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Sexual selection paves the road to sexual isolation during ecological speciation

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ABSTRACT

Background: Divergent natural selection between ecologically different environments often drives speciation. An unanswered question concerns the importance of sexual selection in generating sexual isolation during this process. We illustrate that four distinct mechanisms can drive sexual isolation: (I) divergent sexual selection, (II) uniform sexual selection, (III) intra-population assortative mating, and (IV) divergent mate recognition signals. Currently, we lack empirical studies designed to disentangle their relative importance in the wild.

Question: What is the relative importance of the four mechanisms in generating sexual isolation during ecological speciation in the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*)?

Organisms: Bahamas mosquitofish (*Gambusia hubbsi*) from five inland blue holes on Andros Island, the Bahamas (three without predatory fish, two with predatory fish). Prior work has demonstrated replicated evolution of adaptive phenotypes in the different predation regimes, with strong sexual isolation between divergent predation regimes that is associated with body shape differences between populations.

Methods: We conducted a mate-choice experiment using video animations to test for within-population mating preferences for male body shape. Holding all other traits constant, we digitally manipulated body shapes of animations to present each female with two subtly different options: (1) a male with a relatively streamlined body shape (10% confidence limit for the female's respective population along a multivariate morphological axis), and (2) a male with a relatively large mid-body/caudal region (corresponding 90% confidence limit). These shapes mirror the differences observed between predation regimes, but only utilize within-population variation.

Results and conclusions: We found that divergent within-population mating preferences (not any other mechanism) explain observed patterns of sexual isolation between populations. Male body shape and female preference for male body shape have co-evolved across populations, resulting in sexual isolation between divergent predatory environments. In combination with previous research, this study reveals that divergent natural and sexual selection on body shape between predation regimes has incidentally increased reproductive isolation as a by-product.

Keywords: adaptive radiation, mate recognition, mating behaviours, natural selection, predation, sexual selection, speciation.

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INTRODUCTION

Divergent natural selection often underlies the evolution of reproductive isolation (e.g. Coyne and Orr, 2004; Grant and Grant, 2008; Price, 2008; Schluter, 2009; Nosil, 2012; Langerhans and Riesch, 2013). Sexual isolation (= behavioural isolation) describes assortative mating between populations (i.e. reduced probability of inter-population mating), and comprises one common barrier to gene flow during speciation by divergent selection. Sexual selection could play an important role in this process, but its influence in speciation is a matter of debate (e.g. Lande, 1981; West-Eberhard, 1983; Kirkpatrick and Ravigne, 2002; Coyne and Orr, 2004; Ritchie, 2007; Price, 2008; van Doorn *et al.*, 2009; Kraaijeveld *et al.*, 2011; Maan and Seehausen, 2011; Servedio, 2012). The critical need to understand the interaction between natural selection and sexual selection during speciation has long been widely acknowledged. Yet while considerable research has addressed both the role of divergent natural selection in speciation and the general role of sexual selection in speciation, few studies have examined the relationship between within-population mating preferences and between-population sexual isolation (see Ptacek, 2000; Chamberlain *et al.*, 2009). Empirical demonstration of within-population mating preferences affecting sexual isolation between populations would provide some of the strongest, unequivocal evidence for sexual selection's role in speciation, but we currently have little evidence in this regard.

Previous work has frequently assumed that the occurrence of assortative mating between populations reflects divergence in mating preferences, purportedly demonstrating sexual selection's influence in speciation. But this is not necessarily the case. Such a pattern only reflects divergence in mating preferences in one particular context: mate choice between populations. We argue that this phenomenon can arise from four conceptually distinct mechanisms (Table 1).

The first mechanism in Table 1 represents the classic case of sexual isolation resulting from divergent sexual selection between populations (e.g. mating preferences for different trait values in different populations), but all four mechanisms implicate a role for mate choice in speciation, assuming their operation enhanced sexual isolation before speciation was completed. Mechanism II avoids any need for linkage disequilibrium since a single

Table 1. Four ways that sexual isolation can arise between ecologically divergent environments (see text for details)

Mechanism of sexual isolation	Potential models of mate-choice evolution
I. Divergent sexual selection: divergent within-population mating preferences	Direct benefits, indicator traits, sensory drive, sexy sons, reinforcement
II. Uniform sexual selection: uniform within-population mating preferences, but only locally adapted forms adequately produce marker trait(s)	Direct benefits, indicator traits, sensory drive, reinforcement
III. Intra-population assortative mating: assortative mating within populations, combined with divergence in marker trait(s) between populations	Direct benefits, indicator traits, genetic compatibility, reinforcement
IV. Divergent mate recognition: between-population mate choice that relies on marker traits not involved in within-population mating decisions	Direct benefits, indicator traits, sensory drive, genetic compatibility, reinforcement

mating preference within multiple populations can facilitate assortative mating between populations when marker-trait production relies on local adaptation, and thus sexual isolation can evolve more easily in sympatry by this mechanism than by mechanism I (for details, see van Doorn *et al.*, 2009). For example, females in all populations may similarly prefer males with a large condition-dependent ornament, but only locally adapted males can produce such a trait. This results in increased sexual isolation between populations adapted to different environments. Mechanism III only involves intra-population assortative mating, not sexual selection *sensu stricto*. Sexual selection can alter frequencies of traits and alleles, while assortative mating only reorganizes existing variation by altering genotype frequencies, and these two different evolutionary forces may have different implications for the evolution of sexual isolation (Kirkpatrick and Ravigne, 2002; van Doorn *et al.*, 2004; Maan and Seehausen, 2011; Bolnick and Kirkpatrick, 2012). This mechanism encompasses phenomena such as genetic linkage between the marker trait and preference, and self-referent phenotype matching, such as size-based assortative mating (see Jiang *et al.*, 2013). Mechanism IV involves traits traditionally viewed as important in 'species recognition', and may commonly reflect necessary mate recognition signals (Mendelson and Shaw, 2012). For instance, mating preferences could centre on male body size within populations, but male coloration might serve as an important mate recognition signal in between-population contexts. Because mechanisms III and IV do not necessarily invoke within-population sexual selection, one could argue that only the first two mechanisms truly represent sexual selection's role in speciation, as these fulfil the spirit of the idea that sexual selection drives speciation. Regardless, all four mechanisms describe how mating behaviours can facilitate the evolution of sexual isolation, thus driving speciation.

For all four mechanisms, the most likely route to sexual isolation involves mate choice based on marker traits that effectively indicate local adaptation. This automatically results in a reduction of inter-population mating for populations adapting to different environments. Any of the four mechanisms can play a role in speciation within any geographic context, including the possible inclusion of reinforcement if populations interact. While selection directly favours reproductive isolation in the case of reinforcement, sexual isolation can evolve as an incidental by-product through any of the four mechanisms in the absence of reinforcement. Which mechanism might be more likely in nature requires empirical investigation. The little empirical research that has examined both within-population mating preferences and sexual isolation has yielded varied results. For instance, divergence in within-population mating preferences can evolve during adaptation to different environments (Rundle *et al.*, 2005), and may underlie sexual isolation in *Enchenopa* treehoppers (Rodríguez *et al.*, 2004, 2006; Cocroft *et al.*, 2008), benthic–limnetic species pairs of threespine stickleback (Boughman, 2001, 2007), and *Laupala* and *Gryllus* crickets (Gray and Cade, 2000; Shaw, 2000; Grace and Shaw, 2012). However, mating preferences sometimes focus on different traits during within-population and between-population contexts (Boake *et al.*, 1997; Pauers and McKinnon, 2012), sometimes do not diverge and can even inhibit speciation (Ryan and Wagner, 1987; Hill, 1994; Ryan and Rand, 1995; Slabbekoorn and Smith, 2002; Kozak *et al.*, 2008; Price, 2008; Verzijden *et al.*, 2012), and sometimes do diverge yet fail to result in sexual isolation (Endler and Houde, 1995; Magurran, 1998; Brooks, 2002). Clearly, additional empirical studies are needed to gain a better understanding of the relative importance of these four mechanisms in driving sexual isolation in the wild.

Here we take an experimental approach to pinpoint the major mechanism(s) underlying sexual isolation during ecological speciation using the model system of the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting inland blue holes on

Andros Island, the Bahamas. Bahamas mosquitofish have repeatedly diverged in body shape between environments with and without predatory fish, and previous work found that the strength of assortative mating between populations correlated with body shape differences (Langerhans *et al.*, 2007). Thus, this system presents a clear pattern of sexual isolation between ecologically divergent environments that appears related to a trait under divergent selection: body shape. Since this scenario effectively rules out mechanism II, we designed our study to uncover the importance of the remaining three mechanisms, providing the first test to date that attempts to disentangle the relative importance of the various mechanisms that can generate sexual isolation.

In this study, we tested within-population mating preferences of female Bahamas mosquitofish to specifically address four questions:

- **Q1:** Have populations inhabiting divergent predation regimes evolved divergent within-population mating preferences for body shape? This directly tests for the presence of mechanism I, and the absence of mechanism IV with respect to male body shape.
- **Q2:** Does female mating preference for male body shape within populations depend on the female's own body shape? This directly tests for the presence of mechanism III.
- **Q3:** Have female mating preferences and male body shapes co-evolved across populations? This directly tests for a pattern predicted only by mechanism I.
- **Q4:** Do within-population mating preferences explain between-population patterns of sexual isolation? The strength of this relationship directly tests the overall importance of mechanism I, and reveals the potential importance of other mechanisms, as unexplained variance reflects other mechanisms.

METHODS

Study system and experimental organisms

Bahamas mosquitofish are small, livebearing fish that colonized and adaptively radiated across inland blue holes on the northern half of Andros Island during the past ~15,000 years (Langerhans *et al.*, 2007; Langerhans, 2009a, 2010). Blue holes are isolated, vertical caves that filled with water as rising sea levels lifted the freshwater lenses of the island (freshwater aquifers floating atop marine groundwater), flooding the voids (Mylroie *et al.*, 1995). Bahamas mosquitofish inhabit distinct low-predation and high-predation populations distinguished by the absence or presence of a predatory fish, the bigmouth sleeper, *Gobiomorus dormitor* (Langerhans *et al.*, 2007; Heinen *et al.*, 2013). Blue holes comprise a 'natural experiment' to test how variation in predation risk may drive adaptive diversification, as no known environmental factor co-varies systematically with predation regime [e.g. productivity, turbidity, water transparency, pH, dissolved oxygen, salinity, temperature, depth (Langerhans *et al.*, 2007; Heinen *et al.*, 2013)]. Bahamas mosquitofish have repeatedly evolved adaptive phenotypes in the respective predation regimes, including divergence in life histories, locomotor performance, genital morphology, and body shape (Langerhans *et al.*, 2005, 2007; Langerhans, 2009a, 2010; Riesch *et al.*, 2013).

Differences in body shape represent a particularly obvious visual difference between predation regimes, and prior work has centred on its role in mediating female mate choice in this system. *Gambusia hubbsi* have evolved relatively streamlined bodies in low-predation environments – a body form that enhances steady-swimming performance important for resource competition – and possess a larger mid-body/caudal region in high-predation environments – a body form that enhances unsteady-swimming performance important for escaping predatory strikes (Langerhans *et al.*, 2004, 2007; Langerhans, 2009a, 2009b, 2010). Females prefer males from their own population over foreign males in mate-choice trials, and the strength of this preference increases as the body shape of the foreign male becomes more divergent from that of the female (Langerhans *et al.*, 2007). This pattern of assortative mating between populations results in greater sexual isolation between populations from different predation regimes than between populations from the same predation regime, irrespective of genetic relatedness. The experiment reported here examined whether mating preferences based on relatively subtle variation in male body shape within populations can explain this pattern observed between populations.

For this study, we collected Bahamas mosquitofish from five inland blue holes on Andros Island in 2009 (three without predators, two with predators). For three populations, we additionally reared F1 offspring that were born in the laboratory after holding parents in the lab for at least 2 months. The F1 offspring were reared without a view of parental-generation fish and separated by sex upon reaching sexual maturity to eliminate any opportunity for mate-choice copying. Fish from each combination of sex/population/generation were held in 38-litre aquaria. All fish were housed at the University of Oklahoma Biological Station at 25 ± 3°C under a 12:12 h light/dark cycle and fed brine shrimp nauplii, *Daphnia*, bloodworms, and commercial flake food. Before experimentation, wild-caught fish were held in the laboratory for approximately 3 months.

To address the four questions described above, we conducted a mate-choice experiment employing video-animation methodology designed to explicitly test female mating preference for male body shapes within populations. Video playback has the critical advantage over live animals in that variation in the trait of interest can be examined while holding other behaviours and morphologies constant, and has been commonly and successfully employed in many animals, including *G. hubbsi* and other poeciliid fishes (e.g. Kodric-Brown and Nicoletto, 1997; Rosenthal, 1999; Trainor and Basolo, 2000; Morris *et al.*, 2003; Langerhans *et al.*, 2005, 2007; Woo and Rieucau, 2011; Polverino *et al.*, 2013; Veen *et al.*, 2013). We measured female association behaviours when presented with two alternative digital animations of male Bahamas mosquitofish differing only in body shape, as described below, and performed analyses to directly test each of our study's four questions.

Male body shape and digital animations

To test whether females preferred particular male body shapes within populations, we assessed mating preferences when given the choice between two relatively extreme male body shapes within each population, holding all other traits constant. To accomplish this, we estimated the distribution of male body shape along a multivariate axis of shape for each population, calculated the 10% and 90% confidence limits (CL) for each population, and presented each female with the choice of two digital animations reflecting the 10% CL and 90% CL of male body shape for her particular population (Fig. 1) (see below for details).

We measured the body shape of 130 male *G. hubbsi* using lateral photographs of live fish (see sample size information in Table 2). We digitized 10 homologous landmarks on each image using tpsDig (Rohlf, 2010a), and used geometric morphometric methods to evaluate body shape variation (for details, see Langerhans *et al.*, 2007). We generated shape variables (partial warps and uniform components) from landmark data using Generalized Procrustes Analysis in

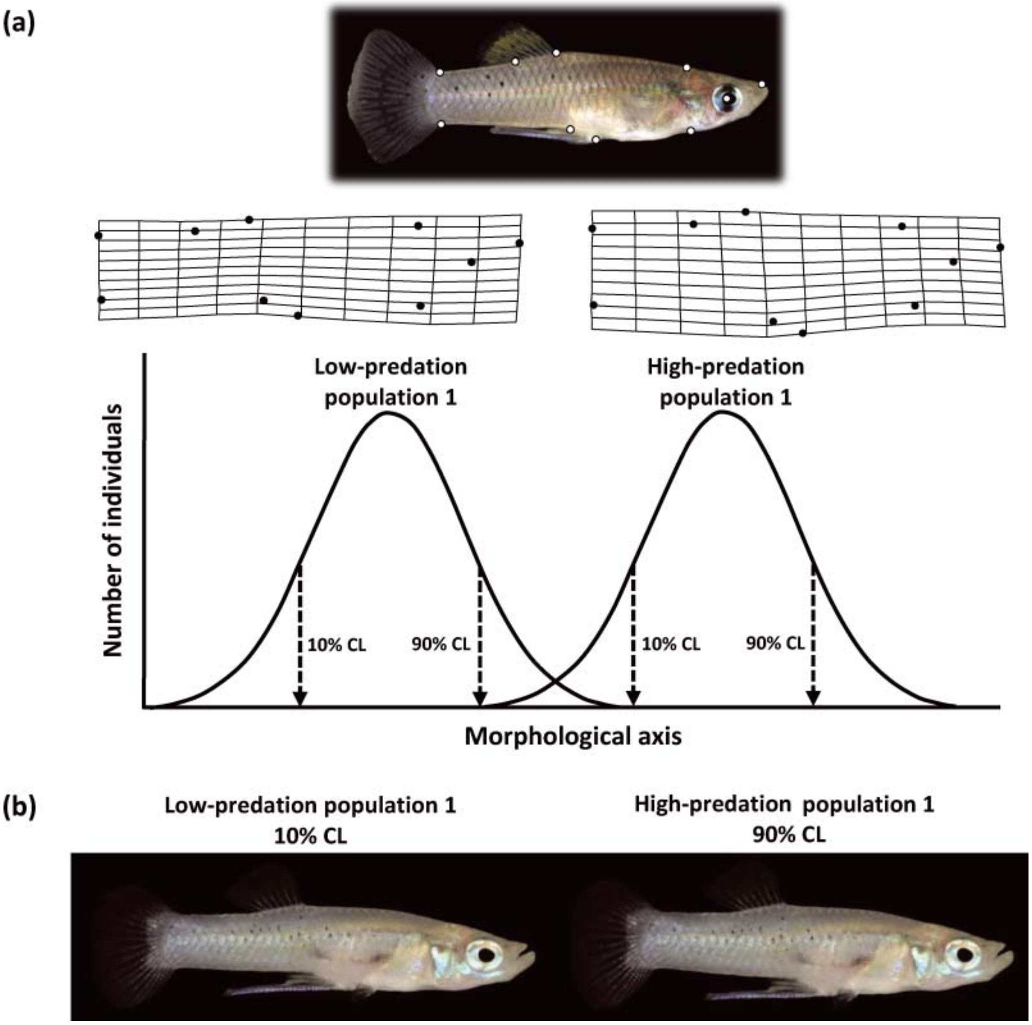

Fig. 1. Illustration of our method for generating male body shapes for use in mate-choice trials, including (a) digitization of 10 landmarks, estimation of the morphological axis (visualization presented for observed variation using thin-plate splines), calculation of the 10% and 90% confidence limits for each population along the morphological axis (two hypothetical populations depicted), and (b) production of thin-plate spline transformed photographs for each population that represent a male that is relatively streamlined (10% CL) and a male that has a relatively enlarged mid-body/caudal region (90% CL) (photographs for Gollum population depicted).

tpsRegr 208 (Rohlf, 2010b). Using these shape variables, we assigned each fish a score on a canonical axis derived in Langerhans *et al.* (2007) describing shape variation between predation regimes. The morphological axis ranges from streamlined bodies characteristic of low-predation populations to the posteriorly robust bodies characteristic of high-predation populations (Fig. 1a).

We used 1000 bootstraps of the male shape data within each population to estimate the 10% CL and 90% CL separately for each population (Table 2). In this way, we estimated two

Table 2. Sample sizes for female *G. hubbsi* examined in mate-choice trials, and sample sizes and morphological information for males used to construct digital animations employed in mate-choice trials (scores on the morphological axis)

				Male body shape			
Predation regime	Population	Wild-caught females (*n*)	Lab-born females (*n*)	Mean	10% CL	90% CL	*n*
Predator absent	East Twin (E)	10	0	−0.99	−2.29	0.31	15
Predator absent	Gollum (G)	4	8	−2.11	−3.24	−0.99	23
Predator absent	Rainbow (R)	8	0	−1.39	−2.72	−0.08	36
Predator present	Cousteau (C)	11	4	2.05	0.57	3.53	26
Predator present	Stalactite (S)	10	16	1.51	−0.03	2.96	30

body shape scores for each population along the morphological axis: (1) 10% CL – a body shape more ‘low-predation shaped’ than 90% of the given population, and (2) 90% CL – a body shape more ‘high-predation shaped’ than 90% of the given population. While differences between predation regimes in body shape are relatively obvious, variation within populations is much more subtle (Fig. 1b).

Using these CL values, we digitally transformed a photograph of an approximately average-shaped male individual from each population into two different images, one for each CL. First, we selected a single male from each population with a morphological axis score as near as possible to the population mean, and digitally trimmed the fish body in the image so that the backgrounds of all images were identical to one another (using Adobe Photoshop Elements 6). We then used these photographs, and their respective landmark coordinates, to transform each image using thin-plate splines so that its landmark coordinates matched those of either the 10% CL or 90% CL value for its particular population using tpsSuper (Rohlf, 2004). In this way, we created two images for each of the five populations, representing a relatively low-predation shaped male and a relatively high-predation shaped male for each population (see Fig. 1b).

We used Pencil traditional animation software (version 0.4.4b) to animate these photographs for use in mate-choice trials. For each photograph, we produced two videos to simulate the fish swimming from left to right and from right to left, on a black background. The two videos were digitally spliced together with SplitFuse software (version 2.2) to create a single AVI video file. Males in the final videos had a body size on screen of approximately 21 mm standard length.

Mate-choice trials

We examined the mating preferences of 71 female *G. hubbsi* (see Table 2). Mate-choice trials were conducted in a laboratory mate-choice arena (77 × 32.5 × 31.7 cm) with a black, opaque back and bottom, a computer monitor at either end (Sony SDM-S53 TFT LCD screen, 1024 × 768 resolution), and one side transparent for observations (Fig. 2). Water temperature was maintained at approximately 25°C for all trials.

We measured female mating responses by recording the time each female spent within five zones: a neutral zone (centre region of tank, 26 cm long), two opportunity zones (within 25.5 cm from the left end and right end, where the monitors were positioned), and two

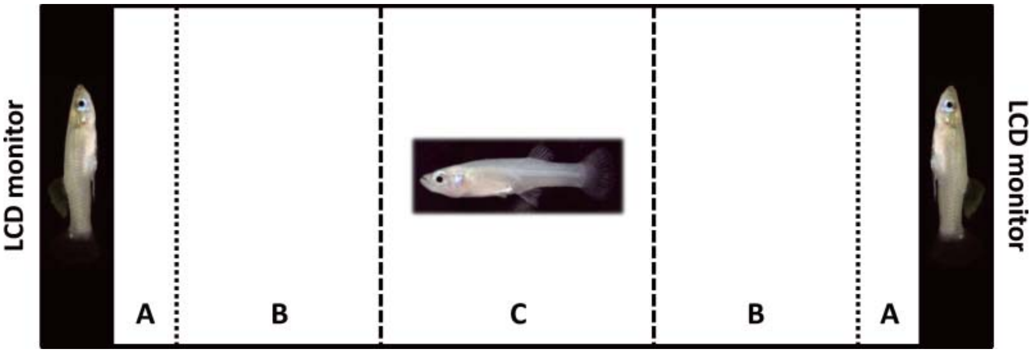

Fig. 2. Illustration of mate-choice arena (77 × 32.5 × 31.7 cm), depicting the interaction zones (A: 6 cm long), opportunity zones (A + B: 25.5 cm long), neutral zone (C: 26 cm long), and the two LCD monitors displaying alternative male animations. Fish photographs not to scale.

interaction zones (within 6 cm of either end) (Fig. 2). At the initiation of each trial, a single female was placed into the centre of the arena within a clear Plexiglas cylinder and allowed to acclimate for 5 min. During the acclimation period, each monitor displayed a video animation in a continuous loop (one displayed a 10% CL male animation, the other displayed a 90% CL male animation). After 5 min, we removed the cylinder and allowed the female to swim freely and inspected the two videos. We recorded the time spent in each zone during the next 10 min, and then returned the female to the Plexiglas cylinder for a second 5-min acclimation period. At this point, the left/right presentation order of the two video animations was reversed. After this acclimation period, we again recorded the time spent in each zone for 10 min, and then concluded the trial. The sum of time spent in each zone across the two sessions was used in analysis.

We calculated a single metric for each female to characterize the overall mating preference. This preference represented the difference between the mating response directed towards the 90% CL male animation and that directed towards the 10% CL male animation. First, the mating response for each male animation (MR90%, MR10%) was measured as the proportion of time the female spent in a given male animation's interaction zone out of the total time she spent in that male animation's opportunity zone (i.e. interaction-zone time/opportunity-zone time). Mating preference was calculated as MR90% – MR10%. This resulted in mating preference scores that can range from –1 (absolute preference for animation of relatively low-predation shaped male) to 1 (absolute preference for animation of relatively high-predation shaped male), with zero representing no preference. Results were qualitatively similar when using two other, alternative mating-preference measures: (1) response index of side association time (difference in opportunity-zone times divided by sum of opportunity-zone times) or (2) response index of interaction time (difference in interaction-zone times divided by sum of interaction-zone times). We focus exclusively on the former estimate of mating preference described above for our statistical analyses presented herein, but for comparison with other studies, we additionally present the population means for all three measurements.

All females were photographed and examined for body morphology following the method described above for males. Using data derived from the 10 homologous landmarks digitized on each female photograph, we assigned a morphological axis score to each female. These morphological scores were used in statistical analysis to evaluate assortative mating for body shape.

Statistical analysis

To address Q1 and Q2 (see Introduction), we conducted a general linear mixed model using restricted maximum likelihood examining variation in female mating preference due to effects of predation regime, female body shape (morphological axis scores), and the interaction between predation regime and female body shape. Population nested within predation regime was included as a random effect. The predation regime term directly tested for divergence in within-population mating preferences; significant differences would provide evidence for mechanism I and reject mechanism IV (see Table 1). The female body-shape term directly tested for assortative mating within populations (mechanism III), which would manifest as a positive slope (i.e. if relatively streamlined females prefer streamlined males and vice versa). The interaction term tested whether assortative or disassortative mating differed between predation regimes. We calculated η^2 as an estimate of the effect size of each model term (percent of total variance explained by each effect).

To address Q3, co-evolution of female mating preferences and male body shape across populations (consistent only with mechanism I), we performed two tests. First, we examined the Pearson correlation between average male body shape (morphological axis scores) and average female mating preference across the five populations. Second, we used a partial Mantel test to examine this correlation while controlling for genetic relatedness among populations. We calculated statistical significance by comparing the *z*-statistic of the actual matrices to the *z*-statistics from 99,999 random permutations. For this analysis, we measured Euclidean differences in male body shape and female mating preference for each population pair, and calculated genetic relatedness as uncorrected percent nucleotide differences (p-distance) based on previously published sequences of an 886-bp fragment of the NADH subunit 2 (ND2) mitochondrial gene (see Langerhans *et al.*, 2007; Riesch *et al.*, 2013).

To address Q4, we used a partial Mantel test as described above to examine the association between pairwise population differences in female mating preferences and sexual isolation between populations, controlling for genetic relatedness. Data for sexual isolation were taken from Langerhans *et al.* (2007), and were only available for pairwise combinations of four populations.

Prior to analysis of female mating preferences to address Q1 and Q2, we first wished to confirm that responses were similar among lab-born and wild-caught females. Because two populations did not include lab-born females, we did not directly include a term for birth status in our model. Rather, we performed separate analyses for lab-born and wild-caught females, and compared results across analyses to determine whether responses differed between the two groups (each model followed the description above). We evaluated consistency in model parameters (partial regression coefficients) across groups, and found parameter values similar in both sign and magnitude across the two groups. Results within each birth-status group were also qualitatively similar to patterns observed when all females were pooled for analysis. These findings indicate broadly similar patterns within lab-born and wild-caught females, and thus we only present results from analyses using all females pooled.

Table 3. Results of general linear mixed model examining female mating preferences

Source	*F*	d.f.	*P*	η^2 (%)
Predation regime	11.53	1,6.24	0.0137	21.77
Female body shape	7.62	1,66.92	0.0074	8.30
Predation regime × Female body shape	3.86	1,66.92	0.0535	4.26

RESULTS

Our general linear mixed model revealed significant effects of predation regime and female body shape on female mating preferences, with a marginally non-significant difference between predation regimes in the way that female body shape was associated with mating preference (Table 3). We uncovered clear evidence for divergent within-population mating preferences between predation regimes: females from low-predation populations tended to prefer relatively low-predation shaped males within their respective populations, while females from high-predation populations tended to prefer relatively high-predation shaped males within their respective populations (Fig. 3a; Table 4). With respect to male body shape, this indicates the operation of mechanism I, and not mechanism IV. Moreover, not only did we not find assortative mating within populations based on body shape – thus rejecting mechanism III – but we instead revealed evidence for disassortative mating for body shape within populations, especially in low-predation populations (Fig. 3b); although the strength of this relationship is much lower than the effect of predation regime (Table 3). As a means of confirming the consistency of this pattern of disassortative mating within populations, we performed linear regression of mating preference on female body shape within each population, and combined probabilities using the weighted Z-transform test (Whitlock, 2005). All populations exhibited a trend of disassortative mating (Fig. 3b), with a significant combined probability ($P = 0.0166$). This indicates that as females become more 'maladaptively' shaped within their given population, their preference for the adaptive male body shape tends to strengthen.

We found a strong correlation between average male body shape and average female mating preference ($r = 0.99$, $P = 0.0006$; Fig. 4a). This trend remained highly significant when controlling for genetic relatedness using a partial Mantel test ($P < 0.0001$).

There was a positive association between pairwise differences in within-population mating preferences and sexual isolation, controlling for genetic relatedness ($P = 0.0394$; Fig. 4b).

DISCUSSION

We uncovered unambiguous evidence for divergence in within-population mating preferences of female *G. hubbsi* inhabiting blue holes with different predation regimes. These differences in mating preferences provide a sufficient explanation for observed levels of sexual isolation between populations. Thus, differences in body shape between predation regimes due to divergent natural selection on locomotor performance (Langerhans *et al.*, 2007; see Langerhans, 2009a, 2010), coupled with divergent sexual selection on body shape (this study), has incidentally resulted in increased reproductive isolation between populations inhabiting

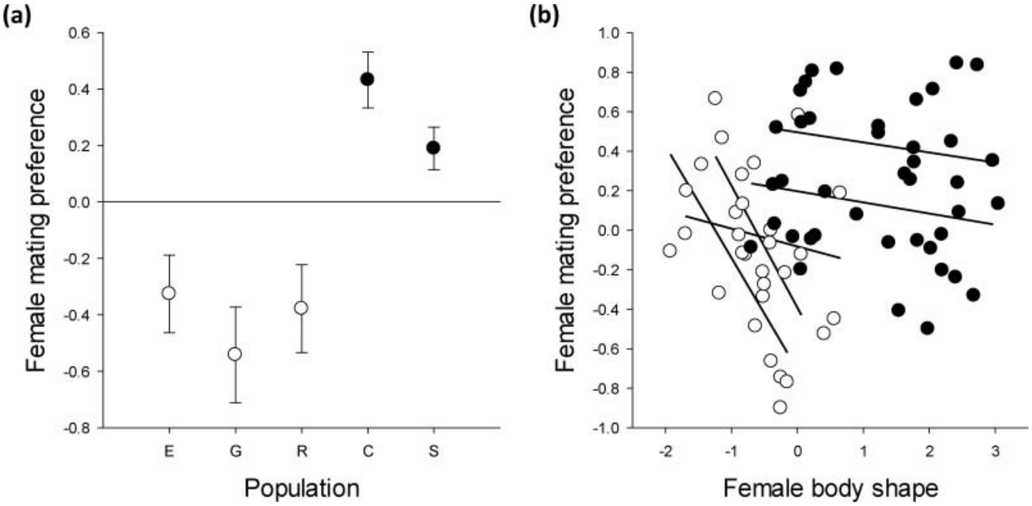

Fig. 3. (a) Divergence in female mating preference for male body shape between predation regimes (positive values indicate preference for 90% CL male, negative values reflect preference for 10% CL male; least-squares means ± 1 s.e.). (b) Association between female body shape (morphological axis scores) and mating preference within each population. Population abbreviations follow Table 2. Open symbols: low-predation populations; solid symbols: high-predation populations.

Table 4. Summary of mating preferences based on three separate measurements (see text for details; least squares means presented, with 1 s.e. in parentheses)

Predation regime	Population	Proportional interaction-zone time*	Opportunity-zone time	Interaction-zone time
Predator absent	East Twin (E)	−0.33 (0.14)	−0.27 (0.23)	−0.40 (0.29)
Predator absent	Gollum (G)	−0.54 (0.17)	−0.68 (0.29)	−0.84 (0.36)
Predator absent	Rainbow (R)	−0.38 (0.16)	−0.45 (0.26)	−0.67 (0.33)
Predator present	Cousteau (C)	0.43 (0.10)	0.90 (0.17)	0.94 (0.21)
Predator present	Stalactite (S)	0.19 (0.08)	0.24 (0.12)	0.20 (0.15)

* Measurement used in all analyses presented in the text (all three estimates are highly correlated with one another, all $r > 0.98$).

different predation regimes. Below we discuss the implications of our findings for the study of speciation.

Why might divergent mating preferences evolve across divergent ecological environments, driving sexual isolation via mechanism I? Considerable research has demonstrated that ecological variation can commonly alter the context of sexual selection in diverse taxa (e.g. Emlen and Oring, 1977; Rowe *et al.*, 1994; Zuk and Kolluru, 1998; Grether *et al.*, 1999; Candolin *et al.*, 2007; Schwartz and Hendry, 2007; Cornwallis and Uller, 2010; Botero and Rubenstein, 2012; Scordato *et al.*, 2012). For instance, divergence in mating preferences and marker traits may often occur between populations differing in predation risk (e.g. Stoner and Breden, 1988; Lima and Dill, 1990; Magnhagen, 1991; Magurran and Seghers, 1994; Sih, 1994; Godin, 1995; Candolin, 1997; Langerhans *et al.*, 2005; Lima, 2009) or background

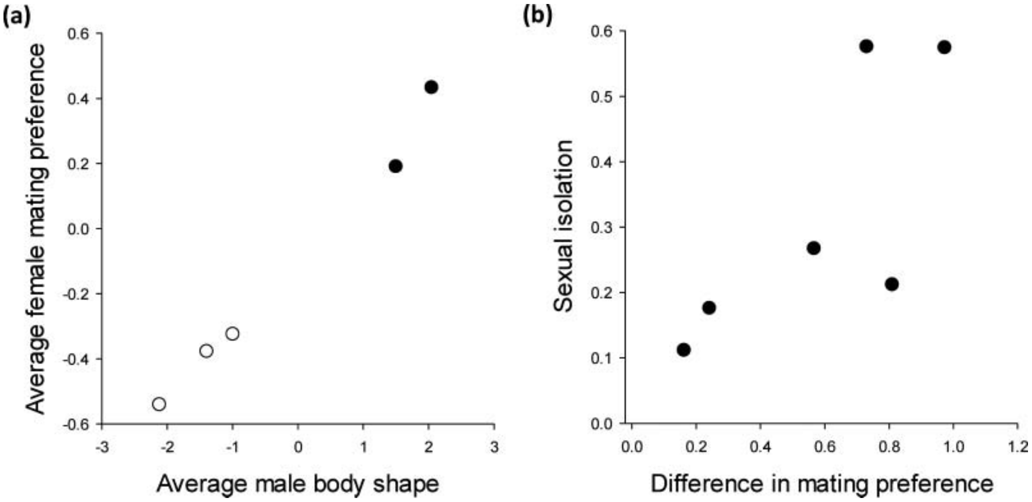

Fig. 4. (a) Co-evolution of male body shape and female mating preference for male body shape across populations (open symbols: low-predation populations; solid symbols: high-predation populations). (b) Positive association between divergence in within-population mating preference and sexual isolation between populations.

environment, i.e. sensory drive (e.g. Endler, 1992; Boughman, 2002; Fuller, 2002; Leal and Fleishman, 2004; Slabbekoorn and den Boer-Visser, 2006). Thus, divergent sexual selection between populations inhabiting different ecological environments may indeed be common. Surprisingly, very few studies have directly examined how this divergent sexual selection might result in sexual isolation.

If mating preferences rely on marker traits that reflect locally high levels of individual fitness (via components independent of mating preference, e.g. viability, fecundity), then sexual isolation can rapidly evolve between populations experiencing divergent natural selection on those marker traits. Such a scenario may be likely if sexual selection evolves via direct benefits or indicator traits, or if populations interact and reinforcement occurs. In the present system, we can effectively rule out the actions of reinforcement (we focus on allopatric populations), sensory drive (similar communication environments), and sexy sons (marker traits not arbitrary and should not reduce female fecundity), leaving the likely underlying causes of divergent sexual selection as direct or indirect benefits offered to females by males with preferred body shapes. In Bahamas mosquitofish, males with locally adaptive body shapes most obviously confer indirect benefits to females through the inheritance of favourable alleles for body shape. Thus, male body shape may serve as an indicator trait, with female preference evolving through a ‘good genes’ mechanism (Kirkpatrick, 1987; Pomiankowski, 1988; Maynard Smith, 1991; Andersson, 1994; Kotiaho *et al.*, 2001). In addition to indirect benefits, male *G. hubbsi* with locally adaptive body shapes could also confer direct benefits to females, such as provide protection from harassment from non-preferred males, reduce predation risk by association with fast fish in the presence of predators, or enhance ability to locate foraging patches by association with high-endurance fish in the absence of predators. However, no clear evidence currently exists for any such direct benefits in this system. Whether divergent sexual selection often acts on markers of local adaptation in other systems, and if so whether this often reflects direct or indirect benefits, requires further investigation.

We found no evidence for assortative mating by body shape within populations (mechanism III); on the contrary, we uncovered a trend of disassortative mating. That is, even though females from different predation regimes preferred different body shapes on average, the strength of the preference for adaptive male body shapes tended to increase in females with relatively maladaptive body shapes. This finding runs counter to the hypothesis that a positive genetic correlation might exist between the preference and trait, as expected to result from Fisherian sexual selection, assuming a strong correlation between male and female body shapes in the present system (Lande, 1981; Andersson, 1994; Mead and Arnold, 2004). But this pattern could occur if the genes underlying male body shape have different morphological effects in females, as may be expected in systems with extreme sexual dimorphism in anatomy like *G. hubbsi* (R.B. Langerhans and E. Rosa-Molinar, unpublished). This pattern could also result under direct benefits or indicator traits (Kirkpatrick and Ryan, 1991; Schluter and Price, 1993; Iwasa and Pomiankowski, 1999; Houle and Kondrashov, 2002). Selection might more strongly favour mating preferences for adaptive male body shapes in females with relatively maladaptive body shapes if they receive particularly large direct or indirect fitness benefits. This pattern of disassortative mating could potentially derive from experiential effects of females, in which females with relatively maladaptive body shapes experience different social interactions than females with relatively adaptive body shapes, and exhibit a plastic response of increased mating preferences. However, this does not seem especially likely for lab-reared fish in this study.

We found strong co-evolution of male body shape and female mating preference for body shape across the five populations examined here. We suggest this indicates that as natural selection drives greater divergence in body shape between populations, a corresponding increase in female mating preference evolves in these populations. Thus, populations experiencing stronger natural selection on body shape may additionally experience greater sexual selection on body shape (perhaps resulting from the stronger link between the indicator trait, male body shape, and viability). This pattern could also partially reflect a by-product of within-population genetic correlations between male body shape and mating preference, although we currently do not have evidence to support this mechanism.

With respect to male body shape in Bahamas mosquitofish, divergent sexual selection (mechanism I), not any other mechanism (see Table 1), appears responsible for driving sexual isolation in this system. The generality of this finding awaits further investigation in other systems, as very few studies have to date examined which mechanism outlined in Table 1 underlies the evolution of sexual isolation in the wild. The only other study system with analogous research to date, benthic–limnetic species pairs of threespine stickleback, also shows a correlation between sexual isolation between populations and divergent within-population mating preferences (Boughman, 2001, 2007). While the evidence so far indicates divergent sexual selection may prove most important in driving sexual isolation, several facts suggest we should be cautious with this conclusion.

First, we simply have extremely little empirical data at our disposal to make any sweeping generalizations about what mechanisms are most important in driving sexual isolation in nature. Second, our study (and the threespine stickleback studies) only considered a small subset of traits that could potentially contribute to sexual isolation. Other mechanisms acting on other traits might drive sexual isolation alongside the presently documented process involving male body shape. For instance, male *G. hubbsi* exhibit sexually dimorphic orange-coloured dorsal fins. This trait's role in sexual isolation has not yet been studied, but uniform sexual selection on this mating signal could potentially contribute to sexual isolation if locally adapted forms can produce greater orange coloration (mechanism II). Furthermore, intra-population assortative mating (mechanism III) is common in many taxa, especially fish (Jiang *et al.*, 2013), and may contribute to sexual isolation between benthic and limnetic sticklebacks (Snowberg and Bolnick, 2008, 2012).

Moreover, divergent mate recognition (mechanism IV) seems plausible in many systems, including Bahamas mosquitofish. During population divergence between ecologically different environments, strong differences in multiple traits often evolve, and within-population variation in putative marker traits often appears quite subtle compared with among-population variation. Owing to such strong phenotypic differences between populations compared to phenotypic variation within populations, the involvement of mate recognition signals in the evolution of sexual isolation seems especially likely. Yet, we found here that female *G. hubbsi* can distinguish subtle differences in body shape within populations, and use this information during mating decisions. Grace and Shaw (2012) also recently demonstrated that female *Laupala cerasina* crickets can discriminate between subtle differences in male songs during what may be the early stages of sexual isolation in the group. Further research should focus on elucidating what role mate recognition signals play during speciation, and whether sexual selection is truly more important for sexual isolation.

This study has confirmed that body shape in Bahamas mosquitofish represents a classic magic trait, a trait under divergent natural selection that also acts as a mating cue, resulting in increased sexual isolation between environments (Servedio *et al.*, 2011; Servedio and Kopp, 2012). Altogether, research in this system indicates that: (1) divergent selection between predation regimes on locomotor demands favours different body shapes (Langerhans *et al.*, 2004; Langerhans, 2009a, 2009b, 2010; Langerhans and Reznick, 2010); (2) this divergent selection has repeatedly led to divergent evolution of body shape between predation regimes (Langerhans *et al.*, 2007; Langerhans, 2009a; R.B. Langerhans and E. Rosa-Molinar, unpublished); and (3) divergent mating preferences within populations (this study) results in increased sexual isolation between populations inhabiting different predation regimes (Langerhans *et al.*, 2007). Importantly, this association between differences in body shape and sexual isolation has been observed both among blue-hole populations of *G. hubbsi*, as well as among closely related species in the *Gambusia* genus (Langerhans *et al.*, 2007). This suggests that divergent sexual selection on body shape has not only facilitated ongoing ecological speciation in Bahamas blue holes, but also promoted speciation events in the past, which left descendent, extant species.

Does sexual selection often promote speciation? Much research has addressed this question using comparative approaches (e.g. Barraclough *et al.*, 1995; Arnqvist *et al.*, 2000; Panhuis *et al.*, 2001; Mendelson, 2003; Ritchie, 2007; Seddon *et al.*, 2008; Kraaijeveld *et al.*, 2011), going back to Charles Darwin himself (Darwin, 1871). While useful in elucidating patterns of association between proxies of sexual selection and estimates of speciation rates, comparative analyses alone cannot answer this question. For instance, comparative analyses typically cannot distinguish between mechanisms I, III or IV (see Table 1), and will generally fail to detect mechanism II altogether. Furthermore, this approach generally cannot unravel whether sexual selection contributed to the evolution of reproductive isolation, occurred alongside speciation without contributing, or merely drove divergence in secondary sexual characters after speciation. Thus, empirical case studies that can directly address the mechanistic role of sexual selection during speciation are needed to complement comparative studies and gain a richer understanding of the role of mating behaviours in speciation.

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