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

Ecological Speciation Among Blue Holes in Mosquitofish

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Abstract

▼ 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. Morphological, molecular, and behavioral data are examined here to investigate ecology's role in incipient speciation for a post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting inland blue holes (water-filled, vertical caves). It is shown 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 independently evolved in similar ecological environments. These results suggest ongoing ecological speciation among blue holes, resulting as a by-product of divergence in ecologically important traits.

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Genetics and Ecology

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; Coyne and Orr, 2004; Dieckmann *et al.*, 2004; Gavrilets, 2004; Rundle and Nosil, 2005; Funk *et al.*, 2006). 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 examples where this may occur 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 where 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 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). This study tests the hypothesis of ecological speciation via the by-product mechanism using allopatric populations of a livebearing fish, and in so doing, further addresses 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 specific phenotypes influencing reproductive isolation between 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, livebearing) than for larger predatory fish, mosquitofish currently inhabit many blue holes, while 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; Langerhans, unpublished data). 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) because blue holes with divergent predator regimes do not systematically differ in abiotic environmental variables (Appendix 1).

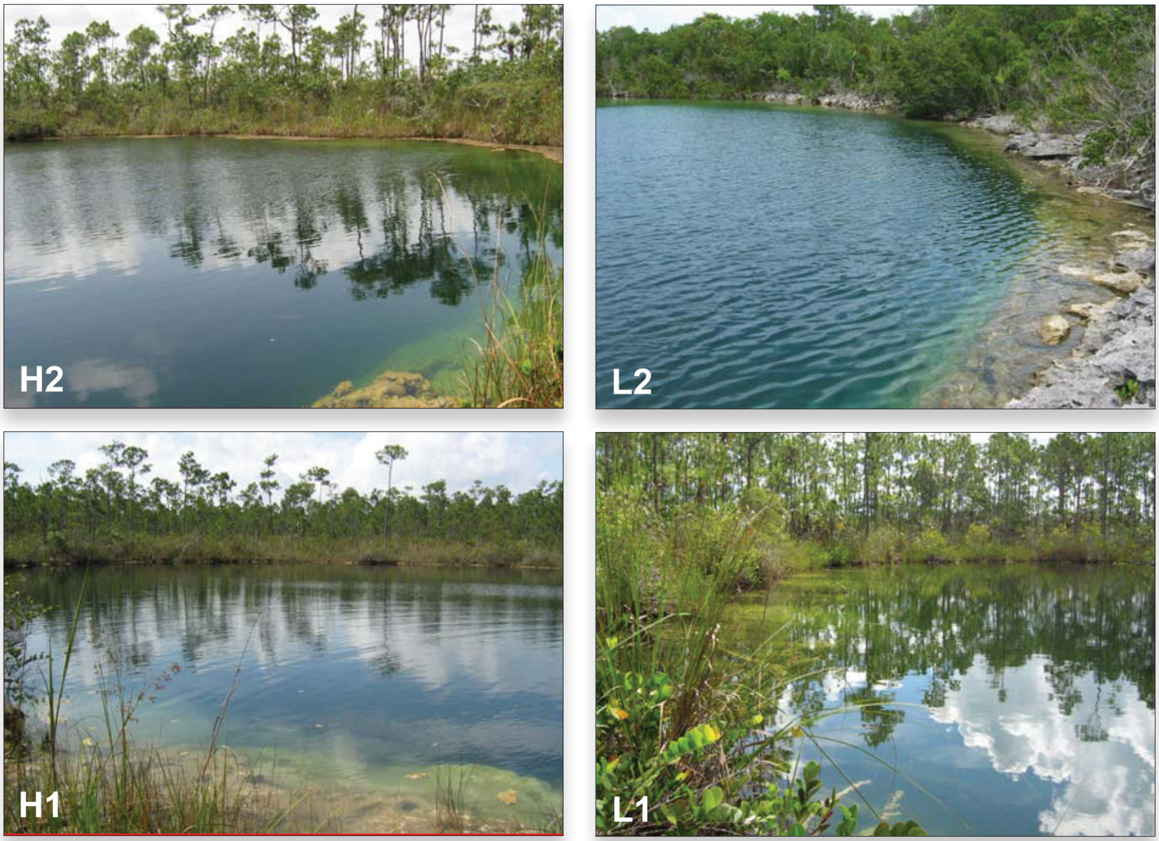

Figure 1. Four of the blue holes examined on Andros Island, The Bahamas. **A:** Blue holes without any piscivorous fish. **B:** Blue holes with the predatory fish *Gobiomorus dormitor*. Population names follow Fig. 2.

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 between 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, morphological data were used to test for divergent natural selection, molecular data to test for evolutionary independence among populations exhibiting similar phenotypes, and mate-choice trials were conducted to test for ecologically-associated premating isolation.

MATERIALS AND METHODS

Collections

More than 600 *G. hubbsi* individuals from a total of 12 blue holes (six with predators, six without) on Andros Island, the Bahamas (Fig. 2) were collected in August 2004 for examination. Additional fish were collected from four of these blue holes in August 2005 for use in the mate-choice experiment. Populations were classified "low-predation" or "high-predation" based on the absence or presence of the bigmouth sleeper., Detection of this predatory fish was easily accomplished using underwater visual observations because of high water clarity and the fish's active behavior.

While piscivorous fish serve as major predators of mosquitofish, avian predators pose an additional potential threat (Kushlan, 1973; Britton and Moser, 1982). Wading birds (*e.g.*, egrets, herons) are virtually excluded from these sites (Downhower *et al.*, 2000) because blue holes are steep-sided and deep. 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).

Morphological Analyses

Clear *a priori* predictions exist regarding divergent selection on mosquitofish morphology between predatory environments. Mosquitofish use body / caudal-fin propulsion for both prolonged and fast-start swimming modes (see Webb, 1984, Blake, 2004). Optimizing one swimming mode necessarily compromises the other, because this locomotor system is mechanically coupled (*i.e.*, same propulsors used for different swimming activities), and because prolonged and fast-start swimming are

APPENDIX 1.

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*

Study site information (population labels follow Fig. 2; L = low predation, H = high predation). Salinity and transparency for the four blue holes in bold text (*i.e.,* those used in the mate choice experiment) 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 were measured using a drop line. None of the four abiotic variables significantly differed between predator regimes (ANOVA, all $P > 0.05$). Dissolved oxygen content and pH was measured 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*), while all other species primarily consume algae, detritus, and small invertebrates (*e.g.,* Randall, 1967; Robins and Ray, 1986; Motta, 1995; Bacheler *et al.*, 2004; Langerhans, unpublished data).

optimized with different propulsor arrangements. Prolonged swimming performance is optimized with a relatively shallow caudal peduncle (see posterior shaded region in Figs. 3 and 4) 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; Langerhans *et al.*, 2005). Environments favoring alternative swimming modes should generate divergent selection on morphology due to this performance tradeoff. 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). Divergent selection on body shape in Bahamas mosquitofish was evaluated by testing for the predicted differences in morphology between blue holes with divergent predator regimes.

Three morphological datasets were utilized: 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 blue holes collected in 2005. Ten landmarks on each image (see Fig. 3) were digitized using the software program tpsDig (Rohlf, 2004a). Landmarks were selected to provide adequate coverage of the lateral body profile as previously described (*e.g.*, Langerhans and DeWitt, 2004; Langerhans *et al.*, 2004). Geometric morphometric analyses (*e.g.*, Rohlf and Marcus, 1993; Adams *et al.*, 2004; Zelditch *et al.*, 2004) were conducted using the digitized landmarks. 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), tpsRegr software (Rohlf, 2004b) was used. Superimposed landmark configurations were used to calculate geometric shape variables — uniform components and partial warps — describing affine and non-affine shape variation. Body shape variation among predator regimes was analyzed 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).

A discriminant function analysis (DFA) was conducted for each dataset to provide an intuitive metric regarding the magnitude of morphological divergence (*i.e.*, percent 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 tested *a priori* hypotheses concerning differences in caudal peduncle and head size by calculating caudal-peduncle area and head area using the convex polygon area of interconnected landmarks in those regions (see Figs. 3 and 4). Differences in caudal-peduncle area and head area between predator regimes, controlling for body size, were analyzed by 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. Analyses were conducted for all datasets, and percent differences in least-squares means between predator regimes calculated to provide quantitative metrics of the magnitude of divergence for particular traits.

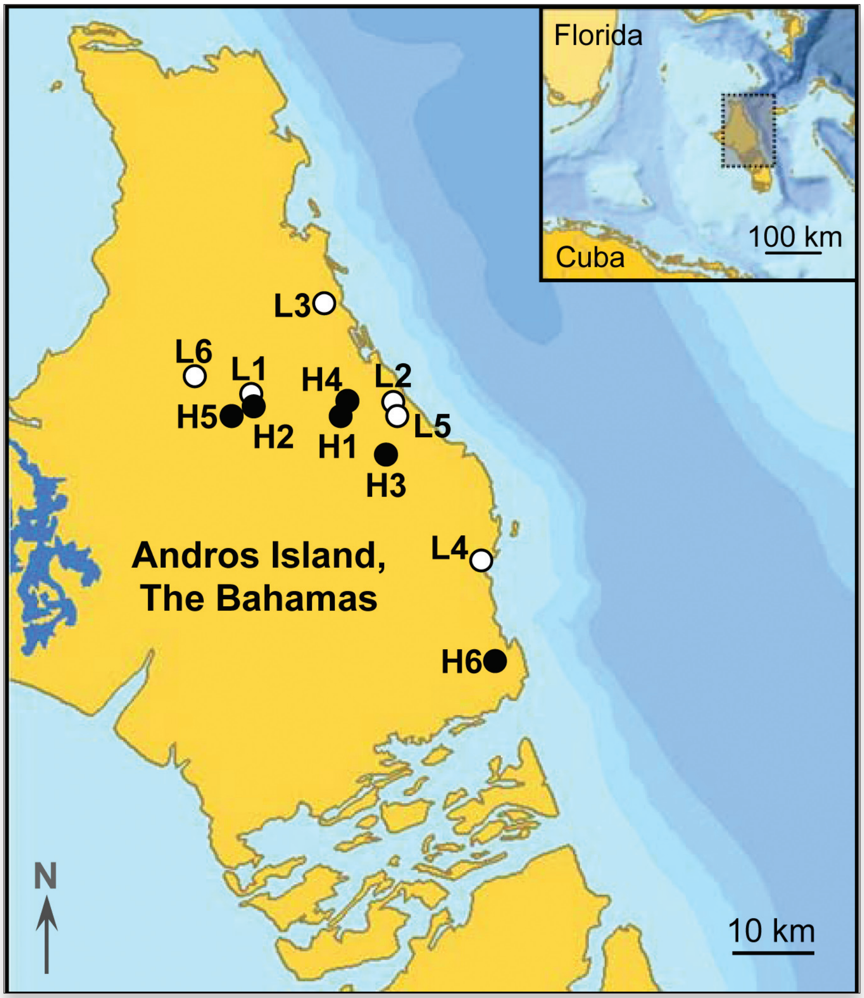

Figure 2.
Map of study sites. Low-predation (open circles; labels beginning with "L") and high-predation (filled circles; labels beginning with "H") blue holes where *G. hubbsi* were collected. Geographic distance between populations does not differ between blue holes with similar (mean ± 1 standard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

mtDNA Analyses

To test the alternative hypothesis that morphological similarities among populations in similar predator regimes reflect shared ancestry rather than replicated evolution, mtDNA sequences were examined for five *G. hubbsi* specimens from each of the 12 blue holes. A fragment (886 bp) of the NADH subunit 2 (ND2) gene was amplified 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

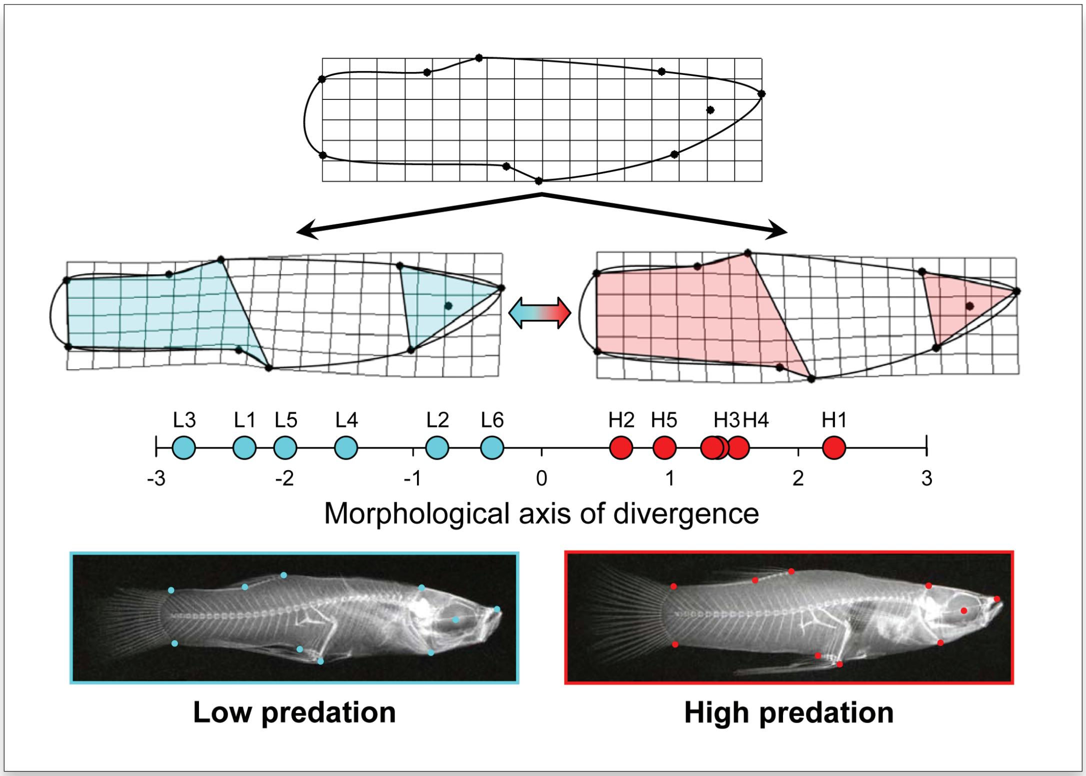

Figure 3.
Morphological divergence between low- and high-predation blue hole populations of *Gambusia hubbsi*. Body shape variation described by the canonical variate axis derived from MANCOVA, illustrated using thin-plate spline transformation grids relative to mean landmark positions (observed range 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 (blue: low-predation, red: 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 examined x-ray radiographs of male *G. hubbsi* from 12 blue holes (see statistical results in Table 1).

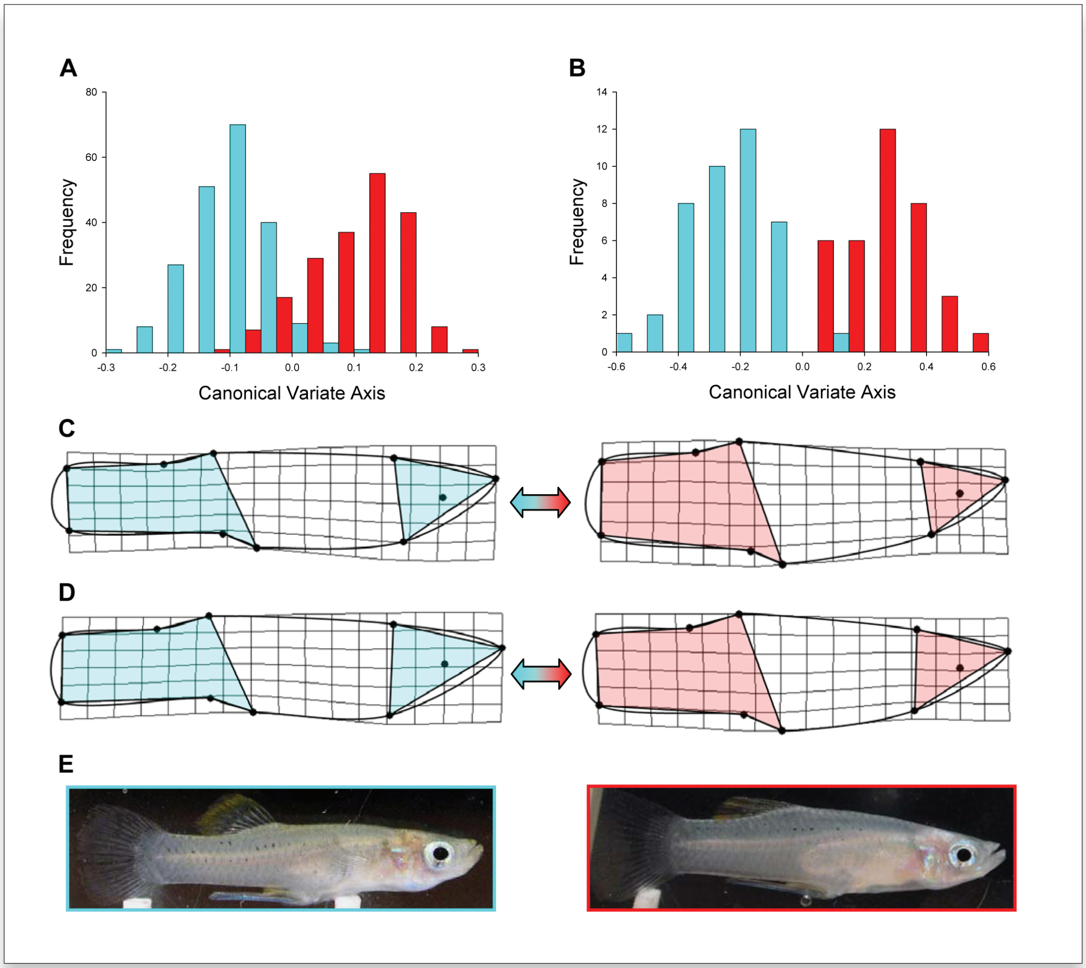

Figure 4.
Morphological divergence between low- and high-predation environments in *Gambusia hubbsi* for the four focal blue holes. Body shape variation is described by the canonical variate axis derived from each MANCOVA. **A:** Frequency histogram of *G. hubbsi* individuals along the canonical variate 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).

at 94°C for 120 seconds followed by 40 cycles of denaturation at 94°C for 35 seconds, annealing at 50°C for 35 seconds, and extension at 72°C for 90 seconds. The amplification protocol concluded with a final extension at 72°C for 300 seconds following the final cycle. Sequences were aligned by eye. No insertions or deletions were observed.

A haplotype network with the mtDNA sequences was constructed using the computer program TCS (Clement *et al.*, 2000), and an analysis of molecular variance (AMOVA) conducted 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.

The independence of ecological variables from patterns of genetic and geographic divergence was assessed using Mantel tests (Mantel, 1967). The tests examined 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), where 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, previously published allozyme allele frequencies were examined for 13 blue hole populations of *G. hubbsi* (6 low-predation, 7 high-predation; 17 polymorphic inferred loci, 47 total alleles) (Schug, 1995). Four of these populations are also studied here (L3, L6, H1, H2); predator-regime classifications for the remaining populations were taken from Downhower *et al.* (2000). For each locus, AMOVA was used to assess possible structuring of genetic variation between predator regimes, populations nested within predator regimes, and within populations. Population variation in multidimensional allele frequency space was investigated by performing principal components analysis (PCA) using all allele classes exhibiting ≥ 5% frequency in at least one population ($n = 37$ allele classes). ANOVA was performed with each PC to test for possible differences in allele frequencies between 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. Mating preferences of female *G. hubbsi* from four blue holes (two with predators, two without predators) were investigated using 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 randomly offered to each female in separate trials. For trials involving a foreign male from the opposite predator regime as the female, 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 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). A pilot study conducted with *G. hubbsi* prior to experimentation confirmed that females exhibit qualitatively similar mating responses with live fish compared to videos.

Videos were made 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. Males were carefully selected for use in video playback to minimize potentially confounding differences between males from different predator regimes other than body shape. Males were similar in body size (ANOVA, $F_{1,6} = 2.55$, $P = 0.16$), relative gonopodium size (ANCOVA, $F_{1,5} = 0.14$, $P = 0.73$), and behavior during the video segment (*i.e.*, average speed, maximum speed, cumulative displacement, maximum displacement; ANOVA, all $P > 0.27$), but differed greatly in body shape (ANOVA with canonical axis, $F_{1,6} = 44.06$, $P = 0.0006$).

Females were isolated from males 24 h before experimentation. For each trial, a female was placed into the mate-choice arena and acclimated for 10 min. Video playback was then initiated and 5 min allowed for the female to inspect the male videos. (If a female did not interact with either male during this time, she was removed and not further examined). Mating responses were recorded for 10 min, the left–right presentation order of the two video males reversed, the female 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. There was a total of 57 useful mate-choice trials because nine fish did not exhibit mating responses during both trials. 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]).

For statistical analyses, one-tailed P-values were used and are noted in the text wherever we have *a priori* directional hypotheses. Three hypotheses were tested: 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, two approaches were employed for analysis: 1) using females as the unit of replication, and 2) using populations as replicates. In this way, the consistency of results among populations was evaluated and potential concerns of pseudoreplication (*i.e.*, females from the same population may not be viewed as statistically independent) alleviated.

Assortative mating was tested using Wilcoxon's signed-ranks test, with females as blocks, conducted separately for comparisons between videos of males from similar ($n = 29$) and different environments ($n = 28$). For these tests, greater mating response (*i.e.*, proportional interaction time) for native males than foreign males was predicted. While these tests used females as replicates, analyses were also conducted 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), each test was weighted by the reciprocal of its squared standard error (see Whitlock, 2005). The percent difference in mating response between native and foreign males (*i.e.*, dividing the larger

TABLE 1.

			Centroid Size			Predator Regime			Pop (Predator Regime)			DFA
	Morphological Dataset	*n*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	Results
1	2004 Radiograph 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 Radiograph 4 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 4 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%

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.

average value by the smaller) was calculated to provide an intuitive metric regarding the magnitude of mating preferences.

To test for ecologically-associated premating isolation, an assortative mating index (AMI; equivalent to the "response index" of Johnson and Basolo [2003]) was calculated, and the strength of assortative mating between similar and different environments compared. 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. The index is not influenced by variation among females in overall mating propensity (cf. Casares *et al*., 1998). To test whether premating isolation was stronger between populations in different environments than between populations in similar ones, a paired *t*-test was used. 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. *T*-tests within each population were also conducted and probabilities combined 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, 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) was examined. Assortative mating for body *size*, rather than shape, was tested by examining the relationship between relative mating response and body size difference (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, linear regressions within each population were also conducted and probabilities combined using the weighted *Z*-transform test to provide an analysis using populations as replicates.

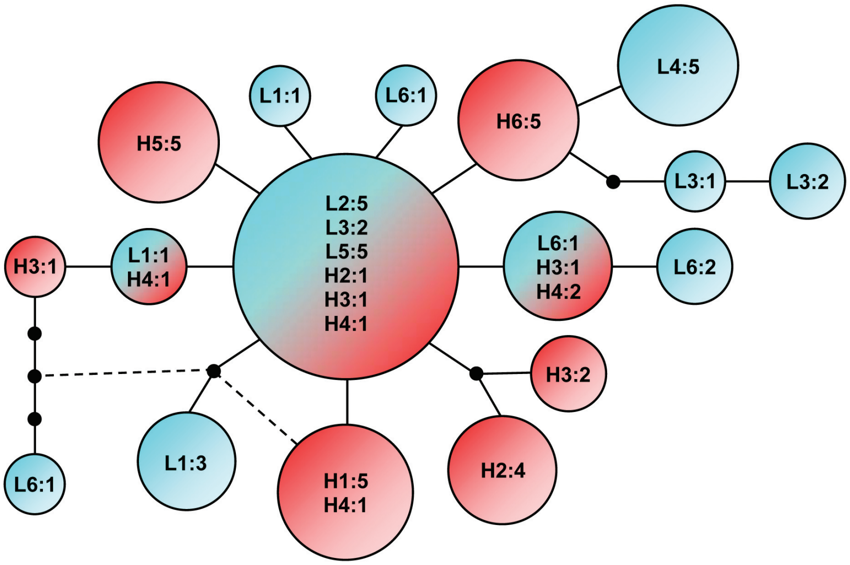

Figure 5.
mtDNA haplotype network. The network is based on 60 mtDNA sequences (5 individuals from each of 12 blue holes), and is shaded according to predator regime: low-predation (blue), high-predation (red), and both low- and high-predation populations (gradient shaded from blue to red). The number 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).

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 (Figs. 3 and 4). 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 are consistent with *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. 5). 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 shared ancestry. A weak, non-significant relationship was found 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 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, females exhibited significant preference for native males when given a choice between males from similar predator regimes (Wilcoxon's signed-ranks test, z = 113.5, one-tailed $P = 0.0057$; 55% greater mating response for native male) or different predator regimes (Wilcoxon's signed-ranks 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).

There was significant evidence for ecologically-associated premating isolation in Bahamas mosquitofish, regardless of whether females or populations were used 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. 6a), providing strong support for the ecological speciation hypothesis. Across populations, we also found a significant trend for stronger assortative mating between populations inhabiting different predator regimes than between populations in similar predator regimes (weighted Z-transform test, one-tailed

TABLE 2.

Source of variation	df	% of variation	P	F-statistic
Among predator regimes	1	3.02	0.2063	FCT = 0.03
Among populations within predator regimes	10	56.86	< 0.0001	FSC = 0.59
Within populations	48	40.12	< 0.0001	FST = 0.60
Total	59			

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.

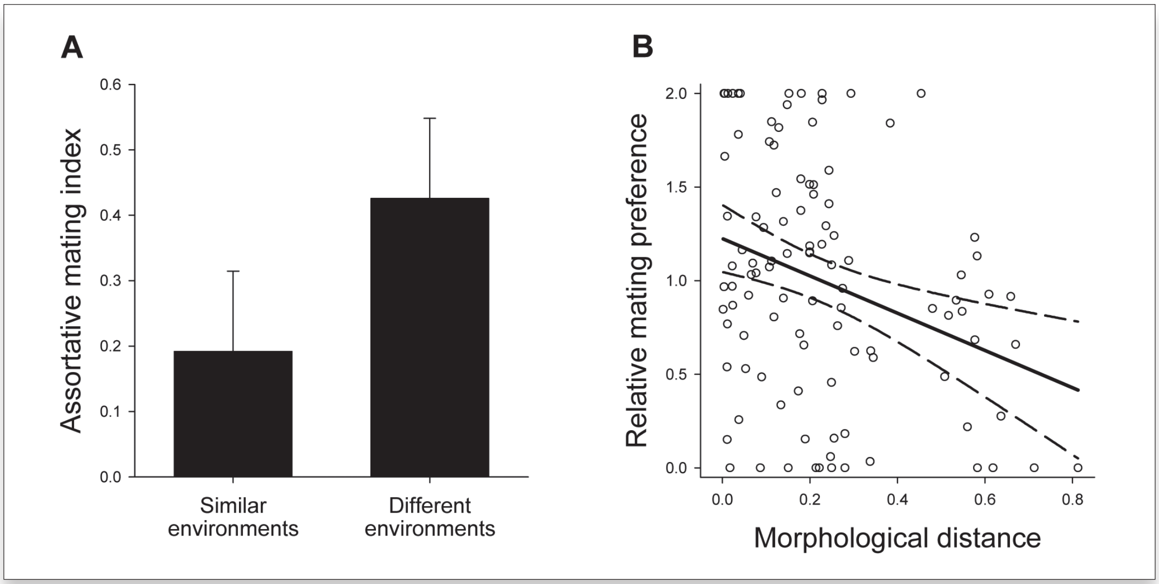

Figure 6. Female mate preference in *G. hubbsi*. **A:** Strength of assortative mating (preference for native male) is stronger between divergent predator regimes than between similar predator regimes (mean ± 1 standard error presented; paired *t*-test, one-tailed $P = 0.03$). This test compared assortative 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.

$P = 0.0397$; 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. 6b). In contrast, there was no relationship between relative mating response and body *size* (linear regression, $P = 0.72$). A consistent trend across populations was found when probabilities of linear regressions conducted within each population (weighted Z-transform test, mean $\beta = -1.0$, one-tailed $P = 0.0021$) were combined, indicating that females from all populations tended to exhibit mating preferences for morphologically similar males.

DISCUSSION

Through the combination of the "natural experiment" conditions of Bahamian blue holes and an integrative examination of morphological, molecular, and behavioral data, this study provides one

TABLE 3.

		Assortative Mating Index	
Scale of Analysis	Population / Species	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

Average levels of assortative mating for each G. 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.

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 greater sexual isolation between ecologically divergent pairs of populations than ecologically similar ones.

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 have been shown to generate ecologically important differences in swimming performance in mosquitofishes (Langerhans *et al*., 2004; Langerhans, 2006, Langerhans, unpublished data). In comparison with another *Gambusia* species (*G. affinis*) known to exhibit morphological differences among predator regimes, *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, pg. 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 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; Greenfield and Wildrick, 1984; Greenfield, 1985; Langerhans *et al*., 2004; Langerhans *et al*., 2005, Langerhans, unpublished data). Indeed, suggestive results were found using laboratory-born *G. hubbsi* from three populations examined in this study (1 low-predation, 2 high-predation): individuals retained their morphological distinctiveness after 8 weeks of rearing under common laboratory conditions (n = 10; using a discriminant function derived from wild fish, all lab-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. While 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, a consistent trend of assortative mating for body shape across all populations was found, indicating that mating preferences are based on the same traits under divergent selection. These 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 & 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, Langerhans, unpublished data). Based on the depth of blue holes examined in this study, these localities were dry caves prior to 15,000 - 4,000 years ago (Fairbanks, 1989). Thus, our results suggest that reproductive isolation can rapidly evolve as a by-product of ecological adaptation before the occurrence of any reinforcement.

CONCLUSIONS

This investigation of Bahamas mosquitofish suggests the possible parallel evolution of sexual isolation between populations inhabiting divergent predator regimes. 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, strong evidence is presented 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.

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