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Predator-induced phenotypic plasticity of shape and behavior: parallel and unique patterns across sexes and species

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Abstract

Phenotypic plasticity is often an adaptation of organisms to cope with temporally or spatially heterogeneous landscapes. Like other adaptations, one would predict that different species, populations, or sexes might thus show some degree of parallel evolution of plasticity, in the form of parallel reaction norms, when exposed to analogous environmental gradients. Indeed, one might even expect parallelism of plasticity to repeatedly evolve in multiple traits responding to the same gradient, resulting in integrated parallelism of plasticity. In this study, we experimentally tested for parallel patterns of predator-mediated plasticity of size, shape, and behavior of 2 species and sexes of mosquitofish. Examination of behavioral trials indicated that the 2 species showed unique patterns of behavioral plasticity, whereas the 2 sexes in each species showed parallel responses. Fish shape showed parallel patterns of plasticity for both sexes and species, albeit males showed evidence of unique plasticity related to reproductive anatomy. Moreover, patterns of shape plasticity due to predator exposure were broadly parallel to what has been depicted for predator-mediated population divergence in other studies (slender bodies, expanded caudal regions, ventrally located eyes, and reduced male gonopodia). We did not find evidence of phenotypic plasticity in fish size for either species or sex. Hence, our findings support broadly integrated parallelism of plasticity for sexes within species and less integrated parallelism for species. We interpret these findings with respect to their potential broader implications for the interacting roles of adaptation and constraint in the evolutionary origins of parallelism of plasticity in general.

Key words: boldness, common garden, geometric morphometrics, reaction norm, sexual dimorphism, size at maturity.

Environmentally induced phenotypic plasticity is commonly viewed as an alternative to divergent adaptation, particularly in reference to mechanisms that produce phenotypic variation across a heterogeneous landscape (Bradshaw 1965; Via et al. 1995; Dybdahl and Kane 2005). However, this viewpoint is somewhat oversimplified in that phenotypic plasticity can be considered an adaptation in its own right when the range of environmentally induced phenotypes expressed by a given genotype, or reaction norm, enhances the relative fitness of those genotypes in the corresponding environment compared to other phenotypes (reviewed in Gotthard et al. 1995, Ghalambor et al. 2007). Given that phenotypic plasticity functions

similar to other adaptations, it may in theory show analogous patterns of parallel or convergent evolution in the form of parallel reaction norms within and among species (West-Eberhard 2005; Ghalambor et al. 2007; Wund et al. 2008). Like other adaptations, one would predict that the degree to which adaptive reaction norms are parallel or unique for 2 species, populations, or even 2 sexes, will be determined both by the extent that the species, populations, or sexes face similar patterns of selection across that gradient, and the degree that history (contingency) and shared genetic architecture constrain or promote analogous responses to that selection. In this study, we consider parallel and unique aspects of phenotypic

plasticity in behavior, size, and morphology of males and females of 2 very closely related and ecologically similar fish species that are adapted to the same environmental gradient of predation risk.

Predator-mediated phenotypic plasticity is common in nature. Phenotypes of prey species respond to physical cues of predation attempts (Trussell et al. 2003; Hammill et al. 2010), chemical cues of predators or injured conspecifics (Brossman et al. 2014), visual cues (Sih et al. 2011), or predator alterations to the community (Alonzo et al. 2003). Physical changes in prey size or shape often confer a fitness benefit associated with a reduced ability of predators to consume an individual (Dewitt et al. 2000) or an increase in an individual's escape performance (Langerhans et al. 2004). Behavioral antipredator defenses are highly variable among taxa but usually center around alterations to time allocation, activity levels or habitat use when feeding (Chalfoun and Martin 2010; Rodgers et al. 2013) or conducting other risky behaviors like mating (Johnson and Basolo 2003; Eggers et al. 2005). Importantly, these various adaptive responses to presence of predators come with trade-offs in environments without predators (Palkovacs et al. 2011; Lacasse and Aubin-Horth 2012), placing a selective premium on an individual's capacity to produce the right phenotype under the right predation risk conditions.

In fishes and other animals, most work on predator-driven phenotypic plasticity, as well as adaptive divergence, has focused on responses in behavior, body size, and body shape. Many risk-response behaviors center around "within-situation" time budgeting and activity levels (Sih et al. 2004), assuming an important trade-off between the risky but profitable benefits of activities that facilitate foraging or acquisition of mates and safer behaviors that afford concealment or otherwise reduce the odds of being preyed upon. The different correlated sets of behaviors that mediate this trade-off are commonly conceptualized in terms of relative boldness or shyness (Sih et al. 2004). Traditionally, bold behaviors associated with increased exploration, aggressiveness, or activity levels (Frost et al. 2007; Wisenden et al. 2011; Sih and Del Giudice 2012; King et al. 2013) are thought to come at an increased risk of predation relative to more reserved shy behaviors (Sih et al. 2004, 2012; Bell and Sih 2007). However, some research indicates that boldness can afford its own benefits for reducing predation risk, such as when bolder individuals are more vigilant of predators (O'Steen et al. 2002; Pascual and Senar 2014). Importantly for the current study, while species, population, and sex differences in boldness are well documented (Harris et al. 2010; Wilson et al. 2010; Sih et al. 2012), studies also show that individuals often adjust their boldness with respect to perceived predation risks, consistent with adaptive phenotypic plasticity (Bell and Sih 2007; Sih et al. 2011).

Many fishes are able to adjust their effective body size in response to predation risk in 1 of 2 ways. Species that increase body size often do so specifically in response to gape-limited predators. As a result, some individuals will grow very quickly or develop body projections to surpass the maximum size a predator can consume (Januszkiewicz and Robinson 2007). Alternatively, some species show decreased overall body size (Bell et al. 2011; Välimäki et al. 2012) often in association with earlier maturation and greater early investment into reproduction (Torres-Dowdal et al. 2012; Handelsman et al. 2013). As in cases of adaptive population divergence, the degree to which such size plasticity is parallel or unique among species (or sexes) might depend upon the degree that size has the same effect on predation risk in different species or sexes. In addition, such parallel or uniqueness of plasticity may also depend on size trade-offs associated with processes like competition and

reproduction that also influence fitness (Hjelm and Persson 2001; Farley et al. 2015).

Independently of size, body shape in fishes can also influence the likelihood of prey escaping attacks by predators (Borazjani 2013; Scharnweber et al. 2013). Various studies of population divergence, plasticity, or functional performance suggest 2 major ways in which body shape might often influence escape potential. First, individuals with more streamlined and hydrodynamic body shapes (Fu et al. 2013) experience less drag and can move more efficiently and quickly through water when pursued by predators. Additionally, individuals may develop body proportions favoring greater relative area of the caudal region and its important role in burst starts (Langerhans et al. 2004; Scharnweber et al. 2013) to avoid a lunging attack. As with other traits, however, the relative degree of parallel or unique plasticity in fish body shape is expected to depend on both the specific attributes of predators and potential trade-offs with other aspects of performance. Notably, these same aspects of body shape in fishes are often important for foraging and reproduction (Ghalambor et al. 2004).

Most studies of phenotypic plasticity in fishes or other species focus on single traits or trait types. However, overall adaptation to a given environmental gradient might in principle involve concerted plastic responses in all or many of these trait categories as part of an integrated plastic phenotype. This raises the possibility that species (or sexes) showing parallel patterns of plasticity in 1 set of traits might generally show parallel patterns in other traits, resulting in an overall integrated pattern of parallelism. Although it might be possible to infer such integrated parallelism of plasticity by comparing multiple studies of plasticity in various traits, sexes, and species, the most rigorous assessment of integrated parallelism would involve simultaneous quantification of multiple forms of plasticity within a single study and set of individuals from 2 species or 2 sexes. In the current study, we adopt this integrated approach for assessing parallelism, both across species and across sexes of mosquitofish (*Gambusia* spp.). The sexes and species of mosquitofish represent an interesting contrast for understanding factors determining parallelism of plasticity given that the sexes of our focal species are more overtly different in terms of size, shape, and many aspects of behavior than are members of the same sex across species, raising the question of whether similarity of trait backgrounds or genetic interdependence are more prone to produce parallel plasticity across the various components of an integrated phenotype.

We examined potential parallel and unique aspects of predator-induced phenotypic plasticity of 2 closely related and ecologically analogous species of prey fish (eastern mosquitofish: *Gambusia affinis* and western mosquitofish: *G. holbrooki*). Specifically, we assessed 1) whether predator cues produce size, shape, or behavioral plasticity within the integrated phenotypes of mosquitofishes; 2) the degree that plastic responses are parallel or unique by species; and 3) the degree that responses are parallel or unique by sex. We in turn consider whether our data are consistent with integration of parallelism at different scales (sex vs. species) and what that might tell us about the relative roles of selection, contingency, and constraint in the origins of parallelism of plasticity in general.

Materials and Methods

Study species

The eastern and western mosquitofish are widespread, and often abundant, species of North American poeciliid fishes. These species have very similar morphologies, behaviors, life histories, and

ecological niches, and were considered a single species or subspecies until as recent as 1988 (Wooten et al. 1988). Both species are sexually dimorphic with females larger than males, and males possessing gonopodia for internal fertilization.

Both species have also been introduced extensively for biological control of disease-carrying invertebrates (Meffe 1985) and have a combined introduced range that now includes all continents except Antarctica (Brown 1987; Cote et al. 2011). The high local abundance of these fishes in their native and introduced ranges is driven by high fecundity accompanied with short generation times and a viviparous reproductive strategy (Vondracek et al. 1988; Haynes and Cashner 1995). The colonizing capacity, abundance, and ecological habits of these fishes that made them appealing for disease vector control also make them a high concern IUCN Red List invasive species (Pyke and White 2000). In particular, mosquitofishes are voracious consumers of aquatic insects and zooplankton (Goodsell and Kats 1999; Matveev et al. 2000) and can be aggressive competitors that displace other species (Carmona-Catot et al. 2013).

Importantly for the present study, mosquitofishes are also common prey for piscivorous fishes and aquatic birds (Britton and Moser 1982; Meffe and Snelson 1989), and evidence suggests that fish predators in particular are important in driving adaptive trait divergence of mosquitofish populations. For instance, the presence of predators is associated with population differences in color patterns (Horth 2004) and body shape (Langerhans and DeWitt 2004). Both of the species in this study coevolved with a diversity of Centrarchid predators, including largemouth bass *Micropterus salmoides*, other bass species, and some sunfishes (*Lepomis* spp.). Populations of *Gambusia* that coexist with piscivorous fish predators exhibit streamlined body shapes with greater investment in the size of the caudal region important for generating high escape velocity in response to predator attacks (Langerhans et al. 2004). Body size and shape in mosquitofish can also be correlated with boldness and sociability behaviors (Cote et al. 2011). Where the ranges of *G. holbrooki* and *G. affinis* overlap, *G. holbrooki* commonly displaces *G. affinis* due to a suite of demographic, behavioral, and genetic factors (Scribner and Avise 1994).

Collection and breeding

Gambusia holbrooki were collected using dip nets from a pond site in the Croatan National Forest lands near New Bern, NC (Lilly Pond 34.79°N, 76.86°W). At the time of collection, the pond was reported to have long-standing populations of eastern mosquitofish and centrarchid predators (largemouth bass *M. salmoides* and bluegill sunfish *Lepomis macrochirus*). *Gambusia affinis* were provided by the Contra Costa Mosquito and Vector Control District in Concord, CA (37.93°N, 121.95°W) Although this region is outside the native range of this species, mosquitofish introduced to California derive from portions of the native range with centrarchid predators (Texas) and the introduced region used for sources by Contra Costa is characterized by pervasive introduced populations of bass and sunfish. Ponds in the source and introduced regions for both species are generally shallow with extensive macrophyte growth along the littoral zone and algal growth in the pelagic zone during the summer. The shallow nature of these systems makes them subject to occasional disturbance and colonization events in the form of droughts, floods, and anthropogenic fish introductions, suggesting both mosquitofish lineages have a long evolutionary history of predator and prey metapopulation dynamics favoring evolution of phenotypic plasticity. Finally, both regions where our fish derived have similar thermal environments, with average annual

highs of 21.2–22.3 °C and annual lows of 8.8–9.5 °C, however, Contra Costa, CA receives significantly less rainfall than New Bern, NC (418 mm vs. 1221 mm).

All brood fish were quarantined and captive reared in a single lab for 1 year in the absence of any predator cues. Brood fish were thus extensively acclimated to shared laboratory conditions before being used to produce common-garden offspring (1 generation) for experimentation. All fish rearing and breeding took place under conditions of 26.5 °C water temperature and 15L:9D photoperiod. Aquaria, breeding pools, and rearing pools were filled with treated and conditioned well water and water levels were maintained with deionized water. Fish were fed *ad libitum* 1–2 times daily with a diverse diet, including dried tubifex worms, dried krill, spirulina flakes, and live bearer flake food.

Breeding and offspring production took place in 3 replicate 295 L wading pools per species with a central, circular refuge area of artificial plants surrounded by 5 mm mesh that could be accessed by fry but not by cannibalistic adults. Each breeding tank employed 10–12 adults of 1 species, with a sex ratio of 60–70% females. Pools were checked daily for fry that were then removed from the breeding tanks for allocation to the rearing treatments. Any fry >12 mm were discarded to ensure all fry were exposed to rearing treatments at a similar size and development stage.

The group of fry from a given parturition event were equally and randomly allocated to exposure treatment pools with or without predator cues. All of the exposure pools (again 295 L wading pools with 3 replications) included a central chamber constructed of 1 mm polyurethane coated square mesh that contained either a predator or remained empty depending on the exposure treatment. The remaining area of each pool was further subdivided with 1 mm polyurethane mesh into 2 halves, into which individuals of the 2 species were, respectively, introduced. Hence, both species and sexes experienced identical exposure conditions within each treatment. Both the predator and nonpredator pools (each subdivided for 2 species) were replicated 3 times to further account for any potential pool effects. The predator used in our exposure pools was a live largemouth bass fed live mosquitofish of both species throughout the study period. Predator cues thus included a cohesive set of potential visual, chemical, and physical (e.g., auditory or movement) signals associated with both predators and predation upon prey (conspecific and heterospecific). Fry of both species remained in these respective pools until the appearance of secondary sexual characteristics at approximately 4–12 weeks, at which time they were assayed for behavior, size at maturity, and morphology.

Behavioral assays

Mature fish from the rearing treatments were assayed for risk-taking and foraging behaviors after a 72-h fasting period. Assays were performed in a 114 × 36 cm risk-reward arena that contained a largemouth bass enclosed behind 5-mm polyurethane coated diamond mesh wide at 1 end to provide visual and chemical cues of predation risk and a shoaling group of 3–5 conspecifics (depending on the size of the individuals) enclosed behind similar mesh that was 10 cm wide at the opposite end. The central 71 cm region of the arena was subdivided into a 2 × 5 grid to quantify movements within the tank (Figure 1). The space closest to the shoal group end of the chamber was designated as the “safe zone.” Conversely, the final row on the grid closest to the predator enclosure was designated the “danger zone.” A food reward was used to encourage exploration in the arena. The food reward consisted of a mixture of freeze-dried krill or spirulina flakes that were distributed among all of the grid

squares. Fish were introduced individually into an acclimation tube at the safe end nearest to the shoal group since this species of fish is social and this most closely mimics realistic biological conditions. Individuals were acclimated for 2 min before the tube was lifted and the assay began. This arena design allowed us to simultaneously score the time individuals spent in presumptively safe versus risky activities (i.e., holding vs. exploring) and in safe versus risky locations (near a shoal vs. near a predator). Behaviors were continuously recorded using the JWatcher software (Ver. 1.0, Macquarie University and UCLA) for 10 min and used to quantify the amount of time required to first leave the release area and begin exploring (*lag time*), the subsequent amount of time spent swimming around the arena or feeding (*exploration time*), the percentage of time spent in portion of the arena closest to the shoal (*time in safe zone*, diagonal dashed area), and the percentage of time in the portion of the arena closest to the predator (*time in danger zone*, vertical dashed area) (Figure 1). Fish spent very little if any time feeding during the trials, so feeding time was included in exploration time. Behaviors were analyzed with a multivariate analysis of variance (MANOVA) on a linear mixed effects (LME) model with replicate as a random variable and predator treatment, species, and sex as fixed effects. Subsequent comparisons within treatment groups (sex, species, or exposure) were performed using a *t*-test with unequal variances and a Bonferroni correction.

Body size and shape

After behavioral assays, individuals were euthanized with a lethal dose of MS-222 ($>250 \text{ mg L}^{-1}$), weighed (to $\pm 4 \text{ mg}$), and photographed against a grid background with a ruler for scale. Individuals were measured in ImageJ (Ver. 1.6.0_20, Rasband and NIH) for total length (mm from tip of snout to tip of caudal fin), and body depth (mm of deepest vertical measure of the body). These 3 different size indexes (mass, length, and depth) were analyzed with MANOVA followed by post hoc *t*-tests within treatment levels.

Shape data were collected from the photographs based on 15 fixed landmarks (Appendix A) adapted from a set used by Palkovacs et al. (2011), excluding some spinal and cranial markers, and adding caudal region markers. These landmarks were used for geometric morphometric analyses to summarize overall fish body shape using the program tpsDig2 (Ver. 2.17, Rohlf and SUNY). Because mosquitofish are strongly sexually dimorphic, relative warps were obtained for each sex in separate geometric morphometric analyses. Relative warps were obtained from a principal component analysis of thin-plate spline shape variation using tpsRelw (Ver. 1.49, Rohlf and SUNY). Sex-specific MANOVA and discriminant function analyses (DFA) were performed on the relative warps using predator

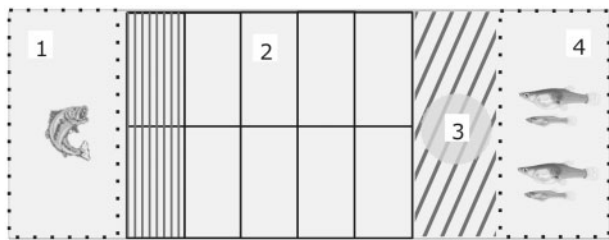


Figure 1. Behavioral assay experimental design. Area (1) is the largemouth bass enclosure, Area (2) is the risk assessment grid, Area (3) is the acclimation tube, and Area (4) is the shoal group. The “danger zone” is represented by the vertical-lined area. The “safe zone” is represented by the diagonal-lined area.

exposure treatment as the primary (discriminating) factor for each sex. Interacting factors that were not significant ($P < 0.05$) were removed and the reduced model was used. DFA scores were then visualized in terms of landmark deformations using tpsRegr (Ver. 1.40, Rohlf and SUNY).

Inference

In all analyses, presence of phenotypic plasticity was inferred where there was statistical evidence for either a direct effect of predator exposure or for its interaction with sex or species. A significant sex-by-exposure or species-by-exposure interaction was considered support for nonparallel (unique) plastic responses. Conversely, presence of a significant exposure effect without a significant sex-by-exposure or species-by-exposure interaction was considered evidence of parallel norms of reaction. All statistical analyses were performed using the R Programming Environment (Ver. 3.1.1, R Core Team), using the libraries *vegan* (Oksanen, Ver. 2.0), *car* (Fox and Weisberg, Ver. 2.0), *MASS* (Ripley, Ver. 7.3), *nlme* (Oenheiro and Bates, Ver. 3.1), *heplots* (Fox, Friendly, and Monette, Ver. 1.0–1.6), and *ape* (Paradis et al., Ver. 3.3).

Results

Behavioral assays

Predator exposure had a significant effect on the behavior of mosquitofish. Importantly, there was a significant interaction effect between species and exposure treatment ($F_{1,216} = 8.10$, $P < 0.01$), but no interaction effect for sex and exposure ($F_{1,214} = 0.54$, $P = 0.49$), and no fixed effect for sex ($F_{1,216} = 0.94$, $P = 0.34$), indicating unique plastic responses by the 2 species but parallel reaction norms for the sexes (Appendix B). Hence, the following description of species effects apply in common to males and females of both species. Considered separately within the LME framework, both components of time allocation were affected by the species-by-exposure regime interaction (lag time: $F_{1,216} = 8.10$, $P < 0.01$, percentage of time exploring: $F_{1,216} = 7.51$, $P = 0.02$). *Gambusia affinis* exhibited a slightly, but not significantly ($t_{114} = 1.58$, $P = 0.11$), longer lag time to begin exploring the risk-reward environment compared to *G. holbrooki* when reared in the absence of predator cues. However, *G. affinis* greatly increased this lag time when reared in the presence of the predator, relative to the *G. holbrooki* ($t_{95} = 4.51$, $P < 0.001$) that responded with a slightly reduced lag time (Figure 2a). Once a fish began exploring, individuals of both species that had not been exposed to predator cues spent similar time actively moving about the arena ($t_{118} = 0.16$, $P = 0.87$). However, predator exposed *G. holbrooki* and *G. affinis* showed different ($t_{101} = -3.08$, $P = 0.002$) and opposing patterns of exploration. *G. holbrooki* tended to increase their exploration time, whereas *G. affinis* showed somewhat reduced exploration (Figure 2b).

As with time allocation, where fish spent their time was also plastically influenced by prior exposure to predator cues. Time spent in the “safe zone” was significantly affected by an interaction between species and exposure ($F_{1,216} = 4.06$, $P = 0.05$), but not by sex ($F_{1,214} = 2.72$, $P = 0.10$) or between sex and exposure ($F_{1,214} = 0.001$, $P = 0.97$). This again indicates a unique aspect to plasticity of the 2 species. While percentage of time spent in the “safe zone” was similar for the 2 species in the absence of predator exposure during development ($t_{114} = -0.41$, $P = 0.68$), exposed *G. affinis* spent a significantly greater percentage of time in the “safe zone” when compared with exposed *G. holbrooki* ($t_{100} = 2.42$,

$P=0.01$) (Figure 2c). Although we did not detect a significant species-by-exposure interaction for time in the “danger zone” ($F_{1,214}=1.24$, $P=0.27$), this was likely due to reduced power due to greater variation among individuals in their use of this region of the arena. Supporting this, we did detect an overall species effect ($F_{1,217}=4.78$, $P=0.03$), and the trends for exposure effects on

relative species use of the “danger zone” was essentially opposite that observed for time in the “safe zone” (Figure 2d). Indeed, unlike the LME, separate t -tests at each exposure treatment indicate that whereas nonexposed members of these species do not differ in their time spent in the “danger zone” ($t_{115}=1.98$, $P=0.53$), exposed *G. holbrooki* spent significantly more time there than exposed *G. affinis* ($t_{104}=-2.19$, $P=0.03$).

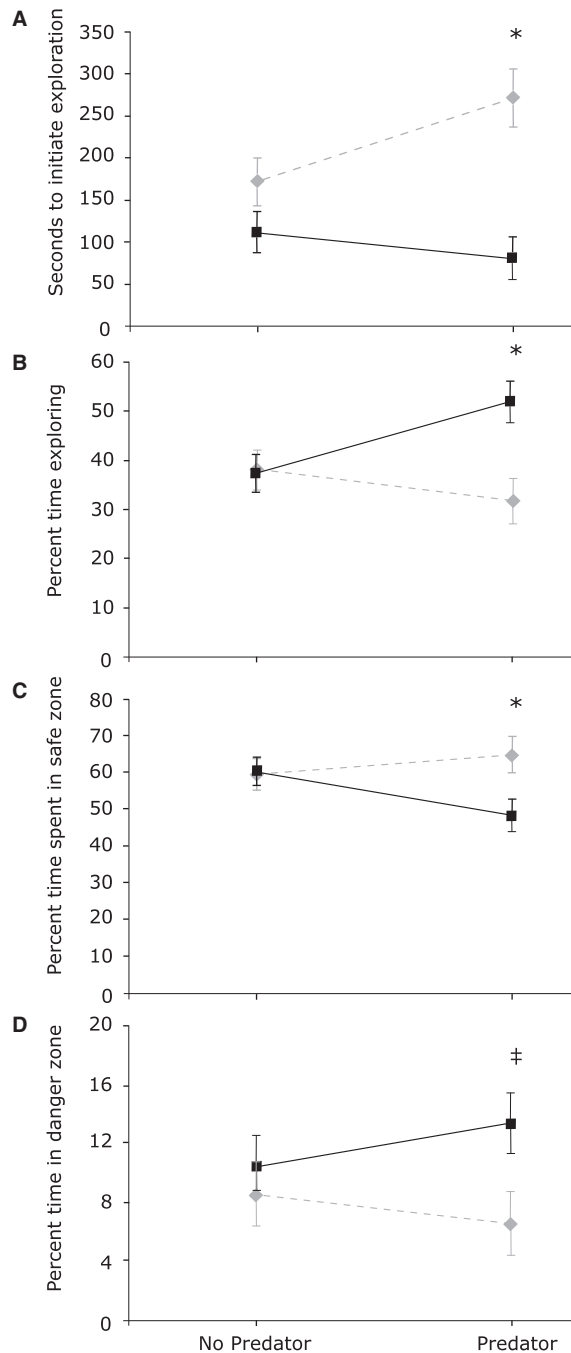


Figure 2. Behavioral responses for *G. affinis* (dashed line) and *G. holbrooki* (solid line). Panel (A) is the time for a fish to leave the “safe zone” the first time. Panel (B) is the percentage of time a fish spent outside of the “safe zone.” Panel (C) is the percentage of time a fish spent near the shoal group. Panel (D) is the percentage of time a fish spent near the bass enclosure. Asterisks represent significant differences with unique interactions (species by predator). Double crosses represent significant effect of species. All error bars are ± 1 standard error.

Body size and shape

The multivariate length, body depth, and mass model showed no significant interactions among sex, species, or exposure groups on body size metrics, as well as no separate effect of predator exposure, indicating that size was not phenotypically plastic (Appendix C). The subsequent reduced model did reveal significant separate effects of both sex ($F_{1,217}=39.3$, $P<0.001$) and species ($F_{1,217}=9.81$, $P<0.01$). The effect size of sex was double that of species (sex = 0.16, species = 0.08), confirming the substantial sexual size dimorphism in these fishes. Overall, females were 11% longer ($t_{207}=-5.33$, $P<0.001$), 30% deeper ($t_{201}=-10.5$, $P<0.001$), and 50% heavier ($t_{201}=-6.40$, $P<0.001$) than males. Univariate comparisons indicated that *G. holbrooki* were 6% longer ($t_{223}=-2.60$, $P<0.01$) than *G. affinis*, but the species did not differ in body depth ($t_{225}=-0.79$, $P=0.43$) or mass ($t_{221}=-1.30$, $P=0.20$).

Unlike body size, we did find support for phenotypic plasticity of body shape. Female body shape responded to predator cues with a parallel norm of reaction across species, as reflected by a significant effect of exposure treatment ($F_{1,114}=34.9$, $P<0.001$, $\eta^2=0.23$) but not species ($F_{1,114}=2.48$, $P=0.11$), with no interaction between the two ($F_{1,114}=3.63$, $P=0.06$). Male body shape differed between species ($F_{1,105}=18.2$, $P<0.001$) and changed with predator exposure ($F_{1,105}=24.9$, $P<0.001$), but still with a parallel reaction norm (i.e., nonsignificant interaction: $F_{1,105}=1.14$, $P=0.26$). Indeed, exposure effects among male fishes were twice as strong as species differences (exposure: $\eta^2=0.19$, species: $\eta^2=0.10$). Within each sex and species, discriminant function scores based on relative warps differed significantly by exposure treatment, with treatment accounting for 33–46% of variation in the DFA scores (females: *G. affinis*: $F_{1,65}=54.4$, $P<0.001$, *G. holbrooki*: $F_{1,49}=38.8$, $P<0.001$; males: *G. affinis*: $F_{1,53}=38.0$, $P<0.001$, *G. holbrooki*: $F_{1,52}=25.7$, $P<0.001$). Regression of DFA scores back on relative warps to depict associated deformations indicated that predator-exposed individuals of both sexes and species tended to show more slender and streamlined bodies, a body shape difference that is particularly apparent in the region of the anal fin insertion (Figure 3). The cranium also showed a reduction in depth, with a dorsal-ventral flattening in both the jaw and the posterior extent of the cranium. Fish from exposure treatments also had eyes more ventral in the cranium than those from nonexposure treatments. Caudal regions in predator-exposed fish were expanded anterior-posterior, particularly along the dorsal side from the posterior dorsal fin insertion to the dorsal side caudal fin insertion.

In addition to these generally parallel reaction norm morphological effects of predator exposure for both sexes and species, there were some sex-specific patterns of plasticity. Male mosquitofish of both species responded to predator cues in the angle and width of their anal fin insertions (Figure 3). Exposure ($F_{1,108}=4.41$, $P=0.04$), but not species ($F_{1,108}=2.27$, $P=0.14$), significantly affected the width of the insertion, with predator-exposed males developing fin insertions 10% wider than nonexposed males. The angle of gonopodial insertion was also marginally affected by

predator exposure ($F_{1,108}=2.95$, $P=0.08$), but not species ($F_{1,108}=2.20$, $P=0.14$). Gonopodia angle was approximately 16% flatter in exposed individuals compared to nonexposed individuals (Figure 3). Given that exposure groups did not differ in any body size measurements, these shape differences are not associated with differences in body size and analyses of DFA scores revealed no significant correlations with size measures.

Discussion

Our study clearly demonstrates that mosquitofishes show marked phenotypic plasticity in response to predator associated cues. However, not all traits showed this response to the same extent and some traits showed more evidence of parallel plastic responses than others. Behavioral responses were parallel for both sexes but unique within species, with the generally bolder *G. holbrooki* becoming bolder after rearing in the presence of predator cues. Body shapes of both species and sexes shifted toward greater caudal peduncle investment and an overall slimmer body profile in the presence of predator cues, a pattern that is very analogous to what has been suggested for adaptive divergence of mosquitofish from habitats lacking or containing predators, respectively (Langerhans et al. 2004). Size was not plastic with predator exposure.

Parallel and unique plasticity of behavior

The 2 species of mosquitofish showed fairly similar behavior when reared without predator cues. In contrast, the behavior of the species

became very different when they were exposed to predator cues, with *G. holbrooki* becoming notably bolder and *G. affinis* becoming somewhat shyer. Indeed, *G. holbrooki* increased seemingly risky behaviors by 20–29% under predator exposure, whereas *G. affinis* reduced such behaviors by 8–37%. Divergence in behavioral norms of reaction between species is not entirely unexpected given prior work showing that this can even occur among populations within species of other taxa (reviewed in Sih et al. 2012). Interestingly, prior studies also suggest that bolder behavioral types might often be more plastic in their tendencies, altering their behaviors more in the presence or absence of a predator or food conditions (Thomson et al. 2012), which is generally consistent with our findings, although there were exceptions (e.g., greater change in lag time by *G. affinis*).

It may not seem intuitive that the opposite responses of both *G. holbrooki* and *G. affinis* to predator cues could both be adaptive, but there is reason to think that may be the case. The fact that bold and shy individuals persist in most populations of fish and other organisms has led to the theory that both phenotypes can be adaptive by expressing a cohesive set of intraspecific and interspecific behaviors (Sih et al. 2004; Wilson et al. 2010; Cote et al. 2011). Support for this premise comes from studies of boldness in fishes, including poeciliids, where it has been shown that shorter lag times, greater exploration, and willingness to approach predators can be associated with predator vigilance and ability to more efficiently gauge risk (O'Steen et al. 2002; Brown and Braithwaite 2004; Brown et al. 2005; Leblond and Reeb 2006; Harris et al. 2010; Reed et al. 2010;

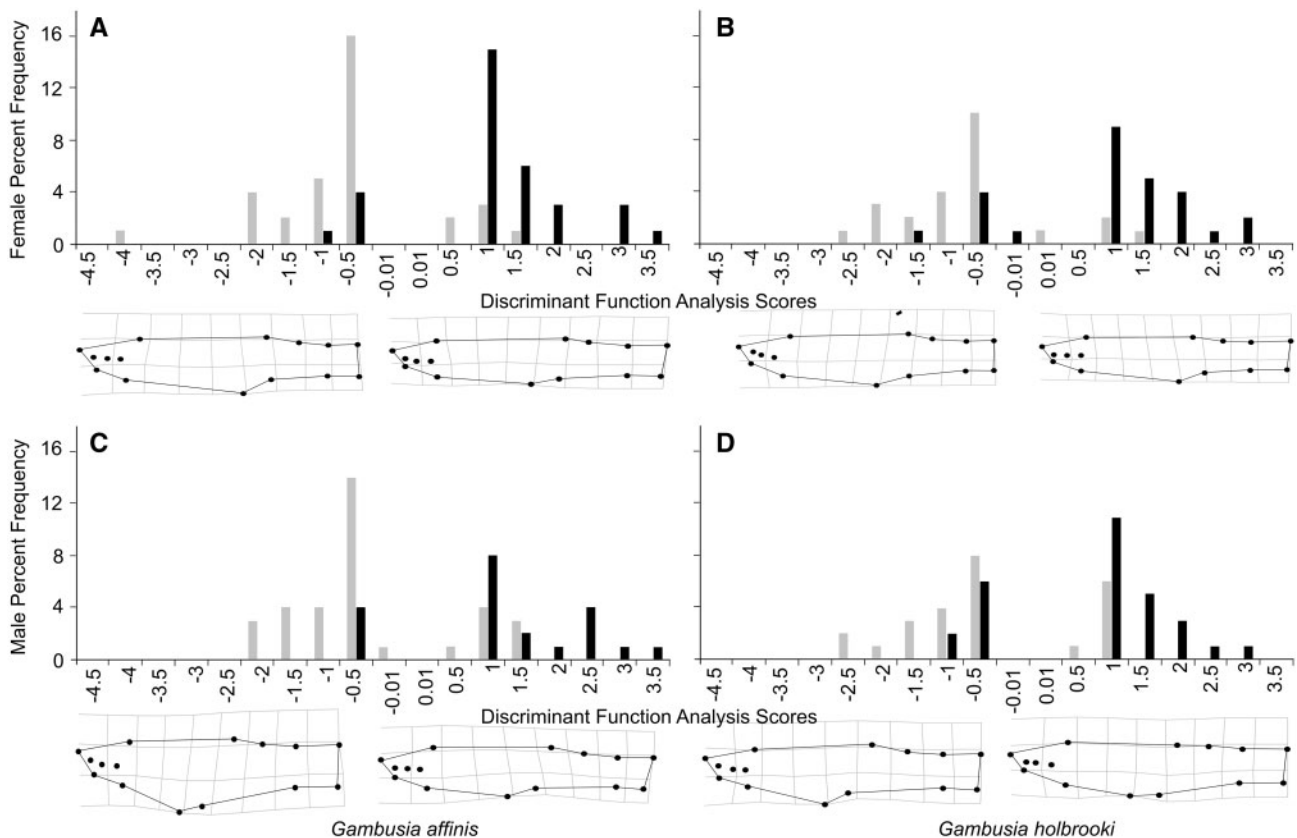


Figure 3. Frequency distributions of discriminant function scores summarizing geometric morphometric deformations of 26 relative warps. Summary deformations are shown at each end of the function axis using a visualization exaggeration factor of 4. Gray bars are non-exposure populations and black bars are the exposed populations. Panel (A) is female *G. affinis*, (B) is female *G. holbrooki*, (C) is male *G. affinis*, and (D) is male *G. holbrooki*.

Brown and Irving 2014). A similar line of reasoning might thus explain why 2 species with somewhat different innate tendencies toward boldness or shyness might evolve plasticity to predators that exaggerates those tendencies to exploit two different adaptive peaks in the fitness landscape (Sih et al. 2004; Briffa et al. 2008).

In contrast to comparison of the 2 species, the 2 sexes showed largely parallel behavioral plasticity within species. This was somewhat surprising given the marked sexual size and shape dimorphism in these species, as well as known differences in how males and females allocate their activity and energy budgets in mosquitofish and other poeciliids (Magurran and Seghers 1994; Basolo and Alcaraz 2003). For many small prey fishes the larger sex can experience increased predation (Britton and Moser 1982) and present shyer traits (Harris et al. 2010; Wilson et al. 2010) than the smaller sex. In mosquitofish, males and females may be somewhat locked together in their plastic responses to predators through both their social and genetic associations tied to mating. Interbreeding could constrain the opportunity for genetic divergence in reaction norms. Socially, mature males almost continuously pursue females and because of this divergent plastic responses to predators might be incompatible where predator attention depends on joint behaviors of interacting males and females (e.g., female shyness negated by attention drawn by bolder males).

Lack of plasticity of size

We did not detect plasticity of size in either mosquitofish species despite the presence of such plasticity in other fishes (i.e., Januszkiewicz and Robinson 2007; Burns et al. 2009; Preisser and Orrock 2012). Larger size is often assumed to enhance prey escape ability through aspects of predator gape limitation or greater escape velocity, but these advantages can be offset if predators target the larger prey for their greater energetic value (Brooks 1968; Britton and Moser 1982). While some prey may be able to surpass a given predator's gape limitation (Cowan et al. 1996; Abate et al. 2010), if the predator has no functional gape limitation for that prey (reviewed in Sogard 1997) or cost of prey capture varies little with size (Gill and Hart 1994), larger size might make prey more attractive target, as is likely the case with mosquitofish (Britton and Moser 1982). Conversely, smaller prey may at times be more difficult for predators to detect and present lower per capita value to predators (Werner and Hall 1974; Goss-Custard 1977), leading to the prediction that predation risks might favor expression of smaller size (reviewed in Blanckenhorn 2000). However, being poeciliid fishes, mosquitofish are already among the smallest fish species in North America, with some of the shortest times to maturation (weeks) and highest investment in maternal provisioning (live bearers). Hence, there may simply be limited scope for plasticity favoring even smaller size in response to predator cues.

Parallel plasticity of shape

We found a strong degree of parallelism of shape plasticity in these 2 species as well as evidence of parallel plasticity of shape across the sexes, even given their substantial sexual dimorphism. Parallelism in divergence or plasticity might be predicted to be greatest where species or sexes with similar genetic and phenotypic makeup are subjected to a genetically or adaptively constrained fitness landscape. Consistent with this prediction, the 2 species of mosquitofish in this study are exceedingly similar in morphology and ecological niche, to the point that they are commonly confused and were initially treated as a single species (Wooten et al. 1988; Pyke 2005). At the same

time, the great size disparity and lunging predatory attacks of bass likely places a premium on burst escape abilities that approach the physical and hydrodynamic limitations of small fishes (Bainbridge 1957; Domenici and Blake 1997). Hence, unlike behavior where the 2 species might have somewhat different behavioral tendencies and alternative adaptive optima might exist to cope with predators (i.e., bolder or shyer), these 2 observations for morphology imply strong adaptive constraints and less opportunity for alternative body conformations to aid escape. Assessing whether morphological plasticity is more generally parallel across species and sexes than behavioral plasticity awaits future studies of the type conducted here, but the importance of adaptive constraints acting on initially analogous body plans is reinforced by another aspect of parallelism observed within this study—parallelism of adaptive divergence and phenotypic plasticity.

Morphological plasticity in the present study showed remarkably analogous predator-associated patterns of overall streamlining, dorsal-side longitudinal expansion of the caudal peduncle and shifts in eye position to patterns observed among wild populations of *G. affinis* living in the presence or absence of fish predators, particularly for females (Langerhans et al. 2004) (Figure 4). Indeed, these patterns of trait divergence appear remarkably conserved among wild populations of mosquitofish, males and females, and even other fish species facing this same ecological selection gradient (González and Gianoli 2004; Langerhans and DeWitt 2004; Hendry et al. 2006; Fu et al. 2013). Moreover, functional studies have established that caudal peduncle size is linked with burst speed and quick escape behaviors in mosquitofish, and differs among populations with and without fish predators (Langerhans et al. 2004; Langerhans and Makowicz 2009; Borazjani 2013). Although many of these prior studies of geographic variation in body shape did not compare populations under common-garden conditions, and thus likely confound adaptive divergence with the phenotypic plasticity that we demonstrate here (our design precludes the opposite), it seems probable that these 2 forms of trait determination are adaptively constrained to be largely analogous. Moreover, these 2 forms of trait determination may not even be independent. Where traits are initially plastic, selection may act to intensify or flatten the slopes and intercepts of associated norms of reaction leading to heritable population divergence (Crispo 2008).

Although sexual parallelism of plasticity predominated, it was not complete. The primary exception was a trait strongly associated with the different reproductive anatomy of males and females. Anal

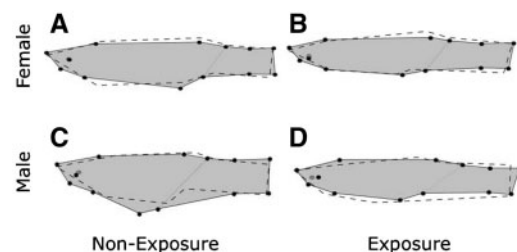


Figure 4. Superimposition of morphological deformations due to plasticity (this study) and due to population divergence (Langerhans et al. 2004). All panels are of *G. affinis*. Thin plate spine images of plasticity from the present study are exaggerated by 4 units (shaded, black points). Images are paired with corresponding sex and predator exposure outlines from Langerhans et al. (2004) with 2-unit exaggeration (dashed, gray points). Different point sets from the 2 studies contribute to some differences in shape resolution, but overall deformation patterns are broadly analogous.

fin morphology is sexually dimorphic for mosquitofish with males having anal fins modified into gonopodia for internal fertilization. Male mosquitofish of both species showed much greater plasticity in this region than females, with males exposed to predator cues showing an expansion in the width and a reduction in the angle of this region. Prior work has found that mosquitofish populations in systems with predators generally have shorter gonopodia with a smaller overall area (Langerhans et al. 2005; Heinen-Kay and Langerhans 2013). We did not measure gonopodia length or area directly, but it seems likely that an overall reduction in the angle of gonopodia could also reflect a reduction in gonopodial investment. Certainly, it seems reasonable that traits that are very tightly linked to alternative sexual anatomy and mating systems, like gonopodia in mosquitofish, might often be subject to more unique patterns of plasticity.

Integration of plasticity across traits

In the current study, the 2 sexes exhibited generally integrated patterns of plasticity in the sense that they showed parallelism of plasticity for nearly all plastic traits, with the possible exception of actual reproductive anatomy (gonopodia in males). In contrast, the 2 species showed less integration of parallelism, with parallel patterns of morphological plasticity but nonparallel patterns of behavioral plasticity. This outcome is again noteworthy in that males and females within each of these species are more overtly divergent in morphology, size, and many aspects of behavior (e.g., sex roles) than are members of the same sex across species. In light of this observation, we advance the hypothesis that initial similarity of phenotypes is less apt than genetic and selective interdependence to produce broad integration of parallelism across various plastic traits. However, we only considered 1 species pairing in our study, and while our approach is unique in combining both multiple traits, sexes and species in 1 study, the generality of our observation and this hypothesis must await additional future studies of phenotypic plasticity that adopt a similarly broad comparative approach. This should be possible in that many closely related species or populations of fish and other taxa show greater sexual dimorphism than interspecific or interpopulation divergence within sexes.

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Conflict of Interest

The authors have declared no conflicts of interest.

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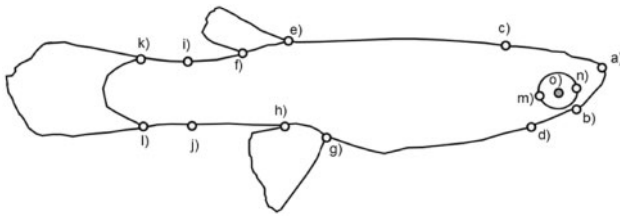
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Appendix A

Body landmarks used in shape analysis: (a) tip of snout, (b) premaxilla, (c) superior posterior extent of cranium, (d) inferior posterior extent of cranium, (e) anterior extent of dorsal fin insertion, (f) posterior extent of dorsal fin insertion, (g) anterior extent of anal fin insertion, (h) posterior extent of anal fin insertion, (i) superior tip of caudal peduncle, (j) inferior tip of caudal peduncle, (k) superior edge of caudal fin insertion, (l) inferior edge of caudal fin insertion, and (o) eye centroid.



Eye landmarks used in eye size: (m) posterior edge of ocular socket and (n) anterior edge of ocular socket.

Appendix B

Complete reduced LME model for behavioral traits. Model represents the effects of sex, species, and exposure treatments on a suite of behavioral traits. Behaviors were a combination of time to initiate exploration, total time spent exploring, percentage of time exploring the “danger zone” (nearest to bass), and percentage of time exploring the “safe zone” (nearest to shoal group). Models were reduced via backwards selection. Interaction terms were removed if not significant

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($P > 0.05$). Table values are from an MANOVA on the final LME model. Effect sizes are as a proportion (partial association in the linear model). Significance is indicated at < 0.01 (**) and < 0.0001 (***).

Population term	Degrees of freedom	F-value	P-value	Significance	Effect size
Sex	1,216	0.94	0.33		0.02
Species	1,216	12.82	0.0004	***	0.10
Exposure treatment	1,216	2.46	0.12		0.02
Species by exposure interaction	1,216	8.10	0.005	**	0.04

Appendix C

Complete reduced LME model for size traits. Model represents the effects of sex, species, and exposure treatments on a suite of independently measure size characteristics. Size metrics were a combination of total length (snout to tip of tail), body depth (vertical measure of deepest portion of the body), and weight. Models were reduced via backwards selection. Interaction terms were removed if not significant ($P > 0.05$). Table values are from an MANOVA on the final LME model. Effect sizes are as a proportion (partial association in the linear model). Significance is indicated at < 0.01 (**) and < 0.0001 (***).

Population term	Degrees of freedom	F-value	P-value	Significance	Effect size
Sex	1,217	39.29	<0.0001	***	0.47
Species	1,217	9.81	0.002	**	0.09
Exposure treatment	1,217	1.52	0.22		0.07