 0.894$, all $P < 0.003$). Estimates of relative head and caudal peduncle size (residuals of convex polygon area size, controlling for standard length) were also highly repeatable (log head area residuals: $r = 0.848$, $P = 0.0003$; log caudal peduncle area residuals: $r = 0.993$, $P < 0.0001$). Thus, all morphological variables exhibited significant repeatability.

mtDNA ANALYSES

Because it is possible that morphological similarities among different populations in similar predator regimes could reflect shared ancestry rather than replicated evolution, we examined mitochondrial DNA sequences to test this alternative hypothesis. We examined mtDNA sequences for five *G. hubbsi* individuals from each of the 12 blue holes. We amplified a fragment (886 bp) of the NADH subunit 2 (ND2) gene in 25 μL reactions using the following primers: L3975 (5′-AAG CTT TCG GGC CCA TAC CC-3′) and H4917 (5′-CGC AAT AGC ATT AAC CAT-3′). The letters in the primer names signify the light and heavy strand, respectively, and the numbers indicate their 5′ position in the *G. affinis* mitochondrial genome (Miya et al. 2003). PCR amplification conditions included an initial denaturation at 94°C for 120 sec followed by 40 cycles of denaturation at 94°C for 35 sec, annealing at 50°C for 35 sec, and extension at 72°C for 90 sec. The amplification protocol concluded with a final extension at 72°C for 300 sec following the final cycle. Sequences were aligned by eye. No insertions or deletions (indels) were observed. GenBank accession numbers for each unique mtDNA haplotype are presented in Table S2 Supplementary Material online.

We constructed a haplotype network with the mtDNA sequences using the computer program TCS (Clement et al. 2000), and conducted an analysis of molecular variance (AMOVA) with Arlequin 3.01 (Excoffier et al. 2005) to summarize the proportion of total genetic variation attributable to variation among predator regimes, variation among populations within predator regimes, and variation within populations.

To assess the independence of our ecological variables from patterns of genetic and geographic divergence, we conducted Mantel tests (Mantel 1967) examining the relationship between matrix pairs describing various distances between populations: mean genetic distance (percent nucleotide divergence using the TrN + I model of nucleotide substitution selected using the Akaike information criterion with Modeltest [Posada and Crandall 1998]), geographic distance (straight-line distance), mean morphological distance (using the canonical axis derived from dataset 1, illustrated in Fig. 3), and ecological distance (0 = same predator regime, 1 = different predator regimes). All Mantel tests were conducted using the computer program Passage (Rosenberg 2001), in which significance was assessed by comparing the *z*-statistic of the actual matrices to the *z*-statistics from 99,999 random permutations.

ALLOZYME ANALYSES

As an additional test of independent evolution among predator regimes, we examined previously published allozyme allele frequencies for 13 blue hole populations of *G. hubbsi* (six low-predation, seven high-predation; 17 polymorphic inferred loci, 47 total alleles) (Schug 1995). Four of these populations are also examined in this study (L3, L6, H1, H2); predator-regime classifications for the remaining populations were taken from Downhower et al. (2000). We conducted AMOVA for each locus, assessing possible structuring of genetic variation between predator regimes, populations nested within predator regimes, and within populations. We further investigated population variation in multidimensional allele frequency space by performing principal components analysis (PCA) using all allele classes exhibiting $\geq 5\%$ frequency in at least one population ($n = 37$ allele classes). We performed ANOVA with each PC to test for possible differences in allele frequencies among predator regimes.

MATE-CHOICE EXPERIMENT

We designed a mate-choice experiment to test the key prediction of the ecological speciation hypothesis that populations adapted to different environments exhibit greater reproductive isolation than populations inhabiting similar environments. Further, our analysis explicitly examined the link between natural selection and speciation by evaluating whether mating preferences were based on the same phenotypes experiencing divergent selection. We examined mating preference of female *G. hubbsi* from four blue holes (two with predators, two without predators) by conducting two mate-choice trials with each of 33 females (eight from L1, 10 from L2, six from H1, nine from H2). For each trial, a female was presented a choice between videos of two males, one from their native population and one from a foreign population either

inhabiting the same or different predator regime; both types of choices were offered to each female in separate trials (order of comparison type randomly chosen). For trials involving a foreign male from the opposite predator regime as the female, there were two possible populations available for use; one population was randomly selected for the first trial of each of the female populations, and then subsequent trials alternated between the two possible populations. Trials were conducted in a laboratory mate-choice arena (25 × 15 cm, three sides opaque, one side with a video monitor, the bottom divided into four equal-sized quadrants), and filmed from above using a Canon (Tokyo, Japan) ES6000 Hi8 video camera. In each trial, two video recordings of males were presented side-by-side on the monitor.

Video playback has been successfully employed in mate-choice experiments for many animals, and is particularly common in poeciliid fishes (e.g., Nicoletto and Kodric-Brown 1999; Rosenthal 1999; Basolo and Trainor 2002; Johnson and Basolo 2003; Langerhans et al. 2005; Witte and Klink 2005; Morris et al. 2006). An important advantage of video playback is that behavioral variation among experimental treatments can be minimized by selecting particular video segments. Behavioral responses of fish to videos are often highly similar to responses to live fish (Kodric-Brown and Nicoletto 1997; Landmann et al. 1999; Trainor and Basolo 2000; Morris et al. 2003). Supporting this prior work, a pilot study conducted with *G. hubbsi* prior to experimentation confirmed that females exhibit qualitatively similar mating responses with live fish versus videos.

We constructed videos for two males from each population (eight total videos); a video was chosen at random to represent each population for each trial in which that population was used. Videos were constructed to minimize differences in behaviors and comprised 12 sec continuously looping sequences. Because we were specifically interested in the role of morphology in mate choice, males were carefully selected for use in video playback to minimize potentially confounding differences between males from different predator regimes other than body shape. To this end, males chosen for the videos were similar in body size (ANOVA, $F_{1,6} = 2.55$, $P = 0.16$), relative gonopodium size (previously shown to influence female mating preference in *G. affinis* [Langerhans et al. 2005]; ANCOVA, $F_{1,5} = 0.14$, $P = 0.73$), and behavior during the video segment (ANOVA, average speed: $F_{1,6} = 1.22$, $P = 0.31$, maximum speed: $F_{1,6} = 0.65$, $P = 0.45$, cumulative displacement: $F_{1,6} = 1.46$, $P = 0.27$, maximum displacement: $F_{1,6} = 0.01$, $P = 0.94$), but differed greatly in body shape (ANOVA with canonical axis, $F_{1,6} = 44.06$, $P = 0.0006$). In this manner, our analysis of mating preferences may be conservative if behavior covaries with morphology among populations (i.e., if predation drives parallel evolution of both behavior and body shape) because natural population-level variation in behavior has been experimentally removed from the trials.

Females were isolated from males 24 h before experimentation. For each trial, a female was placed into the mate-choice arena and allowed to acclimate for 10 min. We then initiated the video playback and allowed 5 min for the female to inspect the male videos (if a female did not interact with either male during this time, she was removed from the arena and not examined further). Mating responses were then recorded for 10 min, the left–right presentation order of the two video males reversed, the female was allowed to acclimate with the new video presentation for 5 min, and then female mating behavior was recorded for another 10 min. For each female, the second mate-choice trial began approximately 40 min after the first trial ended. Mating response was summed across the two observation periods within each trial. Because nine fish did not exhibit mating responses during both trials, there was a total of 57 useful mate-choice trials (see online Supplemental Material Table S3 for sample sizes of each population pair).

Female mating response was measured as the proportion of time spent by the female directly interacting with a given male while in the quadrant of the arena closest to that male (i.e., interaction time divided by opportunity time, following Johnson and Basolo [2003]). A female was directly interacting with a male when she made obvious motions toward the male within one body length of the video monitor, following Langerhans et al. (2005). Because such interaction can only occur when a female is in the quadrant closest to the particular video, our use of proportional interaction time provides a measure of male attractiveness based on the propensity of females to interact with them, while having the opportunity to interact. (Note that results are similar using total interaction time, or using categorical values of mate choice in which male videos are scored either 0 or 1 within each trial based on which male experienced greater proportional or total interaction time.)

For all statistical analyses described below, we use one-tailed *P*-values wherever we have a priori directional hypotheses. All one-tailed *P*-values are noted in the text. We test three particular hypotheses: (1) assortative mating (preference for native male), (2) ecologically associated premating isolation (stronger isolation between populations inhabiting different predator regimes than between populations in similar ones), and (3) the by-product mechanism (mating preference based on traits under divergent selection). For each hypothesis, we employ two approaches for analysis: (1) using females as the unit of replication, and (2) using populations as replicates. In this way, we evaluate the consistency of results among populations and alleviate potential concerns of pseudoreplication (i.e., females from the same population may not be viewed as statistically independent).

We tested for assortative mating using the nonparametric Wilcoxon's signed-ranks test (data did not meet assumptions of normality), with females as blocks, conducted separately for

comparisons between videos of males from similar ($n = 29$) and different environments ($n = 28$). For these tests, we predicted greater mating response (i.e., proportional interaction time) for native males than foreign males. Although these tests used females as replicates, we also conducted analyses using populations as replicates by performing Wilcoxon's signed-ranks tests within each population and combining probabilities using the weighted *Z*-transform test (also known as Stouffer's method; Whitlock 2005). In all cases (except where otherwise noted), we weighted each test by the reciprocal of its squared standard error (see Whitlock 2005). We calculated the percent difference in mating response between native and foreign males (i.e., dividing the larger average value by the smaller) to provide an intuitive metric regarding the magnitude of mating preferences.

To test for ecologically associated premating isolation, we calculated an assortative mating index (AMI; equivalent to the "response index" of Johnson and Basolo [2003]), and compared the strength of assortative mating between similar and different environments. The index was calculated for each trial as the difference in mating response between the native and foreign male divided by the sum of their mating responses. This index can range from -1 (perfect negative assortative mating) to $+1$ (perfect assortative mating), with 0 representing no mate preference. We used this estimate of assortative mating because it provides an intuitive index of assortative mating and is not influenced by variation among females in overall mating propensity (cf. Casares et al. 1998). We tested whether premating isolation was stronger between populations in different environments than between populations in similar ones using a paired *t*-test. This test used females as replicates ($n = 24$), comparing the strength of assortative mating between the two types of choices (males from similar predator regimes, and males from different predator regimes) for each female. We also conducted *t*-tests within each population and combined probabilities using the weighted *Z*-transform test. This test used populations as replicates, combining results from these independent tests of the same hypothesis.

To test the hypothesis of the by-product mechanism, we examined the relationship between relative mating response and morphological distance (distance between a given female and male along the canonical axis derived using morphological dataset 3, the dataset including these individuals). We also tested for assortative mating based on body *size*, rather than shape, by examining the relationship between relative mating response and body size difference (measured as the absolute difference between a given female and male in standard length). Relative mating response was calculated for each male video within each trial as the mating response for a given male video (i.e., proportional interaction time) divided by the average mating response of the two male videos used in the trial. This metric provides an estimate of relative attractiveness for each male video compared to the alternative video within each trial, and eliminates variation among females in mating propensity (cf. Casares et al. 1998). The relationship between relative mating response and morphological distance was examined using linear regression (slopes were homogenous among populations; nonsignificant interaction term in ANCOVA, $P = 0.66$). This analysis used trials as blocks, treating females from the same population as independent; thus, we also conducted linear regressions within each population and combined probabilities using the weighted *Z*-transform test to provide an analysis using populations as replicates.

To test whether within-population mating behaviors might have led to sexual isolation between populations, we conducted a further analysis within each population using a subset of the data. This test provides further scrutiny regarding the mechanism underlying mate choice, and was meant to evaluate whether females based mating decisions within populations using the same traits they use when choosing mates among morphologically divergent populations. Specifically, we examined the relationship between relative mating response and morphological distance using only the native males—ignoring foreign males—from trials in which males from the same predator regime (and thus, similar in body shape) were presented to females. If within-population mating behaviors are responsible for sexual isolation between populations, then females should exhibit greater preference for native males that are more similar to their body shape compared to other native males that are more morphologically dissimilar. Significance was assessed using the weighted *Z*-transform test, combining probabilities across the four tests.

PREMATING ISOLATION AMONG *GAMBUSIA* SPECIES

Data concerning premating isolation exist for four *Gambusia* species: *G. affinis*, *G. geiseri*, *G. heterochir*, and *G. hurtadoi* (Hubbs and Delco 1960). Each of these species can be readily classified as inhabiting either low- or high-predation environments based on densities of co-occurring piscivorous fish species. The two species classified as low-predation, *G. geiseri* and *G. hurtadoi*, inhabit spring environments either completely lacking any major predatory fishes (*G. hurtado*i; Hubbs and Springer 1957; C. Hubbs, pers. comm.; O. Domínguez, pers. comm.) or harboring very low densities of piscivorous fish that occasionally penetrate the lower reaches of the spring headwaters (*G. geiseri*; Hubbs and Springer 1957; Hubbs 2001; C. Hubbs, unpubl. data). The native range of *G. geiseri* comprises the San Marcos Spring and Comal Spring headwaters in Texas, whereas *G. hurtadoi* is endemic to a small spring, Ojo de la Hacienda Dolores, in Chihuahua, Mexico. The two species classified as high-predation, *G. affinis* and *G. heterochir*, are common prey items for sympatric predatory fishes either throughout their entire range (*G. heterochir*; Hubbs 1957, 2001; C. Hubbs, unpubl. data), or throughout much of their extensive range (*G. affinis*; Bonham 1941; Meffe and Snelson 1989;

Matthews et al. 1992). Although *G. affinis* experiences geographic variation in predator regime, the vast majority of populations coexists with predatory fish, and thus inhabits environments higher in predation pressure than the species in low-predation environments. The native range of *G. affinis* is widespread, stretching from Mexico through the southern United States, whereas *G. heterochir* is endemic to the Clear Creek spring system in Texas.

We examined a subset of data from Hubbs and Delco (1960) to test for ecological speciation. We extracted data describing mate choice of males presented with two females (one conspecific, one heterospecific from either the same or different predator regime) from tables 1, 2, 4, and 5 of Hubbs and Delco (1960). The only trials from that study that we did not include in our analysis either involved a *G. heterochir* × *G. affinis* hybrid individual or did not involve a conspecific female. The study provided estimates of premating isolation based on three separate mating behaviors: gonoporal nibbles, gonopodial thrusts, and gonopodial swings. We incorporated results from all three behaviors in our analyses described below. Just as in the mate-choice experiment with *G. hubbsi*, we examined three hypotheses: assortative mating, ecologically associated premating isolation, and the by-product mechanism.

To test for assortative mating (preference for conspecific female), we performed a *Z*-transform test using species as replicates (species were weighted equally, see Whitlock 2005), conducted separately for comparisons between females from similar and different predator regimes. These tests combined *P*-values from the original publication, averaging across the three mating behaviors for each species. We calculated the percent difference in mating response between conspecific and heterospecific males (i.e., dividing the larger value by the smaller; averaging across the three mating behaviors) to provide an intuitive metric regarding the magnitude of mating preferences.

To test for ecologically associated premating isolation, we calculated measures of assortative mating (AMI) as described above for *G. hubbsi* (with the exception that it was calculated using three different mating behaviors), and employed two approaches for analysis. First, we conducted a paired *t*-test using all species-mating behavior combinations as replicates. That is, each of the three estimates of premating isolation (based on the three different mating behaviors) for all four species ($n = 12$) were used as datapoints. This analysis tested for differences in assortative mating between comparisons involving species from similar and different predator regimes. Because this test assumed that estimates from different mating behaviors of the same species were independent, we also conducted a *Z*-transform test treating species as replicates. For this test, we first conducted the *t*-tests within each species comparing assortative mating between similar environments to assortative mating between different environments across the three mating behaviors ($n = 3$ for each species), and then combined these probabilities using the *Z*-transform method.

To test the by-product mechanism, we assessed the relationship between relative mating response and body shape. First, we examined body morphology of adult male specimens for each species following methods described for *G. hubbsi*. Specimens examined for morphology included 46 *G. affinis* collected from three populations in Brazos County, Texas (∼200 mi from type locality; R. B. Langerhans, personal collection), 30 *G. heterochir* from Clear Creek, Texas (type locality; Texas Natural History Collection [TNHC] 24120), 39 *G. geiseri* from San Marcos Spring, Texas (type locality; TNHC 7030, 9146), and 17 *G. hurtadoi* from Ojo de la Hacienda Dolores, Mexico (type locality; TNHC 7298, University of Michigan Museum of Zoology [UMMZ] 211112). We examined all adult males within each collection.

Relative mating response and morphological distance were calculated as described for *G. hubbsi* with two exceptions: (1) relative mating responses for females were calculated using average values across the three measures of mating response, and (2) morphological distance between males and females were calculated using mean canonical values for each species, rather than individual-level measures of body morphology. We examined the relationship between relative mating response and morphological distance using linear regression. This analysis used species-pair presentations during mate-choice trials as blocks (i.e., three possible species-pair presentations for each species), treating separate trials from the same species as independent; thus, we also conducted linear regressions within each species and combined probabilities using the *Z*-transform test to provide an analysis using species as replicates.

We used published sequences of the mitochondrial cytochrome *b* gene (402 bp) (Lydeard et al. 1995) to examine whether phylogeny might have influenced analyses involving these four species. Specimens for each species came from either the same population (type localities; *G. geiseri*, *G. heterochir*, *G. hurtadoi*) or the same county (Travis County, Texas; *G. affinis*) as those used in the mate-choice experiment. To examine whether species inhabiting the same predator regime were more closely related to one another than to species inhabiting different predator regimes, we calculated genetic distances among species (using Tamura-Nei genetic distances).

ECOLOGICAL SPECIATION IN *GAMBUSIA*

To provide overall tests of ecological speciation in *Gambusia* fishes, we used two approaches that combine results from both scales of analysis (i.e., intraspecific and interspecific). First, we used populations/species as replicates ($n = 8$; four *G. hubbsi* populations and four *Gambusia* species) and performed a paired *t*-test comparing average assortative mating values (AMI) for

Table 1. Nested multivariate analysis of covariance (MANCOVA) and discriminant function analysis (DFA) results examining body shape variation (uniform components and partial warps) among populations of *Gambusia hubbsi*. *F*-ratios were approximated using Wilks's lambda values for the population nested within predator regime term. DFA results reflect the percent of fish correctly classified to predator regime using jackknife sampling. The interaction between centroid size and predator regime was included in models when significant.

	Morphological dataset	*N*	Centroid size			Predator regime			Pop (predator regime)			DFA results
			F	df	*P*	*F*	df	*P*	*F*	df	*P*	
1	2004 Radiographs for 12 blue holes, males only	199	5.71	16, 170	<0.0001	19.98	16, 170	<0.0001	4.96	160, 1470.9	<0.0001	88.4%
2	2004, 2005 Radiographs for four blue holes, both sexes	408	73.26	16, 387	<0.0001	61.44	16, 387	<0.0001	15.27	32, 774	<0.0001	90.4%
3	2005 Live images for four blue holes, both sexes	77	4.68	16, 56	<0.0001	14.40	16, 56	<0.0001	2.86	32, 112	<0.0001	94.8%

similar versus different predator regimes. Second, we employed a recently described approach (Funk et al. 2002, 2006) using population/species pairs as replicates ($n = 6$ for each scale; six possible pair combinations for *G. hubbsi* populations and for *Gambusia* species) and conducted correlation analyses within each scale examining the relationship between AMI and ecological distance (i.e., 0 or 1 representing either the same or different predator regimes). At each scale, we statistically controlled for genetic distance (using estimates described above) between population/species pairs by using residuals of AMI and residuals of ecological distance, after removing any possible effects of genetic distance. We employed both parametric (Pearson correlation) and nonparametric (Spearman rank correlation) approaches. For significance testing, we performed three-way Mantel tests because significance tests from correlation approaches improperly treat population/species pairs as independent datapoints. These analyses evaluated the correlation between two matrices (AMI and ecological distance), while statistically holding a third matrix constant (genetic distance; Smouse et al. 1986; Manly 1991; Legendre and Legendre 1998; Thorpe 2002; Harmon et al. 2005; Langerhans et al. 2006). We combined probabilities from these partial Mantel tests using the *Z*-transform test. These tests provide overall assessments of the hypothesis that ecology and premating isolation are associated in *Gambusia*.

Results

MORPHOLOGICAL ANALYSES

Although body size of mosquitofish is similar between divergent predator regimes (ANOVA, $P > 0.18$ for all morphological datasets), body *shape* significantly differs (Fig. 3; see online Supplementary Material Fig. S1 in for results with datasets 2 and 3). Using a discriminant analysis, the vast majority of fish can be correctly assigned to their predator regime of origin based on body morphology (Table 1). Fish inhabiting low-predation environments exhibited a smaller caudal peduncle (9–17% smaller lateral area, depending on dataset) and larger head (4–6% larger lateral area, depending on dataset) than fish in high-predation environments. These results match our a priori predictions of divergent natural selection on body shape.

mtDNA ANALYSES

All mtDNA haplotypes detected were closely related (mean percent nucleotide divergence, 0.26%), with no evidence suggesting that different populations inhabiting the same predator regime are more closely related to one another than to populations in the alternative predator regime (Fig. 4). Genetic variation was not significantly associated with predator regime, but rather nearly all of the genetic variance was ascribed to variation among populations within predator regimes and within populations (Table 2). The observed overall F_{ST} value of 0.60 indicates considerable divergence and limited gene flow among populations, consistent with previous allozyme analysis (Schug et al. 1998).

Mantel tests revealed that genetic distance exhibited no association with ecological distance (i.e., same or different predator regimes; $r = -0.05$, $P = 0.9223$) or morphological distance ($r = 0.02$, $P = 0.4601$), although morphological distance and ecological distance were strongly correlated ($r = 0.78$, $P = 0.0026$). These results indicate that replicated evolution of similar phenotypes in similar environments provides a much better explanation (in the statistical sense) for morphological evolution than a shared ancestry. We further found a weak, but nonsignificant, relationship between genetic distance and geographic distance ($r = 0.26$, $P = 0.1237$), suggesting a slight trend of isolation-by-distance among blue hole populations. Morphological distance and geographic distance were not associated ($r = -0.09$, $P = 0.7386$). Together, these results indicate that predator regime—and not genetics or geography—best predicts morphology.

ALLOZYME ANALYSES

Locus-by-locus AMOVA revealed that variation in allozyme allele frequencies was not significantly associated with variation

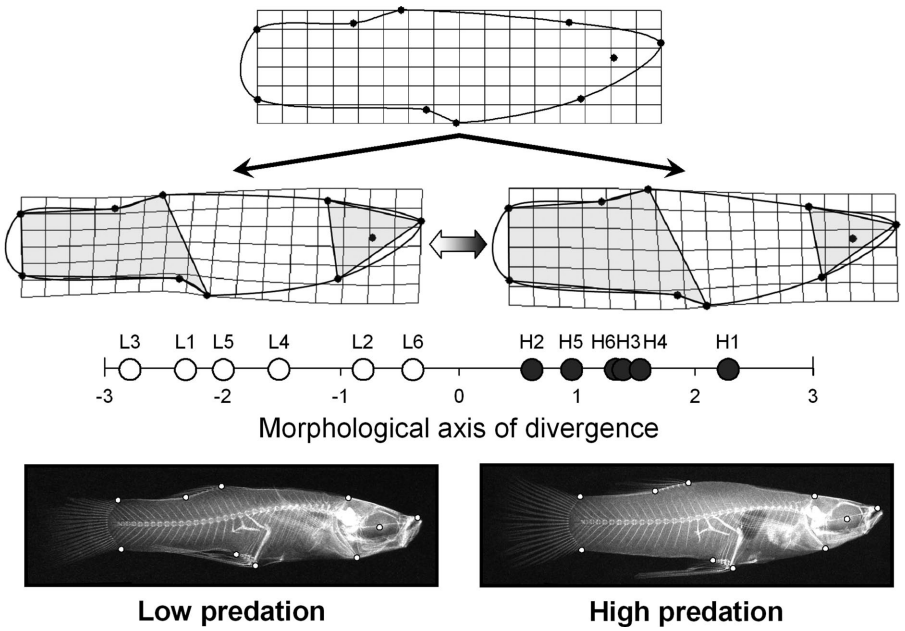

Figure 3. Morphological divergence between low- and high-predation blue hole populations of *Gambusia hubbsi*. Body shape variation described by the canonical variate axis derived from MANCOVA, illustrated using thin-plate spline transformation grids relative to mean landmark positions (observed range of variation depicted). Solid lines connecting outer landmarks are drawn to aid interpretation. Lateral areas of the caudal peduncle and head are highlighted to emphasize major differences matching a priori predictions. Circles along the canonical axis represent population means (open: low-predation, filled: high-predation; labels follow Fig. 2). Radiographs of low- and high-predation individuals are provided below the axis (individuals selected near the lower and upper 5% of canonical variate distribution). Results depicted examine x-ray radiographs of male *G. hubbsi* from 12 blue holes (see statistical results in Table 1); detailed analysis of four blue holes revealed consistent results among years, sexes, and image type (radiograph vs. standard photograph) (see online Supplementary Material Fig. S1).

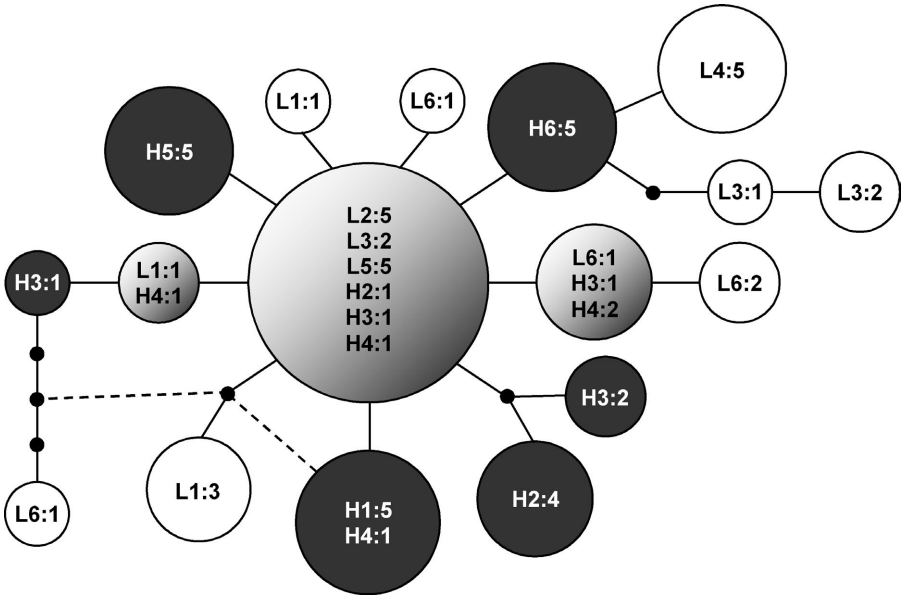

Figure 4. mtDNA haplotype network. The network is based on 60 mtDNA sequences (five individuals from each of 12 blue holes), and is shaded according to predator regime: low-predation (open), high-predation (filled), and both low- and high-predation populations (gradient shaded from open to filled). The number of specimens from each population is provided within each haplotype (population labels follow Fig. 2). Circle sizes reflect the frequency of each haplotype in the dataset. Small black circles indicate unobserved haplotypes, each solid line connecting haplotypes represents a single nucleotide substitution, and dashed lines represent equally parsimonious linkages among haplotypes. Genetic variance was not associated with predator regime, but was attributable to variation among populations within predator regimes and within populations (see Table 2).

Table 2. Analysis of molecular variance (AMOVA) based on mtDNA. Percentage of variation, *P*-values, and *F*-statistics were calculated according to Excoffier et al. (1992). All *F*-statistics are intraclass correlations. F_{CT} is the correlation for random pairs of haplotypes within a predator regime, relative to that of random pairs of haplotypes drawn from the whole system. F_{SC} is the correlation for random pairs of haplotypes within populations, relative to that of random pairs of haplotypes drawn from the same predator regime. F_{ST} is the correlation for random pairs of haplotypes within populations, relative to that of random pairs of haplotypes drawn from the whole system.

Source of variation	df	% of variation	*P*	*F*-statistic
Among predator regimes	1	3.02	0.2063	F_{CT}=0.03
Among populations within predator regimes	10	56.86	<0.0001	F_{SC}=0.59
Within populations	48	40.12	<0.0001	F_{ST}=0.60
Total	59			

among predator regimes (all $P > 0.15$), but rather was typically attributable to variation among populations within predator regimes and within populations (both terms, $P < 0.05$ for 13 of 17 loci). Further, no principal component showed significant effects of predator regime (ANOVA, all $P > 0.098$). These results provide no evidence that populations inhabiting the same predator regime are more closely related to one another than to populations in the alternative predator regime. Note that allozyme data do not provide any evidence for isolation-by-distance, as genetic distance based on allozymes is not associated with geographic distance (Schug et al. 1998).

MATE-CHOICE EXPERIMENT

In our tests of assortative mating, we found that females exhibited significant preference for native males when given a choice between males from similar predator regimes (Wilcoxon's signed-rank test, $z = 113.5$, one-tailed $P = 0.0057$; 55% greater mating response for native male) or different predator regimes (Wilcoxon's signed-rank test, $z = 130.0$, one-tailed $P = 0.0008$; 212% greater mating response for native male). Using populations as replicates, we also found significant assortative mating between similar environments (weighted *Z*-transform test, one-tailed $P = 0.0425$; 66% greater mating response for native male) and between different environments (weighted *Z*-transform test, one-tailed $P = 0.0152$; 251% greater mating response for native male).

We further found significant evidence for ecologically associated premating isolation in Bahamas mosquitofish, regardless of whether we used females or populations as replicates. Across all females, assortative mating was, on average, 122% stronger between different predator regimes than between similar predator regimes (paired *t*-test, $t = 1.91$, df = 23, one-tailed $P = 0.0344$; Fig. 5A), providing strong support for the ecological speciation hypothesis. Across populations, we also found a significant

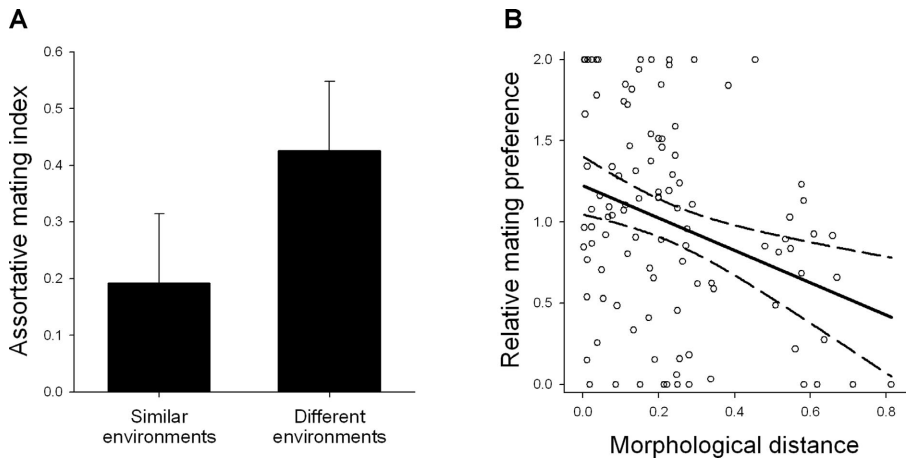

Figure 5. Female mate preference in *Gambusia hubbsi*. (A) Strength of assortative mating (preference for native male) is stronger between divergent predator regimes than between similar predator regimes (mean ± 1 standard error presented; paired *t*-test, one-tailed $P = 0.03$). This test compared assortative mating index values (an index that can range from −1, complete preference for the foreign male, to +1, complete preference for the native male; see details in text) between trials of two types (videos of males from either similar or different predator regimes) for each female. (B) Assortative mating based on body shape (linear regression, one-tailed $P = 0.0007$). Datapoints represent relative mating response values for each male video within each trial (all trials presented). Slopes were negative within each population (i.e., homogenous slopes, ANCOVA, $P = 0.66$), and the trend persists when examining only one trial per female or when combining probabilities across populations (see text for details). The solid line represents the regression line, and the dashed lines indicate the 95% confidence interval of the regression line.

Table 3. Average levels of assortative mating (preference for native/conspecific individual) for each *Gambusia hubbsi* population (population labels follow Fig. 2) and *Gambusia* species (LP=low predation, HP=high-predation) when given the choice between individuals of the opposite sex derived from either similar or different predator regimes. See text for details regarding the assortative mating index.

Scale of analysis	Population/ Species	Assortative mating index	
		Same predator regime	Different predator regime
Intraspecific	L1	0.49	0.58
	L2	0.11	0.34
	H1	−0.01	0.13
	H2	0.22	0.58
Interspecific	*G. geiseri* (LP)	0.95	0.85
	G. hurtadoi (LP)	0.76	0.81
	G. affinis (HP)	0.17	0.70
	G. heterochir (HP)	0.03	0.39

trend for stronger assortative mating between populations inhabiting different predator regimes than between populations in similar predator regimes (weighted Z-transform test, one-tailed $P = 0.0397$; see Table 3 for average AMI values). In the latter case, premating isolation was, on average, 100% stronger between different predator regimes than between similar predator regimes.

If assortative mating is based on the same traits under divergent selection (i.e., body shape), this would indicate that premating isolation has largely evolved as a by-product of natural selection. Indeed, relative mating response was significantly associated with morphological distance (linear regression, $\beta = -1.0$, one-tailed $P = 0.0007$, $R^2 = 0.10$; Fig. 4B). Because many females were represented by multiple trials in this analysis (i.e., causing possible pseudoreplication), we further conducted our analyses with reduced samples sizes including only one trial per female according to four separate criteria. We found similar results using only the first trial conducted with each female (linear regression, $\beta = -0.7$, one-tailed $P = 0.0623$, $R^2 = 0.04$), only the second trial (linear regression, $\beta = -1.3$, one-tailed $P = 0.0014$, $R^2 = 0.18$), only the trial with the maximal mating response (linear regression, $\beta = -0.6$, one-tailed $P = 0.0557$, $R^2 = 0.04$), and only the trial with the minimal mating response (linear regression, $\beta = -1.3$, one-tailed $P = 0.0026$, $R^2 = 0.12$). This suggests that the observed significant relationship between relative mating response and morphological distance is not due to artificially inflated statistical power caused by pseudoreplication at the level of individual females. Thus, females tend to exhibit greater mating preference for males possessing a body shape similar to their own. In contrast, there was no relationship between relative mating response and body *size* (linear regression, $P = 0.72$).

We found a consistent trend across populations when we combined probabilities of linear regressions conducted within each population (weighted Z-transform test, mean $\beta = -1.0$, one-tailed $P = 0.0021$), indicating that females across populations tend to exhibit mating preferences for morphologically similar males. Moreover, we found evidence that within-population mating behaviors might have produced the observed sexual isolation among populations as a by-product. That is, examining only mating responses for native males from trials in which males from the same predator regime were presented to females, we still found a consistent relationship between relative mating response and morphological distance across all populations (weighted Z-transform test, mean $\beta = -2.1$, one-tailed $P = 0.0197$). This preference for morphologically similar males was observed despite the fact that such a pattern might be difficult to detect because there is a relatively small difference in morphology among males from similar predator regimes, and because sample sizes are reduced when only examining this smaller subset of mating trials. Although detailed studies within populations are needed to more thoroughly examine within-population mating behaviors, these results suggest that females within each population tend to prefer mates with morphologies similar to their own, leading to increased levels of reproductive isolation between populations exhibiting divergent body shapes.

PREMATING ISOLATION AMONG *GAMBUSIA* SPECIES

Combining probabilities from Hubbs and Delco (1960), we found significant assortative mating between species from similar environments (Z-transform test, one-tailed $P = 0.0411$; 117% greater mating response for conspecific female) and between species from different environments (Z-transform test, one-tailed $P = 0.0006$; 231% greater mating response for conspecific female).

Treating species-mating behavior combinations as replicates ($n = 12$; three estimates of premating isolation based on different mating behaviors for each of four species), we found that assortative mating for conspecific individuals was, on average, 45% stronger among species inhabiting different predatory environments than among species inhabiting similar predator regimes (paired t-test, $t = 2.44$, df $= 11$, $P = 0.0165$), consistent with the hypothesis of ecologically associated premating isolation. Although this test treated species-mating behavior combinations as replicates, the results are supported when alternatively using species as replicates (Z-transform test, one-tailed $P = 0.0203$; see Table 3 for average AMI values).

As in the case with *G. hubbsi*, morphological divergence between environments appears to largely explain this pattern, as body

shape consistently differs between *Gambusia* species inhabiting different predator regimes (MANCOVA, $F_{16,111} = 34.46$, $P < 0.0001$; DFA correctly assigned 98.5% of fish to predator regime of origin), and relative mating response is significantly associated with morphological distance (linear regression, $\beta = -2.0$, one-tailed $P < 0.0001$, $R^2 = 0.64$). Combining probabilities from linear regressions conducted within each species, we also found a consistent relationship between relative mating response and morphological distance across species (*Z*-transform test, mean $\beta = -2.3$, one-tailed $P < 0.0001$). These results suggest that premating isolation between species has partially resulted as a by-product of divergent selection on body shape. As could be expected by their longer times since divergence (and thus greater genetic distances), premating isolation between *Gambusia* species tended to be greater than between *G. hubbsi* populations (see Table 3); although the signature of divergent selection's role in enhancing premating isolation is present in both cases.

Pairwise genetic distances were not greater for species inhabiting different predator regimes (mean percent sequence divergence $\pm$ 1 standard error, 9.4% $\pm$ 1.9) than for species inhabiting the same predator regime (8.7% $\pm$ 1.4). In support of this result, the current hypothesis for the *Gambusia* phylogeny indicates that different species inhabiting the same predator regime do not form monophyletic groups (Lydeard et al. 1995). This suggests that phylogenetic relatedness did not confound any of our results.

ECOLOGICAL SPECIATION IN *GAMBUSIA*

Across both scales of analysis (i.e., intraspecific and interspecific), we found significant evidence for ecological speciation in *Gambusia*: (1) assortative mating for each population/species tended to be stronger between different predator regimes than between similar ones (paired *t*-test, $t = 2.91$, df = 7, one-tailed $P = 0.0114$; Table 3), and (2) assortative mating tended to be greater for population/species pairs in different predator regimes than similar ones, controlling for genetic distance (*Z*-transform test, one-tailed $P = 0.0127$; mean $r = 0.61$, mean $\rho = 0.51$; Fig. 6).

Discussion

Through the combination of the "natural experiment" conditions of Bahamian blue holes and our integrative examination of morphological, molecular, and behavioral data, this study provides one of the strongest tests to date for ecological speciation via the by-product mechanism in the wild. Altogether, our results are consistent with ongoing ecological speciation among Bahamas mosquitofish populations. First, marked morphological differences between ecologically divergent blue holes match predictions based on divergent natural selection, supporting previous evidence for strong divergent selection between predator regimes in *G. hubbsi* (Krumholz 1963; Sohn 1977; Downhower et al. 2000; Langerhans et al. 2005; Langerhans 2006). Second, molecular analyses demonstrate that phenotypic differences between populations are best predicted by predator regime, not genetic relatedness, suggestive of replicated trait evolution in multiple independent populations. Finally, premating isolation has apparently evolved largely as a by-product of divergent selection on morphology, where assortative mating for body shape results in

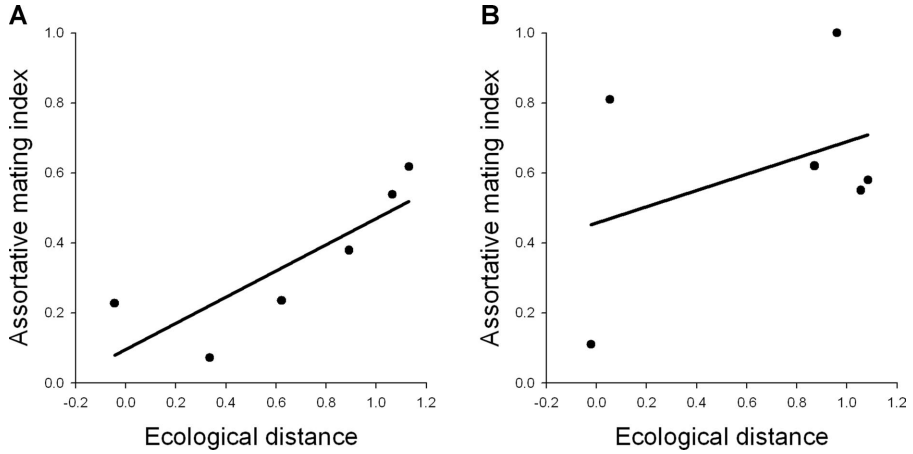

Figure 6. Results for both scales of analysis using population/species pairs to examine the relationship between the assortative mating index and ecological distance (same or different predator regime), controlling for genetic distance. Premating isolation tended to exhibit a positive correlation with ecological distance (A) among blue holes in *G. hubbsi* ($r = 0.83$; $\rho = 0.94$) and (B) among *Gambusia* species ($r = 0.40$; $\rho = 0.09$). Combining probabilities (from partial Mantel tests) across the two scales reveals a significant association (*Z*-transform test, one-tailed $P = 0.0127$). Datapoints in figures represent back-transformed residuals, controlling for genetic distance among population/species pairs. Note that using morphological distance in place of ecological distance produces similar, although marginally significant, results (*Z*-transform test, one-tailed $P = 0.0986$; mean $r = 0.51$, mean $\rho = 0.49$).

greater sexual isolation between ecologically divergent pairs of populations than ecologically similar ones. Moreover, it appears that this mechanism of speciation may be historically important in *Gambusia*, as the signature of such ecological speciation was observed among four *Gambusia* species. This suggests that the ongoing processes detected within *G. hubbsi* may have been instrumental in past speciation events in the genus.

DIVERGENT SELECTION DRIVES DIVERGENCE IN BODY SHAPE

The magnitude of morphological differences observed in this study between populations of *G. hubbsi* inhabiting divergent predator regimes has been shown to generate ecologically important differences in swimming performance in mosquitofishes (Langerhans et al. 2004; Langerhans 2006; R. B. Langerhans, unpubl. data). In comparison with another *Gambusia* species known to exhibit morphological differences among predator regimes (*G. affinis*), *G. hubbsi* displays a greater difference in caudal peduncle size (9–17% vs. 2–4%), but a smaller difference in head size (4–6% vs. 9–11%) between populations inhabiting divergent predatory environments (*G. affinis* data from Langerhans et al. 2004). The nature of this strong morphological divergence is consistent with a recently described "general ecomorphological prediction based on biomechanical principles: fish coexisting with piscivorous fish should evolve a larger caudal region and a shallower anterior body/head region" (Langerhans et al. 2004, p. 2314). This correspondence between evolutionary predictions based on first principles and empirical observations using comparative data strongly suggests that divergent natural selection is the primary causal mechanism (e.g., Endler 1986; Wainwright 1988, 1996; Losos 1990; Williams 1992; Walker 1997; Domenici 2003). The present study adds to the growing evidence that the observed pattern of morphological divergence (i.e., larger caudal region, smaller anterior body/head region in high-predation environments) represents a general ecomorphological paradigm (see Langerhans and DeWitt 2004; Langerhans et al. 2004), and more generally that predation plays a critical role in phenotypic divergence and speciation (e.g., Vermeij 1987; McPeek et al. 1996; Reznick 1996; Jiggins et al. 2001; Vamosi 2005; Nosil and Crespi 2006; Langerhans 2006).

Observed morphological differences between *G. hubbsi* populations are unlikely to merely reflect environmentally induced phenotypic variation, as morphological differences between mosquitofish species, and between populations within species, typically exhibit a strong genetic basis (e.g., Hubbs and Springer 1957; Greenfield et al. 1982; Greenfield 1983, 1985; Greenfield and Wildrick 1984; Langerhans et al. 2004, 2005; R. B. Langerhans, unpubl. data). Indeed, suggestive results were found using laboratory-born *G. hubbsi* from three populations examined in this study (one low-predation, two high-predation): individuals retained their morphological distinctiveness after eight weeks of rearing under common laboratory conditions ($n = 10$; using a discriminant function derived from wild fish, all laboratory-reared individuals were correctly assigned to their predator regime of origin, sign test $P = 0.0020$). These results are consistent with the numerous previous studies, and provide cautious support for the hypothesis that divergence in body shape between populations largely derives from genetic differentiation. A more detailed examination of the genetic basis and possible contribution of phenotypic plasticity to population differences in body morphology and swimming performance is currently underway for multiple *G. hubbsi* populations, as well as several other *Gambusia* species.

INDEPENDENT EVOLUTION AMONG PREDATOR REGIMES?

Neither mtDNA nor allozyme analyses provide evidence that mosquitofish in blue holes with the same predator regime are more closely related to one another than to populations in blue holes with the alternative predator regime. A potential problem often raised in such cases is that introgression between ecologically divergent populations might obscure a true signal of monophyly by environment (Coyne and Orr 2004). However, this is unlikely in the present case as gene flow appears restricted based on both mtDNA ($F_{ST} = 0.60$; this study) and allozymes ($F_{ST} = 0.38$; Schug et al. 1998), and both the history of sea-level change (implying recent colonization) and physical isolation of blue holes (implying little migration) are consistent with genetic results. Our results are consistent with replicated origins of similar phenotypes in similar predation environments. This scenario provides an ideal setting in which to test whether premating isolation has evolved in parallel with divergent phenotypes.

ENHANCED PREMATING ISOLATION AS A BY-PRODUCT OF ECOLOGICAL ADAPTATION

Mate-choice trials demonstrated that sexual isolation has indeed evolved in parallel with body shape. Although females typically preferred males from their native population over foreign males from any other population, premating isolation was strongest between populations with divergent predator regimes (and thus, divergent morphologies). Moreover, we found a consistent trend of assortative mating for body shape across all populations, indicating that mating preferences are based on the same traits under divergent selection. Our results suggest that features of sexual selection within populations can promote sexual isolation between populations, a process recently receiving both empirical corroboration and contradiction (e.g., Wiernasz and Kingsolver 1992; Boake et al. 1997; Blows and Allan 1998; Ptacek 2000; Panhuis et al. 2001; Maan et al. 2004; Boughman et al. 2005). Further examination of mating preferences within multiple *G. hubbsi* populations will be required to more fully address the

intricacies of how within-population mating preferences might produce isolating mechanisms between populations (see Boake 2002; Schwartz and Hendry 2006).

Due additionally to the likely importance of natural selection against migrants—which would further reduce fitness of individuals transplanted into the alternative environment beyond that incurred from the loss of mating opportunities—reproductive isolation may be quite strong between *G. hubbsi* populations inhabiting different predator regimes (Hendry 2004; Nosil et al. 2005; R. B. Langerhans, unpubl. data). Based on the depth of blue holes examined in this study, these localities were dry caves prior to 15,000–4000 years ago (Fairbanks 1989; deeper blue holes began to fill with water earlier). Thus, our results suggest that reproductive isolation can rapidly evolve as a by-product of ecological adaptation before the occurrence of any reinforcement.

ECOLOGICAL SPECIATION IN *GAMBUSIA*

Our investigation of Bahamas mosquitofish suggests the possible parallel evolution of sexual isolation between populations inhabiting divergent predator regimes. We further found the fingerprint of such ecological speciation when examining interspecific data in the genus *Gambusia*. These results suggest that the microevolutionary processes examined in this study—divergent selection and assortative mating—can produce interspecific trends that persist long after speciation events have completed. Further examination of divergent selection between predator regimes at the genus-wide scale should provide important insight into the historical significance of predation-mediated divergent selection on mosquitofish diversification.

Owing to the remarkable opportunity offered by the "natural experiment" of Bahamian blue holes, we present strong evidence that predation can play a critical role in the early stages of speciation. We further elucidate the specific traits under divergent selection, which consequently drive reproductive isolation as a by-product. For many organisms, divergent selection between environments often targets morphology because of its intimate relationship with ecological performance (e.g., Arnold 1983; Wainwright and Reilly 1994; Schluter 2000). If assortative mating based on simple morphological attributes, such as body size, color, or shape, is common in nature—which accumulating evidence suggests may be the case (e.g., Jiggins et al. 2001; Cruz et al. 2004; Maan et al. 2004; Boughman et al. 2005; Schwartz and Hendry 2006)—then speciation via this by-product mechanism may be a frequent phenomenon.

ACKNOWLEDGMENTS

We thank R. Albury and the Department of Fisheries of the Bahamas Government for permission to conduct this work; M. Blackwell and the Bahamas Environmental Research Center (BERC) for support in the field; A. Langerhans, C. Layman, and the BERC field assistants for help collecting specimens; M. Sobotka and D. Oran for laboratory assistance; and D. Funk, J. Johnson, J. Losos, and three anonymous reviewers for comments on a previous draft. This work was funded by the Environmental Protection Agency, National Science Foundation, Society of Systematic Biologists, American Museum of Natural History, Explorers Club, American Society of Ichthyologists and Herpetologists, and Society of Wetland Scientists.

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Associate Editor: D. Funk

Appendix

Study site information (population labels follow Fig. 2; L = low predation, H = high predation). Salinity and transparency for the four focal blue holes (in bold text) represent averages of measurements conducted in three separate years (2004, 2005, 2006); all other measurements were conducted only once in 2004, or as otherwise described below. Both salinity and transparency were highly repeatable across years (salinity: intraclass correlation coefficient, $r = 0.86$, $P = 0.0005$; transparency: $r = 0.90$, $P = 0.0002$). Surface diameter was calculated using latitude/longitude values along the shoreline at each cardinal direction. Maximum depth was taken from the literature (Proudlove 1984; Brown and Downhower 1993; Schug et al. 1998; Gluckman and Hartney 2000) for all blue holes except L1, L3, L4, and H6, which we measured using a drop line. None of the four abiotic variables significantly differed between predator regimes (ANOVA, all $P > 0.05$). We also measured pH and dissolved oxygen content for all blue holes, however these variables were very similar among sites, with greater variance within blue holes (across time) than between them; thus, we do not present those values. Fish species other than *Gambusia hubbsi* observed in blue holes were *Cyprinodon variegatus* (sheepshead minnow), *Lophogobius cyprinoides* (crested goby), *Poecilia latipinna* (sailfin molly), *Eucinostomus* sp. (mojarra sp.), *Gerres cinereus* (yellowfin mojarra), *Gobiomorus dormitor* (bigmouth sleeper), and *Strongylura notata* (redfin needlefish). Two of these species are piscivorous (*G. dormitor*, *Strongylura notata*), whereas all other species primarily consume algae, detritus, and small invertebrates (e.g., Randall 1967; Robins and Ray 1986; Motta et al. 1995; Bacheler et al. 2004; R. B. Langerhans, unpubl. data).

Blue hole	Salinity (ppt)	Transparency (m)	Diameter (m)	Depth (m)	Other fish species present
L1	0.30	6.5	52	12	*C. variegatus*
L2	3.89	8.2	102	50	*C. variegatus*
L3	1.21	1.6	10	6	–
L4	0.89	2.1	22	>16	–
L5	3.50	4.4	69	35	*C. variegatus*
L6	1.37	20.0	117	87	*C. variegatus*, *P. latipinna*, *L. cyprinoides*
H1	0.56	9.7	79	101	*G. dormitor*
H2	0.77	15.9	66	52	*G. dormitor*
H3	1.82	7.7	189	20	*C. variegatus*, *Eucinostomus* sp., *G. cinereus*, *G. dormitor*, *S. notata*
H4	0.48	2.1	122	15	*G. dormitor*
H5	0.00	14.4	62	50	*G. dormitor*
H6	1.40	8.9	161	>16	*C. variegatus*, *G. dormitor*

Supplementary Material

The following supplementary material is available for this article:

Figure S1. Morphological divergence between low- and high-predation environments in *Gambusia hubbsi* for the four focal blue holes.

Table S1. Sample sizes of *Gambusia hubbsi* for morphological and molecular analysis.

Table S2. GenBank accession numbers for each unique mitochondrial ND2 haplotype of *Gambusia hubbsi* observed in this study.

Table S3. Sample sizes for mate-choice trials conducted for each population.

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SUPPLEMENTARY MATERIAL FOR:

ECOLOGICAL SPECIATION IN *GAMBUSIA* FISHES

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Supplementary Material Included in File:
Online Table 1
Online Table 2
Online Table 3
Online Figure 1

ONLINE TABLE 1. Sample sizes of *Gambusia hubbsi* for morphological and molecular analysis. Population labels follow Figs. 1-4 in the text. Sample sizes for females and males are denoted by (F) and (M) respectively. Populations in bold text are the four focal blue holes in which mating preferences were examined.

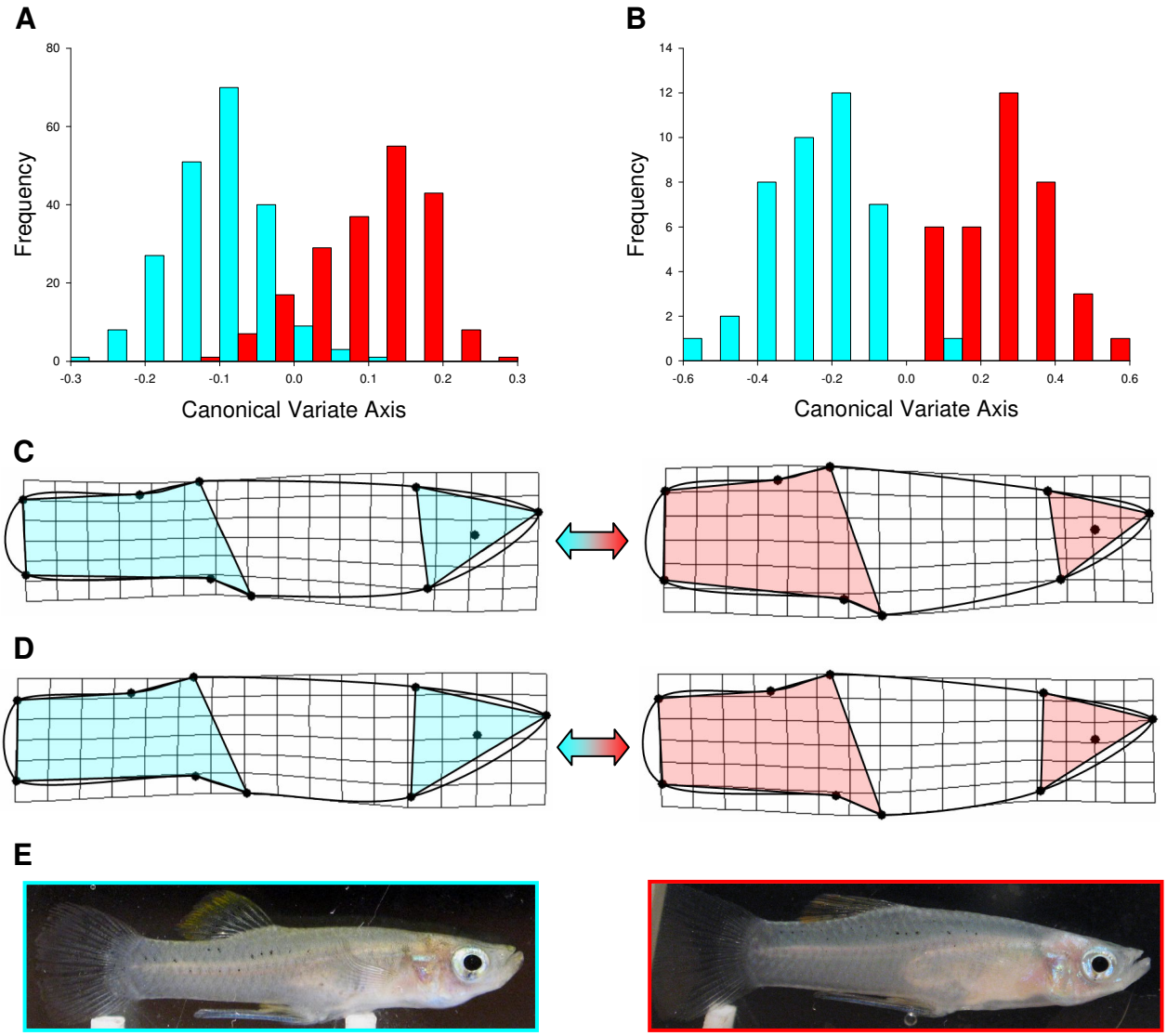
	Morphological Dataset			
	1	2	3	
Population	2004 radiograph males	2004, 2005 radiograph both sexes	2005 live images both sexes	mtDNA (ND2, 886 bp)
L1	15	51 (F), 43 (M)	11 (F), 9 (M)	5
L2	30	50 (F), 66 (M)	11 (F), 10 (M)	5
L3	10			5
L4	9			5
L5	13			5
L6	9			5
H1	30	47 (F), 83 (M)	10 (F), 7 (M)	5
H2	6	43 (F), 25 (M)	10 (F), 9 (M)	5
H3	20			5
H4	17			5
H5	20			5
H6	20			5

ONLINE TABLE 2. GenBank accession numbers for each unique mitochondrial ND2 haplotype of *Gambusia hubbsi* observed in this study. Population labels follow Figs. 1-4 in the text.

Haplotype	Blue Hole(s)	Accession Number
1	L2, L3, L5, H2, H3, H4	EF534741
2	L6	EF534742
3	H5	EF534743
4	L1, H4	EF534744
5	H3	EF534745
6	L1	EF534746
7	L6	EF534747
8	H1, H4	EF534748
9	H2	EF534749
10	H3	EF534750
11	L6, H4	EF534751
12	L6	EF534752
13	H6	EF534753
14	L4	EF534754
15	L3	EF534755
16	L3	EF534756
17	L1	EF534757

ONLINE TABLE 3. Sample sizes for mate-choice trials conducted for each population (population labels follow Figs. 1-4 in the text). Each number gives the number of trials in which a video of a male from a particular population was presented to a female. All trials involved a choice between a native male and a foreign male from one of three alternative populations. Because native males were presented in all trials, the numbers on the diagonal also represent the total number of trials for each population.

	Male Population			
Female Population	L1	L2	H1	H2
L1	12	7	3	2
L2	10	20	6	4
H1	2	2	9	5
H2	5	4	7	16



ONLINE FIG. 1. Morphological divergence between low- and high-predation environments in *Gambusia hubbsi* for the four focal blue holes. Body shape variation is described by the canonical variate axis derived from each MANCOVA. (A) Frequency histogram of *G. hubbsi* individuals along the canonical variate axis derived using morphological dataset 2 (see Table 1). (B) Frequency histogram using morphological dataset 3 (see Table 1). Blue symbols represent low-predation populations, red symbols represent high-predation populations. Thin-plate spline transformation grids in (C) and (D) illustrate body shape variation in the negative (left; low-predation) and positive (right; high-predation) directions along each canonical axis; grid deformations are relative to mean landmark positions (observed variation depicted). Solid lines connecting outer landmarks are drawn to aid interpretation. (C) Thin-plate spline visualization of morphological variation described by the canonical axis in (A). (D) Similar visualization for the canonical axis in (B). Lateral areas of the caudal peduncle and head are highlighted to emphasize major differences matching *a priori* predictions. (E) Representative live photographs of males from low-predation (left) and high-predation (right) populations (individuals selected near the mean body shape for low- and high-predation environments).