



A Novel Body Plan Alters Diversification of Body Shape and Genitalia in Live-Bearing Fish

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Major evolutionary innovations can greatly influence subsequent evolution. While many major transitions occurred in the deep past, male live-bearing fishes (family Poeciliidae) more recently evolved a novel body plan. This group possesses a three-region axial skeleton, with one region—the ano-urogenital region—representing a unique body region accommodating male genitalic structures (gonopodial complex). Here we evaluate several hypotheses for the evolution of diversity in this region and examine its role in the evolution of male body shape. Examining *Gambusia* fishes, we tested *a priori* predictions for (1) joint influence of gonopodial-complex traits on mating performance, (2) correlated evolution of gonopodial-complex traits at macro- and microevolutionary scales, and (3) predator-driven evolution of gonopodial-complex traits in a post-Pleistocene radiation of Bahamas mosquitofish. We found the length of the sperm-transfer organ (gonopodium) and its placement along the body (gonopodial anterior transposition) jointly influenced mating success, with correlational selection favoring particular trait combinations. Despite these two traits functionally interacting during mating, we found no evidence for their correlated evolution at macro- or microevolutionary scales. In contrast, we did uncover correlated evolution of modified vertebral hemal spines (part of the novel body region) and gonopodial anterior transposition at both evolutionary scales, matching predictions of developmental connections between these components. Developmental linkages in the ano-urogenital region apparently play key roles in evolutionary trajectories, but multiple selective agents likely act on gonopodium length and cause less predictable evolution. Within Bahamas mosquitofish, evolution of hemal-spine morphology, and gonopodial anterior transposition across predation regimes was quite predictable, with populations evolving under high predation risk showing more modified hemal spines with greater modifications and a more anteriorly positioned gonopodium. These changes in the ano-urogenital vertebral region have facilitated adaptive divergence in swimming abilities and body shape between predation regimes. Gonopodium surface area, but not length, evolved as predicted in Bahamas mosquitofish, consistent with a previously suggested tradeoff between natural and sexual selection on gonopodium size. These

results provide insight into how restructured body plans offer novel evolutionary solutions. Here, a novel body region—originally evolved to aid sperm transfer—was apparently co-opted to alter whole-organism performance, facilitating phenotypic diversification.

Keywords: adaptive radiation, body plan, exaptation, morphological evolution, genital evolution, novel traits, Poeciliidae, predation

INTRODUCTION

Evolutionary change is contingent on past evolutionary history (Gould and Vrba, 1982; Price et al., 2000; Gould, 2002; Losos, 2009; Barve and Wagner, 2013; Blount et al., 2018). Novel traits or body plans can lead to new evolutionary routes with far-reaching consequences. For instance, feathers initially evolved for reasons other than flight (Sumida and Brochu, 2000; Xu et al., 2009; Foth et al., 2014; Foth and Rauhut, 2020), while general body plans like bilateral symmetry strongly biased future trajectories of evolutionary diversification (Raff, 2000; Newman, 2016; Isaeva, 2018).

Major transitions in body plan clearly set the stage for much subsequent morphological evolution. But since the Cambrian explosion, very few novel body plans have appeared (Gould, 1989; Levinton, 1992; Erwin et al., 1997; Davidson and Erwin, 2006; Maxwell and Wilson, 2013). For fish, primary literature and textbooks in ichthyology and comparative vertebrate anatomy describe the teleost axial skeleton body plan and vertebral formulae as a two-part system: anterior trunk and posterior caudal vertebral regions [see Rivera-Rivera et al. (2010) and references therein]. However, recent work revealed male live-bearing fishes in the family Poeciliidae have altered this ancestral body plan with a novel three-part body plan composed of the two aforementioned regions plus a third region known as the ano-urogenital vertebral region (Rosa-Molinar et al., 1994, 1996; Rivera-Rivera et al., 2010). This body plan appears to have evolved at least 44 million years ago (Hrbek et al., 2007). Because of the relatively little research conducted on this novel body plan to date, and the emphasis on Poeciliidae so far, its origination and extent is not fully understood—this body plan could be more widespread throughout much of Atherinomorpha.

While many organisms have a general body region encompassing the anus, urinary tract, and reproductive organs, the “ano-urogenital vertebral region” is unique to male poeciliid fishes. This region reflects a marked remodeling of the axial and appendicular skeleton to accommodate development of male genitalia and associated structures. The novel region includes a highly modified anal fin used for sperm transfer (the gonopodium) along with its musculoskeletal components. This unique region comprises vertebrae with ribs and hemal spines (Rosa-Molinar et al., 1994, 1998) and is thought to reflect an adaptation for rapid and effective sperm transfer and internal fertilization in the group (Rivera-Rivera et al., 2010; Serrano-Velez et al., 2014). The ano-urogenital region is situated between the anterior trunk and posterior caudal regions, from vertebrae 11 to 16. Development of this region most notably involves the elongation, thickening, and anterior bending of hemal spines (typically of vertebrae 14–16; sometimes

termed “gonapophyses”) as well as the anterior transposition of the gonopodium and the gonopodial appendicular support (**Figure 1A**). As a consequence, poeciliid fishes exhibit striking sexual dimorphism in adults, with the male anal fin (i.e., gonopodium) positioned more anteriorly than the female anal fin (**Supplementary Figure 1**).

Because the evolution of novel body plans can have important consequences for further morphological and behavioral evolution, it is perhaps not surprising that poeciliid fishes exhibit great variability in the various features of the gonopodial complex, such as the number of modified hemal spines (typically between 1 and 3), their size and extent of modification (e.g., thickening, bending, uncinat processes), the degree of gonopodial anterior transposition (i.e., relative placement of the gonopodium along the body), and the size of the gonopodium (Rosen and Gordon, 1953; Rosen and Bailey, 1963; Chambers, 1987; Ghedotti, 2000; Langerhans, 2011). Yet, to date, explanations for the origins of such diversity and the potential consequences for the evolution of other body regions are lacking (Rosa-Molinar, 2005; Martinez-Rivera et al., 2010; Langerhans, 2011). When novel innovations evolve, can we predict their subsequent evolution based on our functional understanding of the structures, and how might novel traits influence adaptive diversification after their initial emergence?

Here we address several outstanding questions regarding the evolution of this understudied, unique region and its role in facilitating adaptive diversification in poeciliid fishes. We directly tackled three broad questions: (Q1) How are various components of the gonopodial complex developmentally or functionally integrated, and how might they evolve in a correlated manner? (Q2) What forms of selection shape diversity of this region? and (Q3) Has the novel ano-urogenital region been co-opted for adaptive diversification in lateral body shape? To address Q1 (blue region in **Figure 1**), we used a laboratory mating experiment to test whether correlational selection favors particular combinations of two gonopodial-complex traits (**Figure 1B**, see below) and used comparative analyses to test predictions of correlated evolution of particular traits in the ano-urogenital region due to developmental linkages and selection on copulatory performance (**Figure 1C**, see below). We addressed Q2 (yellow region in **Figure 1**) by testing among predatory environments specific predictions of how divergent selection on body shape and gonopodium size should drive evolutionary divergence of gonopodial-complex traits (**Figure 1D**, see below). Finally, we addressed Q3 (green region in **Figure 1**) by directly testing the co-optation prediction of correlated evolution between gonopodial anterior transposition and lateral body shape during predator-driven diversification in an adaptive radiation (**Figure 1E**, see below).

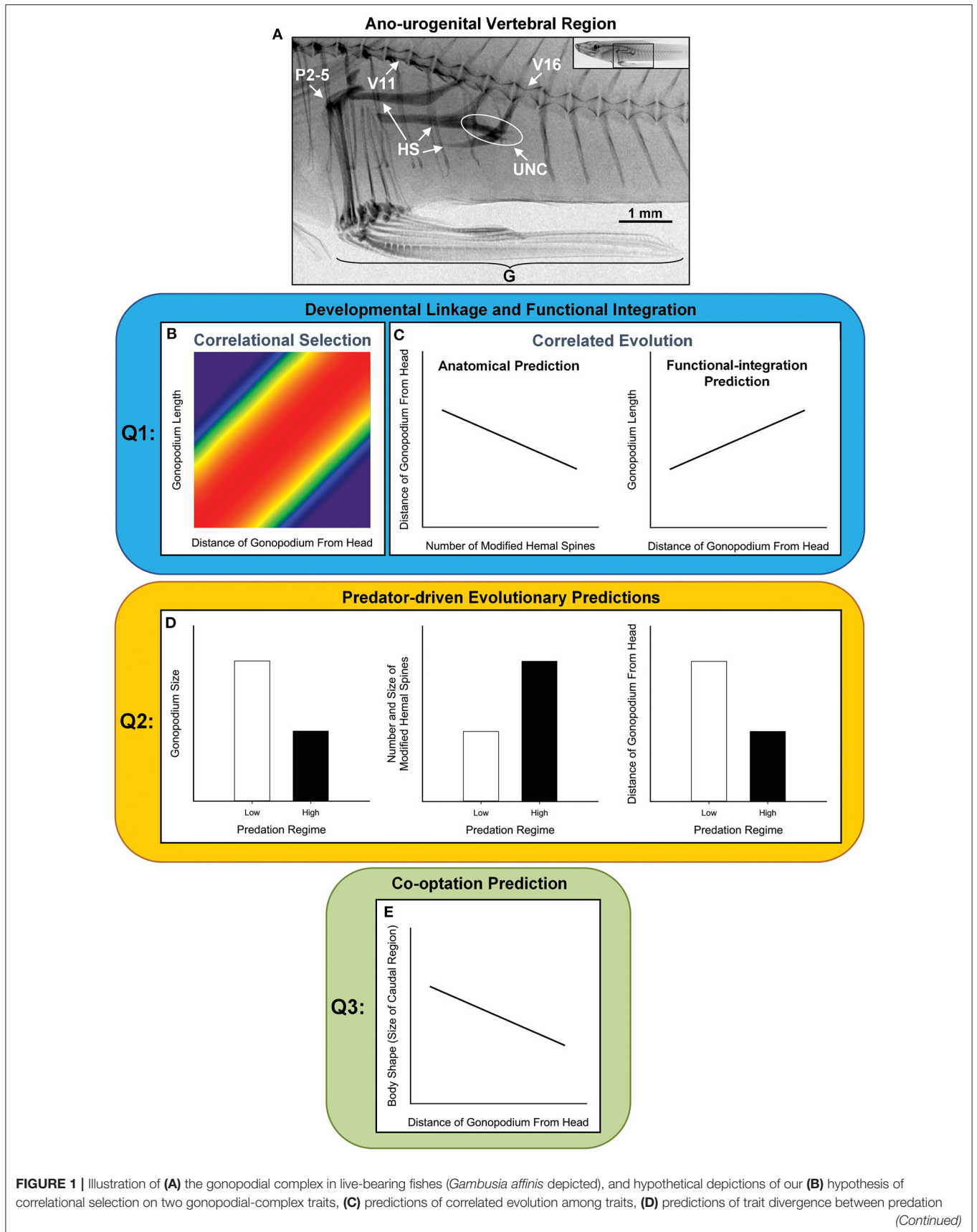


FIGURE 1 | regimes, and **(E)** prediction of co-optation of the ano-urogenital region for the evolution of different body forms (see text for details). Labels in **(A)** indicate key components of the ano-urogenital region: vertebrae 11 and 16 (V11, V16; demarcating the ano-urogenital vertebral region), the gonopodium (G), and its appendicular skeletal support [hemal spines (HS), the uncinat process on the 15th hemal spine (UNC), the columnar fusion of pterygiophores 2-5 (P2-5)]. Anterior/rostral is toward the left in **(A)**. Fitness in **(B)** ranges from low (cool colors) to high (warm colors). The three colored panels reflect the three core questions addressed in the study (Q1–Q3, see text).

MATERIALS AND METHODS

Overview of Hypotheses and Predictions

All hypotheses evaluated in this study derive from previous research, with testable evolutionary predictions being derived from the hypotheses. We first tested a copulatory-performance hypothesis of functional integration between gonopodial anterior transposition and gonopodium length. We specifically hypothesized that when the gonopodium is located closer to the head (greater gonopodial anterior transposition), high performance during copulation should be achieved by a shorter gonopodium, and vice versa. This hypothesis describes positive correlational selection on the two traits, with a ridge-shaped selection surface where trait combinations along the top of the ridge have similarly high mating performance values (**Figure 1B**). Copulation in most live-bearing fishes is very rapid, involving complex locomotor maneuvers, and selection favoring efficient sperm transfer may be strong in many species (Rosa-Molinar, 2005; Martinez-Rivera et al., 2010; Rivera-Rivera et al., 2010; Langerhans, 2011; Devigili et al., 2015; Head et al., 2015). As revealed in high-speed video (1000 Hz) of western mosquitofish (*Gambusia affinis*), the male circumducts the gonopodium, with the distal tip pointing anteriorly, and performs a rapid, complex torque-thrust behavior in an attempt to inseminate the female during copulation (Rosa-Molinar, 2005; Martinez-Rivera et al., 2010; Rivera-Rivera et al., 2010). When the gonopodium is positioned more posteriorly along the body (less transposition), a male may exhibit reduced maneuverability and acceleration [owing to a reduced caudal region (see below) and more posterior center of mass] and require a longer gonopodium to enhance insemination or fertilization success during copulation. Additionally, if males use visual cues for gonopodial placement during copulation (Langerhans, 2011), then an optimal position of the gonopodial tip relative to the eye would reinforce this functional integration. Thus, males' mating success should depend on the combination of the two traits, and we tested this prediction of correlational selection using a mating experiment.

We then examined two predictions regarding correlated evolution of genital traits among species (**Figure 1C**). First, we have an anatomical hypothesis that the magnitude of gonopodial anterior transposition is largely governed post-developmentally by variation in the extensive sexually dimorphic ligament system associated with the modified hemal spines (Rosa-Molinar et al., 1994). This anatomical hypothesis leads to the evolutionary prediction that species having a greater number of modified hemal spines will additionally exhibit greater gonopodial anterior transposition (gonopodium closer to the head, resulting in a smaller abdominal region). That is, an increased number of modified hemal spines should essentially

push the gonopodium anteriorly to a greater extent through direct movement of the columnar fusion of pterygiophores 2–5 (see **Figure 1A**). Second, if our copulatory-performance hypothesis described above is accurate and captures the major aspects of selection on the traits (i.e., other aspects of natural and sexual selection are comparatively of little importance), this leads to an evolutionary prediction: correlational selection on gonopodial anterior transposition and gonopodium length will cause the two traits to exhibit correlated evolution among species/populations. Using phylogenetic comparative analysis of 10 closely related species in the genus *Gambusia* (mosquitofishes) known to exhibit variation in these genital traits, we tested the predictions that (1) a greater number of modified hemal spines is associated with greater gonopodial anterior transposition and that (2) a more posteriorly positioned gonopodium is associated with a longer gonopodium length (**Figure 1C**).

Next, we tested three general predictions regarding evolutionary divergence of genital traits between predatory environments (**Figure 1D**). Previous work has demonstrated the importance of two particular factors in generating diversity of gonopodium size and general body form in live-bearing fishes: (1) sexual selection via female mating preferences and (2) natural selection via predation from piscivorous fish [both reviewed in Langerhans (2010), Langerhans (2011)]. Specifically, females of some poeciliid species exhibit mating preferences for males with particular body shapes and larger gonopodia (Langerhans et al., 2005, 2007; Kahn et al., 2010; Langerhans and Makowicz, 2013), while natural selection in the presence of fish predators favors larger caudal regions and smaller gonopodia due to their positive effects on locomotor escape behaviors (Langerhans et al., 2005; Langerhans, 2009a, 2010, 2011). This has led to divergence in these traits between environments with and without piscivorous fish. For example, male poeciliids of a number of species exhibit smaller gonopodia and larger caudal regions in localities with higher intensities of fish predation [reviewed in Langerhans (2010), Langerhans (2011), Langerhans (2018)]. To date, no study has investigated the role the gonopodial complex might play in these observed patterns of morphological divergence.

Divergent selection between environments with and without predatory fish arising from this interplay of natural and sexual selection leads to three general predictions of phenotypic divergence in the gonopodial complex: males in high-predation environments should exhibit (1) smaller gonopodia, (2) more modified hemal spines that are larger and more strongly modified, and (3) a greater magnitude of gonopodial anterior transposition compared to males in low-predation localities (**Figure 1D**). The first prediction derives from previous work demonstrating both female preference for larger gonopodia and

detrimental effects of gonopodium size on escape performance and survivorship with a predatory fish. The latter two predictions derive from the functional and developmental hypotheses that increasing gonopodial anterior transposition should facilitate both shorter gonopodia and larger caudal regions. If selection on general body shape has driven evolution in the ano-genital region, we should see correlated divergence of gonopodial anterior transposition and lateral body shape across populations (**Figure 1E**). Here we test these predictions using the model system of the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) inhabiting inland blue holes.

Experimental Test of Functional Integration of Gonopodial-Complex Traits

Using laboratory-born Bahamas mosquitofish from a single population on Andros Island, The Bahamas (Cousteau's), we conducted 39 mating trials to test for correlational selection via copulatory performance on two male traits: gonopodial anterior transposition and gonopodium length. All fish were raised under common laboratory conditions in 10-L aquaria within a recirculating system (providing biological, mechanical and UV-filtration) at $\sim 25^{\circ}\text{C}$ in a temperature-controlled room and fed a varied diet of live brine shrimp, freeze-dried daphnia and bloodworms, and TetraMin Pro flakes. Each trial comprised one virgin female placed with one virgin male in a 2.5-L polycarbonate aquarium ($22 \times 9.5 \times 12$ cm, L \times W \times H) with no structure for 1 h. The first 30 min were video recorded from the side with a Sony DCR-SR68 camera (Sony, Tokyo, Japan). From the video, we counted the number of copulation attempts and the number of apparently successful copulations where appropriate genital contact seemed to occur. We watched videos frame-by-frame during copulation attempts, and recorded genital contact when unambiguous physical contact was made between the male gonopodium tip and the female body in the immediate vicinity of the urogenital opening. Fish were euthanized immediately following the trial, photographed for morphological measurements (standard length and the two aforementioned traits), and preserved in 95% ethanol. Using tpsDig2 (Rohlf, 2017), we measured gonopodial anterior transposition as the distance from the anterodorsal tip of anal fin ray 1 to the center of the eye orbit, and gonopodium length as the distance from the anterodorsal tip of anal fin ray 1 to the distal tip of the gonopodium. We estimated mating success in three ways, as it was not obvious which measure might be most appropriate: genital contact success (0 or 1), number of genital contacts, and genital contact efficiency (number of genital contacts divided by number of copulation attempts). Using sperm retrieval, we confirmed that these estimates provided adequate surrogates for insemination success (see **Supplementary Material**).

To test for positive correlational selection on gonopodium length and gonopodial anterior transposition, we employed three statistical approaches, one for each estimate of mating success. Prior to analysis, we regressed each \log_{10} -transformed trait on \log_{10} -transformed standard length and saved residuals to examine size-independent variables. Note that results are virtually identical if we measured relative gonopodial anterior

transposition as its proportion of standard length, as described below (correlation among the two trait estimates: $r = 0.999$, $P < 0.0001$). In each case, we tested for significance of the interaction between the two traits on variation in mating success, and calculated the standardized correlational selection gradient (γ) using multiple regression of standardized trait values (mean = 0, standard deviation = 1) on relative fitness (mating success score divided by average mating success score) (Lande and Arnold, 1983). To accurately and thoroughly characterize selection on these traits, we additionally tested for directional selection (linear selection acting directly on each trait) and quadratic selection (i.e., stabilizing/disruptive selection on each trait). Note that the hypothesized ridge-shaped selection surface implies both positive correlational selection on the traits, as well as stabilizing selection acting on both traits. For significance testing, we conducted a logistic regression for genital contact success, a generalized linear model with a Poisson distribution and log link for the number of genital contacts, and a multiple regression for genital contact efficiency. We arcsin square-root transformed genital contact efficiency, as this transformation of the proportional values increased normality of model residuals. In each model, gonopodium length, gonopodial anterior transposition, their squared terms, and their interaction served as independent variables. The significance of the interaction term from these models provided the relevant tests of correlational selection. To interpret any observed correlational selection, we visualized the quadratic selection surface.

Correlated Evolution of Gonopodial-Complex Traits Among Species

To test predictions of correlated evolution among species, we chose to focus on the *Gambusia* genus, as this is the most species-rich poeciliid genus and has experienced the greatest scrutiny to date in understanding the development and anatomy of the gonopodial complex (Langerhans, 2011). We generated phylogenetic hypotheses for 10 *Gambusia* species using mitochondrial and nuclear gene sequences, measured the relevant traits using radiograph images, and calculated standardized phylogenetic independent contrasts. First, we used PCR to amplify a 975-bp fragment of the NADH subunit 2 mitochondrial gene (ND2) (following Langerhans et al., 2012), a 402-bp fragment of the cytochrome *b* mitochondrial gene (cyt *b*) (following Lydeard et al., 1995), and a 760-bp fragment of the first intron of the S7 ribosomal protein nuclear gene (S7) (following Langerhans et al., 2012) for each of 10 species of *Gambusia*, as well as two outgroup species, *Heterophallus rachovii* (*Gambusia rachovii*) and *Belonesox belizanus*. We sequenced a single specimen for most species (outgroups, *G. clarkhubbsi*, *G. gaigei*, *G. geiseri*, *G. heterochir*, *G. hurtadoi*, *G. speciosa*) but included multiple samples (each from geographically distinct populations) for four species (*G. affinis*: 4, *G. aurata*: 3, *G. holbrooki*: 3, *G. quadruncus*: 4). Sequences were aligned by eye and deposited in GenBank (**Supplementary Table 1**). We constructed phylogenetic trees using two separate Bayesian analyses with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003),

one for concatenated mitochondrial genes and one for the nuclear gene, as the different datasets revealed significantly different evolutionary histories (partition homogeneity test, $P = 0.02$). For additional details, see the **Supplementary Material**.

We obtained radiograph images of 4–50 adult males for each species to obtain estimates of the number of modified hemal spines on vertebrae 14–16, the magnitude of gonopodial anterior transposition, and the relative length of the gonopodium. The number of modified hemal spines, defined as hemal spines exhibiting obvious anterior bending, uncinat processes, or both, was counted on each radiograph. Although most species exhibit a fixed number of modified hemal spines (Rosen and Bailey, 1963; Rauchenberger, 1989), some species are polymorphic. Of the 10 species examined in the interspecific component of this study, two species (*G. clarkhubbsi*, *G. holbrookii*) were found to exhibit variation in modified hemal spine number (either 2 or 3) and were classified as 2.5 for the analyses performed here. We measured the magnitude of gonopodial anterior transposition as the relative placement of the columnar fusion of pterygiophores 2–5 along the body: distance from the anterodorsal tip of the second pterygiophore to the center of the eye orbit divided by standard length (larger ratio values indicate relatively farther distances from the eye, and thus *less* transposition). We employed this ratio metric as our estimate of relative gonopodial anterior transposition because we were specifically interested in the relative placement of the gonopodium on the body (results are qualitatively similar using residuals of log-log regression on standard length instead, as the two estimates of relative gonopodial anterior transposition were highly correlated, $r = 0.99$, $P < 0.0001$). Gonopodium length was measured as the distance from the anterodorsal tip of anal fin ray 1 to the distal tip of the gonopodium. To control for the possible confounding effects of body size, we tested for associations between the three traits described above and standard length using linear regression and statistically removed effects of size where necessary. Relative gonopodial anterior transposition was not correlated with body size, and thus no additional size correction was performed for this variable. Correlation with body size was evident for the other two traits. We conducted phylogenetic size correction (following Revell, 2009) for both modified hemal-spine number and gonopodium length to eliminate size effects, i.e., calculated residuals from linear regression of hemal spine number and \log_{10} -transformed gonopodium length on \log_{10} -transformed standard length, controlling for non-independence due to phylogenetic history.

We calculated standardized phylogenetic independent contrasts using the *ape* package (Paradis et al., 2004) in the R statistical program (R Core Team, 2019) for both the mtDNA and nDNA phylogenetic hypotheses (after pruning the tree so that each species was represented by a single tip). Because normality of residuals was evident in all cases, we used linear regression through the origin to test for correlated evolution among (1) the number of modified hemal spines and gonopodial anterior transposition and (2) gonopodial anterior transposition and relative gonopodium length. For analyses using the nDNA phylogeny, we adjusted degrees of freedom based on the

number of polytomies (Purvis and Garland, 1993; Garland and Diaz-Uriarte, 1999).

Predator-Driven Divergence in Gonopodial-Complex Traits in Bahamas Blue Holes

To evaluate the predictability of evolution in the gonopodial complex between divergent predation regimes, we investigated morphological divergence among male Bahamas mosquitofish inhabiting blue holes on Andros Island, The Bahamas. Bahamas mosquitofish colonized these relatively isolated blue holes (water-filled vertical caves) during the past ~15,000 years, with some blue holes being additionally colonized during this period by a larger predatory fish (bigmouth sleeper, *Gobiomorus dormitor*). Because no environmental factor is known to co-vary with the presence of the fish predator (e.g., salinity, water transparency, surface diameter, depth, resource availability; Langerhans et al., 2007; Heinen et al., 2013; Riesch et al., 2013), this system represents an ideal “natural experiment” with which to test the effects of predation on evolutionary diversification (e.g., Langerhans et al., 2007; Langerhans, 2010, 2018).

Based on hypothesized divergent selection between predation regimes, it is predicted that Bahamas mosquitofish should evolve larger caudal regions and smaller gonopodia in high-predation blue holes [see Langerhans (2010) for details], changes that might be facilitated by phenotypic modifications of the gonopodial complex (see **Figure 1D**). Prior work revealed that fish inhabiting blue holes with predatory fish have indeed evolved larger caudal regions, facilitating increased locomotor escape performance and survivorship during predator encounters (Langerhans, 2009a, 2010, 2018). Using the males from 18 populations examined in this study (see below), we confirmed prior studies’ results regarding this pattern of morphological divergence using a partially overlapping dataset (see **Supplementary Figure 2**). Previous work also demonstrated a pattern of smaller gonopodia in high-predation localities of Bahamas mosquitofish across a range of habitat types (as measured by the gonopodium lateral surface area; Langerhans et al., 2005), but no direct test of differences in gonopodium size has yet been performed for inland blue hole populations. Here we perform such a test. It is important to note that although the hypothesis of divergent selection on gonopodium size focuses on effects of its *surface area* on fitness components (i.e., mating preferences for lateral projection area, frictional drag incurred by gonopodia during locomotion), the hypothesis of correlational selection on gonopodial anterior transposition and gonopodium size centers on its *length*. Thus, here we examine both gonopodium length (measured as described above) and its lateral surface area (measured as area inside the gonopodium’s outer boundaries, comprising anal-fin rays 1–5) in our assessment of divergence in gonopodium size among predation regimes. The two aspects of gonopodium size are not very tightly correlated with one another ($r = 0.34$, $P < 0.001$). We test whether changes in the ano-urogenital region might be partially responsible for mediating responses to selection on lateral body shape and gonopodium

size by comparing trait values among predation regimes (see **Figure 1D**).

We obtained radiograph images of 272 males from 18 blue holes: nine populations that evolved in the absence of fish predators and nine that evolved in the presence of predatory fish (**Supplementary Figure 3**). The following nine traits were measured on each image: number of modified hemal spines, length of the 14th hemal spine, length of the 15th hemal spine, length of the uncinat process on the 15th hemal spine, length of the 16th hemal spine, angle of the 16th hemal spine, magnitude of gonopodial anterior transposition, length of the gonopodium, and lateral surface area of the gonopodium. We selected these traits due to their hypothesized effects on gonopodial anterior transposition, and thus body shape, as well as the observation that these traits exhibited considerable variation in these fish. Length of hemal spines was measured using straight-line distances between 2 and 3 landmarks on each bone, depending on the presence of bone curvature. Angle of the 16th hemal spine (degrees) was measured from the angle formed by lines connecting each end of the bone with the vertex at the most posterior edge along the length of the bone (smaller angles depict greater curvature, with stronger anterior bending of the bone). Gonopodial anterior transposition was again measured as the distance from the second pterygiophore to the center of the eye orbit divided by standard length, to capture the relative position of the gonopodium along the body; however all results were again virtually identical if we used log-log residuals of the distance regressed on standard length rather than the ratio. Other traits were measured as previously described. We made all measurements using tpsDig2 software (Rohlf, 2017). Because prior work has shown that blue hole populations are relatively isolated and that fish from similar predation regimes are not more closely related to one another than to populations in different predation regimes (Schug et al., 1998; Langerhans et al., 2007; Heinen-Kay and Langerhans, 2013; Riesch et al., 2013), populations were treated as independent replicates in statistical analyses.

To test for differences among predation regimes, we first conducted a mixed-model nested multivariate analysis of covariance (MANCOVA) using all traits as dependent variables [e.g., see Riesch et al. (2013)], predation regime and population nested within predation regime (random factor) as independent variables, and \log_{10} -transformed standard length as a covariate to control for effects of body size. We found no evidence for heterogeneity of slopes among predation regimes ($P = 0.55$). This analysis provided the overall test for differences in gonopodial-complex traits between predation regimes. To inspect trait-specific patterns of among-population variation, we additionally conducted mixed-model nested analysis of covariance (using restricted maximum likelihood estimation) separately for each trait, mirroring the model structure of the MANCOVA described above. Body size did not differ among predation regimes (mixed-model nested ANOVA, $P = 0.95$), indicating that predation regime was not confounded with variation in body size. Prior to analysis, the length and area measurements were \log_{10} -transformed to meet assumptions of normality of residuals; the count data for hemal spines, the ratio metric for gonopodial

anterior transposition, and the angle of the 16th hemal spine were left untransformed. For the univariate model examining variation in the number of modified hemal spines, we employed a generalized linear mixed model with a Poisson distribution and log link.

To more directly test the role of the ano-urogenital vertebral region in divergence of body shape and gonopodium length among populations, we calculated population means of our measurements of hemal-spine morphology (6 hemal-spine variables), gonopodial anterior transposition, gonopodium length, and the morphological divergence vector (**d**, describing multivariate lateral body shape variation, see **Supplementary Material**). Mean values represent least-squares means, statistically adjusted for effects of body size. We tested our anatomical prediction that hemal-spine morphology underlies divergence in gonopodial anterior transposition using canonical correlation analysis. We tested for an association between the six hemal-spine variables and gonopodial anterior transposition, and visualized the relationship using the canonical axis derived from the statistical model. Using linear regression between multivariate body shape (**d**) and gonopodial anterior transposition, we then tested our co-optation prediction that gonopodial anterior transposition partially underlies this primary axis of divergence in lateral body shape among populations, reflecting a novel mechanism for adaptive diversification of body form. Finally, we used linear regression to test the functional-integration prediction that populations with greater gonopodial anterior transposition will also exhibit shorter gonopodium length to enhance copulatory performance. We used the statistical program JMP (v. 14.2, 2018, SAS Institute Inc.) for most analyses, but used SAS (v. 7.15, 2017, SAS Institute Inc.) for the mixed-model MANCOVA, and the R package *lme4* (Bates et al., 2011) for the generalized linear mixed model. In all cases, one-tailed P values are employed when we have *a priori* directional hypotheses (Rice and Gaines, 1994).

Because variation among populations in gonopodial-complex traits could reflect either genetically-based differentiation, phenotypic plasticity, or both, we tested for a genetic basis to trait divergence using a common-garden experiment. We raised lab-born offspring ($n = 158$) from eight populations (four without predators, four with predators) to adulthood under common laboratory conditions in a large, recirculating aquarium system comprising 72 115-L aquaria with biological, mechanical, and ultraviolet filtration. Water was maintained at $\sim 25^{\circ}\text{C}$, a conductivity of 2,850 $\mu\text{S}/\text{cm}$, and a pH of 8.3, with a 14-h light/10-h dark photoperiod. Fish were fed daily with a mixture of TetraPro Tropical Crisps, Fluval Bug Bites for Tropical Fish, and Hikari freeze-dried *Daphnia*, blood worm, and brine shrimp. We collected the parental generation (F0) from the wild as newborns (in an attempt to minimize maternal and environmental effects), transported them to the laboratory at North Carolina State University, raised them to adulthood, and mated each female with one unique male from the same population (~ 19 mated pairs per population). Offspring (F1) were then raised to adulthood, with sexes separated as they matured (males raised in 4–5 separate tanks per population; 5–15 males per tank).

For F1 fish, we measured all traits as described above for wild-caught fish. We then performed statistical analyses using a dataset that included for these eight populations both wild-caught fish that had been immediately euthanized and preserved after field collection and F1 lab-born fish that had been euthanized and preserved at ~225 days of age. We conducted MANCOVA using all traits as dependent variables and ANCOVAs separately for each trait. In all models, \log_{10} -transformed standard length served as a covariate; population (testing for genetic differences), rearing environment (lab-born or wild-caught), and their interaction served as independent variables. Of particular interest in this study is whether fish born and raised in a common laboratory environment retained trait differences between populations consistent with those observed in wild-caught individuals (population term of model), indicating evolutionary divergence in gonopodial-complex traits among populations. In all cases, we did not adjust P values for multiple tests; however all significant results would remain significant when adjusted to maintain a false discovery rate of 5% (Benjamini and Hochberg, 1995).

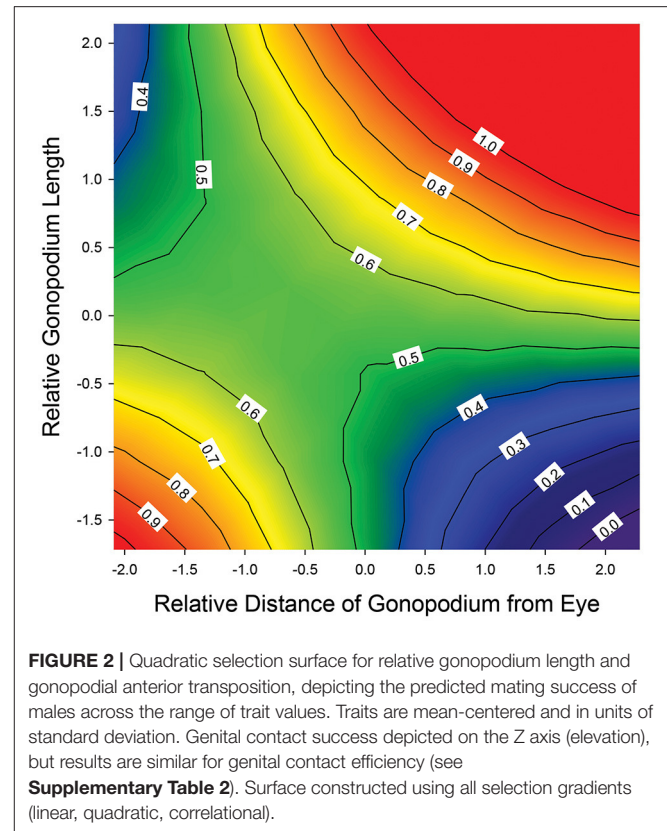
RESULTS

Experimental Test of Functional Integration of Gonopodial-Complex Traits

We found significant positive correlational selection on gonopodium length and gonopodial anterior transposition when using either genital contact success or efficiency as our estimate of mating success, but not when using the number of genital contacts as the mating-success surrogate (Supplementary Table 2). We found a saddle-shaped selection surface rather than the predicted ridge-shaped selection surface (Figure 2). This shape arose from the presence of correlational selection and absence of stabilizing selection (Supplementary Table 2). Consistent with expectations, males with a long gonopodium and a long distance between their gonopodium and eye had relatively high mating success, as did males with a short gonopodium and a short distance between their gonopodium and eye. But males with intermediate values of both traits had slightly reduced mating success, while males with mismatched trait values (long gonopodia with short distance to eye or short gonopodia with long distance to eye) had especially low mating success (Figure 2). This latter effect was particularly pronounced for the combination of short gonopodia and a far distance from the eye.

Correlated Evolution of Gonopodial-Complex Traits Among Species

Bayesian analysis of mtDNA gene sequences produced a well-supported phylogeny for the 10 *Gambusia* species examined (Figure 3A), while analysis of nDNA gene sequences yielded a less resolved phylogeny, differing in topology primarily with regard to the placement of *G. aurata*, *G. speciosa*, and *G. holbrooki* (suggesting possible historical mtDNA introgression; Figure 3B).



Species were found to vary in the number of modified hemal spines present, with five species exhibiting two such spines (*G. aurata*, *G. gaigei*, *G. geiseri*, *G. heterochir*, *G. hurtadoi*), three species having three (*G. affinis*, *G. speciosa*, *G. quadruncus*), and two species having variable spine numbers (see above). Using phylogenetic independent contrasts, we found a consistent trend for correlated evolution of modified hemal spines and gonopodial anterior transposition (mtDNA: $F_{(1, 8)} = 4.46$, one-tailed $P = 0.0338$; nDNA: $F_{(1, 5)} = 3.87$, one-tailed $P = 0.0532$). As predicted from previous anatomical work, species with more modified hemal spines tended to exhibit greater gonopodial anterior transposition (Figures 3C,D). On the other hand, phylogenetic independent contrasts did not uncover a strong relationship between gonopodial anterior transposition and gonopodium length (mtDNA: $F_{(1, 8)} = 1.41$, one-tailed $P = 0.13$; nDNA: $F_{(1, 5)} = 0.01$, one-tailed $P = 0.47$).

Predator-Driven Divergence in Gonopodial-Complex Traits in Bahamas Blue Holes

In Bahamas mosquitofish, we found considerable among-population variation in male genital traits, with males from divergent predation regimes differing in mean trait values for most measured traits (Table 1). Consistent with predictions, male *G. hubbsi* inhabiting blue holes with predatory fish had a greater number of modified hemal spines (Figure 4A), a longer 14th, 15th, and 16th hemal spine

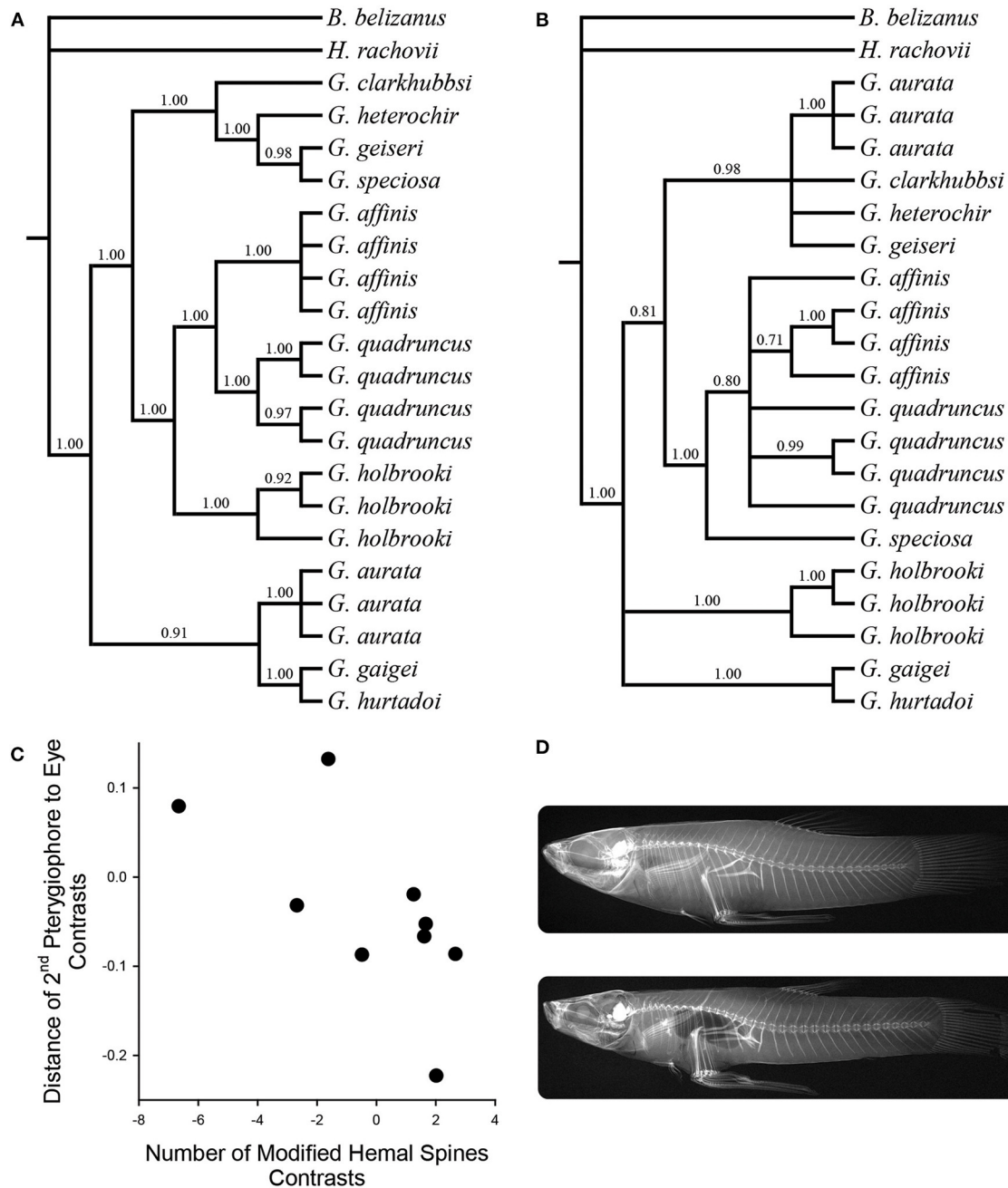


FIGURE 3 | Bayesian phylogenetic hypothesis using **(A)** concatenated mtDNA gene sequences and **(B)** the nuclear encoded S7 intron. Topologies represent 50% majority-rule consensus trees, and numbers represent Bayesian posterior probabilities of nodes. **(C)** Phylogenetically independent contrasts based on the S7 phylogeny, depicting correlated evolution of the relative number of modified hemal spines and gonopodial anterior transposition. **(D)** Representative radiographs of *G. aurata* (top; two modified hemal spines, weak gonopodial anterior transposition) and *G. affinis* (bottom; three modified hemal spines, strong gonopodial anterior transposition).

(Supplementary Figures 4A–C), a longer uncinat process on the 15th hemal spine (Supplementary Figure 4D), a smaller angle (greater anterior bending) of the 16th hemal spine (Supplementary Figure 4E), greater gonopodial anterior transposition (Figure 4B), and a smaller lateral surface area

of the gonopodium (Figure 4C) than did males from blue holes without piscivorous fish. In contrast with our prediction, populations in blue holes with predatory fish did not exhibit relatively shorter gonopodia than those in predator-free blue holes (Supplementary Figure 4F).

TABLE 1 | Statistical results examining variation in gonopodial-complex traits across predation regimes in Bahamas mosquitofish (see statistical details in text).

Dependent Variable	Predation regime		Log ₁₀ standard length	
	Test statistic	1-tailed <i>P</i>	Test statistic	<i>P</i>
All Traits (MANCOVA)	$F_{(8, 899)} = 29.21$	<0.0001	$F_{(9, 220)} = 308.34$	<0.0001
Number of modified hemal spines	$z = 3.70$	0.0001	$z = 1.88$	0.0600
Length of 14th hemal spine	$F_{(1, 16.0)} = 5.26$	0.0179	$F_{(1, 263.1)} = 961.54$	<0.0001
Length of 15th hemal spine	$F_{(1, 16.2)} = 12.04$	0.0016	$F_{(1, 243.1)} = 1031.29$	<0.0001
Length of uncinat process on 15th hemal spine	$F_{(1, 15.0)} = 14.41$	0.0009	$F_{(1, 259.1)} = 193.58$	<0.0001
Length of 16th hemal spine	$F_{(1, 16.6)} = 24.98$	<0.0001	$F_{(1, 214.8)} = 370.91$	<0.0001
Angle of 16th hemal spine	$F_{(1,17.0)} = 19.44$	0.0002	$F_{(1, 171.2)} = 5.81$	0.0170
Distance of 2nd pterygiophore to eye	$F_{(1,14.1)} = 7.36$	0.0084	$F_{(1,195.6)} = 5.12$	0.0247
Gonopodium lateral surface area	$F_{(1, 14.9)} = 9.75$	0.0035	$F_{(1, 204.4)} = 496.52$	<0.0001
Gonopodium length	$F_{(1, 17.0)} = 2.42$	0.9308	$F_{(1, 244.5)} = 1819.53$	<0.0001

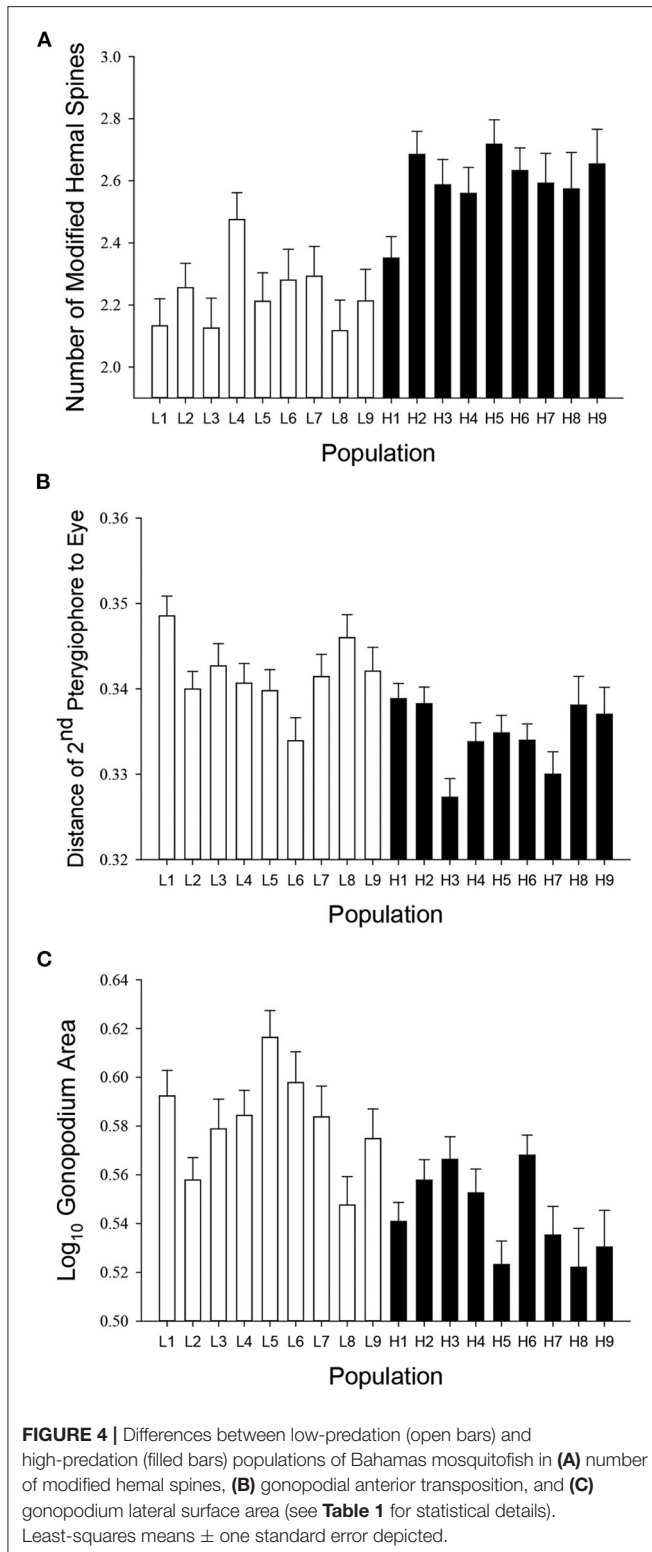
Using population means, we found support for our anatomical and co-optation predictions, but not for our functional-integration prediction. Hemal-spine morphology was indeed significantly associated with gonopodial anterior transposition ($F_{(6, 11)} = 4.61$, one-tailed $P = 0.0070$): populations with a greater number of modified hemal spines, longer hemal spines, a longer uncinat process on the 15th hemal spine, and greater curvature of the 16th hemal spine exhibited a gonopodium positioned more anteriorly (**Figure 5A, Supplementary Table 3**). Populations exhibiting greater gonopodial anterior transposition also had relatively larger caudal regions (one-tailed $P = 0.0003$, **Figure 5B**), matching predictions. However, populations with greater gonopodial anterior transposition did not have shorter gonopodia (one-tailed $P = 0.93$).

Results from the common-garden experiment suggested that divergence in gonopodial-complex traits among populations, at least partially, reflects genetic differentiation. Our multivariate model revealed highly significant effects for all model terms, indicating that although genetically-based differences were evident, some traits for some populations exhibited differences between wild and laboratory conditions (**Supplementary Table 4**). Examining each trait separately revealed that some traits showed clear, genetically-based differences among populations with little-to-no effects of a laboratory environment on their expression, while others showed both genetic differences and influences of laboratory rearing (**Supplementary Table 4**). For instance, we found no evidence for consistent differences between wild and lab-raised fish for the length of the 15th hemal spine, length of the uncinat process of the 15th hemal spine, or gonopodial anterior transposition, but found strong effects of laboratory rearing on gonopodium size and number of hemal spines (smaller gonopodia and more hemal spines in lab-raised fish). In most cases, we found significant evidence of population-specific environmental effects (i.e., $G \times E$), although its strength was always weaker than overall effects of population (see **Supplemental Material**). Thus, environmental influences on observed trait values in the wild likely exist for some traits, but genetically-based differentiation in gonopodial-complex traits was evident.

DISCUSSION

The evolution of novel traits and body plans can dramatically alter subsequent evolutionary trajectories (see Introduction). Here we uncovered several important insights into the evolutionary causes and consequences of an understudied novel body region, the ano-urogenital vertebral region of male live-bearing fish in the family Poeciliidae. Our findings revealed that developmental linkages among components of this gonopodial complex underlie correlated evolution among populations and species, but that correlational selection for functional integration of traits has not driven correlated evolution. We further discovered that predator-associated natural and sexual selection has driven evolutionary diversification of this region: novel traits intimately related to copulation have been co-opted to facilitate evolutionary changes in locomotor performance and body form. This work supports the notion that major evolutionary innovations fundamentally alter future evolutionary patterns, and one need not look into the deep past to find such examples.

In the mating experiment, we uncovered significant evidence for functional integration of two key gonopodial-complex traits. We predicted a ridge-shaped selection surface, where a combination of stabilizing selection and correlational selection would favor a positive association between relative gonopodium length and its distance from the eye—i.e., successful sperm insemination would benefit from longer gonopodia when positioned more posteriorly and from shorter gonopodia when positioned more anteriorly. The saddle-shaped correlational selection surface we observed is only partially consistent with this expectation. Our finding of positive correlational selection indeed suggests that insemination is highest for fish with the appropriate combination of gonopodium length and gonopodial anterior transposition, and particularly low for mismatched combinations of the traits; however males with intermediate values of both traits suffered slightly reduced fitness. This dip in fitness for intermediate trait values arose because of the lack of stabilizing selection on the traits—it is unclear why this fitness valley might occur. Moreover, when using the number of genital contacts as our estimate of mating success, we found no evidence

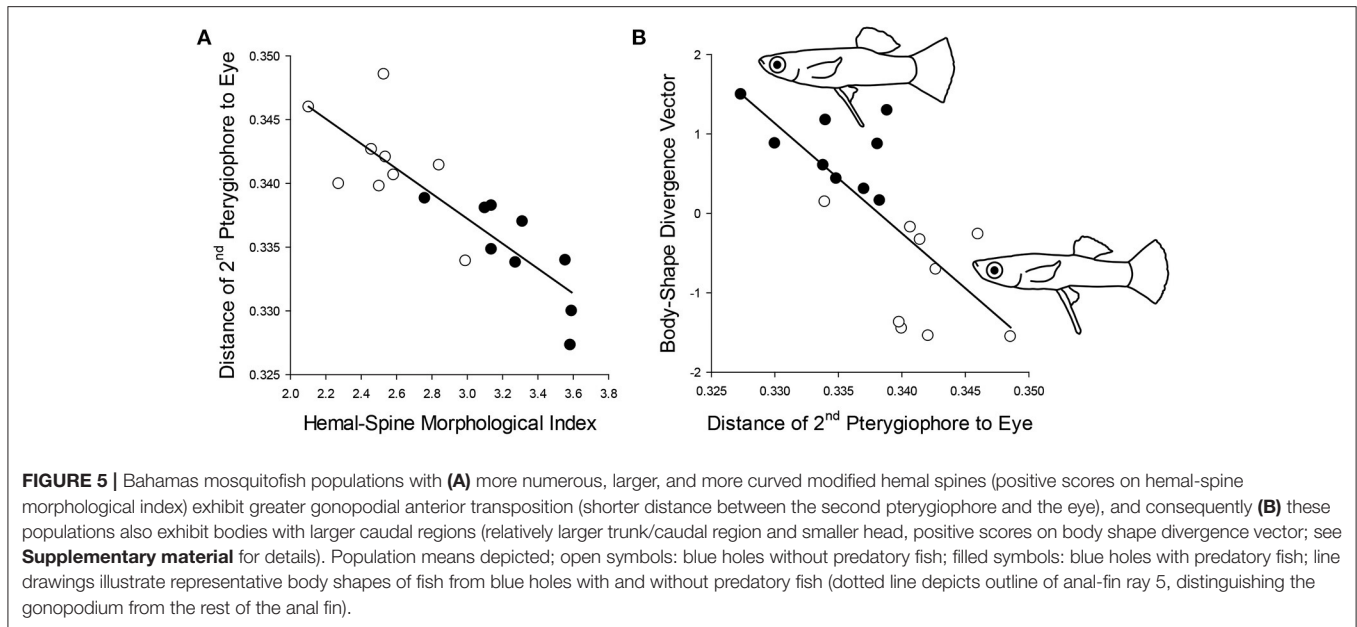


for correlational selection. This implies that this fitness estimate captures something different than the others (i.e., genital contact success and efficiency), perhaps reflecting male behavior, rather

than mating success, more so than the others. For instance, males with more genital contacts might simply attempt more copulations or have larger sperm reserves, and not necessarily reflect sperm insemination the same way the other estimates do. Indeed, the number of genital contacts observed here was highly correlated with the number of coerced copulation attempts ($P < 0.0001$), while genital contact efficiency was not ($P = 0.52$). The functional integration of gonopodial anterior transposition and gonopodium length during mating is apparently more related to the ability and efficiency of achieving mating success than the overall number of times that a successful copulation might occur during a given mating encounter.

Although a number of studies have examined effects of gonopodium length and distal-tip elements on mating success in poeciliid fishes (Evans et al., 2011; Gasparini et al., 2011; Kwan et al., 2013; Devigili et al., 2015; Head et al., 2015, 2017; Hernandez-Jimenez and Rios-Cardenas, 2017; Chung et al., 2020), no prior study has investigated the relationship examined here. Our findings add to the evidence that complex selection surfaces may characterize insemination and fertilization success in *Gambusia* fishes (Devigili et al., 2015; Head et al., 2015) and suggest we still have much to learn. That said, further work in additional populations and species, with larger sample sizes, will help us understand the generality and robustness of these findings. Overall, our results indicate that gonopodium length and its position along the body are functionally related to one another, and if this copulatory function captures the primary link between these traits and fitness, then this predicts correlated evolution of the two traits across populations and species.

Examining 10 closely related *Gambusia* species, and investigating 18 populations of the post-Pleistocene radiation of Bahamas mosquitofish, we did not find this predicted correlated evolution—we found no evidence that gonopodium length and gonopodial anterior transposition coevolve in the expected manner. The evolution of a more anteriorly positioned gonopodium apparently does not necessarily lead to the evolution of a shorter gonopodium, even though these trait combinations seem to enhance mating success. One possible explanation for this finding is that correlational selection might vary over time or among populations/species (e.g., Siepielski et al., 2009, 2013; Weese et al., 2010). Further work is needed to evaluate this putative cause. However, we suggest a likely explanation for this finding is these traits experience complex forms of selection from multiple agents other than their co-dependency during copulation and their genetic basis probably involves different genes (i.e., little-to-no pleiotropy). One possible complicating factor is selection on testes size (e.g., via sperm competition), which could result in reduced gonopodial anterior transposition to accommodate larger testes. However, we have found no evidence supporting this notion (see **Supplementary Material**). Still, considering the disparate forms of selection, such as natural selection on locomotor performance and sexual selection via female preference, that might act on gonopodium length and gonopodial anterior transposition, it is perhaps not surprising that their evolutionary trajectories are not tightly correlated.



On the other hand, at both micro- and macroevolutionary scales, we revealed correlated evolution of hemal-spine morphology and the magnitude of gonopodial anterior transposition, matching our *a priori* anatomical prediction. As *Gambusia* species evolve more modified hemal spines, they additionally evolve a positioning of the gonopodium closer to the head. Similarly, populations of Bahamas mosquitofish with more numerous, larger, and more curved modified hemal spines also show a more anteriorly positioned gonopodium. The anatomical/developmental linkage between growth of the modified hemal spines and the anterior transposition of the gonopodium has so far been described in only one species (*G. affinis*; Rosa-Molinari et al., 1994, 1998), but the findings here suggest this may represent an evolutionarily conserved mechanism of gonopodial transposition in poeciliid fishes. Thus, one of the most obvious external differences in anatomy between the sexes in the family Poeciliidae may be largely explained by the modified hemal spines (and their associated ligament system) present in males.

In the post-Pleistocene radiation of Bahamas mosquitofish inhabiting blue holes, we uncovered clear evidence for predator-driven change in the gonopodial complex, pointing to ecological agents partly responsible for the diversity of morphological variation associated with the novel ano-urogenital vertebral region in poeciliid fishes. Findings largely corresponded to our *a priori* predictions. Specifically, male Bahamas mosquitofish evolved predictable differences between predation regimes in hemal-spine structure, gonopodial anterior transposition, and lateral surface area of the gonopodium. We argue that these differences largely resulted from divergent natural and sexual selection on locomotor performance, body shape, and gonopodium size.

First, we found that diversification of male body shape in Bahamas mosquitofish—a well-known example of predator-driven parallel evolution—appears to be partially governed by changes in the ano-urogenital vertebral region. Natural selection favoring more energetically efficient steady-swimming abilities in low-predation environments where resource competition is particularly high has resulted in more streamlined bodies in these populations, while selection favoring greater acceleration during fast-start escape behaviors in the presence of predatory fish has resulted in bodies with larger mid-body caudal regions in high-predation populations (Langerhans et al., 2007; Langerhans, 2009a,b; Langerhans, 2018; Riesch et al., 2020; this study). Moreover, females show mating preferences within populations based on male body shape, with low-predation females preferring more streamlined males and high-predation females preferring males with larger caudal regions (Langerhans and Makowicz, 2013). We hypothesized that one way to partially develop these divergent morphologies in males involves modification of the hemal-spine morphology and transposition of the gonopodium: a more anteriorly positioned gonopodium creates a larger caudal region, while a more posteriorly positioned gonopodium creates a larger anterior region and may delay the separation of the boundary layer during steady swimming (Langerhans, 2010). Consistent with this notion, we found here that high-predation populations exhibited a suite of differences in hemal-spine morphology that apparently resulted in greater anterior transposition of the gonopodium compared to low-predation populations. This highlights the role of predation regime in driving variation in this novel body region and suggests the region was co-opted to facilitate diversification of body shape after its initial evolution for genital support and mating functions.

The strongest evidence in support of this co-optation hypothesis was the observation that among populations there was a clear relationship between gonopodial anterior transposition

and lateral body shape. The most parsimonious explanation for our results is that the ano-urogenital vertebral region, originally evolved for effective sperm transfer, served as an exaptation for body form and locomotor performance, and then evolved in response to selection on these attributes, reflecting secondary adaptation for a new function. That is, once the novel body region had evolved as an adaptation for rapid and effective sperm transfer, it inadvertently affected other attributes which selection later acted upon, leading to greater diversification in the region and novel evolutionary solutions. In this light, re-examination of past work on predator-driven morphological divergence in poeciliid fishes reveals that males often exhibit greater gonopodial anterior transposition in high-predation populations: e.g., *G. affinis*, *G. caymanensis*, *Poecilia reticulata*, *B. rhabdophora* (Langerhans and DeWitt, 2004; Langerhans et al., 2004; Langerhans and Makowicz, 2009; Langerhans, 2010; Ingley et al., 2014). Meanwhile, female poeciliids also often exhibit morphological differences between predation regimes, but not a shift in the position of the anal fin (e.g., Langerhans, 2009b; Langerhans and Makowicz, 2009; Ingley et al., 2014), perhaps because they lack a readily available means of anteriorly transposing their anal fin. Combined with the widespread pattern of habitat-associated lateral morphology in poeciliid fishes (e.g., Hendry et al., 2006; Gomes and Monteiro, 2008; Tobler et al., 2008; Langerhans and Reznick, 2010; Torres-Mejia, 2011; Hassell et al., 2012; Ingley et al., 2014; Landy and Travis, 2015; Riesch et al., 2016, 2018; Langerhans, 2018; Moody and Lozano-Vilano, 2018), it seems that the co-optation of the novel ano-urogenital region could represent a common, but previously unrecognized, means of male body shape evolution in the family.

Prior work suggests females of several poeciliid fishes, including *G. hubbsi*, prefer males with larger gonopodia, but larger gonopodia increase drag and thus reduce fast-start escape performance (e.g., Langerhans et al., 2005; Kahn et al., 2010; Langerhans, 2011). Consistent with our prediction, and with empirical work in other habitat types and species, we found that low-predation populations exhibited a larger lateral surface area of the gonopodium. The evidence that a tradeoff between natural and sexual selection underlies this divergence was further strengthened by the fact that we did not find divergence in gonopodium length between predation regimes. This suggests that the results for the two estimates of gonopodium size were not confounded, and divergent selection acts on the surface area of the organ (via mating preference of lateral projection area and locomotor effects of frictional drag), not on its length. This further means that the changes observed between predation regimes in hemal-spine morphology and gonopodial anterior transposition cannot be explained as accommodations for changes in gonopodium length. Some prior studies have found longer gonopodia in high-predation environments [Kelly et al., 2000; Jennions and Kelly, 2002; but see Evans et al. (2011) for results similar to this study], perhaps explained by positive effects of length on insemination during coercive matings in some species (Evans et al., 2011; Devigili et al., 2015; Head et al., 2015, 2017). However, patterns of gonopodium length divergence between predation regimes appear inconsistent. The marked variation in gonopodium length across the family Poeciliidae has long attracted considerable research attention. Our findings

add to this body of research, but suggest the primary causes of this variation are neither functional integration with gonopodial anterior transposition, nor the influence of either female mating preferences or natural selection via predation. Because most prior studies examining size of the gonopodium have only measured length, we caution researchers to consider whether in certain cases surface area might provide a more relevant measure.

We found that a combination of both genetically based differences and environmental influences appear to explain among-population variation in components of the gonopodial complex. Depending on the trait, we found moderate to strong population differences persisted in a common laboratory environment, suggesting evolutionary differentiation has occurred in all traits examined. Yet, the laboratory environment altered the expression of most traits, in at least some populations—the exception being gonopodial anterior transposition. Consistent with work in other poeciliid fishes (*G. affinis*: Langerhans, 2009b; *Heterandria formosa*: Landy and Travis, 2015), this particular trait showed strong genetically based population differences with little-to-no effects of laboratory rearing. On the other hand, the laboratory environment clearly had the greatest influence on gonopodium size, consistent with recent work showing that higher food levels result in shorter gonopodia relative to body size (Broder et al., 2020)—we observed relatively smaller gonopodia in the lab compared to the wild, and food was very likely in higher quality and quantity in the laboratory. But still, even gonopodium size showed genetically-based differences as well, matching prior work on heritability of gonopodium size within populations (Evans et al., 2013; Booksmythe et al., 2016) and genetically-based differences among populations in gonopodium morphology (Langerhans et al., 2005; Heinen-Kay and Langerhans, 2013; Broder et al., 2020). Future work can more carefully disentangle the roles of genetic divergence, plasticity, and population-specific plasticity in these traits, but it is clear all sources of variation may prove important for different traits and evolutionary changes in these traits have occurred among populations.

CONCLUSION

Among vertebrates, poeciliid fishes have evolved a unique body plan, and the ano-urogenital region appears to provide novel means of diversification. Subsequent to the evolution of the ano-urogenital region in poeciliids, the region has apparently been co-opted as a means of responding to natural and sexual selection on locomotor performance and lateral body shape, but the length of the gonopodium appears to evolve somewhat independently of the rest of the gonopodial complex. Changes in gonopodial anterior transposition may represent a common means of responding to selection on body shape in male live-bearing fishes, but peripheral consequences of such changes include a restricted trunk region which could lead to reshaping and repositioning of organs, decreased organ sizes, altered diets, and even changes in habitat use and foraging behaviors. These potential effects represent new hypotheses pointing to future research directions. Population divergence in body shape and genitalia can potentially influence reproductive compatibility, suggesting that evolution of diversity in the novel body region could play an important

role in speciation. Evolution of the ano-urogenital region made new evolutionary solutions possible in poeciliid males. Poeciliid fishes exhibit marked variation in mating systems and habitat types, and future investigations at intraspecific and interspecific scales should yield important insights into the influence of the ano-urogenital region in diversification patterns in the family.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. GenBank accession numbers are provided in the article/**Supplementary Material**, and other datasets are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.fbg79cnt8> (Langerhans and Rosa-Molinar, 2021).

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of North Carolina State University (protocols 13-101-O, 16-193-O).

AUTHOR CONTRIBUTIONS

RL and ER-M conceived the study and wrote the manuscript. RL collected and analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.619232/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Online Supplementary Material for *A novel body plan alters diversification of body shape and genitalia in live-bearing fish*

R.B. Langerhans and E. Rosa-Molinar

SUPPLEMENTARY TEXT

Sperm Extraction

We confirmed that visually determined genital contact during copulation attempts provides an adequate surrogate for mating success using sperm retrieval from the female reproductive tract. For each preserved female, we followed standard protocols for sperm extraction in female poeciliid fishes (e.g., Evans et al., 2003; Schupp and Plath, 2005). Briefly, under a stereomicroscope we inserted the tip of a Drummond micropipette into the female urogenital opening to flush and retrieve 3 mL of physiological solution (NaCl 0.9%), and repeated this procedure five times. The solution was transferred to an Eppendorf tube, gently shaken to break up the spermatozeugmata, and then examined using a haemocytometer. We scored each female as either having (1) or not having (0) sperm present in the reproductive tract. While this technique may not have fully recovered sperm in all cases, and it is possible that sperm rejection could have occurred, positive cases are unambiguous evidence of successful insemination.

Using this estimate of insemination success as the dependent variable, we conducted a logistic regression to determine whether genital contact significantly predicted insemination success. We performed three separate models, one for each measurement of mating success (genital contact success, number of genital contacts, and genital contact efficiency). We found that all three estimates of mating success significantly predicted sperm presence in the female reproductive tract (genital contact success: $P = 0.0276$, number of genital contacts: $P = 0.0199$, and genital contact efficiency: $P = 0.0030$). Not only did we only find sperm in females where we had observed genital contact, but based on the odds ratios from the models, the odds of insemination increased by 37% for each genital contact observed, and a male with perfect contact efficiency had 197 times higher likelihood of insemination compared to a male showing no appropriate genital contact. Altogether, this provides strong evidence that visually-assessed genital contact during the 30-min mating trials can serve as a meaningful approximation of insemination success after 1 hr of mating.

Phylogenetic Analyses

We used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) to construct phylogenetic hypotheses using the two molecular datasets. We employed six data partitions for the mtDNA analysis (one for each codon of the ND2 and cyt *b* genes) and a single partition for the nDNA analysis. The optimal maximum-likelihood model of sequence evolution was determined for each data partition using jModelTest 0.1.1 (Posada, 2008). We performed partitioned mixed-model Bayesian analyses, where each data partition was assigned its own evolutionary model, with model parameter values being “unlinked” among partitions assigned the same molecular evolutionary model. MrBayes 3.1.2 was run for 5,000,000 generations, sampling trees every 100 generations. We discarded the lower 25% of the trees as burn-in trees in the computation of a 50% majority rule consensus tree. Support values for inferred clades were calculated from Bayesian posteriors (percent of times a clade occurred among post burn-in trees).

Body Shape Divergence Between Predation Regimes in Bahamas Blue Holes

To evaluate lateral body shape variation across predation regimes in Bahamas mosquitofish, we digitized ten landmarks on x-ray radiograph images (following Langerhans et al., 2007) and employed geometric morphometric methods and a statistical approach described by Riesch et al. (2013). Briefly, we conducted mixed-model nested multivariate analysis of covariance (MANCOVA) using geometric shape variables (relative warps) as dependent variables, and centroid size, predation regime, and population nested within predation regime (random effect) as independent variables. To evaluate the nature of morphological variation associated with predation regime, we performed canonical analysis of the predation regime term in the model to derive a morphological vector of divergence (**d**, following Langerhans, 2009). This vector describes the linear combination of dependent variables exhibiting the greatest differences between predation regimes, controlling for other factors in the model, in Euclidean space. We visualized shape variation along this axis by regressing landmark coordinates (superimposed using Generalized Procrustes Analysis) onto **d** using tpsRegr (Rohlf, 2016). 199 of 272 specimens examined here were previously examined using the same landmarking method in Langerhans et al. (2007), but this new analysis includes new individuals collected during the same time period from six additional populations and performs a more appropriate statistical procedure developed after the original publication. Here, we found that body shape differences between predation regimes were strongly evident ($F_{15,1563} = 15.27$, $P < 0.0001$), with Bahamas mosquitofish inhabiting blue holes with the piscivorous bigmouth sleeper exhibiting a larger mid-body / caudal region and smaller head (Fig. S2). These patterns mirror those previously documented in this system, indicating that the shape divergence between predation regimes is robust across numerous populations, and consistent among multiple statistical approaches.

Results of Common-garden Experiment

We found significant effects of population, environment, and their interaction on variation in gonopodial-complex traits (Table S4). We were particularly interested in whether populations largely maintained their characteristic differences observed in the wild after rearing in a common laboratory environment (i.e. the population term), and whether the strength of these genetically-based differences was similar or greater in magnitude to environment-dependent variation among populations (i.e. the interaction term). Using Wilks's partial η^2 as an estimate of multivariate effect size (Langerhans and DeWitt, 2004), we found that the overall strength of genetically-based differences (partial $\eta^2 = 16.93\%$) exceeded that of environment-dependent variation among populations (partial $\eta^2 = 10.20\%$). By far, the strongest effect of laboratory rearing was found for the length and surface area of the gonopodium, while laboratory rearing had no influence on the expression of gonopodial anterior transposition. Most traits showed population-dependent environmental effects (Population \times Environment term). The strongest population-dependent environmental effects were observed for gonopodium size and length of the 16th hemal spine, where these effects were 63-86% as strong as the main effect of population (using η^2 as an estimate of effect size). Meanwhile, some traits showed moderate but significant population-dependent environmental effects (number of hemal spines, angle of the 16th hemal spine), and other traits showed highly significant population-dependent environmental effects that were relatively small in magnitude compared to the main effect of population differences (length of 14th hemal spine, length of the 15th hemal spine, and length of the uncinuate process of the 15th hemal spine).

Relative Testes Size and Gonopodial Anterior Transposition

An alternative explanation for diversity in gonopodial anterior transposition is that it is correlated with testes size, with reduced transposition accommodating larger testes. This could arise from indirect selection or architectonic associations, resulting in within-population and among-population associations between the two traits. For Bahamas mosquitofish inhabiting blue holes, prior work can help us address this notion. Previous research has demonstrated that fish in high-predation blue holes exhibit relatively larger testes than those in low-predation blue holes (Riesch et al., 2013; Langerhans, 2018). Thus, even though high-predation populations exhibit greater gonopodial anterior transposition (shown in this study), they do not consequentially have smaller testes—they actually show the opposite pattern. Moreover, Riesch et al. (2020) specifically examined the relationship between lateral body shape and testes size, and found no association between the two traits even though lean weight and fat content did show significant relationships with general body form. Using previous data, we can further provide new tests that directly investigate the relationship between gonopodial anterior transposition and relative testes size (GSI: gonadosomatic index). Using population mean values for male GSI from these prior studies, we can examine among-population associations across 17 of the 18 populations studied here, testing whether populations with larger testes show reduced gonopodial anterior transposition. We found no such association (linear regression, one-tailed $P = 0.73$). Moreover, we can calculate gonopodial anterior transposition (relative distance of anterior interior insertion of gonopodium [anal fin ray 1] to the center of the eye) for 138 individuals using these prior data on body shape (landmarks previously digitized, but this metric not calculated), and test for the predicted association with relative testes size at the inter-individual level (individuals from 14 populations). We again found no such association (one-tailed $P = 0.29$; general linear model with relative gonopodial anterior transposition as dependent variable, \log_{10} SL as covariate, arcsin square-root transformed GSI as independent variable, Population as random effect). Thus, it does not appear that testes size is involved in patterns of diversification in gonopodial anterior transposition.

SUPPLEMENTARY TABLES

Table S1 | Information for specimens used in phylogenetic analyses (LLSTC = Langerhans Laboratory Specimen and Tissue Collection, housed at North Carolina State University; CPUM = Colección de Peces de la Universidad Michoacana de San Nicolás de Hidalgo).

Species	Collection locality	GenBank accession number			Voucher
		Cyt <i>b</i>	ND2	S7	
<i>Belonesox belizanus</i>	Amalgres, México	HM443900	HM443919	HM443938	LLSTC 04587
<i>Heterophallus rachovii</i>	Amalgres, México	HM443901	HM443920	HM443939	LLSTC 04584
<i>Gambusia affinis</i>	Mainero, México	HM443902	HM443921	HM443940	CPUM 2715
<i>Gambusia affinis</i>	Brazos County, Texas	HM443906	HM443923	HM443941	LLSTC 04578
<i>Gambusia affinis</i>	Cleveland County, Oklahoma	HM443905	HM443925	JN128635	LLSTC 04579
<i>Gambusia affinis</i>	Shannon County, Missouri	HM443903	HM443926	HM443942	LLSTC 04580
<i>Gambusia aurata</i>	Ciudad Mante, Tamaulipas, México	JX275481	JX275467	JX275474	CPUM 2312
<i>Gambusia aurata</i>	El Limón, Tamaulipas, México	JF437627	JF437630	JF437633	LLSTC 11425
<i>Gambusia aurata</i>	Forlon, Tamaulipas, México	JX275482	JX275468	JX275475	CPUM 4409
<i>Gambusia clarkhubbsi</i>	Val Verde County, Texas	JX275483	JX275469	JX275476	LLSTC 01787
<i>Gambusia gaigei</i>	Brewster County, Texas	JX275484	JX275470	JX275479	LLSTC 13468
<i>Gambusia geiseri</i>	Hays County, Texas	GGU18207	JX275471	JX275478	LLSTC 13467
<i>Gambusia heterochir</i>	Menard County, Texas	GHU18208	JX275472	JX275477	LLSTC 01442
<i>Gambusia holbrooki</i>	Miami-Dade County, Florida	HM443916	HM443935	HM443948	LLSTC 04582
<i>Gambusia holbrooki</i>	Manatee County, Florida	HM443917	HM443936	HM443947	LLSTC 04583
<i>Gambusia holbrooki</i>	Richland/Lexington Counties, South Carolina	HM443918	HM443937	JN128636	LLSTC 04586
<i>Gambusia hurtadoi</i>	Nuevo Zaragoza, Chihuahua, México	JX275485	JX275473	JX275480	CPUM 2125
<i>Gambusia quadruncus</i>	Ciudad Mante, Tamaulipas, México	HM443911	HM443928	HM443944	LLSTC 04572
<i>Gambusia quadruncus</i>	El Limón, Tamaulipas, México	HM443909	HM443930	HM443945	LLSTC 04573
<i>Gambusia quadruncus</i>	Llera, Tamaulipas, México	HM443912	HM443932	HM443946	LLSTC 04574
<i>Gambusia quadruncus</i>	Ciudad Victoria, Tamaulipas, México	HM443908	HM443927	HM443943	LLSTC 04571
<i>Gambusia speciosa</i>	Val Verde County, Texas	JF437628	JF437631	JF637634	LLSTC 11426

Table S2 | Standardized directional, quadratic, and correlational selection gradients on relative gonopodium length and gonopodial anterior transposition based on three separate estimates of fitness (mating success). Note that directional selection gradients were calculated and tested for significance with only linear terms in the model, while other gradients employed models with all terms included (Lande and Arnold, 1983). Quadratic terms were doubled to accurately estimate these selection coefficients (Stinchcombe et al., 2008).

Trait	Genital Contact Success			Number of Genital Contacts			Genital Contact Efficiency		
	β / γ	Std. Err	<i>P</i>	β / γ	Std. Err	<i>P</i>	β / γ	Std. Err	<i>P</i>
Relative Gonopodium Length (GL)	0.17	0.13	0.1682	0.14	0.26	0.2685	-0.03	0.15	0.8757
Gonopodial Anterior Transposition (GAT)	-0.05	0.13	0.6636	-0.29	0.26	0.1002	-0.01	0.15	0.9319
GL ²	0.17	0.22	0.2615	-0.49	0.47	0.1547	-0.01	0.27	0.9141
GAT ²	0.04	0.21	0.5355	-0.35	0.44	0.1088	0.20	0.25	0.4137
GL x GAT	0.30	0.16	0.0293	0.00	0.33	0.7118	0.40	0.19	0.0406

Table S3 | Canonical loadings for the six hemal-spine traits examined for association with gonopodial anterior transposition, describing the relationship between greater modification and elaboration of hemal spines and a more anteriorly position gonopodium (all traits *P* < 0.001).

Hemal-spine trait	Canonical Loading
Number of modified hemal spines	0.85
Length of 14th hemal spine	0.79
Length of 15th hemal spine	0.86
Length of uncinat process on 15th hemal spine	0.90
Length of 16th hemal spine	0.90
Angle of 16th hemal spine	-0.93

Table S4 | Statistical results examining the genetic basis of variation in gonopodial-complex traits in Bahamas mosquitofish.

dependent variable	Population		Environment		Population × Environ.		log ₁₀ standard length	
	test statistic	<i>P</i>	test statistic	<i>P</i>	test statistic	<i>P</i>	test statistic	<i>P</i>
All traits (MANCOVA)	$F_{63,1414.1} = 5.82$	<0.0001	$F_{9,250} = 54.92$	<0.0001	$F_{63,1414.1} = 3.21$	<0.0001	$F_{9,250} = 461.82$	<0.0001
Number of modified hemal spines	$\chi^2_7 = 52.58$	<0.0001	$\chi^2_1 = 38.42$	<0.0001	$\chi^2_7 = 32.64$	<0.0001	$\chi^2_1 = 0.38$	0.5371
Length of 14th hemal spine	$F_{7,275} = 10.62$	<0.0001	$F_{1,275} = 8.18$	0.0046	$F_{7,275} = 4.50$	<0.0001	$F_{1,275} = 1913.44$	<0.0001
Length of 15th hemal spine	$F_{7,275} = 8.76$	<0.0001	$F_{1,275} = 0.20$	0.6523	$F_{7,275} = 4.48$	<0.0001	$F_{1,275} = 1970.01$	<0.0001
Length of unc. process on 15th hemal spine	$F_{7,270} = 14.44$	<0.0001	$F_{1,270} = 0.02$	0.8908	$F_{7,270} = 6.09$	<0.0001	$F_{1,270} = 280.58$	<0.0001
Length of 16th hemal spine	$F_{7,275} = 11.00$	<0.0001	$F_{1,275} = 3.89$	0.0495	$F_{7,275} = 6.69$	<0.0001	$F_{1,275} = 375.84$	<0.0001
Angle of 16 th hemal spine	$F_{7,275} = 6.44$	<0.0001	$F_{1,275} = 5.71$	0.0175	$F_{7,275} = 3.85$	0.0005	$F_{1,275} = 0.01$	0.9385
Distance of 2 nd pterygiophore to eye	$F_{7,275} = 2.74$	0.0090	$F_{1,275} = 0.29$	0.5895	$F_{7,275} = 1.91$	0.0679	$F_{1,275} = 6.10$	0.0141
Gonopodium lateral surface area	$F_{4,263} = 9.64$	<0.0001	$F_{1,263} = 384.44$	<0.0001	$F_{4,263} = 6.07$	<0.0001	$F_{1,263} = 1178.55$	<0.0001
Gonopodium length	$F_{4,274} = 5.95$	<0.0001	$F_{1,274} = 206.08$	<0.0001	$F_{4,274} = 5.11$	<0.0001	$F_{1,274} = 1741.94$	<0.0001

SUPPLEMENTARY FIGURES

Figure S1 | Illustration of sexual dimorphism in poeciliid live-bearing fishes: **(A)** male, **(B)** female (*Gambusia affinis* depicted). Note the highly modified anal fin in males (gonopodium), which is positioned more anteriorly than the anal fin in females. While females have retained a relatively little-modified ancestral two-part body plan, male poeciliids have evolved a three-part body plan with the evolution of the novel ano-urogenital vertebral region.

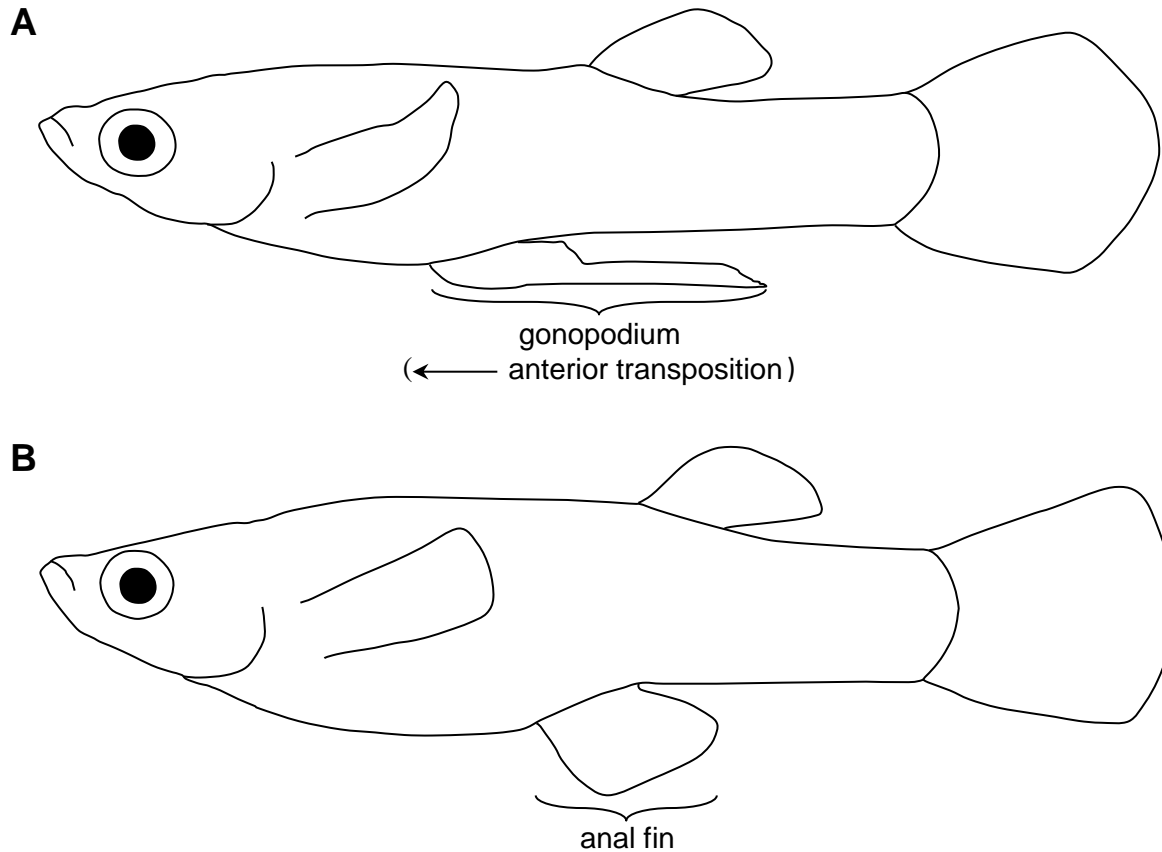


Figure S2 | Morphological divergence in male Bahamas mosquitofish between low-predation (open symbols) and high-predation (filled symbols) blue-hole populations. Body shape variation described by the divergence vector derived from the predation regime term of the MANCOVA, illustrated using thin-plate spline transformation grids relative to mean landmark positions (observed range of variation depicted; i.e., no magnification). Solid lines connecting outer landmarks are drawn to aid interpretation. Circles along the divergence vector represent population means. Representative radiographs of individuals from low-predation and high-predation populations are provided below the axis (individuals selected from two blue holes separated by only 0.3 km of terrestrial habitat). Note the larger mid-body / caudal region, smaller head, more anteriorly positioned gonopodium, and longer and more numerous modified haemal spines in high-predation populations.

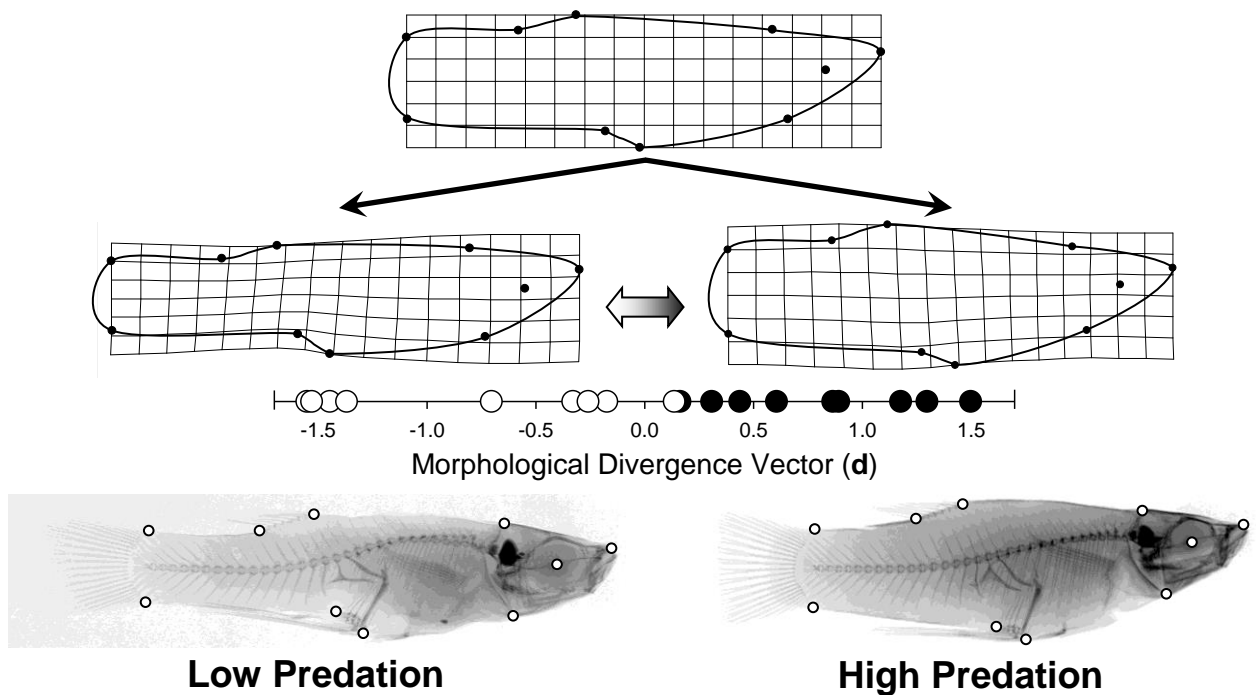


Figure S3 | Map of 18 blue holes where Bahamas mosquitofish were collected (blue holes without piscivorous fish: blue circles, labels beginning with “L”; blue holes with piscivorous fish: red circles, labels beginning with “H”).

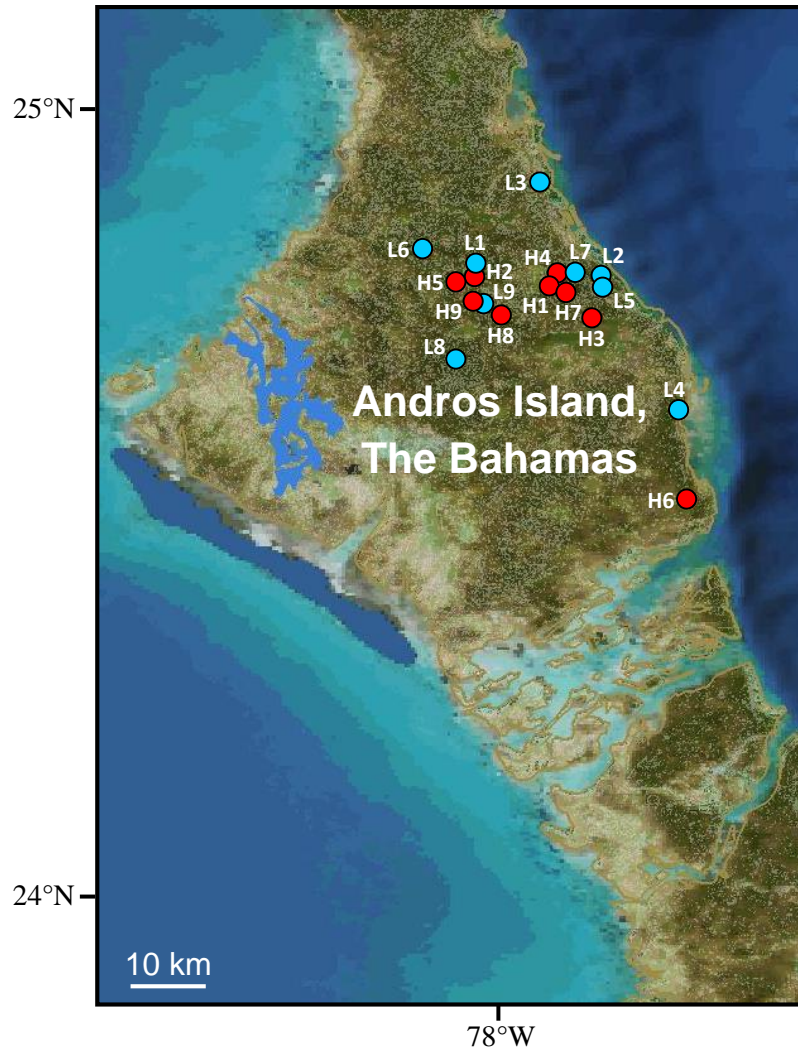
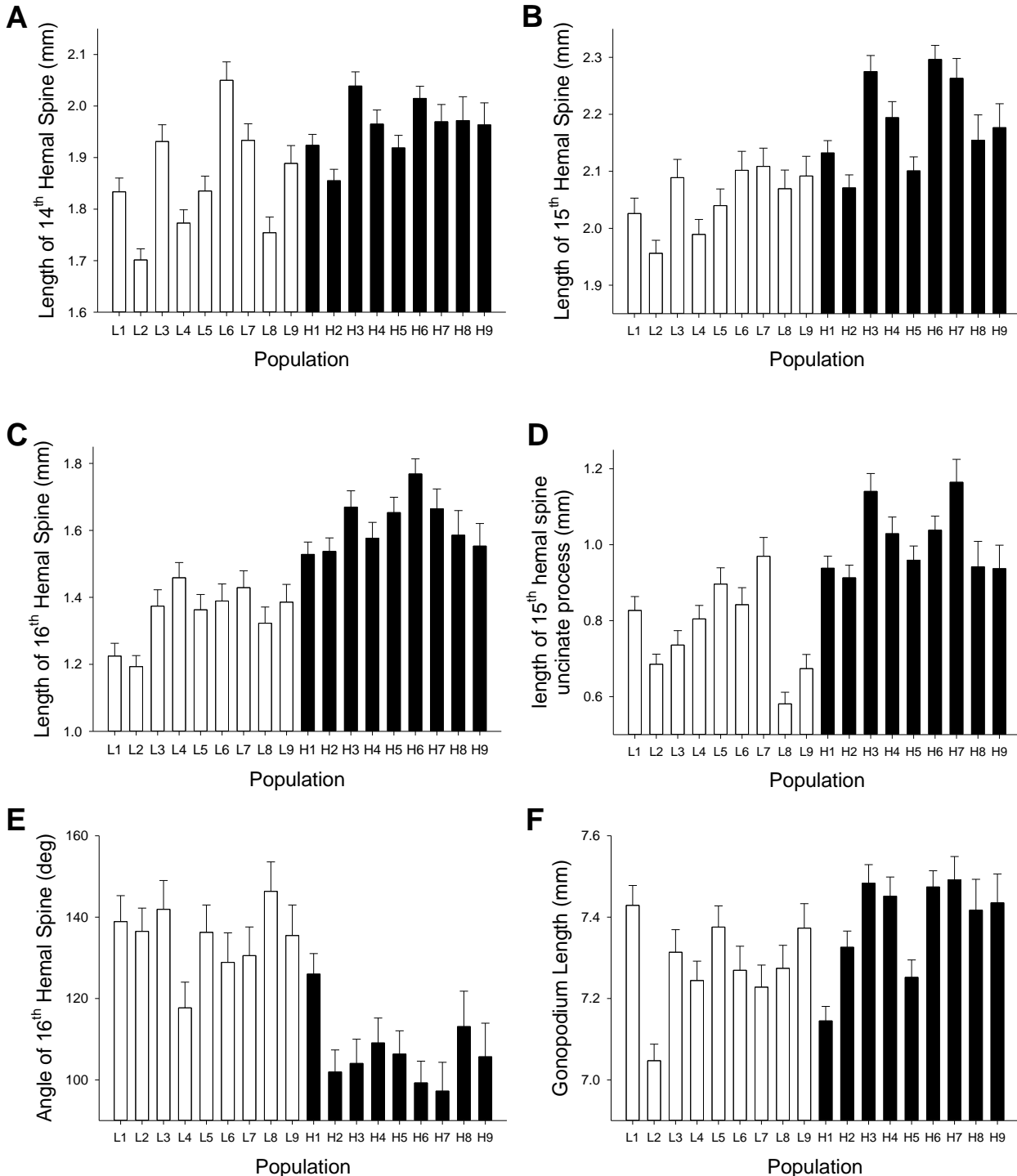


Figure S4 | Differences between low-predation (open bars) and high-predation (filled bars) populations of Bahamas mosquitofish in (A) length of the 14th hemal spine (one-tailed $P = 0.0179$), (B) length of the 15th hemal spine (one-tailed $P = 0.0016$), (C) length of the 16th hemal spine (one-tailed $P = 0.0002$), (D) length of the uncinata process on the 15th hemal spine (one-tailed $P = 0.0009$), (E) angle of the 16th hemal spine (one-tailed $P = 0.0002$), and (F) gonopodium length (one-tailed $P = 0.9308$). Least-squares means \pm one standard error depicted (for length variables, mm units back-transformed from \log_{10} -transformed data used in analyses).



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