Predation-associated divergence of male genital morphology in a livebearing fish

J. L. HEINEN-KAY & R. B. LANGERHANS

Department of Biological Sciences and W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC, USA

Abstract

Male genital morphology is remarkably diverse across internally fertilizing animals, a phenomenon largely attributed to sexual selection. Ecological differences across environments can alter the context of sexual selection, yet little research has addressed how this may influence the rapid, divergent evolution of male genitalia. Using the model system of Bahamas mosquitofish (*Gambusia hubbsi*) undergoing ecological speciation across blue holes, we used geometric morphometric methods to test (i) whether male genital shape (the small, approximately 1 mm long, distal tip of the sperm-transfer organ, the gonopodium) has diverged between populations with and without predatory fish and (ii) whether any observed divergence has a genetic basis. We additionally examined the effects of genetic relatedness and employed model selection to investigate other environmental factors (i.e. interspecific competition, adult sex ratio and resource availability) that could potentially influence genital shape via changes in sexual selection. Predation regime comprised the most important factor associated with male genital divergence in this system, although sex ratio and some aspects of resource availability had suggestive effects. We found consistent, heritable differences in male genital morphology between predation regimes: Bahamas mosquitofish coexisting with predatory fish possessed more elongate genital tips with reduced soft tissue compared with counterparts inhabiting blue holes without predatory fish. We suggest this may reflect selection for greater efficiency of sperm transfer and fertilization during rapid and often forced copulations in high-predation populations or differences in sexual conflict between predation regimes. Our study highlights the potential importance of ecological variation, particularly predation risk, in indirectly generating genital diversity.

Introduction

The diversity of male genital morphology across internally fertilizing animals is striking, with marked variation among even closely related species (Eberhard, 1985, 1996; Hosken & Stockley, 2004). Post-copulatory sexual selection has been widely implicated as a primary driver of the remarkably rapid evolution of male genitalia (Arnqvist, 1998; Arnqvist & Rowe, 2005;

Correspondence: Justa L. Heinen-Kay, Department of Biological Sciences and W. M. Keck Center for Behavioral Biology, North Carolina State University, Campus Box 7617, Raleigh, NC 27695, USA.
Tel.: +612 559 6608; fax: 919 515 5327;
e-mail: justaheinen@gmail.com

Eberhard, 2010). One way that genital morphology can diverge quickly is via between-population differences in the nature or strength of sexual selection. Ecological differences across space and time – such as variation in predation risk, competition, resource availability, parasite community, structural habitat and climate – can commonly alter the context of sexual selection in diverse taxa (e.g. Emlen & Oring, 1977; Rowe *et al.*, 1994; Zuk & Kolluru, 1998; Grether *et al.*, 1999; Candolin *et al.*, 2007; Schwartz & Hendry, 2007; Botero & Rubenstein, 2012; Scordato *et al.*, 2012). Yet, little research to date has investigated the importance of ecological agents in ultimately, though indirectly, driving rapid evolution of male genital morphology by modifying the context of sexual selection. For instance, this

notion was not discussed in several recent reviews of the causes of genital evolution (Hosken & Stockley, 2004; Eberhard, 2010, 2011; Reinhardt, 2010). Here, we investigate the relationship between male genital shape and one particular ecological factor known to alter sexual selection in many systems – predation risk. We additionally examine several other environmental factors that could potentially contribute to genital divergence (i.e. interspecific competition, sex ratio and resource availability), as well as genetic relatedness.

Variation in predation risk can promote divergence in many important reproductive traits including copulation duration, conspicuity of sexual signals, mating tactics, and nature and magnitude of courtship displays (e.g. Stoner & Breden, 1988; Lima & Dill, 1990; Sakaluk, 1990; Endler, 1991; Magnhagen, 1991; Magurran & Seghers, 1994; Sih, 1994; Godin, 1995; Candolin, 1997; Lima, 2009). This phenomenon is common and widespread – for instance, risk-dependent reproductive behaviours have been documented in diverse taxa such as arachnids, insects, crustaceans, fish, amphibians, reptiles, birds and mammals (reviewed in Magnhagen, 1991). However, investigation of the role of predation risk in driving divergence in sexually selected traits has centred almost exclusively on conspicuous sexual signals subject to trade-offs between natural selection and premating sexual selection. Post-copulatory sexual selection might also differ across environments varying in predation risk, driving divergence in aspects of male genitalia with negligible (if any) effects on viability (e.g. locomotor ability, conspicuity to predators) or mate choice – hence, characters primarily subject to post-copulatory sexual selection, not natural selection or premating sexual selection.

Selection in high-predation environments often favours males that mate quickly and efficiently to reduce mortality risk while maximizing number of matings or enhancing the probability of fertilization for a given copulation attempt. This selection may act on genital morphology because of associations with copulation duration, sperm-transfer quantity, and fertilization and paternity success (Arnqvist & Danielsson, 1999; House & Simmons, 2003; Bertin & Fairbairn, 2005; Pilastro et al., 2007; Simmons et al., 2009; Holwell et al., 2010; Evans et al., 2011; Wojcieszek & Simmons, 2011). In low-predation environments, selection for efficient sperm transfer may be considerably weaker as courtship and copulatory behaviours can occur over longer timescales and potentially involve greater intersexual cooperation. Thus, populations experiencing different levels of predation risk may experience divergent selection on genital morphology.

Other factors that could affect evolution of genital morphology by altering the context of sexual selection include inter- and intraspecific competition for limited resources and the sex ratio of breeding adults (Emlen & Oring, 1977). Resource limitation can foster sexual selection because individuals vary in their ability to acquire limited resources and allocate energy towards condition-dependent trait expression or energetically taxing mating behaviours, thus producing the variation on which selection can act. Adult sex ratio comprises a commonly employed estimate of the opportunity for sexual selection, where populations with relatively male-biased sex ratios tend to experience stronger sexual selection (e.g. Clutton-Brock & Parker, 1992; Fairbairn & Wilby, 2001).

Livebearing fishes of the Family Poeciliidae represent a model system for investigating the role of predation risk during genital evolution because they use internal fertilization, mate promiscuously, inhabit varied predation regimes and exhibit diverse genital morphologies across species (Rosen & Gordon, 1953; Rosen & Bailey, 1963; Langerhans, 2011). Male poeciliids use their gonopodium (nonretractable, highly modified anal fin; Fig. 1) to transfer sperm to females. Variation exists between species in the shape of this organ's distal tip and its overall size. The relative size of the gonopodium appears to experience both natural and sexual selection: females of some species prefer males with relatively larger gonopodia (Brooks & Caithness, 1995; Langerhans et al., 2005; Kahn et al., 2010), but males possessing larger gonopodia suffer reduced burst-swimming ability to escape predators and experience lower survivorship in the presence of predatory fish (Langerhans et al., 2005; Langerhans, 2011). Indeed, gonopodium size differs between predation regimes in several poeciliid species (Kelly et al., 2000; Jennions & Kelly, 2002; Langerhans et al., 2005; though see Evans et al., 2011). In contrast, little is known about variation in gonopodial distal-tip morphology between predation regimes in poeciliid fishes (but see Evans et al., 2011).

The distal tip of the poeciliid gonopodium is very small (usually < 1 mm) and highly complex, comprising bony structures (e.g. hooks, spines, serrae) encapsulated in soft tissue (Fig. 1; Rosen & Gordon, 1953; Langerhans, 2011), and directly contacts the female urogenital aperture during copulation. Unlike gonopodium size and most other sexually selected traits, gonopodial distal-tip shape is unlikely to represent a direct target of natural selection (via locomotor ability or conspicuity to predators) or premating sexual selection (via mate choice) because these structures are extremely small and difficult to discern without the aid of microscopy. Instead, gonopodial distal-tip shape more likely experiences sexual selection arising from insemination or fertilization success. Existing hypotheses and preliminary experimental work suggest that hooks on the gonopodial distal tip may serve as holdfast devices during copulation (Rosen & Gordon, 1953; Langerhans, 2011), and recent work suggests that gonopodial distal-tip shape may be associated with quantity of sperm transferred during forced copulations in guppies (Poecilia reticulata; Evans et al., 2011).
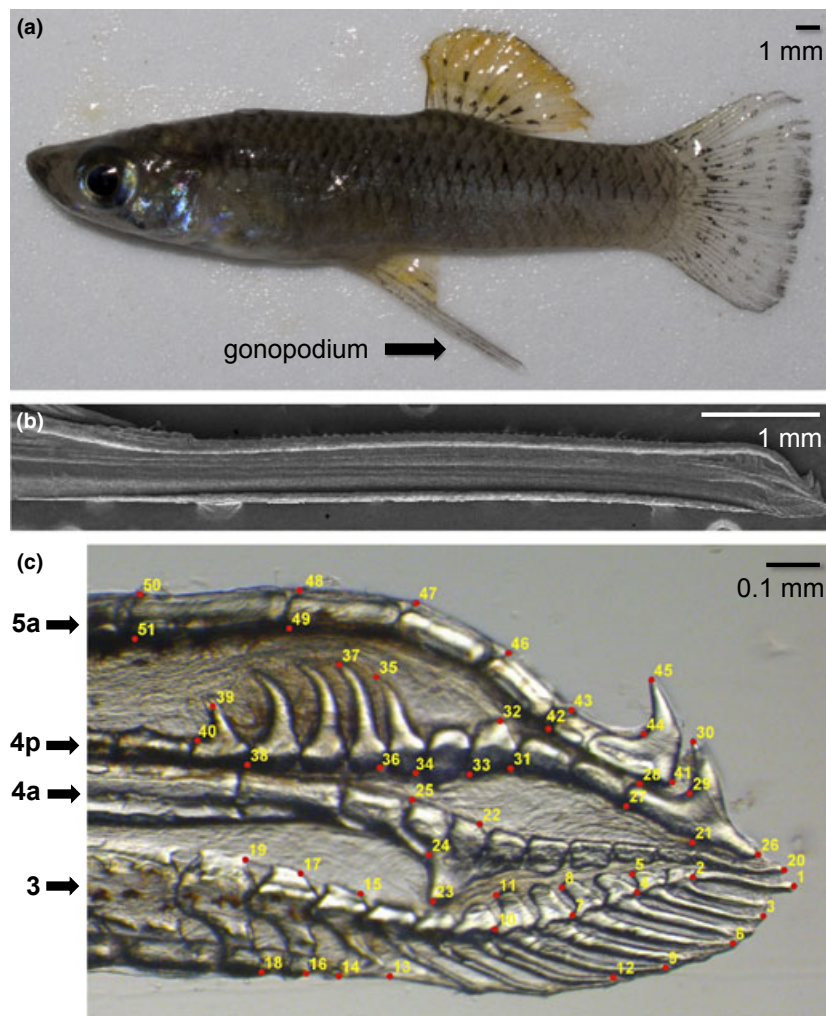
Fig. 1 (a) Lateral photograph of a male *Gambusia hubbsi*; (b) lateral image of a *G. hubbsi* gonopodium using scanning electron microscopy; (c) lateral photograph of the distal tip of the gonopodium illustrating the 51 homologous landmarks digitized on each specimen (see Table S3 for details). Numbers on the left indicate gonopodial fin-ray numbers. Gonopodial-tip structures include spines: landmarks 1–12; elbow: 22, 23 and 25; 4p compound hook: 26–30; serrae: 34–40; and 5a hook: 41–45.

Here, our primary goal is to investigate whether male gonopodial distal-tip morphology of Bahamas mosquito-fish (*Gambusia hubbsi*) has diverged between blue holes with and without predatory fish on Andros Island, The Bahamas. We expect that in blue holes with piscivorous fish – where mortality risk is higher, and conspicuity and duration of courtship and copulation may be reduced – selection will favour genital shapes that enhance insemination efficiency or increase fertilization probability during rapid copulation attempts compared with blue holes without fish predators where this may depend more on female cooperation. Thus, we hypothesize divergence in gonopodial distal-tip morphology between predation regimes, with high-predation males perhaps possessing more rigid, elongate distal tips, with larger hooks than low-predation males because this

morphology should mechanically enhance insemination efficiency and fertilization success during rapid copulations by increasing the accuracy of tip placement, depth of insertion and anchoring capacity in the absence of female cooperation. We test for divergence in male genital morphology between predation regimes using geometric morphometric methods to quantify gonopodial-tip shape of *G. hubbsi* that were (i) wild-caught, providing a comparison among blue holes, and (ii) laboratory-born, testing for a genetic basis. Because shared ancestry and environmental factors other than predation risk could also affect genital shape, we additionally tested for an association between genital shape and genetic relatedness and for potential roles of the presence of an interspecific competitor, adult sex ratio and several aspects of resource availability.

Materials and methods

Study system

Blue holes are vertical, water-filled caves that contain sparse fish communities (Langerhans *et al.*, 2007; Heinen *et al.*, 2013). *Gambusia hubbsi* inhabit distinct low-predation and high-predation populations distinguished by the absence or presence of a predatory fish, *Gobiomorus dormitor* (bigmouth sleeper; Langerhans *et al.*, 2007; Heinen *et al.*, 2013); both fish species colonized blue holes in the last approximately 15 000 years (Fairbanks, 1989). Bahamas mosquitofish populations are genetically isolated, and different populations inhabiting the same predation regime are not more closely related to each other than they are to populations from the alternate predation regime (Schug *et al.*, 1998; Langerhans *et al.*, 2007; Riesch *et al.*, 2013; see Supporting Information). Thus, consistent phenotypic differences observed between predation regimes likely result from divergent selection and not genetic relatedness. Blue holes comprise a 'natural experiment' to test how variation in predation risk may drive adaptive diversification. Although some environmental variation exists between populations, 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). *Gobiomorus dormitor* serves as the primary predator in these blue holes (an additional fish predator, *Strongylura notata*, is present in two high-predation blue holes examined here), as there are no predatory turtles or snakes in blue holes, predatory invertebrates are rare, aerial diving birds have never been observed, and the vertical cave walls prevent predation by wading birds (R. B. Langerhans, personal observation).

Although primary productivity and resource availability do not differ between predation regimes, population density of *G. hubbsi* is significantly reduced in the presence of predatory fish, likely resulting in stronger intraspecific resource competition in low-predation populations (Heinen *et al.*, 2013). Average adult sex ratio also does not differ between predation regimes (Heinen *et al.*, 2013). A competitor fish species, the pupfish *Cyprinodon variegatus* (sheepshead minnow), inhabits some blue holes but does not seem to represent a major selective force in this system as its presence has a demonstrated association with divergence in only a single population-level characteristic in *G. hubbsi* – habitat use (Heinen *et al.*, 2013).

Bahamas mosquitofish have repeatedly evolved similar phenotypes in similar predation regimes (e.g. body morphology, life history; Langerhans *et al.*, 2007; Riesch *et al.*, 2013), and low-predation males generally exhibit greater trait elaboration (e.g. larger gonopodium size, stronger male coloration; Langerhans *et al.*, 2005; R.B.

Langerhans & E. Rosa-Molinar, in review; R.A. Martin, R. Riesch, J.L. Heinen-Kay & R.B. Langerhans, accepted), suggesting a trade-off between strong premating sexual selection in the absence of predators and counter-acting natural selection in the presence of predators. Mating in *G. hubbsi* appears to involve both coercive and cooperative tactics much like other poeciliid fishes (e.g. Rios-Cardenas & Morris, 2011): Bahamas mosquitofish males employ mating behaviours that can include both male-directed and female-directed displays, followed by copulation attempts characterized by very rapid, complex locomotor manoeuvres (J.L. Heinen-Kay & R.B. Langerhans, personal observation). High-predation males exhibit more frequent sexual behaviours (e.g. copulation attempts, female chases; Heinen *et al.*, 2013), suggesting a more coercive mating environment in the presence of predators, similar to guppies (Endler, 1987; Godin, 1995).

High- and low-predation populations of *G. hubbsi* exhibit strong behavioural reproductive isolation and are currently undergoing ecological speciation (Langerhans *et al.*, 2007). Distinguishing between closely related poeciliid species often relies on differences in male genitalia, and most genital evolution studies investigate genital divergence long after speciation has completed. Thus, although rare, studies of genital diversification within the context of ongoing speciation can offer important insight into the timing of genital divergence during speciation.

Morphometrics

We collected *G. hubbsi* from 22 blue holes (12 low predation, 10 high predation; Table S2, Fig. S1) and captured digital photographs of the gonopodial distal tip using a Leica S8 APO stereoscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) equipped with a DFC 425 digital camera and a TL RCI base. Three to five lateral photographs were taken of the left side of the gonopodial tip at 128× magnification and stacked into a single composite image for each specimen using the software Helicon Focus (http://www.heliconsoft.com/). On gonopodial-tip photographs, we used TPSDIG2, version 2.16 (Rohlf, 2010a), to digitize 51 homologous landmarks (Fig. 1c, Table S3) chosen to represent all potentially important areas of the gonopodial tip. We employed geometric morphometric methods for analysis, using TPSRELW, version 1.49 (Rohlf, 2010b), to obtain relative warps (RWs), which are principal components of shape variation. To reduce dimensionality for statistical analysis, we retained only the RWs that cumulatively explained 90% of total shape variance, resulting in the retention of 12 RWs (of 98 total RWs), accounting for 89.5% of total variance. Because gonopodial distal-tip shape could exhibit allometry with distal-tip size, body size or overall size of the gonopodium, we measured each of these variables. Centroid size of

the gonopodial tip (square root of the summed, squared distances of all landmarks from their centroid) provided our estimate of distal-tip size, and we took whole-body lateral photographs using a Canon Rebel XS digital camera (Canon, Melville, NY, USA) to measure standard length (tip of snout to posterior tip of hypural plate) and lateral surface area of the gonopodium (area inside the gonopodium's outer boundaries, comprising anal-fin rays 1–5).

Wild-caught comparison

We used mixed-model multivariate analysis of covariance (MANCOVA) to test for divergence in gonopodial-tip shape between predation regimes in wild-caught fish. The 12 RWs served as dependent variables, predation regime served as the main effect, population nested within predation regime served as a random effect and covariates included log-transformed standard length, centroid size of the gonopodial tip and relative gonopodium surface area (residuals from log–log regression of gonopodium surface area on standard length). All covariates were included to test for allometry, as allometric variation might arise independently from any of these three sources (all variance inflation factors ≤ 3.3, indicating no concerns for high multicollinearity). Statistical significance was determined using an F test based on Wilks's Λ for all terms except predation regime, which used an F test employing restricted maximum likelihood and the Kenward–Roger degrees of freedom adjustment (Kenward & Roger, 1997, 2009; SAS, 2011), which allowed us to use population as the unit of replication, effectively treating population as a random effect (see Wesner et al., 2011; Hassell et al., 2012; Riesch et al., 2013). The latter significance test was conducted using the MIXED procedure in SAS (Cary, NC, USA), whereas all other tests were conducted in JMP (SAS Institute). We evaluated the relative importance of model terms using the effect size measurement of Wilks's partial η^2 (see Langerhans & DeWitt, 2004). There were no significant heterogeneity of slopes, and data met assumptions of MANCOVA.

To evaluate the importance of particular RWs in explaining differences between predation regimes, we inspected eigenvector coefficients of the divergence vector (**d**) derived from the MANCOVA predation regime term following Langerhans (2009). Briefly, this procedure performs an eigenanalysis on the sums of squares cross-products matrix of the predation regime term from the MANCOVA, resulting in a multivariate axis describing the greatest difference between predation regimes, controlling for other terms in the model. We visualized shape variation along RWs using TPSRELW.

To examine whether environmental factors other than predation regime might influence genital divergence between populations, we employed a model selection approach. Population means for RWs were calculated using least-squares means from the MANCOVA so that site means reflected only distal-tip *shape*, statistically controlling for multivariate allometry with standard length, gonopodial-tip centroid size and gonopodium size. We were specifically interested in whether observed differences in distal-tip shape between predation regimes might be additionally (or alternatively) associated with other environmental factor(s). Thus, for each RW identified as important in explaining variation between predation regimes according to **d**, we conducted model selection using general linear models and Akaike information criterion corrected for small sample sizes (AIC$_c$; Akaike, 1992; Burnham & Anderson, 2002) to determine which environmental factor(s) best explained among-population variation in gonopodial distal-tip shape observed in the wild. Model terms were selected based on their potential importance during sexual selection and included predator (*G. dormitor*) presence, competitor (*C. variegatus*) presence, the interaction between predator and competitor presence, adult *G. hubbsi* sex ratio and three separate estimates of resource availability: log-transformed zooplankton density, log-transformed phytoplankton density and relative chlorophyll *a* density. Data for these additional factors were taken from a previous study (Heinen et al., 2013; methods for these measurements found therein). Site means likely adequately captured salient differences among populations because blue holes comprise relatively stable environments with significant repeatability across seasons and years in population demographics and environmental measurements (Heinen et al., 2013).

Because Heinen et al. (2013) only examined 17 of the 22 populations examined in this study, the model selection analyses only included these 17 populations (see Table S2). For each RW examined, we selected the model with the lowest Δ AIC$_c$ value that included at least one term with $P < 0.05$ and other terms with $P < 0.2$. This allowed us to include any suggestive factors that could serve as hypotheses for future testing. For selected models, we calculated η^2 as an effect size estimate of each model term (percent of variance explained by each term).

Genetic basis

To test for a genetic basis of observed gonopodial-tip shape differences among populations, we raised offspring of wild-caught fish from four blue holes (F1 and F2 generations) under common laboratory conditions: fish were raised in 38-L aquaria and fed brine shrimp nauplii, daphnia and bloodworms. Laboratory-born adult males ($n = 10$) were examined as described above for gonopodial-tip shape, standard length, gonopodial-tip centroid size and gonopodium size. We projected each laboratory-raised specimen onto the RWs derived for wild-caught fish and conducted separate general linear models for each RW identified as important in pre-

dation-regime differences in the wild. Both wild-caught and laboratory-born fish for each of these four populations were included in analyses. The statistical models included all covariates as in the MANCOVA, as well as population (testing for genetically-based differences between populations), rearing environment (wild-caught or laboratory-born) and the interaction between population and rearing environment. A significant population effect would indicate that wild-caught and laboratory-born fish from the same population tended to more closely resemble one another than fish derived from different populations (i.e. a genetic basis to observed population differences). As described above, we estimated effect sizes of model terms using η^2. We could not test for differences between predation regimes or populations strictly among laboratory-born fish due to the low sample size. Because of low statistical power in these analyses, this experiment serves as a first step in assessing the genetic basis of gonopodial distal-tip shape divergence – although we have confidence in significant effects, nonsignificant effects should be treated more cautiously and await further research.

Population genetics

Because differences in genital shape among populations could arise from shared ancestry or gene flow rather than replicated evolution driven by variation in predation regime, we additionally tested this hypothesis using molecular genetic data. We used previously published sequence data for an 886-bp fragment of the NADH subunit 2 (ND2) mitochondrial gene (see Langerhans *et al.*, 2007; Riesch *et al.*, 2013). Data were available for 17 of the 22 populations examined here and comprised a total of 25 haplotypes, with five samples from each population ($n = 85$). We tested for an association between male genital shape and genetic relatedness using a Mantel test (Mantel, 1967); we used a one-tailed significance test using 9999 randomizations to specifically test whether genital morphologies were more similar among more closely related populations. Pairwise population differences in genital shape were estimated using Euclidean distances based on the first 12 RWs (results are qualitatively similar when using the geometric estimate of overall shape differences, Procrustes distance). Mean genetic distance between populations was estimated as the percent nucleotide divergence using the GTR + Γ + I model of nucleotide substitution selected using the Akaike information criterion with JMODELTEST 0.1.1 (Posada, 2008).

Results

MANCOVA revealed highly significant effects of all model terms on gonopodial-tip shape except relative gonopodium surface area, which was marginally nonsignificant

Table 1 MANCOVA results examining gonopodial-tip shape of wild-caught *Gambusia hubbsi* among 22 blue holes.

Source	F	d.f.	P	Partial variance (%)
Standard length	6.15	12,87	< 0.0001	45.88
Gonopodial-tip centroid size	5.46	12,87	< 0.0001	42.95
Relative gonopodium surface area	1.82	12,87	0.0568	20.08
Predation regime	3.97	11,563	< 0.0001	56.68
Population (predation regime)	1.86	240,930.22	< 0.0001	28.67

(Table 1). Predation regime represents the strongest predictor of gonopodial-tip shape, and estimated effect sizes also indicated important effects for standard length and centroid size of the gonopodial tip (revealing multivariate allometry). Based on eigenvector coefficients of the divergence vector (**d**), most of the predation effect was due to variation captured by three of the 12 RWs examined (RWs 1, 2 and 4; Table S4).

Model selection results examining other environmental factors that may influence genital morphology for RWs 1, 2 and 4 revealed that predation regime was clearly the most important model term for all three RWs (Tables 2 and S5). Phytoplankton density exhibited a marginally nonsignificant effect for RW4, whereas only weakly suggestive evidence occurred for effects of sex ratio on RW1 and phytoplankton density on RW2 (Table 2). For the two RWs with the clearest effects of predation (RW2 and RW4), higher RW scores were associated with populations with *G. dormitor* present and those with greater phytoplankton density (Fig. S2).

Linear models testing for a genetic basis of the three RWs identified above as important in describing differences between predation regimes indicated that RW2 had a clear genetic basis (Table 3). Because we were interested in evolutionary divergence of male genital shape, we focused on RW2 to interpret the effect of predation regime on gonopodial-tip shape. Independent

Table 2 Results of general linear models examining variation in gonopodial distal-tip shape among 17 blue holes (models selected using AIC$_c$; see Table S5 for details).

Trait	Source	F	d.f.	P	η^2 (%)
RW1	Predation regime	4.59	1,14	0.0501	21.83
	Sex ratio	2.45	1,14	0.1401	11.63
RW2	Predation regime	6.36	1,14	0.0244	28.53
	Phytoplankton density	1.93	1,14	0.1859	8.68
RW4	Predation regime	5.20	1,14	0.0388	21.91
	Phytoplankton density	4.52	1,14	0.0517	19.07

RW, relative warps.

Table 3 Results of linear mixed models testing for a genetic basis of relative warps (RWs) important in describing gonopodial-tip shape differences between predation regimes. Wild-caught and laboratory-born fish from four populations were included in analyses.

Source of variation	RW1				RW2				RW4			
	F	d.f.	P	η^2 (%)	F	d.f.	P	η^2 (%)	F	d.f.	P	η^2 (%)
Standard length	3.13	1,21	0.0913	5.18	0.72	1,21	0.4069	1.57	0.19	1,21	0.6712	0.51
Gonopodial-tip centroid size	2.60	1,21	0.1217	4.30	3.67	1,21	0.0691	8.02	< 0.01	1,21	0.9827	0.00
Relative gonopodium surface area	0.31	1,21	0.5839	0.51	2.59	1,21	0.1227	5.65	0.17	1,21	0.6860	0.47
Population	2.17	3,21	0.1220	10.15	3.82	3,21	**0.0249**	**25.07**	0.38	3,21	0.7711	3.13
Rearing environment	21.82	1,21	**0.0001**	**36.08**	1.68	1,21	0.2089	3.67	4.30	1,21	0.0507	11.91
Population × rearing environment	1.70	3,21	0.1968	8.45	1.55	3,21	0.2316	10.15	3.10	3,21	**0.0488**	**25.76**

For significant effects (P-values ≤ 0.05), P-values and effect sizes are bolded.

of size, male *G. hubbsi* from high-predation blue holes exhibited higher RW2 scores than low-predation males (Figs 2 and S2). High-predation gonopodial distal tips (high RW2 scores) were more elongate and shallow with more densely positioned bony segments, whereas low-predation males (low RW2 scores) possessed more rounded and deep gonopodial tips (Figs 3 and S3). To aid in the interpretation of gonopodial-tip shape differences between predation regimes, we additionally measured several traditional linear measurements to provide intuitive univariate metrics regarding the magnitude of divergence (see Supporting Information). We found that on average, high-predation populations exhibited a gonopodial distal tip with a 6.7% higher aspect ratio and a 13.3% longer elbow on ray 4a. Low-predation populations exhibited a 9.0% deeper and 9.9% longer soft tissue gap between rays 4a and 4p and a 16.2% longer distal hook element on ray 5a. For the description of gonopodial fin-ray numbers and structures, see Fig. 1c.

Population differences in gonopodial distal-tip shape were not positively correlated with genetic distance ($P = 0.8922$), indicating that more closely related populations do not tend to exhibit more similar gonopodial-tip shapes.

Discussion

Research investigating rapid genital evolution has long focused on understanding the proximate mechanisms underlying genital divergence (e.g. sperm competition, cryptic female choice, sexual conflict), largely neglecting the broader ultimate causes of genital diversity. With this study, we wish to highlight a connection between two widely supported premises: (i) environmental variation often alters the context of sexual selection and (ii) sexual selection is a primary driver of rapid male genital evolution. Our results support the logical prediction of these two premises: variation in ecological conditions, specifically the presence of preda-
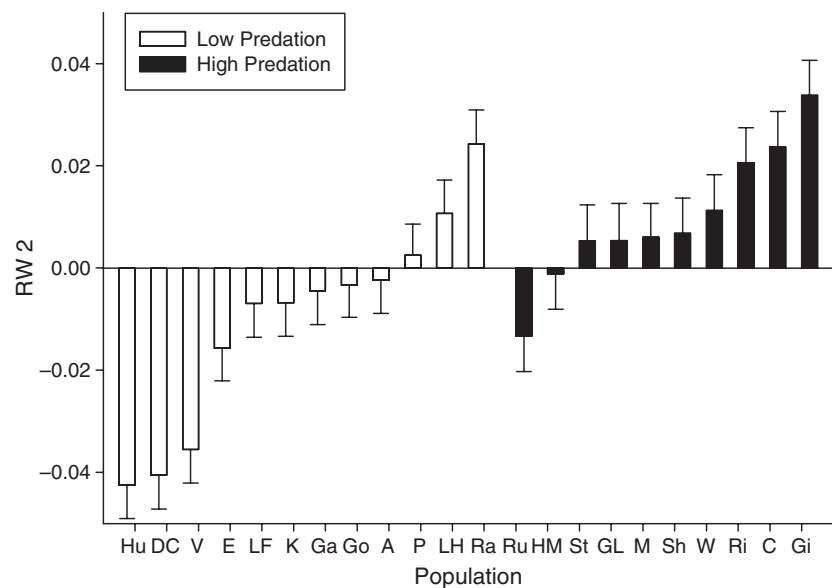

Fig. 2 Variation among *Gambusia hubbsi* populations in gonopodial-tip shape (least-squares means ± 1 SE). Population abbreviations follow Table S2 and are arranged by increasing mean RW2 score within predation regime. RW, relative warps.
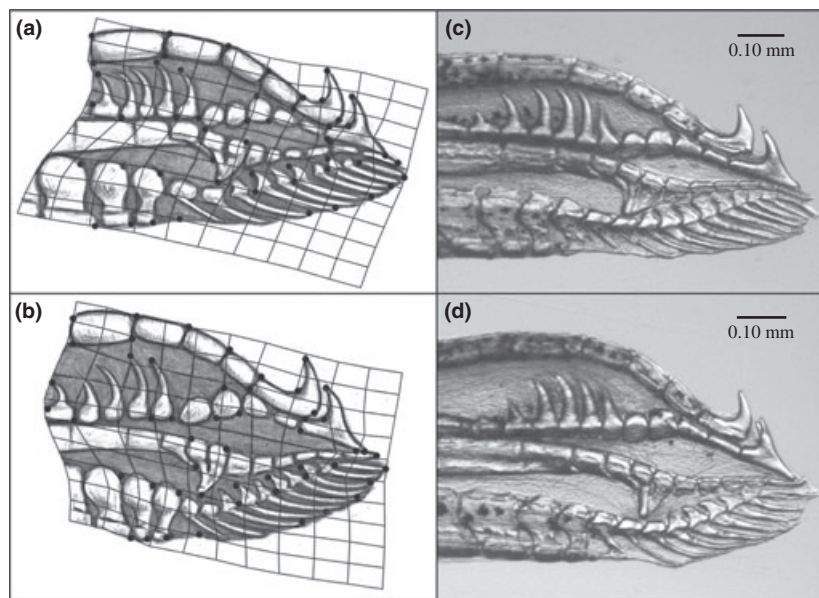
Fig. 3 Thin-plate spline transformation grids depicting (a) positive scores along RW2 (high-predation populations) and (b) negative scores (low-predation populations), with illustration to facilitate visualization of gonopodial-tip shape (light regions = bony structures, shaded regions = soft tissue). Representative gonopodial-tip photographs of *Gambusia hubbsi* from (c) high-predation and (d) low-predation populations. RW, relative warps.

tors, might ultimately play an important but underappreciated role in the widespread phenomenon of rapid evolution of male genitalia.

We found that the shape of the gonopodial distal tip has repeatedly diverged between Bahamas mosquitofish populations with and without predatory fish. These differences at least partially reflect genetically based differentiation because gonopodial distal-tip shape (characterized by RW2) of laboratory-born fish (F1 and F2 generations) was more similar to wild-caught ancestors of their population of origin than to other laboratory-born fish from alternative populations raised in a common environment. Mating in the presence of predators is risky and potentially fatal, and thus, stronger selection may be expected in these environments for genital shapes that facilitate fast and efficient sperm transfer or that increase the probability of successful fertilization during a given copulation attempt. A number of lines of evidence suggest that the observed association between predation regime and male genital morphology likely reflects differential sexual selection in high- and low-predation environments.

First, genetic drift is inconsistent with the significant pattern of distal-tip shape differences between predation regimes. We further ruled out genetic relatedness and several environmental factors that could potentially influence gonopodial distal-tip shape in this system (i.e. competitor presence, zooplankton density and chlorophyll *a*). However, we did reveal possible roles for phytoplankton density and sex ratio in influencing genital shape, independent of predation regime, which require further investigation. Second, direct natural selection and premating sexual selection unlikely target gonopodial distal-tip shape due to its very small size and doubtful functional consequences for any activity other

than copulation. Third, evidence from other systems, as well as recent work within the *G. hubbsi* system, suggests predation regimes may differ in the opportunity for post-copulatory sexual selection. Fourth, sexual selection is widely regarded as the primary driver of genital morphology in animals with internal fertilization (Hosken & Stockley, 2004; Eberhard, 2010, 2011). Finally, the distal tip of the gonopodium is inserted into the female urogenital sinus during copulation, where sexual selection can act on its morphology via sperm competition, cryptic female choice and sexual conflict; recent work suggests that gonopodial distal-tip shape may influence sperm-transfer rate. We suggest that genital shape divergence between predation regimes arises from divergent sexual selection during (i) sperm transfer or (ii) post-copulatory processes.

Regarding sperm transfer, we hypothesized that more rigid, elongate distal tips with larger hooks might evolve in high-predation environments, as these tips might be sturdier and better able to achieve and maintain appropriate contact with the female for effective sperm transfer during rapid unsolicited copulation attempts. We did indeed observe a more elongate gonopodial distal tip with more densely positioned bony segments in high-predation populations, partially matching predictions. However, high-predation Bahamas mosquitofish did not exhibit larger distal hooks – perhaps selection in the presence of predators does not favour larger hooks because although the hooks could enhance sperm-transfer rate through improved attachment to the female gonopore, they could also prolong copulation duration, which might increase vulnerability to predation. Although no study has yet examined the effects of distal-tip shape on sperm transfer in *G. hubbsi*, recent work has revealed an association between

genital shape and sperm-transfer quantity in guppies, with the effect seeming most apparent during forced copulation (Evans *et al.*, 2011). *Gambusia hubbsi* exhibit highly rapid copulation behaviours and frequently employ forced copulation, particularly in high-predation blue holes where males exhibit sexual behaviours more frequently (Heinen *et al.*, 2013). In low-predation environments, a more elongate and bony gonopodial tip may offer little advantage if copulation can occur over longer timeframes and involve greater female cooperation. Longer copulation duration and courtship prior to copulation are associated with greater quantities of sperm transferred in guppies (Pilastro & Bisazza, 1999; Pilastro *et al.*, 2007), and under this scenario, males may rely more on cooperation and less on genital shape to enhance sperm transfer.

Previous research has demonstrated post-copulatory sexual selection in poeciliid fishes, although much remains to be elucidated about these processes (Evans *et al.*, 2008; Evans & Pilastro, 2011). The greater frequency of copulation attempts in *G. hubbsi* males in high-predation environments may translate to greater opportunity for post-copulatory sexual selection in these populations (Heinen *et al.*, 2013). The more shallow and elongate gonopodial-tip shape observed in high-predation populations could be advantageous during sperm competition by allowing males to achieve deeper penetration and deposit sperm further inside the female reproductive tract, and also potentially by displacing sperm deposited during recent matings with other males. Males are more aggressive towards each other in high-predation blue holes (Heinen *et al.*, 2013), and these elevated levels of competition may continue inside the female reproductive tract. Cryptic female choice could additionally prove important, but predictions regarding this process are more tenuous. Sexual conflict over control of fertilization can often be fostered by post-copulatory sexual selection (Birkhead & Pizzari, 2002), and recent work suggests this may be the case in guppies (Evans *et al.*, 2011; Gasparini *et al.*, 2011). The risky mating context of high-predation blue holes in Bahamas mosquitofish may enhance conflict over control of fertilization between the sexes, selecting for males that invest more in offensive methods to achieve fertilization instead of cooperating with females and for females to evolve counter adaptations (Chapman *et al.*, 2003; Arnqvist & Rowe, 2005). Many animals exhibit reduced premating courtship behaviours, and some show increased coercive mating behaviours in environments with increased predation risk during mating (e.g. Magurran & Seghers, 1994; Godin, 1995; Candolin, 1997; Hruskova-Martisova *et al.*, 2010). Indeed, *G. hubbsi* females possess a large genital papilla, ostensibly serving as an obstruction to gonopodial entry into the urogenital aperture. The more bony, elongate distal tips in high-predation males could enhance a male's ability to circumvent female papillae. Future

work could examine among-population variation in female genital morphology to further investigate the sexual conflict hypothesis. Moreover, future experimental work can determine the functional consequences of variation in gonopodial-tip morphology and uncover which processes play important roles in explaining the observed divergence.

Although our results indicate that variation in predation risk represents the most important environmental factor influencing genital divergence in *G. hubbsi*, our study additionally revealed a suggestive role for phytoplankton density and adult sex ratio. Rearing environment (whether fish were wild-caught or born in the laboratory) also provided an important predictor of some components of genital shape (RWs 1 and 4). Further research examining these environmental factors may prove fruitful, as much among-population variance remained unexplained in this study.

Extensive evidence in diverse taxa indicates that male genital morphology varies markedly among even closely related species (Eberhard, 1985; Hosken & Stockley, 2004), but the extent to which rapid genital evolution directly contributes to reproductive isolation via mechanical/sensory incompatibilities remains unclear (e.g. Sota & Tanabe, 2010; Masly, 2011; Bath *et al.*, 2013). Our results demonstrate that male genital morphology has diverged between reproductively isolated populations (Langerhans *et al.*, 2007), but whether a direct relationship exists between gonopodial distal-tip shape and reproductive isolation remains untested.

Our study represents one of the first to explore a potential link between variation in ecological conditions and rapid evolution of male genital morphology. Most prior studies of genital evolution have focused on uncovering the proximate mechanisms, namely modes of sexual selection, without considering why differences in sexual selection may exist among populations and species. In our case study of Bahamas mosquitofish, we discovered that male genital shape consistently diverged among populations with different levels of predation risk. We suggest differential sexual selection as the likely culprit, but this hypothesis requires further investigation. We advocate testing the role of ecological variation, particularly predation risk, in other systems as internally fertilizing animals inhabit a wide variety of environments. We further suggest that future work in poeciliid fishes focus on enhancing our understanding of the functional significance of gonopodial distal-tip morphology, how gonopodial-tip shape affects fitness and whether genital evolution might contribute to reproductive isolation in this system.

Acknowledgments

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Methods.

Table S1 AMOVA results examining mtDNA sequence variation among 17 *Gambusia hubbsi* populations in blue holes.

Table S2 Sample sizes of wild-caught male *Gambusia hubbsi* from 22 blue holes (n = 123).

Table S3 Description of all 51 homologous landmark locations depicted in Fig. 1c; landmarks were selected to capture shape variation in all potentially important gonopodial-tip regions.

Table S4 Eigenvector coefficients of the divergence vector (**d**) used to determine which relative warps

(RWs) are most important in describing gonopodial-tip shape differences between predation regimes for wild-caught fish.

Table S5 Model selection results examining variation in gonopodial distal-tip shape (3 RWs) among 17 blue holes.

Figure S1 Map of study sites on Andros Island, The Bahamas.

Figure S2 Variation in gonopodial distal-tip shape of male *Gambusia hubbsi* in relation to predation regime (filled circles: predator present, open circles: predator absent) and phytoplankton density for (a) RW2 and (b) RW4.

Figure S3 Representative gonopodial-tip photographs from (A) four different high-predation populations and (B) four different low-predation populations.

Data deposited at Dryad: doi:10.5061/dryad.g9735

Predation-associated divergence of male genital morphology in a livebearing fish
J.L. Heinen-Kay and R.B. Langerhans

Supporting Methods
Population genetics analysis
Previous studies using subsets of the populations examined here have demonstrated strong population genetic structuring among *G. hubbsi* populations in blue holes, with no significant association between the presence of predatory fish and genetic relatedness (12 populations: Langerhans *et al.,* 2007; 14 populations: Riesch *et al.,* 2013). To directly confirm these findings for as many populations as possible, we gathered and analyzed all available and relevant published gene sequences, which resulted in samples for 17 of the 22 populations investigated in this study (see GenBank accession numbers in Langerhans *et al.,* 2007 and Riesch *et al.,* 2013). The data comprised a total of 25 haplotypes for an 886bp fragment of the NADH subunit 2 (ND2) mitochondrial gene, with five samples from each population (n = 85). We first conducted analysis of molecular variance (AMOVA; Excoffier *et al.,* 1992) to confirm that population genetic structure is indeed strong and largely independent of the presence of *G. dormitor*. Consistent with prior work, all mtDNA haplotypes were closely related (mean percent nucleotide divergence = 0.26%), we found strong population genetic structure among blue holes (F_{ST} = 0.57), and populations within a predation regime were not more closely related to one another than they were to populations in the alternative predation regime (Table S1).

Traditional measurements of gonopodial distal tip
We calculated the average percent difference between low-predation and high-predation populations for each of five traditional, univariate measurements. We selected measurements based on findings of our geometric morphometric analysis (see Table S3 and Fig. 1c for landmark locations on the gonopodial tip): aspect ratio of the distal tip (tip length [landmark 20-40] / tip depth [landmark 48-13]), depth of the gap between anal-fin rays 4a and 4p (landmark 22-31), length of the gap between anal-fin rays 4a and 4p distal to the elbow (landmark 21-22), length of the elbow on ray 4a (landmark 22-25), and length of the distal element of ray 5a proximal to the curve of the hook (landmark 43-44). We performed linear mixed models as described in the text for each log-transformed variable (aspect ratio was not log-transformed), i.e., models included all three size covariates, predation regime, and population nested within predation regime (random effect). We then extracted the least-squares means for each trait in low-predation and high-predation populations (controlling for allometric variation), back-transformed the means from log units to original units, and calculated the percent difference in length between predation regimes by dividing the larger value by the smaller value. This allowed us to present percent differences between predation regimes for some intuitive linear measurements. Overall differences between predation regimes in traditional measurements were strongly evident (mixed-model MANCOVA: *P* < 0.0001), and posthoc examination revealed all traits showed significant differences (*P* < 0.05) except length of the ray 5a hook element (*P* = 0.0892).

Table S1 AMOVA results examining mtDNA sequence variation among 17 *Gambusia hubbsi* populations in blue holes. F_{CT} is the correlation for random pairs of haplotypes within a predation regime, relative to random pairs of haplotypes drawn from the whole system. F_{SC} is the correlation for random pairs of haplotypes within populations, relative to random pairs of haplotypes drawn from the same predation regime. F_{ST} is the correlation for random pairs of haplotypes within populations, relative to random pairs of haplotypes drawn from the whole system.

Source of variation	df	% of variation	*F*-statistic	*P*
Among predation regimes	1	2.91	$F_{CT} = 0.03$	0.1017
Among populations within predation regimes	15	53.62	$F_{SC} = 0.55$	< 0.0001
Within populations	68	43.47	$F_{ST} = 0.57$	< 0.0001
Total	84			

Table S2 Sample sizes of wild-caught male *Gambusia hubbsi* from 22 blue holes ($n =$ 123). All fish were collected between 2004-2006 except for Douglas-Christopher (2010) and Voy's (2011). * indicates populations used for laboratory rearing. † indicates populations used in model selection analyses.

Gobiomorus status	*Cyprinodon* status	Population	*n*
Absent	Absent	Archie's (A)†	5
Absent	Absent	East Twin (E)†	8
Absent	Absent	Gabbler (Ga)	5
Absent	Absent	Little Frenchman (LF)	4
Absent	Absent	Pigskin (P)†	11
Absent	Present	Douglas-Christopher (DC)	4
Absent	Present	Gollum's (Go)*†	8
Absent	Present	Hubcap (Hu)†	5
Absent	Present	Ken's (K)†	5
Absent	Present	Lonely Hole (LH)	5
Absent	Present	Rainbow (Ra)*†	5
Absent	Present	Voy's (V)†	5
Present	Absent	Cousteau (C)*†	5
Present	Absent	Hard Mile (HM)†	5
Present	Absent	Murky Brown (M)	8
Present	Absent	Runway (Ru)†	5
Present	Absent	Stalactite (St)*†	4
Present	Absent	West Twin (W)†	5
Present	Present	Gibson (Gi)†	6
Present	Present	Goby Lake (GL)†	2
Present	Present	Rivean's (Ri)†	7
Present	Present	Shawn's (Sh)†	6

Table S3 Description of all 51 homologous landmark locations depicted in Fig. 1c; landmarks were selected to capture shape variation in all potentially important gonopodial-tip regions.

Landmark #	Landmark description
1	most distal tip of the most distal ray 3 spine (spine 1)
2	most proximal tip of the most distal ray 3 spine
3	most distal tip of third most distal ray 3 spine (spine 3)
4	most proximal-anterior point of the third most distal ray 3 spine
5	most proximal-posterior point of the third most distal ray 3 spine
6	most distal tip of fifth most distal ray 3 spine (spine 5)
7	most proximal-anterior point of the fifth most distal ray 3 spine
8	most proximal-posterior point of the fifth most distal ray 3 spine
9	most distal tip of seventh most distal ray 3 spine (spine 7)
10	most proximal-anterior point of the seventh most distal ray 3 spine
11	most proximal-posterior point of the seventh most distal ray 3 spine
12	most distal tip of eighth most distal ray 3 spine (spine 8)
13	most posterior point of indentation along the anterior margin of ray 3
14	most distal-anterior point of the most distal segment of ray 3 possessing lateral projections (first segment proximal to ray 3 spines)
15	most distal-posterior point of the most distal segment of ray 3 possessing lateral projections (first segment proximal to ray 3 spines)
16	most distal-anterior point of the second most distal segment of ray 3 possessing lateral projections (second segment proximal to ray 3 spines)
17	most distal-posterior point of the second most distal segment of ray 3 possessing lateral projections (second segment proximal to ray 3 spines)
18	most distal-anterior point of the third most distal segment of ray 3 possessing lateral projections (third segment proximal to ray 3 spines)
19	most distal-posterior point of the third most distal segment of ray 3 possessing lateral projections (third segment proximal to ray 3 spines)
20	most distal tip of the most distal segment of ray 4a
21	distal closure of the soft tissue gap between rays 4a and 4p
22	most distal-posterior point of the most distal fused elbow element on ray 4a
23	most anterior tip of the elbow on ray 4a
24	most distal-anterior tip of the anterodistal projection on the ray 4a segment

immediately proximal to the elbow

25	most proximal-posterior point of the most proximal fused elbow element
26	most distal tip of the ray 4p hook
27	most proximal-anterior point on the base of the ray 4p hook
28	most proximal-posterior point of the base of the ray 4p hook
29	inflection point of the concave curvature of the left ray 4p hook (the 4p hook is bifurcated)
30	most posterior tip of ray 4p hook
31	most distal-anterior point of the most distal rounded segment on ray 4p distal to the serrae
32	most posterior tip of the most distal rounded segment on ray 4p distal to the serrae
33	most proximal-anterior point of the most distal rounded segment on ray 4p distal to the serrae
34	most distal-anterior point of the most distal serra on ray 4p
35	posterior tip of the most distal serra on ray 4p
36	most distal-anterior point of the second most distal serra on ray 4p
37	posterior tip of the second most distal serra on ray 4p
38	most distal-anterior point of the most proximal serra
39	posterior tip of the most proximal serra
40	most proximal-posterior point of the most proximal serra
41	most distal-anterior point of the hook on ray 5a
42	most proximal-anterior point of the base of the ray 5a hook
43	most proximal-posterior point of the base of the ray 5a hook
44	inflection point of the concave curvature of the ray 5a hook
45	posterior tip of the ray 5a hook
46	most proximal-posterior point of the first segment proximal to the ray 5a hook
47	most proximal-posterior point of the second segment proximal to the ray 5a hook
48	most proximal-posterior point of the third segment proximal to the ray 5a hook
49	most proximal-anterior point of the third segment proximal to the ray 5a hook
50	most proximal-posterior point of the fourth segment proximal to the ray 5a hook
51	most proximal-anterior point of the fourth segment proximal to the ray 5a hook

Table S4 Eigenvector coefficients of the divergence vector (**d**) used to determine which relative warps (RWs) are most important in describing gonopodial-tip shape differences between predation regimes for wild-caught fish. Coefficients in bold indicate the most important variables (coefficients $\geq |0.40|$).

Variable	d
RW 1	**0.63**
RW 2	**0.44**
RW 3	0.30
RW 4	**-0.42**
RW 5	0.15
RW 6	0.27
RW 7	0.15
RW 8	0.05
RW 9	0.11
RW 10	-0.09
RW 11	0.04
RW 12	0.03

Table S5 Model selection results examining variation in gonopodial distal-tip shape (3 RWs) among 17 blue holes. Models with Δ AIC$_c$ < 4.0 presented. Model term abbreviations: Pred = *Gobiomorus* predator presence, Pup = *Cyprinodon* pupfish presence, SR = sex ratio, Zoo = zooplankton density, Phy = phytoplankton density, Chl = chlorophyll *a* density. Selected models in bold type. Models selected based on the lowest Δ AIC$_c$ that also included at least one term with $P \le 0.05$ and other terms with $P < 0.2$.

Trait	Model	AIC$_c$	ΔAIC$_c$
RW1	Pred	-62.20	0.00
	Pred + SR	**-61.45**	**0.75**
	SR	-60.11	2.09
	Phy	-60.04	2.16
	Pup	-59.97	2.23
	Pred + Phy	-59.76	2.44
	Zoo	-59.64	2.55
	Chl	-59.43	2.76
	Pred + Chl	-58.95	3.24
	Pred + Pup	-58.95	3.25
	Pred + Zoo	-58.84	3.35
	Pred + SR + Zoo	-58.27	3.93
RW2	Pred	-82.95	0.00
	Pred + Phy	**-81.67**	**1.29**
	Pred + Chl	-79.85	3.10
	Pred + Pup	-79.57	3.38
	Pred + Zoo	-79.49	3.47
	Pred + SR	-79.48	3.47
RW4	**Pred + Phy**	**-83.40**	**0.00**
	Chl	-82.14	1.26
	Pred	-82.12	1.27
	Pred + Chl + Phy	-81.83	1.57
	Chl + Phy	-81.62	1.78
	Phy	-81.52	1.88
	Pred + Chl	-81.35	2.05
	Pred + SR + Chl	-81.32	2.08
	SR + Chl	-80.27	3.13
	Pred + SR + Chl + Phy	-80.22	3.18
	Pred + Pup + Phy	-80.04	3.36
	Pred + SR + Phy	-80.02	3.38
	Pred + SR	-79.72	3.67
	Pred + Zoo + Phy	-79.71	3.68
	Pred + Pup	-79.57	3.83

Fig. S1 Map of study sites on Andros Island, The Bahamas. Population abbreviations follow Table S2 (red circles: predator present, blue circles: predator absent).
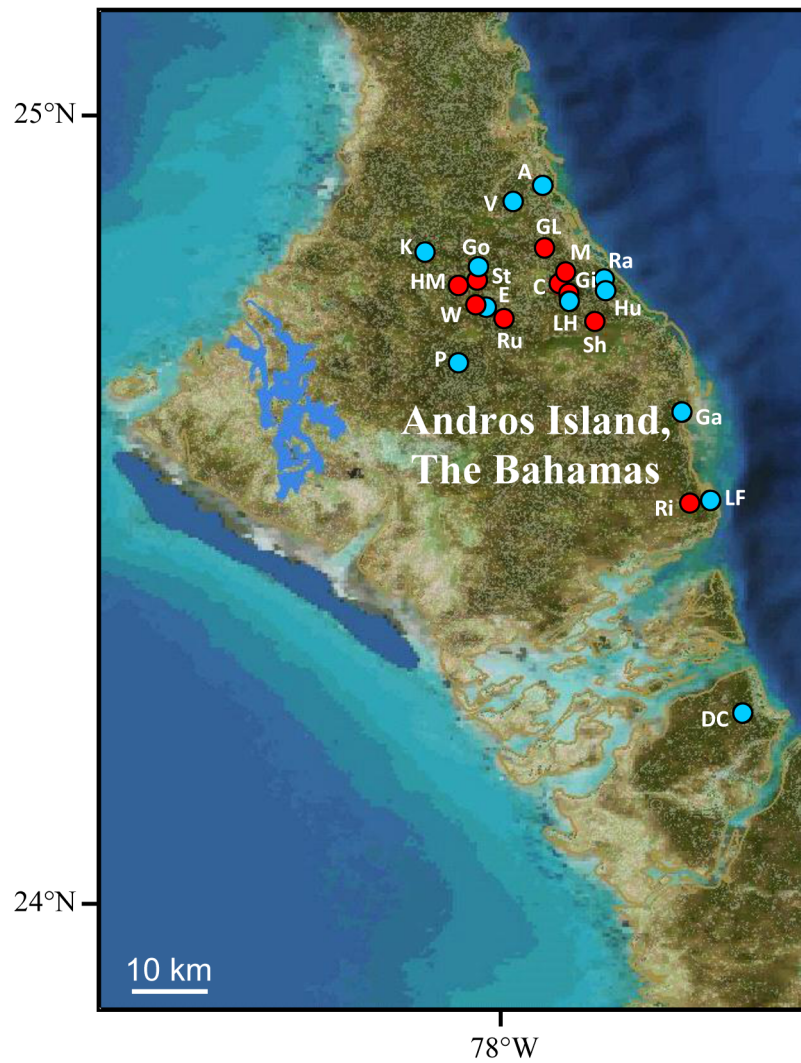
Fig. S2 Variation in gonopodial distal-tip shape of male *Gambusia hubbsi* in relation to predation regime (filled circles: predator present, open circles: predator absent) and phytoplankton density for (a) RW2 and (b) RW4. Thin-plate spine transformation grids depict negative and positive ends of each RW axis (lines drawn to aid interpretation of gonopodial distal-tip shape).
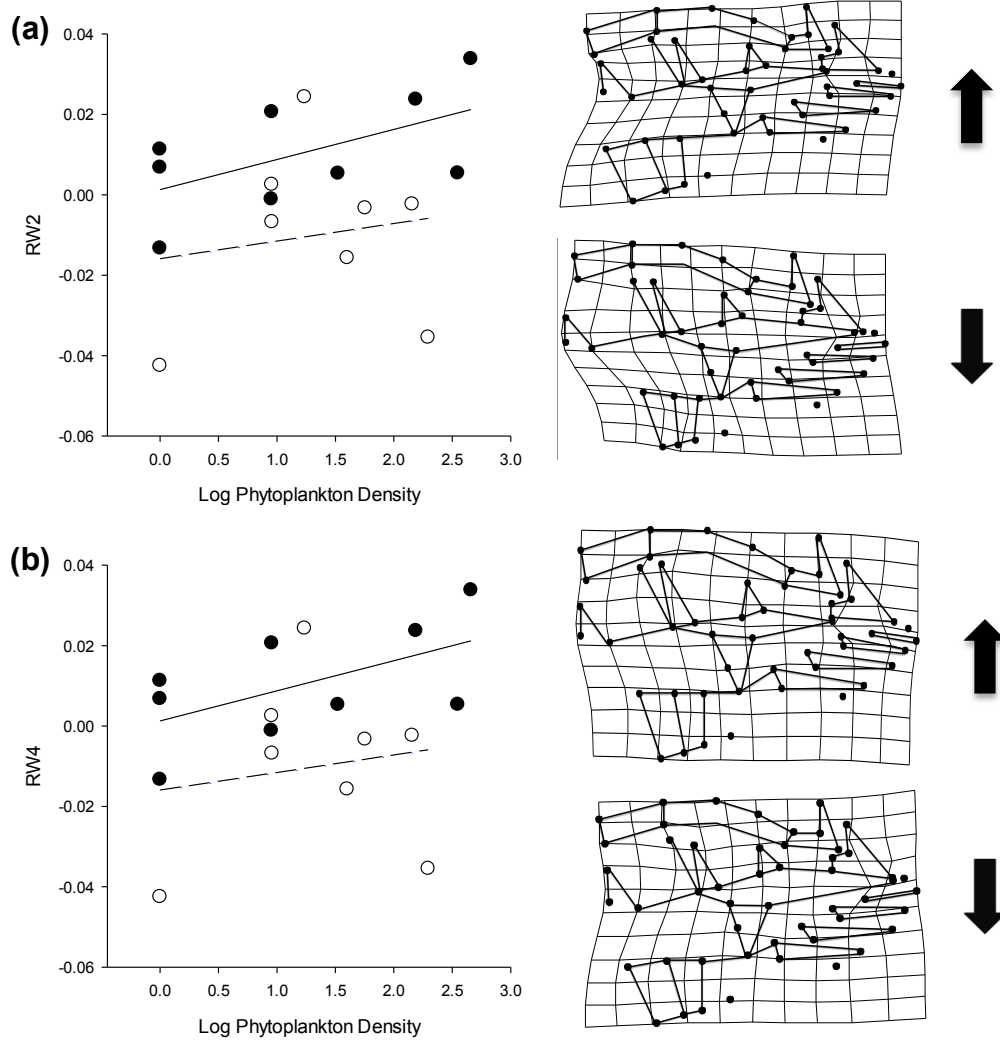
Fig. S3 Representative gonopodial-tip photographs from (A) four different high-predation populations and (B) four different low-predation populations. Population abbreviations given in the lower-right corner of images follow Table S2. Scale bar in top-left image applies to all photographs.