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# Origins of female genital diversity: Predation risk and lock-and-key explain rapid divergence during an adaptive radiation

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**The study of male genital diversity has long overshadowed evolutionary inquiry of female genitalia, despite its nontrivial diversity. Here, we identify four nonmutually exclusive mechanisms that could lead to genital divergence in females, and potentially generate patterns of correlated male–female genital evolution: (1) ecological variation alters the context of sexual selection ("ecology hypothesis"), (2) sexually antagonistic selection ("sexual-conflict hypothesis"), (3) female preferences for male genitalia mediated by female genital traits ("female-choice hypothesis"), and (4) selection against inter-population mating ("lock-and-key hypothesis"). We performed an empirical investigation of all four hypotheses using the model system of Bahamas mosquitofish inhabiting blue holes that vary in predation risk. We found unequivocal support for the ecology hypothesis, with females exhibiting a smaller genital opening in blue holes containing piscivorous fish. This is consistent with stronger postmating female choice/conflict when predators are present, but greater premating female choice in their absence. Our results additionally supported the lock-and-key hypothesis, uncovering a pattern of reproductive character displacement for genital shape. We found no support for the sexual conflict or female choice hypotheses. Our results demonstrate a strong role for ecology in generating female genital diversity, and suggest that lock-and-key may provide a viable cause of female genital diversification.**

KEY WORDS: **Coevolution, cryptic female choice, genital evolution, reproductive character displacement, sexual conflict, sexual selection.**

The rapid divergent evolution of male genital morphologies among internally fertilizing species represents one of the most well-established trends in phenotypic evolution, with closely related species often showing marked variation (Eberhard 1985, 1996; Arnqvist 1998; Hosken and Stockley 2004). Recent work has highlighted the importance of postmating sexual selection (cryptic female choice and sperm competition) and sexual conflict in driving rapid evolution of male genital morphology (Eberhard 1996; Hosken and Stockley 2004; Eberhard 2009, 2010); although other mechanisms, such as the lock-and-key mechanism (Dufour 1844), also play a role (Langerhans 2011; Masly 2012; Wojcieszek and Simmons 2013; Simmons 2014). While most research in this area has focused squarely on male genital morphology, the prevailing hypotheses for its rapid evolution are also generally predicted to cause diversification of female genitalia (Arnqvist and Rowe 2005; Rönn et al. 2007; Ah-King et al. 2014; Simmons 2014). Yet, we currently know very little about the rate and mechanisms of female genital evolution. The comparatively small, but growing literature on the topic suggests that female genitalia can evolve rapidly, and coevolution of male and female genitalia may be far from rare (Brennan et al. 2007; Kuntner et al. 2009; Evans et al. 2011; Langerhans 2011; Simmons and Garcia-Gonzalez 2011; Ah-King et al. 2014; Simmons 2014; Tanabe and Sota 2014). To accurately understand the causes of the remarkable diversity of animal genitalia, and to grasp the evolutionary consequences of genital diversification, such as speciation, we must bring the study of female genital diversity to the fore.

Research during the past several decades has identified four primary, nonmutually exclusive mechanisms that could lead to rapid genital evolution in females, and potentially result in a pattern of correlated evolution of male and female genitalia across populations and species (Table 1). Because previous theoretical and empirical research suggests all four mechanisms are plausible, we now need an elucidation of the specific predictions for divergence of female genitalia among populations/species made by each mechanism, as well as empirical tests of the predictions.

First, ecological variation can ultimately cause female genital divergence by altering the context of sexual selection, such as through changes in the frequency of multiple mating of females, frequency or duration of copulation, or frequency of courtship and coercive mating behaviors (Emlen and Oring 1977; Wing 1988; Lima and Dill 1990; Endler 1991; Magnhagen 1991; Magurran and Seghers 1994; Sih 1994; Candolin 1997; Jennions and Petrie 1997; Botero and Rubenstein 2012). Ecological variation could also alter natural selection on genitalia, such as through parasites or disease (Reinhardt 2010); although sexual selection is generally considered more important. The central feature of this "ecology hypothesis" is that variation in ecological factors, such as predation risk or resource availability, alters selection on genitalia, leading to genital divergence between ecological environments. Although ecological variation could cause changes in sexual conflict, female choice, or selection against hybridization (the other three primary mechanisms), it represents the only mechanism by which no reciprocal selection necessarily occurs among the sexes—that is male genital morphology need not affect selection on female genital morphology, and vice versa. Despite the lack of reciprocal selection in this case, a pattern of correlated evolution of male and female genitalia across populations inhabiting variable environments can result due to each sex independently responding to shared environmental variation. While some evidence for such a role of ecology in driving male genital evolution exists (Langerhans et al. 2005; Neufeld and Palmer 2008; Evans et al. 2011; Oneal and Knowles 2013; Heinen-Kay et al. 2014), we now need to test this hypothesis for female genital differentiation.

Second, sexually antagonistic selection can result in a coevolutionary arms race among male and female genitalia. That is, males and females often have conflicting interests regarding mating and fertilization, which can lead to the evolution of male genitalia that bypass female choice (at a cost to the female), then causing female genitalia to evolve defenses against the male armaments and regain control over mating or fertilization (at a cost to the male), and so on (Arnqvist and Rowe 1995; Rice and Holland 1997; Arnqvist and Rowe 2005; Rönn et al. 2007; Kuntner et al. 2009; Brennan et al. 2010; Perry and Rowe 2014). In contrast to the ecology hypothesis (but similar to the "female-choice hypothesis," see below), this "sexual-conflict hypothesis" predicts correlated evolution of male and female genitalia across populations/species regardless of the ecological environment. According to this hypothesis, reciprocal selection should drive coevolution of genitalia within any environment in which sexual conflict is strong. Because among-population correlation of male and female genitalia could result from shared ancestry or from selection against interpopulation mating for populations experiencing elevated migration (see below), evidence for this mechanism is greatly strengthened when the pattern persists after controlling for genetic relatedness among populations.

Third, female genitalia can provide a means through which females exert preference for male genital morphologies, such as via mechanical or sensory components of genitalia. Similar to the sexual-conflict hypothesis, this "female-choice hypothesis" also posits that females bias insemination or fertilization success of males based on male genital morphology, but in this case females obtain net fitness gains by selecting high-quality males instead of reducing mating costs through restriction of copulation (Eberhard 1996; Arnqvist and Rowe 2005; Eberhard 2010; Simmons 2014). Predictions for among-population divergence of female genitalia through this mechanism follow that of sexual conflict, and thus disentangling these two processes requires more than comparative data of genital phenotypes (Fricke et al. 2009). Again, evidence for this mechanism is strengthened when its predictions are upheld after controlling for possible association with genetic relatedness. Considerable research suggests female choice may play a major role in rapid male genital evolution (Eberhard 1996; Hosken and Stockley 2004; Andersson and Simmons 2006; Eberhard 2010; Simmons 2014), but little attention has focused on its role in female genital evolution to date.

Fourth, the "lock-and-key hypothesis" posits that selection against interpopulation mating (either mating per se, or hybridization, or both) favors genital incompatibilities between populations, resulting in rapid evolution of complementary genitalia in the male (key) and female (lock) within populations/species (Dufour 1844; Shapiro and Porter 1989). This essentially represents the postmating analog of reinforcement, the well-studied phenomenon describing the similar evolution of premating isolation among populations (both mechanisms describe prezygotic isolation). Lock-and-key drives the functional coevolution of male and female genitalia as a means of reducing the probability of interpopulation mating. Under this scenario, correlated evolution among male and female genitalia could occur primarily across dissimilar ecological environments or across similar environments, depending on fitness consequences of interpopulation mating, making it difficult to discern from other hypotheses in this respect. However, the lock-and-key hypothesis also makes

**Table 1.** Description and testable predictions for the four major, nonmutually exclusive hypotheses for female genital diversification.

| Hypothesis | Description | Prediction of among-population patterns of differentiation |
|---|---|---|
| Ecology | Ecological variation across populations/species alters context of sexual selection on genitalia, such as through changes in mating system. | Genital traits diverge between ecological environments, potentially resulting in correlated evolution of male and female genital traits across environments but not within similar environments. |
| Sexual conflict | Sexually antagonistic selection on genitalia, with offensive male genital "weapons" that enhance male fitness and defensive female genital traits that increase female fitness. | Correlated evolution of male and female genital traits, owing to a tight coevolutionary arms race, irrespective of ecological variation. |
| Female choice | Cryptic female choice of male genital morphologies partially determined by female genital traits, such as size/shape of genital opening or tract, and density/location of nerve endings. | Female genital traits diverge between populations with different male genital preferences, resulting in correlated evolution of male and female genital traits, irrespective of ecological variation. |
| Lock-and-key | Selection against interpopulation mating favors genital incompatibilities between populations/species. | Reproductive character displacement, where populations/species that experience more interpopulation mating opportunities differ more strongly in genital traits. |

Note that the sexual-conflict and female-choice hypotheses yield similar predictions of among-population differentiation, and thus cannot be disentangled with comparative data alone.

a unique prediction of genital divergence: reproductive character displacement, where populations/species differ more strongly in genital morphology when experiencing higher probabilities of interpopulation mating opportunities (currently or historically; e.g., sympatry, elevated population mixing or gene flow among largely allopatric populations). Specifically, according to this mechanism, selection against interpopulation mating does not occur for completely allopatric populations, but can drive the evolution of mating incompatibilities for populations experiencing low-moderate frequencies of interpopulation encounters (Servedio and Kirkpatrick 1997; Servedio and Noor 2003; Servedio 2011). While the lock-and-key hypothesis has not gained much support in some taxa (Eberhard 1985; Shapiro and Porter 1989; Arnqvist 1998; Eberhard 2010), recent work has provided supportive evidence in a variety of taxa (McPeek et al. 2009; Langerhans 2011; Masly 2012; Wojcieszek and Simmons 2013; Simmons 2014). The importance of this mechanism in explaining genital diversification remains an open question.

Here, we use comparative data to test these hypotheses in the adaptive radiation of *Gambusia hubbsi* (Bahamas mosquitofish, Family Poeciliidae) inhabiting blue holes. Blue holes are water-filled vertical caves found in some carbonate banks and islands (Mylroie et al. 1995). The small, livebearing Bahamas mosquitofish colonized inland blue holes of Andros Island during the past ~15,000 years, and repeatedly evolved different adaptive traits in either the presence (high-predation) or absence (low-predation) of the predatory fish, *Gobiomorus dormitor* (bigmouth sleeper) (e.g., morphology, color, life history, locomotor performance; Langerhans et al. 2007; Langerhans 2009, 2010; Riesch et al. 2013; Martin et al. 2014). These populations are further undergoing speciation, with significant sexual isolation among many populations, especially populations that differ in the presence of predatory fish (Langerhans et al. 2007; Langerhans and Makowicz 2013). Previous work has clearly identified predation risk as the primary agent of phenotypic diversification in this system, although other environmental factors do vary across blue holes (but do not covary with predation risk) and have apparently influenced at least some phenotypes (Heinen-Kay and Langerhans 2013a; Riesch et al. 2013; Martin et al. 2014).

Bahamas mosquitofish provide a model study system for investigating the causes of female genital evolution as these fish use internal fertilization, mate promiscuously, and previous work has characterized variation among blue holes in ecological parameters (notably predation risk), sexual behaviors (notably copulation frequency), and male genital morphology (notably the shape of the gonopodial distal tip) (Heinen-Kay and Langerhans 2013a; Heinen et al. 2013; Heinen-Kay et al. 2014). Males use their gonopodium—a nonretractable modified anal fin—to transfer sperm to females through the urogenital apertural opening (Fig. 1A,B). The size and shape of male gonopodia vary across

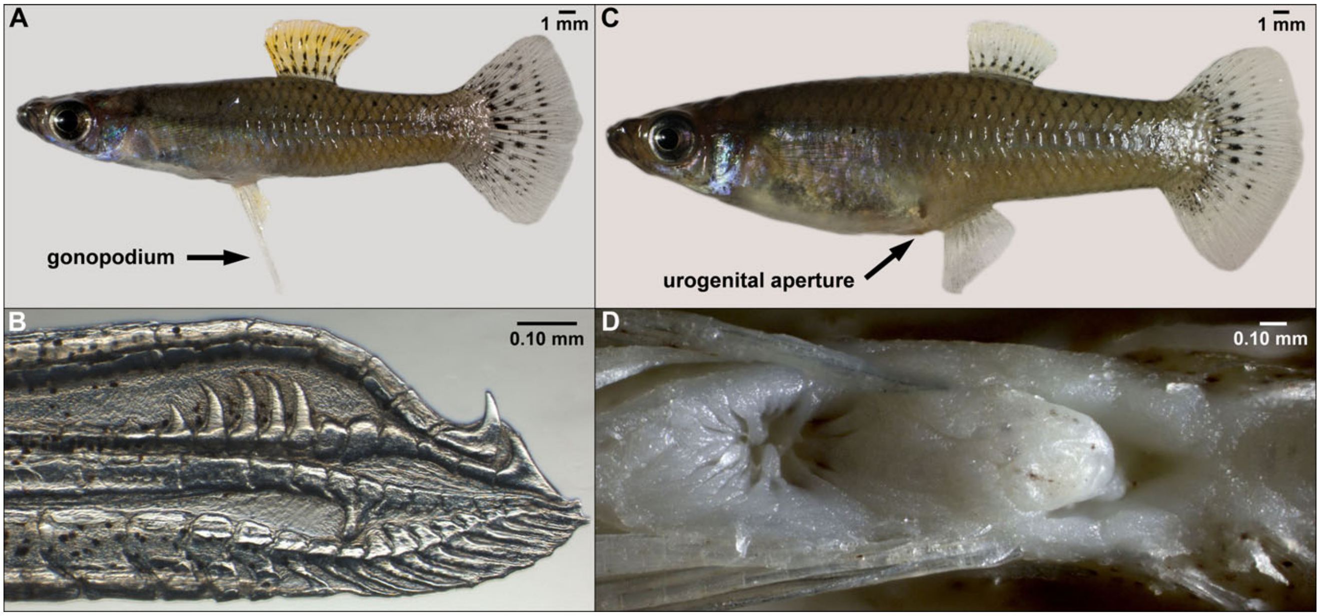

**Figure 1.** Photographs of *Gambusia hubbsi* (A) adult male, (B) lateral view of the distal tip of the male gonopodium, (C) adult female, and (D) ventral view of the female anus (on left, anterior) and urogenital aperture (on right, posterior; see Fig. 3 for details).

populations and species (Kelly et al. 2000; Jennions and Kelly 2002; Langerhans et al. 2005; Langerhans 2011; Heinen-Kay and Langerhans 2013a), with considerable variation in the complex distal tip, which directly contacts the female genitalia during copulation. Gonopodial tips comprise both bony fin-ray elements and membranous tissue, and exhibit a complex array of features such as hooks, spines, serrae, and an elbow (Fig. 1B; Rosen and Gordon 1953; Rosen and Bailey 1963; Langerhans 2011). Gonopodial tip morphology could influence insemination success through mechanical or stimulatory processes (Evans et al. 2011; Kwan et al. 2013), and exhibits differences in shape between predation regimes in Bahamas blue holes (Heinen-Kay and Langerhans 2013a). Comparatively little is known about variation in female genital morphology in poeciliid fishes, but existing work has documented notable variation among populations and species (Peden 1972b; Evans et al. 2011; Greven 2011; Langerhans 2011). The female genitalia of Bahamas mosquitofish comprise the external urogenital aperture and the internal urogenital sinus. The urogenital aperture contains a large papilla and a small opening, within which the gonopodial tip must enter for insemination (Fig. 1C,D). The opening leads to the urogenital sinus, where the urinary and reproductive tracts meet. Because the gonopodium directly interacts with the external features of the female genitalia, we exclusively focus on that region in this study. As with male genital morphology, variation in the size and shape of the female genital aperture, papilla, and opening could analogously influence insemination success of males.

Altogether, several lines of evidence demonstrate the plausibility of all four hypotheses in driving evolutionary divergence of female genitalia within the focal system: the relative strength of postmating sexual selection appears stronger in high-predation environments, physical appearance of genitalia suggests that sexual conflict or female choice could be important, and evidence for reproductive isolation among many populations suggests that selection against interpopulation mating may be strong for populations that experience any appreciable migration. A fifth mechanism could also cause female genital diversification in some systems, but is not discussed here as we can largely rule it out in the present case. That is, female genitalia could diversify owing to shared genetic/developmental bases with male genitalia, which rapidly evolve for separate reasons ("pleiotropy hypothesis"). Because we focus on genital traits of Bahamas mosquitofish that are nonhomologous among the sexes, we do not consider this further in the present study. Here, we empirically test the relative importance of the four major mechanisms described in Table 1 in producing diversification of female genital morphology.

## Methods

To test the four hypotheses of diversification of female genitalia, we employed a four-step approach, testing (1) whether female genital morphology has diverged between predation regimes (test of ecology hypothesis), (2) whether male and female genital morphology is correlated among populations while statistically controlling for potential ecological factors (test for pattern predicted by sexual conflict, female choice, and lock-and-key hypotheses), (3) whether populations with higher migration probabilities exhibit greater divergence in those aspects of genital morphology that we have identified as potentially coevolving among the sexes (test of lock-and-key hypothesis), and (4) whether a pattern of coevolution of male and female genitalia persists after statistically controlling for genetic relatedness (test of sexual conflict and female choice hypotheses). This latter test is designed to rule out mechanisms that can result in male–female genital correlation among populations (shared ancestry, lock-and-key), so as to isolate the effects of sexual conflict and female choice per se (see above). With our tests, we cannot distinguish between the sexual-conflict and female-choice hypotheses, as they make similar predictions for among-population differentiation. Thus, we focus on uncovering the importance of the ecology hypothesis, lock-and-key hypothesis, and sexual-conflict/female-choice hypotheses in driving female genital diversity in Bahamas mosquitofish. To accomplish these objectives, we measured female genital morphology for 14 populations of Bahamas mosquitofish, and examined previously published data on several biotic and abiotic factors in blue holes, male genital morphology, and genetic relatedness among populations (all from Heinen-Kay and Langerhans 2013a). We further conducted a common-garden rearing experiment using four populations to examine the genetic basis of morphological differences observed in wild-caught fish.

We collected 109 female Bahamas mosquitofish from 14 blue holes on Andros Island, The Bahamas (eight low-predation, six high-predation; Table 2, Fig. 2); all fish examined were preserved in 70% ethanol. Images of female genitalia were captured using a Leica S8 APO stereoscope (Leica Microsystems Inc., Buffalo Grow, IL, USA) equipped with a DFC 425 digital camera and a TCL RCI base. For each specimen, three to four photographs were taken of the female genital region at either 48× or 64× magnification, and stacked into a single composite image using the software Helicon Focus (http://www.heliconsoft.com/). We used TPSDIG2, version 2.16 (Rohlf 2010) to measure several aspects of female genital morphology on each image (Fig. 3). We performed five measurements on each image, blind with respect to the predation regime of origin for each individual. From these measurements, we calculated a total of eight genital traits: urogenital aperture area, apertural opening area, apertural papilla area, proportional apertural opening area (apertural opening area/urogenital apertural area), apertural opening width, apertural opening length, apertural elongateness (apertural opening width/length), and apertural opening aspect ratio (apertural opening width$^2$/apertural opening area). Areas were measured by tracing outlines of relevant features on the images.

**Table 2.** Sample size information for measurement of female genital morphology of preserved *Gambusia hubbsi* specimens from 14 blue holes.

| *Gobiomorus* | *Cyprinodon* | Population | n |
|---|---|---|---|
| Absent | Absent | Archie's (A)† | 9 |
| Absent | Absent | East Twin (E)† | 8 |
| Absent | Absent | Gabbler (Ga) | 8 |
| Absent | Absent | Little Frenchman (LF) | 9 |
| Absent | Present | Douglas-Christopher (DC) | 9 |
| Absent | Present | Gollum's (Go)† | 7 |
| Absent | Present | Hubcap (Hu)† | 7 |
| Absent | Present | Rainbow (Ra)† | 10 |
| Present | Absent | Cousteau's (C)† | 9 |
| Present | Absent | Runway (Ru)† | 7 |
| Present | Absent | Stalactite (St)† | 7 |
| Present | Absent | West Twin (W)† | 7 |
| Present | Present | Goby Lake (GL)† | 8 |
| Present | Present | Rivean's (Ri)† | 8 |

Presence of the predatory fish, *Gobiomorus dormitor,* and the potential competitor species, *Cyprinodon variegatus*, provided. Specimens collected in 2004 or 2006, with the exceptions of Douglas-Christopher (2010), Stalactite (2010), and Hubcap (2011).

† indicates populations used in model selection analyses and tests involving genetic relatedness.

Because size or shape of genital components might vary with overall body size and parturition history, we attempted to minimize variation due to these variables by exclusively examining large adult females (> 0.3 g); females of this size collected from the wild are virtually guaranteed to have given birth multiple times in their lives. We measured body mass of each fish using a Core™ compact portable balance CQT202 scale (Adam Equipment Co. Ltd., Danbury, CT, USA). Prior to analysis, we first transformed the variables to meet assumptions of normality of residuals (log-transformed mass, area, and length measurements, arcsine square-root transformed proportional area of apertural opening, and square-root transformed ratio metrics). We then regressed all variables against body size (log-transformed body mass) and saved residuals (after confirming homogeneity of slopes among groups) for all cases exhibiting significant associations (all but measurements of apertural elongateness and apertural opening aspect ratio). To control for body size, we used these eight size-corrected genital traits in analyses described below. Moreover, female body size did not differ between predation regimes (mixed-model ANOVA testing for differences between predation regimes, while treating population as a random effect: $F_{1,12} = 1.22$, $P = 0.2912$). Altogether, our dataset provided a highly appropriate dataset for investigation of among-population variation in female genital morphology, independent of variation in body size or prior parturition experience.

To test for a genetic basis of observed among-population differences in female genital morphology, we raised offspring of wild-caught fish from four blue holes under common laboratory conditions. We acquired offspring from wild-caught, pregnant females that were housed in the laboratory for at least two months

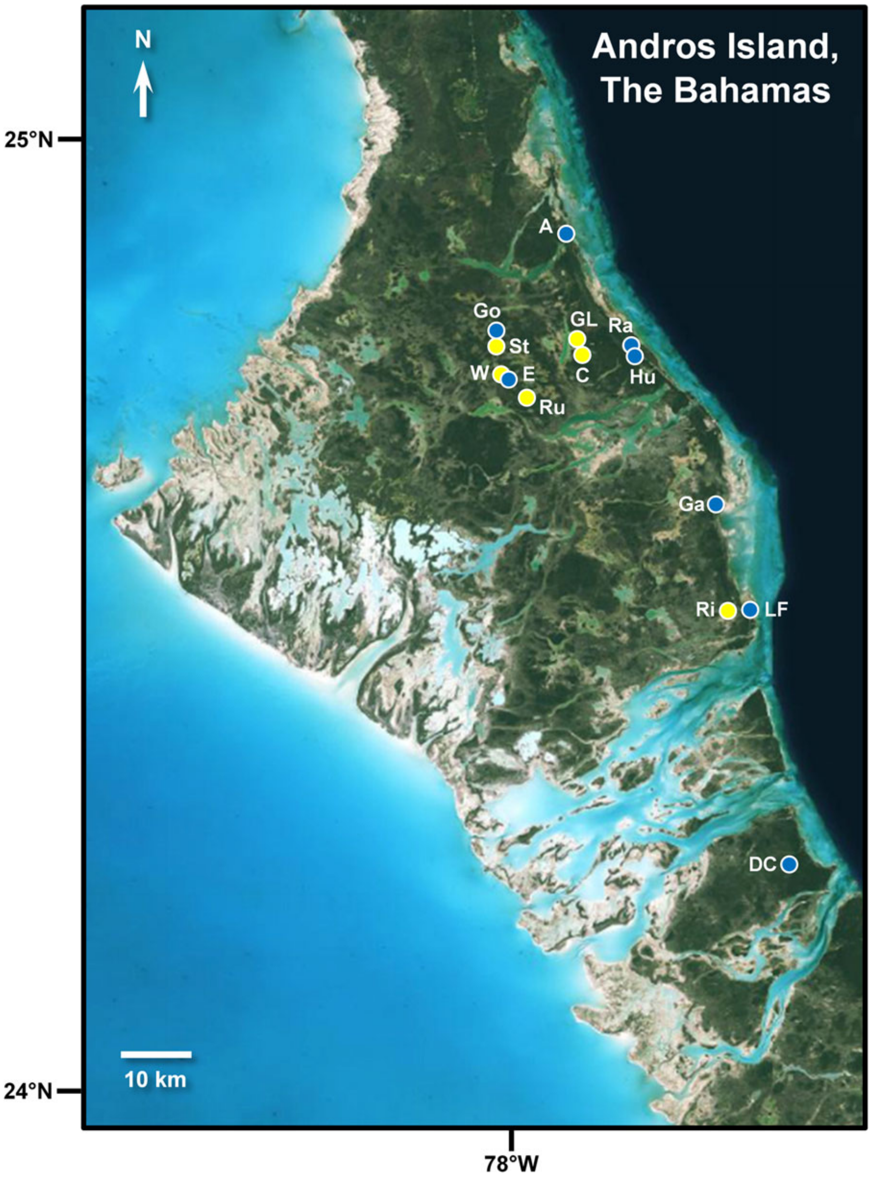

**Figure 2.** Map of 14 study sites on Andros Island, The Bahamas (see Table 2 for population abbreviations). Yellow/light symbols: predatory fish present; blue/dark symbols: predatory fish absent.

prior to parturition in an effort to minimize maternal effects. Lab-born fish were raised in 38-L aquaria at approximately 25°C, and fed brine shrimp nauplii, daphnia, bloodworms, and TetraMin Pro flakes. To avoid confounding any tank effects with population effects, we reared individuals from all populations in multiple aquaria (2–6 tanks per population). We measured laboratory-born adult females ($n = 31$; see Table S1 for details) using the same methods described above for wild-caught fish.

## Statistical Analyses

First, we tested whether female genital morphology, characterized by the eight genital measurements, differed between predation regimes (test of ecology hypothesis) by conducting a mixed-model multivariate analysis of variance (MANOVA). The eight size-corrected genital traits served as dependent variables, predation regime as the fixed effect, and population as a random effect. We conducted an $F$ test employing restricted maximum likelihood and the Kenward–Roger degrees of freedom adjustment (Kenward and Roger 2009) using the MIXED procedure in SAS to test significance of the predation regime term. This procedure enabled us to employ population as the unit of replication, effectively treating it as a random effect (Hassell et al. 2012; Heinen-Kay and Langerhans 2013a; Riesch et al. 2013; Martin et al. 2014). To interpret any significant differences in female genital morphology between predation regimes, we

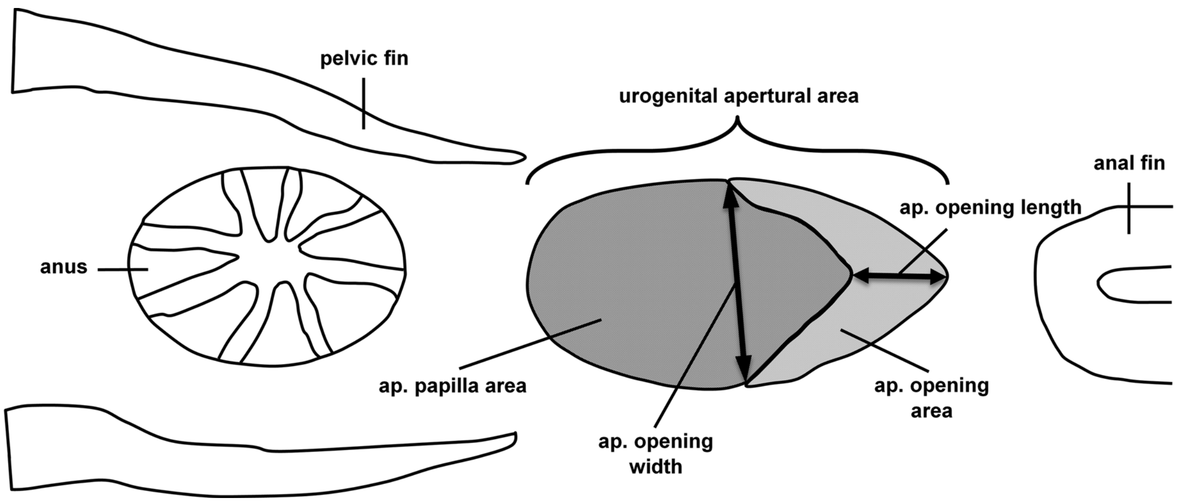

**Figure 3.** Diagrammatic ventral view of the ano-urogenital region of female *Gambusia hubbsi* illustrating the five measurements (three areas, two lengths) used to calculate the eight genital traits examined in this study ("apertural" abbreviated as "ap."; see Fig. 1D for representative photograph). The shaded region denotes the urogenital aperture.

examined canonical variate loadings derived from the predation regime term of the MANOVA, conducted posthoc univariate tests with each genital trait (model structure identical to MANOVA), and calculated the magnitude of trait differences between predation regimes using the standardized effect size of Cohen's *d* (the difference between group means in standard deviation units; Cohen 1988).

Second, we used a model selection approach to test (1) whether ecological factors in addition to predation might influence female genital divergence, and (2) whether male and female genital morphology were correlated among populations while statistically controlling for potential ecological factors (test of pattern predicted by sexual-conflict, female-choice, and lock-and-key hypotheses). We selected five ecological factors in addition to predation regime for investigation due to their potential influence in altering sexual selection on genitalia through changes in sex ratio or resource availability/competition: adult Bahamas mosquitofish sex ratio (F:M), presence of the competitor pupfish *Cyprinodon variegatus* (sheepshead minnow), log-transformed zooplankton density, log-transformed phytoplankton density, and relative chlorophyll *a* density (all data from Heinen-Kay and Langerhans 2013b; see original publication for methodological details). All ecological variables are highly repeatable across time, indicating reasonable estimates for comparison among blue holes (Heinen et al. 2013). To estimate male genital morphology, we extracted population means of gonopodial distal-tip shape (Relative Warp 2 [RW2], a principal component axis of male genital shape) from Heinen-Kay and Langerhans (2013b). We employed this particular estimate of male genital shape because of its known differentiation between predation regimes, known genetic basis, and the fact that this region captures the part of the male genitalia that makes contact with the female genitalia during copulation. Because only 11 populations overlapped between the studies (see Table 2), we used a reduced dataset of 11 populations for these analyses. For each of the eight size-corrected female genital traits, as well as the multivariate canonical variate derived from our mixed-model MANOVA described above, we examined their possible association with ecological factors and male genital shape by conducting general linear models with the possible model terms: presence of the predator *G. dormitor*, presence of the competitor *C. variegatus*, adult Bahamas mosquitofish sex ratio, log-transformed zooplankton density, log-transformed phytoplankton density, relative chlorophyll *a* density, and male genital morphology (RW2) (variance inflation factors $< 3$ for all models). For each female genital trait, we selected top models for presentation based on Akaike information criterion corrected for small sample sizes ($\leq 2$ $\Delta\mathrm{AIC}_c$ units) and Akaike weight ($\geq 0.10$ Akaike weight).

Third, we tested the prediction of the lock-and-key hypothesis that populations with greater opportunities for interpopulation mating should exhibit greater divergence in genital morphology (i.e., reproductive character displacement). We used genetic distance to estimate migration probabilities for each population pair because this provides the most reasonable known surrogate for this study system, as geographic distance does not appear to correspond to gene flow or movement patterns in these fish (Schug et al. 1998; Langerhans et al. 2007; Heinen-Kay and Langerhans 2013a). We conducted a Mantel test (one-tailed significance test using 9999 randomizations) to examine the association between genetic relatedness and both male and female genital morphology (Mantel 1967). We exclusively examined aspects of genital morphology identified in the model selection analyses as showing patterns of coevolution among male and female genitalia. We used previously published estimates of genetic relatedness among populations based on mtDNA (Langerhans et al. 2007; Heinen-Kay and Langerhans 2013a; Riesch et al. 2013). Again, this analysis used a reduced set of 11 populations.

Fourth, we tested for male–female genital coevolution, controlling for genetic relatedness, as predicted by the sexual conflict and female choice hypotheses. To accomplish this, we conducted a partial Mantel test (one-tailed significance test using 9999 randomizations), examining the association between male and female genital morphology (using the aspects identified above as showing evidence for male–female coevolution), while holding the matrix of genetic distance constant.

Finally, we tested for a genetic basis to observed differences in female genital morphology among populations using lab-born fish from four populations raised in a common laboratory environment. Both wild-caught and laboratory-born fish for each of these four populations were included in statistical analyses. Wild-caught fish from these four populations comprised the fish previously examined in analyses described above. We performed MANOVA using the same eight size-corrected genital traits described earlier as dependent variables, and population, rearing environment (wild-caught or laboratory-born), and their interaction as independent variables. We were especially interested in the population term, as a significant population effect would indicate that wild-caught and laboratory-born fish from the same population tended to more closely resemble one another in female genital morphology than fish derived from different populations (i.e., a genetic basis to observed population differences). To interpret the nature of any multivariate differences revealed by MANOVA, we examined canonical variate loadings for the first canonical variate derived from each term of the MANOVA, conducted posthoc univariate tests with each genital trait (model structure identical to MANOVA), and calculated $\eta^2$ as an estimate of the effect size of each model term (percent of variance explained by each term).

Because we conducted multiple significance tests based on the same populations of fish, we can experience inflated Type I error rates. To correct for this without suffering the substantial increase in Type II error rates (reduction of statistical power) associated with Bonferroni correction procedures (e.g., Garcia 2004; Nakagawa 2004; Verhoeven et al. 2005), we controlled the false discovery rate for all of our tests conducted in this study (FDR; Benjamini and Hochberg 1995; Storey 2003; Storey and Tibshirani 2003). FDR describes the proportion of significant tests that are actually null. We used the program QValue (Storey 2003) to control the FDR at 5%, using the bootstrap procedure to estimate $\pi_0$ (probability of a true null hypothesis). We determined tests were significant when both the $P$-value and $q$-value (FDR equivalent of $P$-value; determined by Q-value) were less than or equal to 0.05. In this study, all observed $P$-values $\leq 0.05$ remained significant at the FDR of 5%. Thus, we straightforwardly interpret $P$-values $\leq 0.05$ as significant throughout.

# Results

Mixed-model MANOVA revealed significant differences in female genital morphology between predation regimes ($F_{7,345} = 10.14, P < 0.0001$). Inspection of canonical loadings and results of posthoc tests uncovered widespread differences in female genitalia between predation regimes (significant differences for six of eight traits), with three particular traits showing the strongest and most consistent divergence across predatory environments: apertural opening area, apertural opening width, and urogenital aperture area (Table 3, Fig. 4). This indicated that overall, populations without predatory fish had relatively larger female genital measurements, controlling for body size, than populations that have coevolved with piscivorous fish, with an average of 89% larger apertural opening area, 74% larger apertural opening width, and 48% larger urogenital aperture area. Low-predation populations also tended to exhibit greater elongateness of the apertural opening, reflecting a more crescent shaped opening (greater width relative to length). Descriptive statistics for all variables are provided in Table S2. These findings provide strong support for the ecology hypothesis.

Within our model selection framework, predation clearly had the strongest overall influence on female genital morphology, but we also uncovered other significant predictors (Table 4). Predation regime solely and strongly influenced variation in the canonical variate describing overall female genital shape, indicating that other environmental factors and male genital morphology played little role in influencing this multivariate axis when controlling for variation in predation regime. For the univariate genital traits, predation regime was included within the top model set for seven of eight genital traits, and provided the only significant predictor for four traits: urogenital aperture area, apertural opening area, apertural papilla area, and apertural opening width. In each case, populations without predatory fish exhibited relatively larger genital measurements, reaffirming MANOVA results and indicating that predation risk shows an unambiguous association with female genital morphology. The only trait for which an environmental factor other than predation provided a significant predictor was proportional apertural opening area. This trait exhibited complex results, with a positive association with the presence of the competitor pupfish, chlorophyll *a* density, and Bahamas mosquitofish F:M sex ratio, and a negative association with the presence of predatory fish. We found suggestive effects of phytoplankton density for several traits, but it was never in the top model nor statistically significant. The top model for two female genital traits included only male genital morphology (RW2), providing

**Table 3.** Canonical variate loadings for the predation regime term of the mixed-model MANOVA, significance of differences between predation regimes in post-hoc tests, and standardized univariate effect sizes (Cohen's *d*) between predation regimes.

| Female genital trait | Canonical loading | *P* | Cohen's d (std. error) |
|---|---|---|---|
| **Urogenital apertural area** | **0.72** | **0.0094** | **1.69 (0.69)** |
| **Apertural opening area** | **0.83** | **0.0003** | **2.67 (0.83)** |
| Apertural papilla area | 0.65 | 0.0226 | 1.42 (0.66) |
| Proportional opening area | 0.48 | 0.0245 | 1.40 (0.66) |
| **Apertural opening width** | **0.89** | **0.0044** | **1.89 (0.72)** |
| Apertural opening length | 0.03 | 0.6435 | 0.26 (0.59) |
| Apertural opening width/length | 0.67 | 0.0330 | 1.30 (0.65) |
| Apertural opening aspect ratio | 0.63 | 0.0656 | 1.09 (0.63) |

Bold text highlights the three female genital traits with clearest and strongest differentiation between predation regimes, although many traits exhibited differences.

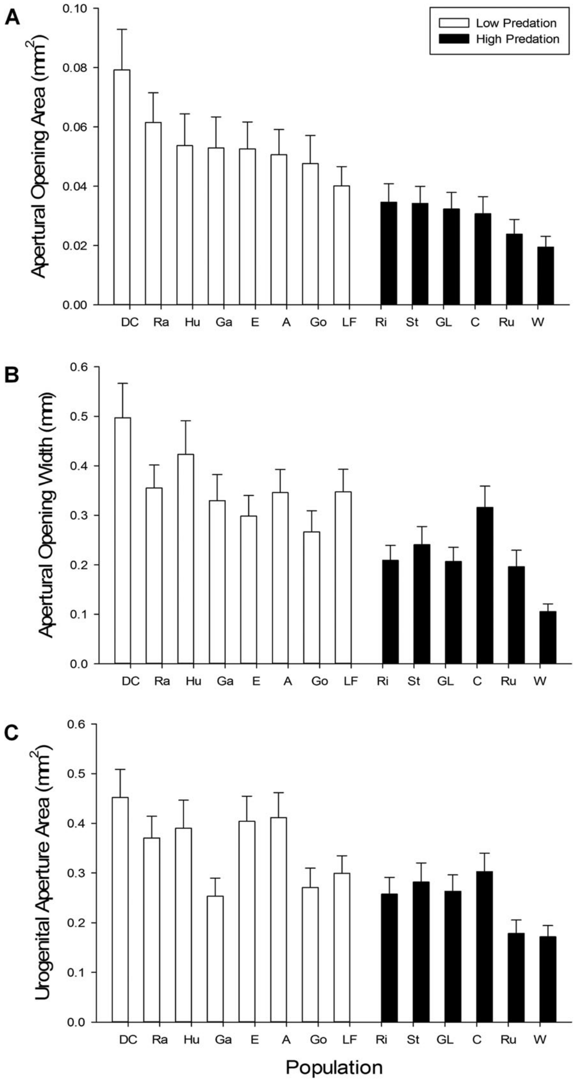

**Figure 4.** Variation among populations in female (A) urogenital apertural opening area, (B) urogenital apertural opening width, and (C) urogenital aperture area. Population abbreviations follow Table 2 and Fig. 2. Back-transformed least-squares means ± SE depicted.

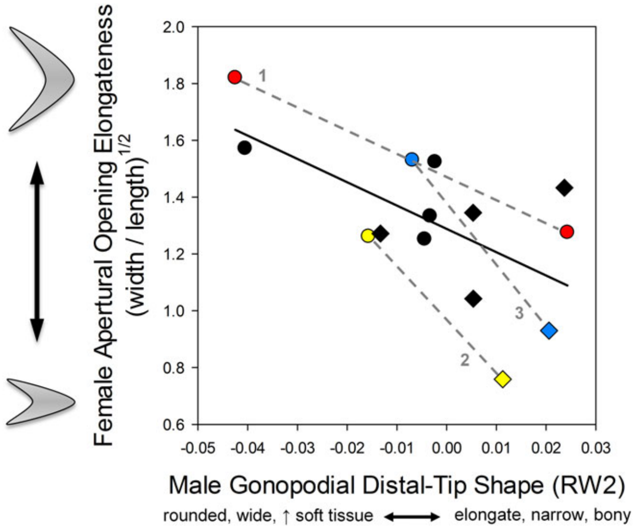

**Figure 5.** Relationship between the shape of male and female genitalia across populations of Bahamas mosquitofish (circles: predator absent, diamonds: predator present). Shape variation in female genital openings depicted along *Y* axis. The three numbered population pairs with dashed lines connecting them denote the three pairs with the highest probability of migrants/gene flow, illustrating the strong differences between populations most likely to have experienced historical or recent mating opportunities: 1: Hu, Ra; 2: E, W; 3: LF, Ri (population abbreviations follow Table 2 and Fig. 2).

significant evidence for correlated evolution of male and female genital morphology for one aspect of female genitalia: apertural opening elongateness (Fig. 5). This finding is consistent with the prediction of coevolution of genitalia among the sexes made by the sexual conflict, female choice, and lock-and-key hypotheses.

For our Mantel tests, we exclusively examined apertural opening elongateness and gonopodial distal-tip shape (RW2), as these traits exhibited a significant among-population correlation. In our test of reproductive character displacement (predicted only

**Table 4.** Summary of results from our model selection analyses.

| Trait | Top model |  | Model 2 |  |  | Model 3 |  |  | Model 4 |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | Term(s) | Ak Wt | Term(s) | $\Delta$AIC*c* | Ak Wt | Term(s) | $\Delta$AIC*c* | Ak Wt | Term(s) | $\Delta$AIC*c* | Ak Wt |
| Canonical variate | $\text{Pred}^{**}$ | 0.61 |  |  |  |  |  |  |  |  |  |
| Urogenital apertural area | $\text{Pred}^{*}$ | 0.33 | $\text{Pred}^{**} + \text{Phy}$ | 0.64 | 0.24 |  |  |  |  |  |  |
| Apertural opening area | $\text{Pred}^{**}$ | 0.38 | $\text{Pred}^{**} + \text{Phy}$ | 0.87 | 0.24 |  |  |  |  |  |  |
| Apertural papilla area | $\text{Pred}^{*}$ | 0.32 | $\text{Pred}^{*} + \text{Phy}$ | 1.16 | 0.18 | $\text{Pred}^{**} + \text{Chl}$ | 1.8 | 0.13 |  |  |  |
| Proportional opening area | $\text{Pup}^{**} + \text{Chl}^{*}$ | 0.23 | $\text{Pred}^{*} + \text{SR}^{*}$ | 1.13 | 0.13 | $\text{SR}^{*}$ | 1.48 | 0.11 | $\text{Pup}^{*}$ | 1.53 | 0.11 |
| Apertural opening width | $\text{Pred}^{*}$ | 0.44 |  |  |  |  |  |  |  |  |  |
| Apertural opening length | MG | 0.31 |  |  |  |  |  |  |  |  |  |
| Apertural opening width/length | $\text{MG}^{*}$ | 0.26 | Pred | 0.35 | 0.21 |  |  |  |  |  |  |
| Apertural opening aspect ratio | Pred | 0.15 | SR | 0.08 | 0.15 | Phy | 0.88 | 0.1 |  |  |  |

Our selected model set included all models with $\Delta\text{AIC}_c \leq 2$ and Akaike weight $\geq 0.10$. Model term abbreviations: Pred = *Gobiomorus* predator presence, Pup = *Cyprinodon* pupfish presence, SR = Bahamas mosquitofish adult sex ratio (F:M), Zoo = zooplankton density, Phy = phytoplankton density, Chl = chlorophyll *a* density, MG = male genital morphology (RW2 from Heinen-Kay and Langerhans [2013b], describing shape variation in the distal-tip of the gonopodium).

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

by the lock-and-key hypothesis), we found significant support for both female and male genital morphology, as these traits exhibited significant, negative matrix correlations with genetic distance (females: $P = 0.0442$, males: $P = 0.0011$). This suggests that population pairs with greater mating opportunities (estimated here as smaller genetic distance) tend to exhibit greater divergence in male and female genital morphology (see Fig. 5). In our test for among-population correlation of male and female genital morphology, while controlling for genetic relatedness, partial Mantel test revealed no evidence for male–female genital coevolution ($P = 0.2197$). This rejects the prediction of overall male–female genital coevolution, irrespective of the effects of reproductive character displacement, as made by the sexual conflict and female choice hypotheses. Moreover, this result unlikely reflects lack of statistical power (Harmon and Glor 2010; Legendre and Fortin 2010), as a partial Mantel test conducted to examine the association between overall female genital morphology (phenotypic distance using all eight genital traits) and predation regime (0: same, 1: different), while controlling for genetic distance, revealed a significant, positive correlation ($P = 0.0263$; female genital morphology more similar among populations within the same predation regime).

We found significant evidence for a genetic basis to differences in female genital morphology ($F_{24,145.62} = 2.06$, $P = 0.0049$), as well as differences between wild-caught and lab-raised individuals ($F_{8,50} = 8.06$, $P < 0.0001$); differences between populations did not vary among rearing environments ($F_{24,145.62} = 1.22$, $P = 0.2372$). Inspection of canonical loadings and posthoc univariate tests indicated that populations differed consistently in the size of the genital opening regardless of whether they were wild-caught or lab-raised (all four estimates of relative opening size had significant differences), while lab-raised fish tended to have smaller genital measurements than wild-caught fish (Table S3). Descriptive statistics for lab-raised fish are provided in Table S4.This indicated that variation among populations in relative size of the genital opening appears largely genetically based (explaining 18.33% of phenotypic variation, on average). Although we did not find significant evidence for genetically based differences for size of the overall aperture or papilla, or for our two shape variables, those traits did not significantly differ in wild-caught individuals from these four particular blue holes. Thus, for all female genital traits that showed differentiation in the field for these four populations, lab-raised fish retained those differences.

# Discussion

We uncovered strong divergence in female genitalia within the post-Pleistocene radiation of Bahamas mosquitofish inhabiting the blue holes of Andros Island, The Bahamas. Among-population variation in female genital morphology appeared to largely reflect genetically based differences based on our laboratory experiment, although we also showed that rearing environment can influence genital morphology. While the evolutionary diversification of female genitalia has so far received much less attention than male genitalia, here we described and tested four mechanisms that could drive rapid changes in female genital morphology, and pinpointed two apparent causes for most of the observed female genital divergence in the present study: (1) variation in predation risk that altered sexual selection on the relative *size* of female genitalia, and (2) selection against inter-population mating that led to divergence in the *shape* of female genitalia and the coevolution of male and female genital shape among populations. We found no evidence that sexual conflict or female choice were responsible for driving correlated evolution of male and female genitalia.

Female Bahamas mosquitofish exhibited larger genital openings with a relatively crescent shape in blue holes without predatory fish, providing strong support for the ecology hypothesis (Fig. 6). Because we did not find evidence of correlated evolution between the size of the female genital opening and male genital morphology when controlling for effects of ecological factors, the observed patterns appear to largely represent an overall response to changes in predation risk, and not a response to selection imposed by male genital morphology through sexually antagonistic selection or as a morphological means of preferring particular male genital shapes through cryptic female choice. For the shape of the genital opening, we found that among-population variation was statistically explained by an association with male genital morphology, not predation risk (see discussion below). For one genital trait (proportional apertural opening area), we also uncovered evidence for the importance of ecological factors other than predation risk, such as interspecific competition, primary productivity, and adult sex ratio. However, our results clearly indicate that the presence of predatory fish represents the strongest and most broadly important ecological agent associated with female genital morphology.

We suggest that divergence in female genital morphology between predation regimes reflects differences in the strength of premating sexual selection (stronger in the absence of predators) and postmating sexual selection (stronger in the presence of predators). In the absence of predation threat, selection can favor increased courtship behaviors and permit females to exhibit considerable choice of mates prior to mating, while under high predation risk, selection can favor reduced courtship and even increased coercive mating behaviors in males and lead to females that attempt to exert choice of males largely during or after copulation (cryptic female choice, sexual conflict) (Magurran and Seghers 1994; Godin 1995; Eberhard 1996; Candolin 1997; Hruskova-Martisova et al. 2010). Indeed, previous work in this system has demonstrated divergence between predatory environments in mating behaviors (e.g., more frequent sexual behaviors in presence of predators), and male traits thought to experience differences in premating sexual selection (e.g., greater male coloration without predators) and postmating sexual selection (elongate, narrow, bony male genital tips with predators) (Heinen-Kay and Langerhans 2013a; Heinen et al. 2013; Martin et al. 2014). Thus, reduced size of the genital opening in the presence of predators might represent greater postmating female choice of males through physical restriction of gonopodial entry during copulation (either via “choice” to enhance fitness gains of male choice, or “conflict” to reduce costs of mating); whereas the larger genital openings observed without predators could reflect relaxed selection for postmating female choice in the nonrisky environment where premating female choice can more freely operate. But can the observed female genitalia actually restrict gonopodial entry?

Based on available evidence, females can likely bias insemination through mechanical restriction of the genital opening. First, copulatory behaviors of *Gambusia* fishes represent some of the most sophisticated and rapid behaviors known in fishes (Rivera-Rivera et al. 2010). For successful copulation, a male must accurately place the tip of the gonopodium into a very small genital opening while performing a complicated torque-thrust maneuver that takes only 20–50 ms. Slight movement by the female during or just prior to the copulation attempt can cause the gonopodial tip to completely miss the urogenital aperture. Thus, even small mechanical constraints on gonopodial entry could potentially have substantial consequences. Second, depending on the depth of insertion into the female apertural opening, male gonopodial tips are typically 0.31–0.44 mm in width for an average-sized male (data from Heinen-Kay and Langerhans 2013b); tip dimensions do not appear to change during copulation [R.B.L. unpubl. data]). During copulation, these tips must insert into openings that are typically only $\sim$0.20 mm wide in high-predation localities and $\sim$0.35 mm wide in low-predation populations, for relatively large-sized females (we examined females $>$0.3 g in this study; opening widths are virtually identical for live and preserved fish [C.M.A. and R.B.L. unpubl. data]). With typical male genital tips ranging from 11% smaller to over 200% larger than female genital openings, this suggests that females might frequently have the morphological capability of restricting insemination by males, perhaps often requiring some degree of behavioral cooperation (or mechanical fit that depends on shape) for the majority of successful copulations. This is consistent with previous work pointing to the importance of cryptic female choice in the evolution of male genitalia, and more specifically to the putative role of sexual conflict in the evolution of male and female genital form in poeciliid fishes (Evans et al. 2011; Gasparini et al. 2011; Langerhans 2011; Kwan et al. 2013; Wang et al. 2015). Future work should test functional hypotheses for male–female genital interactions, and whether female genital morphology affects insemination or

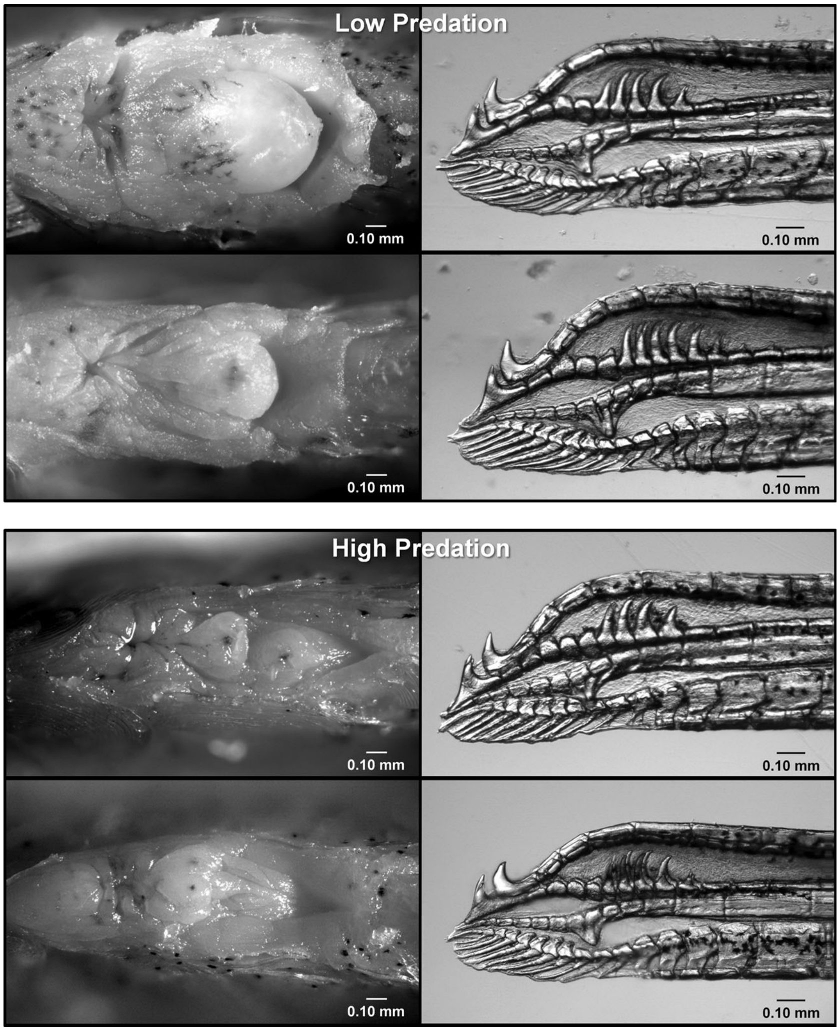

**Figure 6.** Representative photographs of female and male genitalia of Bahamas mosquitofish from low-predation (top panel; upper: LF blue hole, lower: DC blue hole) and high-predation (bottom panel; upper: Ri blue hole, lower: W blue hole) populations. Female ano-urogenital region photographs on left, male gonopodial distal-tip photographs on right.

fertilization success of males (and potentially bias toward certain male phenotypes).

Given the previous research suggesting the possible importance of sexual conflict and cryptic female choice for the rapid evolution of female genital morphology, why did we find no such evidence in this study? First, had we ignored the ecological environment, we would have observed male–female correlation in multiple genital traits, consistent with these mechanisms. Indeed, some aspects of these patterns, such as a negative association between female genital opening size and male genital tip elongateness and boniness, superficially suggests a coevolutionary arms race driven by sexual conflict. However, variation in predation regime explained the bulk of variation in genital morphology, not male–female genital coevolution. Genitalic traits of both sexes exhibited nontrivial among-population variation within each predation regime, and yet they showed little evidence of coevolution. We only found one significant pattern of among-population correlation in male and female genital morphology. However, in this case the correlation was statistically explained by genetic relatedness (lock-and-key hypothesis, see below). Thus, it appears that sexually antagonistic selection has not driven correlated evolution among male and female genitalia within this system, nor has variation in female choice of male genital morphologies relied on changes in external female genital form. Female genital morphology could certainly facilitate preference for particular males (e.g., based on nongenital traits)—and the small opening size might provide ample opportunity for stimulus-based choice—but any preference for certain male genital forms apparently relies on variation in sensory, behavioral, neurological, or internal genital morphological traits.

For one aspect of female genital shape (not relative size), we found significant support for the lock-and-key hypothesis, where selection against interpopulation mating appears to have led to greater genital divergence between populations having higher probabilities of migration—no other mechanism makes such a prediction. Although Bahamas mosquitofish in blue holes represent allopatric populations, for which many seem quite isolated, several lines of evidence suggest at least low-moderate levels of movement among some blue holes, either historically or recently: close geographic proximity, relatively flat topography of Andros Island with abundant seasonal marshes, notoriously high movement and colonization ability of *Gambusia* fishes, and molecular data suggesting gene flow for some populations (Schug 1995; Schug et al. 1998; Langerhans et al. 2007; Riesch et al. 2013). For instance, prior estimates of the theoretical number of migrants per generation exceed 1.0 for 12 of 14 blue holes examined using allozyme frequencies (Schug et al. 1998) and 11 of 17 blue holes examined using mtDNA (Heinen-Kay and Langerhans 2013a). Even low levels of population movement or gene flow can lead to enhanced reproductive isolation via selection against interpopulation mating (e.g., Servedio and Noor 2003). In the present study system, selection against migrants appears strong for many population pairs based on assortative mating and divergent adaptations to variation in predation risk and intra- and interspecific competition (Langerhans et al. 2007; Langerhans 2009; Heinen et al. 2013; Langerhans and Makowicz 2013). We found that genital shape of both sexes of Bahamas mosquitofish exhibited large differences among population pairs with small genetic distances, which apparently resulted in an overall pattern of correlated evolution among male and female genital shape (pattern disappeared after controlling for genetic relatedness). Thus, despite much recent work suggesting little importance of the lock-and-key mechanism in genital diversification (Ware and Opell 1989; Porter and Shapiro 1990; Arnqvist et al. 1997; Arnqvist and Thornhill 1998; Eberhard and Ramirez 2004), it appears to play a role in genital evolution in the present study system, consistent with supportive evidence in some prior work in *Gambusia* fishes (Peden 1972a; Peden 1975; Langerhans 2011). Observed patterns suggest a functional hypothesis: narrow and bony gonopodial tips may mechanically fit better into narrow and long female genital openings, while wide and comparatively soft gonopodial tips may more effectively transfer sperm within wide, curved female genital openings (see Fig. 6). Greater rigidity in the gonopodial tip might hinder its ability to conform to curved openings or properly stimulate certain genital regions, while wider gonopodial tips should have difficulty fitting into more narrow genital openings. Future work should test these functional hypotheses regarding restriction of heterospecific matings based on genital morphology, as well as more thoroughly investigate movement of individuals, and gene flow, among localities.

Results from our common-garden laboratory-rearing experiment suggest that many differences in female genital morphology observed among populations in the field reflect evolutionary divergence, not phenotypic plasticity. Although several traits differed between wild-caught and lab-raised fish, relative differences between populations were unchanged. While we point to the roles of ecology and selection against interpopulation mating as important drivers of female genital variation in this study, an alternative explanation is that it reflects differences in the size of offspring at birth (e.g., delivery of larger offspring requires larger external openings). Indeed, previous work has demonstrated evolutionary divergence in offspring size between predation regimes in this system (larger, but fewer, offspring in blue holes without predatory fish; Downhower et al. 2000; Riesch et al. 2013). However, using data on life-history traits from Riesch et al. (2013), we found no evidence for associations between any female genital trait and offspring size or fecundity while controlling for effects of predation regime, despite ample variation (all $P > 0.32$). We further observed consistent differences among populations in lab-raised fish, regardless of their prior mating or parturition experience. Thus, evidence to date suggests that female Bahamas mosquitofish have evolved different genital morphologies in different populations, depending on the predatory environment, and partially on past mating opportunities with fish from outside populations.

Our findings highlight the need for future work into the evolution of female genitalia, as we uncovered rapid divergence in external female genital morphology, strong importance of ecological variation in driving genital divergence, and a potentially crucial role of genital evolution in the speciation process through selection against interpopulation mating. A better understanding of the causes and consequences of female genital evolution will require both more detailed experimental approaches within systems such as Bahamas mosquitofish, as well as more comparative approaches in disparate taxa to evaluate the generality of evolutionary patterns of female genital diversification.

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## DATA ARCHIVING

Data are provided in the Supporting Information and deposited in Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.4gf06).

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## Supporting Information

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

**Table S1.** Sample size information for laboratory-born females raised in our common-garden experiment.
**Table S2.** Descriptive statistics for wild-caught female *Gambusia hubbsi* genital morphology (back-transformed means ± SD presented, adjusted for mean body size).
**Table S3.** Trait-by-trait evaluation of the genetic basis of differences in female genital morphology among four populations, including the canonical variate (CV) loadings for the first CV derived from each term of the MANOVA, and results from post-hoc ANOVAs conducted separately for each trait. Wild-caught and laboratory-born fish from four populations were included in analyses. Bold text denotes significant effects ($P < 0.05$).
**Table S4.** Descriptive statistics for lab-born female *Gambusia hubbsi* genital morphology (back-transformed means ± SD presented, adjusted for mean body size).

### Supporting Information for

# Origins of female genital diversity: predation risk and lock-and-key explain rapid divergence during an adaptive radiation

Christopher M. Anderson and R. Brian Langerhans

**Table S1.** Sample size information for laboratory-born females raised in our common-garden experiment. Presence within the parental blue hole of origin for the predatory fish, *Gobiomorus dormitor*, and the potential competitor species, *Cyprinodon variegatus*, provided.

| *Gobiomorus* | *Cyprinodon* | Population | Females | # aquaria |
|---|---|---|---|---|
| Absent | Absent | East Twin | 4 | 2 |
| Absent | Present | Rainbow | 8 | 3 |
| Present | Absent | Cousteau’s | 10 | 6 |
| Present | Absent | Stalactite | 9 | 3 |

**Table S2.** Descriptive statistics for wild-caught female *Gambusia hubbsi* genital morphology (back-transformed means ± SD presented, adjusted for mean body size).

| Predation Regime | Population | Urogenital apertural area (mm$^2$) | Apertural opening area (mm$^2$) | Apertural papilla area (mm$^2$) | Proportional opening area (mm$^2$) | Apertural opening width (mm) | Apertural opening length (mm) | Apertural opening width / length | Apertural opening aspect ratio |
|---|---|---|---|---|---|---|---|---|---|
| Low Predation | Archie's | 0.41 ± 0.15 | 0.05 ± 0.03 | 0.36 ± 0.13 | 0.13 ± 0.04 | 0.35 ± 0.14 | 0.16 ± 0.05 | 2.13 ± 0.89 | 2.46 ± 1.08 |
| | Douglas Christopher | 0.45 ± 0.17 | 0.08 ± 0.04 | 0.37 ± 0.14 | 0.18 ± 0.05 | 0.50 ± 0.21 | 0.20 ± 0.06 | 2.20 ± 0.91 | 2.97 ± 1.19 |
| | East Twin | 0.40 ± 0.14 | 0.05 ± 0.03 | 0.35 ± 0.13 | 0.13 ± 0.05 | 0.30 ± 0.12 | 0.20 ± 0.06 | 1.65 ± 0.79 | 1.90 ± 0.95 |
| | Gabbler | 0.25 ± 0.09 | 0.05 ± 0.03 | 0.20 ± 0.07 | 0.21 ± 0.06 | 0.33 ± 0.13 | 0.21 ± 0.06 | 1.59 ± 0.77 | 2.13 ± 1.01 |
| | Gollum's | 0.27 ± 0.10 | 0.05 ± 0.02 | 0.22 ± 0.08 | 0.18 ± 0.05 | 0.27 ± 0.11 | 0.15 ± 0.05 | 1.72 ± 0.80 | 1.56 ± 0.86 |
| | Hubcap | 0.39 ± 0.15 | 0.05 ± 0.03 | 0.33 ± 0.13 | 0.14 ± 0.05 | 0.42 ± 0.18 | 0.13 ± 0.04 | 2.92 ± 1.05 | 3.13 ± 1.22 |
| | Little Frenchman | 0.30 ± 0.11 | 0.04 ± 0.02 | 0.26 ± 0.09 | 0.14 ± 0.05 | 0.35 ± 0.14 | 0.17 ± 0.05 | 2.41 ± 0.95 | 3.37 ± 1.26 |
| | Rainbow | 0.37 ± 0.14 | 0.06 ± 0.03 | 0.31 ± 0.12 | 0.17 ± 0.05 | 0.36 ± 0.15 | 0.22 ± 0.07 | 1.83 ± 0.83 | 2.21 ± 1.02 |
| | Average | 0.35 ± 0.08 | 0.05 ± 0.01 | 0.30 ± 0.07 | 0.16 ± 0.03 | 0.35 ±0.10 | 0.18 ± 0.04 | 2.08 ± 0.69 | 2.47 ± 0.82 |
| High Predation | Cousteau’s | 0.30 ± 0.11 | 0.03 ± 0.02 | 0.27 ± 0.10 | 0.12 ± 0.04 | 0.32 ± 0.13 | 0.16 ± 0.05 | 2.25 ± 0.92 | 3.07 ± 1.21 |
| | Goby Lake | 0.26 ± 0.09 | 0.03 ± 0.02 | 0.23 ± 0.08 | 0.13 ± 0.05 | 0.21 ± 0.08 | 0.20 ± 0.06 | 1.15 ± 0.66 | 1.53 ± 0.85 |
| | Rivean's | 0.26 ± 0.10 | 0.04 ± 0.02 | 0.22 ± 0.08 | 0.14 ± 0.05 | 0.21 ± 0.09 | 0.24 ± 0.07 | 1.01 ± 0.62 | 1.35 ± 0.8 |
| | Runway | 0.18 ± 0.07 | 0.02 ± 0.01 | 0.15 ± 0.06 | 0.13 ± 0.05 | 0.20 ± 0.08 | 0.12 ± 0.04 | 1.37 ± 0.72 | 1.54 ± 0.86 |
| | Stalactite | 0.28 ± 0.10 | 0.03 ± 0.02 | 0.25 ± 0.09 | 0.12 ± 0.04 | 0.24 ± 0.10 | 0.15 ± 0.05 | 1.97 ± 0.86 | 2.15 ± 1.01 |
| | West Twin | 0.17 ± 0.06 | 0.02 ± 0.01 | 0.15 ± 0.05 | 0.12 ± 0.04 | 0.11 ± 0.04 | 0.20 ± 0.06 | 0.55 ± 0.45 | 0.62 ± 0.54 |
| | Average | 0.24 ± 0.05 | 0.03 ± 0.01 | 0.21 ± 0.05 | 0.12 ± 0.03 | 0.20 ± 0.06 | 0.17 ± 0.03 | 1.29 ± 0.63 | 1.61 ± 0.67 |

**Table S3.** Trait-by-trait evaluation of the genetic basis of differences in female genital morphology among four populations, including the canonical variate (CV) loadings for the first CV derived from each term of the MANOVA, and results from post-hoc ANOVAs conducted separately for each trait. Wild-caught and laboratory-born fish from four populations were included in analyses. Bold text denotes significant effects ($P < 0.05$).

| | Population (Pop) | | | Rearing Environment (Env) | | | Pop × Env | | |
|---|---|---|---|---|---|---|---|---|---|
| Female Genital Trait | CV loading | *P* | $\eta^2$ (%) | CV loading | *P* | $\eta^2$ (%) | CV loading | *P* | $\eta^2$ (%) |
| Urogenital apertural area | 0.46 | 0.2639 | 5.26% | **-0.63** | **0.0002** | **20.40%** | 0.36 | 0.8622 | 0.96% |
| Apertural opening area | **0.82** | **0.0004** | **25.86%** | **-0.38** | **0.0435** | **5.13%** | 0.22 | 0.9528 | 0.40% |
| Apertural papilla area | 0.35 | 0.5465 | 2.79% | **-0.65** | **0.0001** | **22.14%** | 0.38 | 0.8300 | 1.14% |
| Proportional opening area | **0.65** | **0.0013** | **21.44%** | **0.35** | **0.0079** | **9.02%** | -0.31 | 0.6954 | 1.72% |
| Apertural opening width | **0.37** | **0.0475** | **12.76%** | -0.08 | 0.8166 | 0.08% | 0.15 | 0.8983 | 0.89% |
| Apertural opening length | **0.61** | **0.0186** | **13.27%** | **-0.57** | **0.0011** | **14.39%** | 0.44 | 0.5645 | 2.52% |
| Apertural opening width / length | -0.08 | 0.6007 | 2.76% | **0.45** | **0.0094** | **10.61%** | -0.40 | 0.5664 | 3.00% |
| Apertural opening aspect ratio | -0.34 | 0.2159 | 7.03% | 0.32 | 0.0800 | 4.86% | -0.04 | 0.8822 | 1.01% |

**Table S4.** Descriptive statistics for lab-born female *Gambusia hubbsi* genital morphology (back-transformed means ± SD presented, adjusted for mean body size).

| Predation Regime | Population | Urogenital apertural area (mm$^2$) | Apertural opening area (mm$^2$) | Apertural papilla area (mm$^2$) | Proportional opening area (mm$^2$) | Apertural opening width (mm) | Apertural opening length (mm) | Apertural opening width / length | Apertural opening aspect ratio |
|---|---|---|---|---|---|---|---|---|---|
| Low Predation | East Twin | 0.25 ± 0.11 | 0.04 ± 0.02 | 0.20 ± 0.10 | 0.20 ± 0.06 | 0.30 ± 0.12 | 0.13 ± 0.04 | 3.06 ± 1.25 | 2.46 ± 1.42 |
|  | Rainbow | 0.28 ± 0.11 | 0.06 ± 0.03 | 0.21 ± 0.09 | 0.22 ± 0.06 | 0.38 ± 0.14 | 0.16 ± 0.05 | 2.61 ± 1.16 | 2.73 ± 1.50 |
| High Predation | Cousteau’s | 0.27 ± 0.11 | 0.04 ± 0.02 | 0.23 ± 0.10 | 0.14 ± 0.05 | 0.34 ± 0.12 | 0.13 ± 0.04 | 2.88 ± 1.22 | 3.51 ± 1.70 |
|  | Stalactite | 0.23 ± 0.09 | 0.03 ± 0.01 | 0.20 ± 0.09 | 0.13 ± 0.05 | 0.30 ± 0.11 | 0.14 ± 0.04 | 2.16 ± 1.05 | 3.33 ± 1.66 |