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Sources of Ecologically Important Trait Variation in Mosquitofish (Gambusia affinis and Gambusia holbrooki)

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By

Heather Ann Arnett

B.S., University of Wisconsin-La Crosse, 2008

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A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Ecology and Environmental Sciences)

The Graduate School

The University of Maine

May 2016

Advisory Committee

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THESIS ACCEPTANCE STATEMENT

On behalf of the Graduate Committee for Heather Ann Arnett I affirm this manuscript is the final and accepted dissertation. Signatures of all committee members are on file with the Graduate School at the University of Maine, 42 Stodder Hall, Orono, Maine.

____ ____________________

Dr. Michael T. Kinnison, Professor Date

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CHAPTER 1: INTRODUCTION

There is a growing body of empirical and theoretical literature that relates heritable trait variation within and among populations to population, community, and ecosystem processes. This eco-evolutionary synthesis has garnered substantial attention in recent years, based in large part on increasing evidence that the traits of many populations change in “contemporary” time, or along the same timescales as ecological processes (Thompson 1998, Hendry and Kinnison 1999, Collyer et al. 2007). This opens the door for phenotypic trait changes to not only occur in response to ecological dynamics, the focus of evolutionary ecology for decades, but also has the potential to reciprocally shape ecological dynamics (Hairston Jr et al. 2005, Kinnison and Hairston Jr 2007, Post and Palkovacs 2009, Ellner et al. 2011).

However, review of the current eco-evolutionary studies reveals a fairly narrow theoretical focus on classically heritable trait variation; wherein, most empirical studies in the field are conducted with wild-sourced individuals for which the exact heritable basis for phenotypic traits is unknown. Evolution has a long history of studying phenotypic variation in nature, but it is almost certainly not accurate to portray all interactions of phenotypes and ecology as evolution in the narrow sense of “changes in allelic frequencies over time” (Lande 1976, Hoffmann and Merila 1999, Futuyma 2014). Indeed, many cases reported results that are not well resolved (Post and Palkovacs 2009) and phenotypic effects are often misclassified (Ellner et al. 2011). There are good reasons to believe that other processes contributing to trait variation may have as large if not larger effects on phenotypes and ecology, exemplifying the importance of understanding the relative contributions of these additional phenotypic effects compared to genetic evolution if we are to build a true synthesis linking the reciprocal dynamics between trait variation and ecological dynamics. My dissertation is motivated by a desire to better understand the extent, and in some cases ecological effects, of these neglected components of population trait variation in an emerging model system for study of eco-evolutionary dynamics in aquatic systems – mosquitofish.

Mosquitofish as a Model System

A good focal species for field and lab studies of eco-evolutionary dynamics would in principle 1) have widespread and large ecological effects, 2) be prone to contemporary trait change, and 3) be logistically manageable in the context of replicable laboratory and field experiments, as well as field surveys. The mosquitofishes (*Gambusia* spp.), a genus of forty-five species of small, sexually dimorphic, live-bearing fishes within the family poeciliidae, possess all of these attributes.

Mosquitofish are native to North and Central America but two species the Eastern and Western Mosquitofish (*Gambusia holbrooki* and *G. Affinis* respectively) are now found on every continent except Antarctica. These fishes are now so widespread, in part, because they have been used extensively as biocontrol agents for mosquitoes and their associated vector-borne diseases (Meffe 1985). Once established in a new region, however, mosquitofish are also extremely successful at invasion and colonization (Grapputo et al. 2006). This is in part facilitated by the live-bearing reproductive mode of these species, which have very short generation times (as short as a few months) and can give birth to an average of 1-101 offspring per brood (Krumholz 1948). A single female can thus establish a new population that could grow to thousands of individuals within a single year. These life history traits facilitate the capacity of the species for control of fast-reproducing insect pests like mosquitos, but are also associated with profound impacts on the ecosystems to which they are introduced.

Despite their reputation for consuming mosquito larvae, *Gambusia* species are voracious, generalist feeders that can very effectively deplete aquatic insect larvae and thus influence ecosystem pathways tied to these consumers (Goodsell and Kats 1999, Matveev et al. 2000). In addition, mosquitofish have been known to eat from several trophic levels, consuming anything from detritus and algae, to zooplankton, insect larva and even other fishes (Rincon et al. 2002, Specziar 2004, Blanco et al. 2004). Hence, mosquitofish serve as mesopredators, linking trophic levels together. Mesopredators have become a focus in ecology due to the theory of mesopredator release, wherein the unabated growth of a mesopredator is linked with mass extirpations in the lower trophic levels in an ecosystem (Crooks and Soulé 1999, Myers et al. 2007, Ritchie and Johnson 2009, Albins and Hixon 2013). In addition to their direct effects as consumers, mosquitofish interact in other important ways with other species in foodwebs. Again, their short generation times and large abundance often make mosquitofish strong competitors with fishes, or other taxa (e.g., amphibians) that occupy similar habitats and consume similar prey. Moreover, mosquitofishes tend to be behaviorally aggressive in interactions with competitors, segregating those species to a lower quality habitat (Rincon et al. 2002, Carmona-Catot et al. 2013). Conversely, the high productivity, small size, and lack of any defensive morphology, make mosquitofish a common and preferred prey for many larger piscivorous fishes and aquatic birds (Britton and Moser 1982, Meffe and Snelson 1989).

In addition to their widespread and strong ecological interactions, mosquitofish and other poeciliids have long been the subject of observational and experimental studies of local adaptation and contemporary evolution. The short generation of times of these animals make it possible for substantial evolution to occur over periods of just years to decades. Historic studies show that mosquitofish show marked patterns of divergence in morphological and life history traits in association with habitat (Stearns 1983a), predator regime (Langerhans and DeWitt 2004, Langerhans et al. 2004, 2005), and abiotic factors (Stockwell and Weeks 1999). Many of these patterns have arisen since mosquitofish were introduced to new habitat ranges and are repeatable, suggesting consistent patterns of selection and trait responses. At the same time, the small size, broad diet, and fast generation times of mosquitofish are logistically useful in that they make these species suited to being reared and assayed in highly replicated lab experiments that can span multiple generations.

The laboratory that I study in at the University of Maine has begun extensive field and lab investigations of eco-evolutionary dynamics in the two closely related Eastern and Western Mosquitofishes. Much of this work is couched in terms of the role that addition or removal of top predators might play in the evolution of prey traits that influence the strength and pattern of trophic cascades. However, predators are known to have a diversity of evolutionary and non-evolutionary effects on their prey (Abrams 2000, Werner and Peacor 2003, Peckarsky et al. 2008). Hence, for this study system to be most informative, one would ideally want to understand not just the capacity of these species for genetic evolution in response to predators, but also the full suite of interacting processes that determine phenotypic trait expression and variation in nature.

Components of Phenotypic Trait Variation

Classic quantitative genetics tells us the variance among phenotypes in a given population can in principle be decomposed into a genetic component (V_G), an environmental component (V_E), their interactions (V_{GxE}), and their covariances (COV_{GE}) (Stearns 1992). The environmental component and genes-by-environment interactions have a long history of study with respect to phenotypic plasticity, with the latter receiving the most focus in the form of phenotypic norms of reaction (Mousseau and Fox 1998b, Crispo et al. 2010, Fusco and Minelli 2010, Viney and Diaz 2012). Within-generation (i.e., intra-generational) changes in phenotypes due to phenotypic plasticity have been documented for a wide range of traits in animals and plants. This plasticity is most often treated as adaptive, in the sense that it represents a mechanism by which organisms can adjust their phenotypes in a fashion that conveys greater fitness in the face of environmental heterogeneity and uncertainty (Via et al. 1995).

This classic framework was developed initially for animal husbandry and thus was often applied to particular sexes or life stages within a carefully managed breeding program and a controlled environment or set of environments. Wild populations present additional complexities in the form of further potentially important sources of trait variation. First, wild populations have more complex demographic structure associated with individuals like age and sex. Given that individuals of different ages and sex often have markedly different traits, this demographic variability can contribute substantially to overall trait variation and in principle the ecological effects of that variation (Ellner et al. 2011). Second, whereas husbandry programs often seek to precisely control for the environments experienced by parents and offspring to better isolate heritable effects of allelic variation, the environments experienced by various lineages can vary widely in nature. This variation in environments experienced by parents, or even grandparents, can be passed on to influence offspring phenotypes through an array of non-allelic transgenerational effects. Mechanisms of transgenerational information transmission range from simple sharing of local environmental conditions with parents (Galloway and Etterson 2007, Shama et al. 2014), to maternal effects (e.g., yolk provisioning) (Marsh-Matthews et al. 2005, McLeod et al. 2007), and epigenetic inheritance (e.g., DNA methylation) (Gapp et al. 2014, Ho 2014, Iwasaki and Paszkowski 2014).

Although mosquitofish have been studied extensively with respect to their ecological effects and evolution in response to predators and other environmental factors, little is known about the degree that mosquitofish respond plastically to predator cues, either within or among generations. Likewise, anecdotal observations and published field studies suggest a large amount of demographic variation among mosquitofish populations in attributes like sex ratios, and that these attributes might vary with predator regimes (Britton and Moser 1982). Hence, there is a clear need for some understanding of whether or how these additional processes might contribute to mosquitofish trait variation, particularly if the ultimate goal of employing these fishes is to understand the reciprocal interactions of trait change and ecological change over contemporary time scales.

Dissertation Approach and Chapters

In this dissertation, I used a combination of surveys, lab rearing experiments and mesocosms to better understand the causes and consequences of a complex suite of mechanisms underlying phenotypic responses of mosquitofish populations to predators. Variation in sex ratios is a potentially widespread demographic feature of prey fish populations where dimorphism between males and females can not only arise due to sexual selection but also reflect different ecological roles and vulnerabilities. In my first research chapter (Chapter 2), I consider intra-generational phenotypic plasticity of two species and both sexes of mosquitofish in response to non-lethal predator cues. In this rearing experiment, I assess plasticity in an exceptionally broad set of traits (size, shape and behavior) and ask whether patterns of plasticity are primarily parallel or unique for the different sexes and species. In my second research (Chapter 3), I use an experimental manipulation of one species of mosquitofish across three generations to evaluate possible transgenerational plasticity of bold-shy behavior associated with parental or grandparental exposure to predators. In my third data chapter (Chapter 4), I use the same multigenerational design to assess transgenerational plasticity of size and morphology in female mosquitofish. In my final research chapter (Chapter 5), I consider the ecological consequences of sex ratio variation in this sexually dimorphic group of fishes.

CHAPTER 2: PARALLEL AND UNIQUE PATTERNS OF PREDATOR-INDUCED PHENOTYPIC PLASTICITY IN TWO SPECES AND SEXES OF MOSQUITOFISH

Introduction

Environmentally-induced phenotypic plasticity is commonly viewed as an alternative to local adaptation, particularly in reference to mechanisms that produce phenotypic variation across a heterogeneous landscape (Bradshaw 1965, Via et al. 1995, Dybdahl and Kane 2005). The pattern of this plasticity is referred to as a reaction norm. This viewpoint however, is drastically over-simplified. Phenotypic plasticity can be considered an adaptation in its own right, particularly as a mechanism to accommodate spatially variable or changing environments (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 (West-Eberhard 2005, Ghalambor et al. 2007, Wund et al. 2008). Like other adaptations, one would predict that the degree to which adaptive plasticity is similar (parallelism of reactions norms) or unique for two species, or even two sexes, will be determined both by the extent that the species or sexes face similar patterns of selection across that gradient, including adaptive tradeoffs, and the degree that history (contingency) and shared or unique genetic architecture promote or constrain 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 two 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 depredation 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 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 tradeoffs in environments without predators (Palkovacs et al. 2011), placing a selective premium on an individual's capacity to produce the right phenotype under the right depredation risk conditions.

In fishes, 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 time spent foraging or seeking mates and safer but less profitable behaviors that afford concealment and reduce the odds of encountering predators. Traditionally, bold behaviors such as aggressiveness and dominance were thought to come at an increased risk of depredation relative to more reserved shy behaviors (Sih et al. 2004, 2012). However, some research indicates that boldness can afford its own benefits for reducing depredation risk, such as when bolder individuals are more vigilant of predators (O'Steen et al. 2002, Pascual and Senar 2014). As with size and shape, there is evidence that different predator environments lead to divergence in boldness.

Many fishes are able to adjust their effective body size in response to predation risk in one of two 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 depredation risk in different species or sexes, but also the degree there is similar size tradeoffs associated with processes like competition and reproduction that also influence fitness (Hjelm and Persson 2001, Farley et al. 2015).

Beyond the effects of size on predation risk, body shape in fishes can also influence the likelihood of prey escape when pursued by different types of predators (Borazjani 2013, Scharnweber et al. 2013). Studies of population divergence, or even plasticity, suggest that escape potential can be affected in two ways. Individuals with more streamlined and hydrodynamic body shapes experience less drag (Fu et al. 2013) and thus potentially able to move more efficiently and quickly through water when pursued by predators. Alternatively, or often additionally, individuals may develop body proportions favoring greater area of the caudal region and its important role in burst starts (Langerhans et al. 2004, Scharnweber et al. 2013) to avoid depredation from a lunging attack by a predator. Again, the relative degree of parallel or unique plasticity in fish body shape is expected to depend on the specific attributes of predators and potential tradeoffs between depredation risk and competition or reproduction.

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 one 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 two species or two sexes within a species. 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 behavior than are members of the same sex across species, raising the question of whether similar trait backgrounds or interbreeding and other interactions that occur with a population of males and females, are more prone to produce parallel plasticity across the various components of an integrated phenotype.

Objectives

Here I examine potential parallel and unique aspects of predator-induced phenotypic plasticity of two 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. I in turn consider whether the data are consistent with integration of parallelism at different scales (sex versus species) and what that might tell us about the relative roles of selection, contingency and constraint in the origins parallelism of plasticity in general.

Methods

Study species

Eastern and Western Mosquitofish widespread and often abundant species of North American poeciliid fishes. These species have very similar morphology, behavior, life histories, and niche and were considered a single species (or group of subspecies) until as recent as 1988 (Wooten et al. 1988). In fact, some authorities (e.g., Integrated Taxonomic Information System [ITIS]) continue to treat them as a single taxon (*G. affinis*), even though they are genetically and meristically distinct (reviewed in Pyke 2005). Both Eastern and Western Mosquitofish are sexually dimorphic with females larger than males, and males possessing gonopodia for internal fertilization.

Both of these mosquitofish species have 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 fish 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, these fish 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, mosquitofish are also common prey for piscivorous fish and aquatic birds (Britton and Moser 1982, Meffe and Snelson 1989) and evidence suggests that predators 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*) and their congeners. 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 has also been correlated to boldness and sociability (Cote et al. 2011). Moreover, where the ranges of *G. holbrooki* and *G. affinis* overlap, *G. holbrooki* commonly displaces *G. affinis* (Walters and Freeman 2000).

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). The pond is a protected freshwater site with long-standing populations of eastern mosquitofish and centrarchid predators. *Gambusia affinis* were collected with the aid of the Contra Costa Mosquito and Vector Control District in Concord, CA (37.93°N, 121.95°W). The source pond is a facility protected site with an open system to aquatic and aerial predation.

All wild brood fish were quarantined and captive reared in a single lab for one year in the absence of any predator cues. Brood fish were thus extensively acclimated to shared laboratory conditions before being used to produce offspring. Offspring of wild fish (F1) were bred to produce the laboratory stock used for experimentation (F2). Mosquitofish do not have superfetation (Turner 1937), nor are they likely to store sperm for more than a few weeks

(Constanz 2012), thus all offspring used for experiments were the product of laboratory matings. 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 untreated deionized water. Fish were fed ad libidum 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 three replicate 295 liter 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 (Figure 2.1a). Each breeding tank employed ten to twelve adults of one 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.

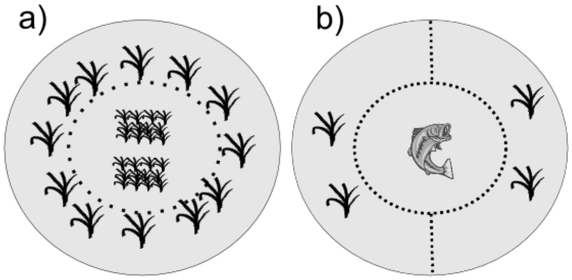

Figure 2.1: Experimental design pools for breeding and exposure treatments. Pool a) is the breeding system with an inner ring of mesh acting as fry refugia. Pool b) is the exposure treatment, with the inner ring of mesh serving as the bass enclosure (for exposed groups). Mesh lines separating the outer ring delineates the two populations, *G. holbrooki* and *G. affinis.*

The group of fry from a given parturition event were equally and randomly allocated to rearing treatments with or without predator cues. All of the exposure pools included a central chamber constructed of 1 mm polyurethane coasted 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 two halves, into which individuals of the two species were respectively introduced (Figure 2.1b). Hence, both species and sexes experienced identical exposure conditions within each treatment. Both the predator and non-predator pools (each subdivided for two species) were replicated three times to further account for any potential pool effects. The predator used in our exposure pools was a live Largemouth Bass fed live mosquitofish 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 prey depredation itself (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 exposure treatments were assayed for risk taking and foraging behaviors after a 72 hour fasting period. Assays were performed in a risk-reward arena that contained a Largemouth Bass enclosed behind 5 mm polyurethane coated diamond mesh at one end to provide visual and chemical cues of predation risk and a shoaling group of three to five conspecifics enclosed behind similar mesh at the opposite end. The central region of the arena was subdivided into a 2x5 grid to quantify movements within the tank (Figure 2.2). The “safe zone” was represented by the space closest to the shoal group before the feeding grid. Conversely, the “danger zone” was the represented by the final row on the grid closest to the predator enclosure. A food reward was used to encourage exploration in the arena. The food reward consisted of freeze-dried krill or spirulina flakes distributed randomly into one or the other side of each pair of grid squares (Figure 2.2). Experimental fish were individually introduced into an acclimation tube at the safe end of the arena for two minutes before the tube was lifted and the assay began. This arena design allowed us to simultaneously score both time spent in risky (predator) versus safe (conspecific shoal) locations, as well as time spent conducting shy but energetically unrewarding (e.g., holding at shoal end of arena) versus bold but potentially rewarding exploration behaviors. Behaviors were continuously recorded using the JWatcher software (ver. 1.0, Macquarie University and UCLA) for ten minutes and included fish activities (swimming or feeding), location (closeness to shoal group), and the time taken to first exit the release zone to explore. Behaviors were analyzed with a multivariate analysis of variance (MANOVA) on a linear mixed effects (LME) model with replicate as a random variable and exposure 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 with a Bonferonni correction.

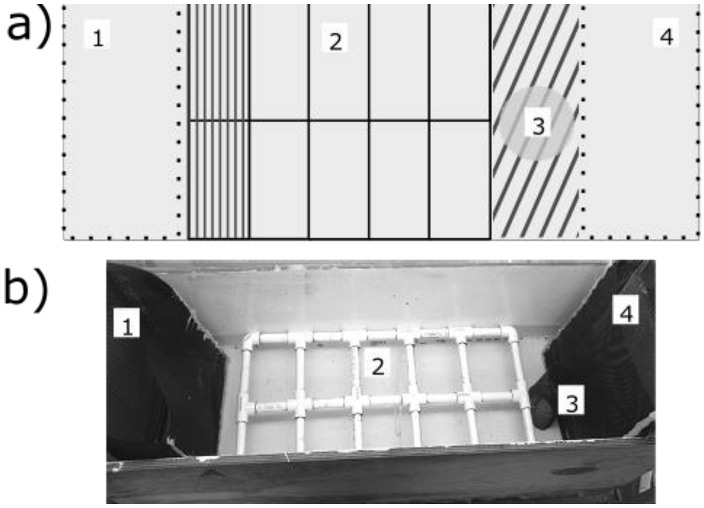

Figure 2.2: Behavioral assay experimental design. Panel a) is a representative diagram of panel b) the actual set-up. Area 1) is the Largemouth Bass enclosure, Area 2) is the risk assessment grid, Area 3) is the acclimation tube, and Area 4) is the conspecific shoaling group. The “danger zone” is represented by the vertical lined area. The “safe zone” is represented by the diagonal lined area.

Body Size

After behavioral assays, individuals were euthanized with a lethal dose of MS-222 (>250 mgL^{-1}), weighed, and photographed against a grid background with a ruler for scale. Individuals were measured in Image J (ver. 1.6.0_20, Rasband and NIH) for total length (tip of snout to tip of caudal fin), and body depth (vertical measure of the deepest portion of the body). These different indexes were statistically compared in the same manner as behavior.

Body Shape

Data were collected for fifteen fixed landmarks (Figure 2.3, 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 collected on photographs of sample fish 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 and species combination in separate geometric morphometric analyses. Relative warps were obtained from a principal component analysis (PCA) of thin-plate spline shape variation using tpsRelw (ver. 1.49, Rohlf and SUNY). Given these species are known to be strongly sexually dimorphic in size and shape, a pattern we confirmed, we also computed and compared discriminant function scores for each sex separately. A MANOVA and discriminant function analysis (DFA) were performed on the relative warps using predator exposure treatment as the primary (discriminating) factor. 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).

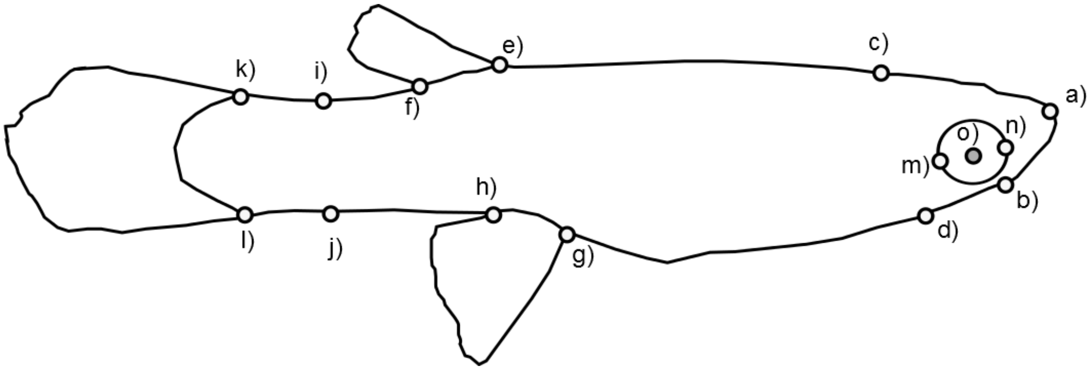

Figure 2.3: Fish outline with fifteen morphometric landmarks. a)-d) are cranial markers, e)-h) and k)-l) are fin positions, i)-j) identify the caudal peduncle, and m)-0) are eye markers. Full descriptions are landmarks positions may be found in Appendix A.

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 non-parallel (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 a parallel norm of reaction. All statistical analyses were performed using the R Programming Environment (Ver. 3.1.1, R Core Team 2014), 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 behavior of mosquitofish (Table 2.1). Importantly, there was a significant interaction effect between species and exposure treatment group (Table 2.1), but no interaction effect for sex and exposure ($F_{1,214}$=0.54, p=0.49) and no fixed effect for sex (Table 2.1) indicating a unique plastic response by the two species but a parallel response among the sexes. Hence, the following description of species effects apply in common to males and females of both species. Considered separately within the LME framework, all three behavior categories were affected by this species-by-exposure regime interaction (lag time: $F_{1,216}$=8.10, p<0.01, percent time exploring: $F_{1,216}$=7.51, p=0.02, and percent time foraging: $F_{1,216}$=9.14, p<0.01). *G. 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 both were reared in the absence of predator cues. However, *G. affinis* greatly increased this lag time when reared in the presence of the predator, particularly compared to *G. holbrooki* (t_{95}=4.51, p<0.001) that responded with opposite behavior and exhibited a slightly reduced lag time (Figure 2.4a). 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, *G. holbrooki* and *G. affinis* that had been previously exposed to predator cues showed significantly 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 2.4b).

Table 2.1: Reduced behavior model expressing the effect of sex, species, and exposure regime. Behavior parameters include lag time and percent exploration.

Population Characteristic	Std Error	DF	F-value	P-Value	Sig.
Sex	1.08	1,216	0.93	0.335	
Species	3.39	1,216	12.81	<0.01	**
Exposure	3.35	1,216	2.46	0.119	
Species:Exposure Interaction	2.17	1,216	8.10	<0.01	**

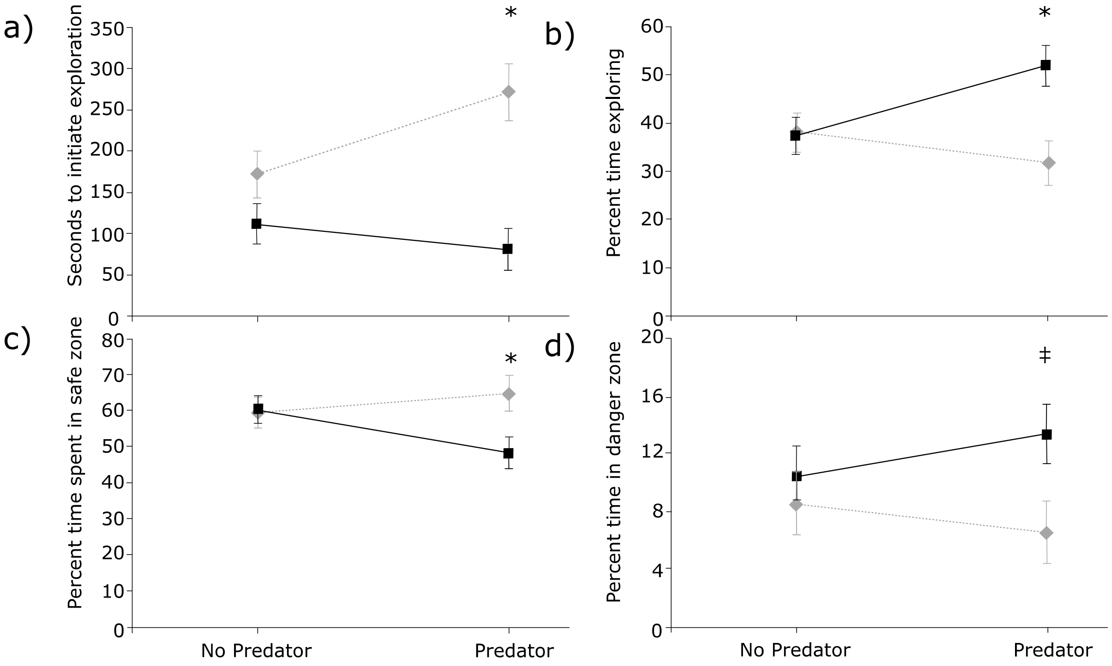

Figure 2.4: Behavioral responses for mosquitofish species. Species partitioned by *G. affinis* (grey diamonds and dashed line) and *G. holbrooki* (black squares and solid line). Panel a) is the lag time for a fish to leave the safe zone the first time. Panel b) is the percent time a fish spent outside of the safe zone swimming. Panel c) is the percent time a fish spent near the shoal group (safe end). Panel d) is the percent time a fish spent near the bass enclosure (risky end). Asterisks (*) represent significant differences with unique interactions (species by predation). Double crosses (ǂ) represents significant effect of species. All error bars are ±1 standard error.

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 but not with sex (Table 2.2) or between sex and exposure ($F_{1,214}$=0.001, p=0.97). Conversely, time in the "danger" zone showed no interaction and was driven by species alone (Table 2.2). This again indicates a unique aspect to plasticity of the two species. While percent time spent in the "safe" zone was similar for the two species in the absence of predator exposure during development (t_{114}=-0.41, p=0.68), exposed *G. affinis* spent a significantly higher percent of time in the "safe" zone when compared to exposed *G. holbrooki* (t_{100}=2.42, p=0.01) (Figure 2.4c). Although we did not detect a significant species-by-exposure interaction for time in the "danger zone" ($F_{1,217}$=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 (Table 2.2), 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 2.4d). Indeed, unlike the LME, separate t-tests at each exposure treatment indicate that whereas non-exposed 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).

Table 2.2: Reduced models for population characteristics' effect on risk-taking behavior. Behaviors include percent time spent in the "safe" zone (nearest the shoal group) and "danger" zone (nearest to the predator). All two-way interactions were included. Interactions were removed if p<0.05.

	Safe Zone					**Danger Zone**				
Population Characteristic	**df**	**F-value**	**p-value**	**Sig.**	**Effect Size**	**df**	**F-value**	**p-value**	**Sig.**	**Effect Size**
Sex	1,216	2.72	0.100			1,217	0.00	0.97		
Species	1,216	1.49	0.224			1,217	4.78	0.03	*	4.80
Exposure	1,216	0.21	0.647			1,217	0.07	0.79		
Species:Exposure Interaction	1,216	4.06	0.045	*	17.07					

Body Size

The multivariate length, body depth, and weight model showed no significant interactions between sex, species, or exposure groups on body size metrics, as well as no separate effect of predator exposure (Table 2.3), indicating that size was not phenotypically plastic. The subsequent reduced model revealed significant effects of both sex and species. The effect size of sex was double that for species or exposure, confirming the substantial sexual dimorphism in these fishes (Table 2.3). 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 assessment of the species effect 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 weight (t_{221}=-1.30, p=0.20) (Figure 2.5a-c). In general males of the two species differed more in size than females.

Table 2.3: Reduced model expressing the effect of sex, species, and exposure on fish body size. Size metrics are total length, body depth, and weight.

Population Characteristic	**Std Error**	**DF**	**F-value**	**p-value**	**Sig.**	**Effect Size**
Sex	0.024	1,217	39.29	<0.001	***	0.16
Species	0.025	1,217	9.81	<0.01	**	0.08
Exposure	0.025	1,217	1.51	0.22		

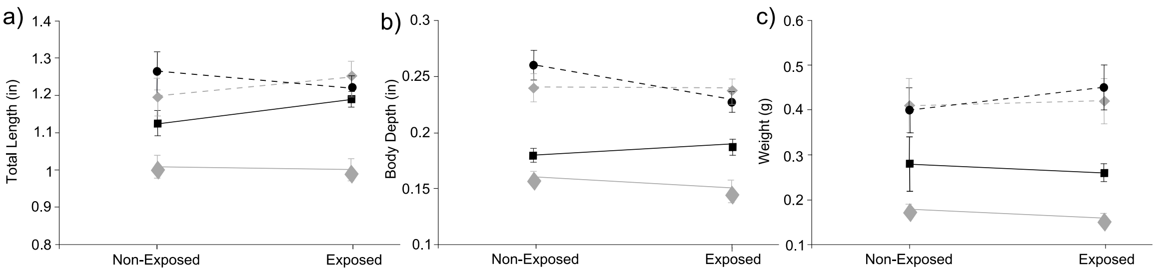

Figure 2.5: Size metrics for mosquitofish groups based on species, sex, and exposure. G. affinis are (grey lines) and G. holbrooki are (black lines). Species are partitioned into males (solid lines) and females (dashed lines). Panel a) is snout to tail length, b) is the deepest part of the belly, and c) is weight. Error bars are all ±1 standard error.

Body Shape

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 (η^2=0.23) but not species, with no interaction between the two (Table 2.4). Male body shape differed by species and changed with predator exposure, but still with a parallel reaction norm (Table 2.4). Indeed, exposure effects among male fishes were twice as strong as species differences (exposure: η2=0.19, species: η2=0.10).

Table 2.4: MANOVA analysis on linear models for body shape DFA scores. Models are subset by sex to control for overt sexual dimorphism.

	Females				**Males**			
Population Characteristic	**MS**	**F-value**	**p-value**	**Sig.**	**MS**	**F-value**	**p-value**	**Sig.**
Species	2.48	2.56	0.11		16.5	18.2	<0.001	***
Exposure	33.8	34.9	<0.001	***	22.5	24.9	<0.001	***

Within each sex and species, discriminant function scores based on relative warps differed significantly by exposure treatment, with treatment accounting for 33% to 46% of variation in the DFA scores (Table 2.5). Regression of DFA scores back on relative warps to depict associated deformations indicate that predator-exposed individuals of both sexes and species tended to show more slender and streamlined bodies, a trait that is most exaggerated by the relative ventral depth of the anterior anal fin insertion point (Figure 2.6). The cranium is also streamlined, with 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

Table 2.5: Simplified models testing the difference between relative warp DFA scores for exposed and non-exposed individuals within sex and species subgroups.

Population Group	MS	DF	F value	P-Value	Sig.	Proportion Variance
Female *G. affinis*	54.4	1,65	54.4	<0.001	***	0.46
Female *G. holbrooki*	38.8	1,49	38.8	<0.001	***	0.44
Male *G. affinis*	38.0	1,53	38.0	<0.001	***	0.42
Male *G. holbrooki*	25.7	1,52	25.7	<0.001	***	0.33

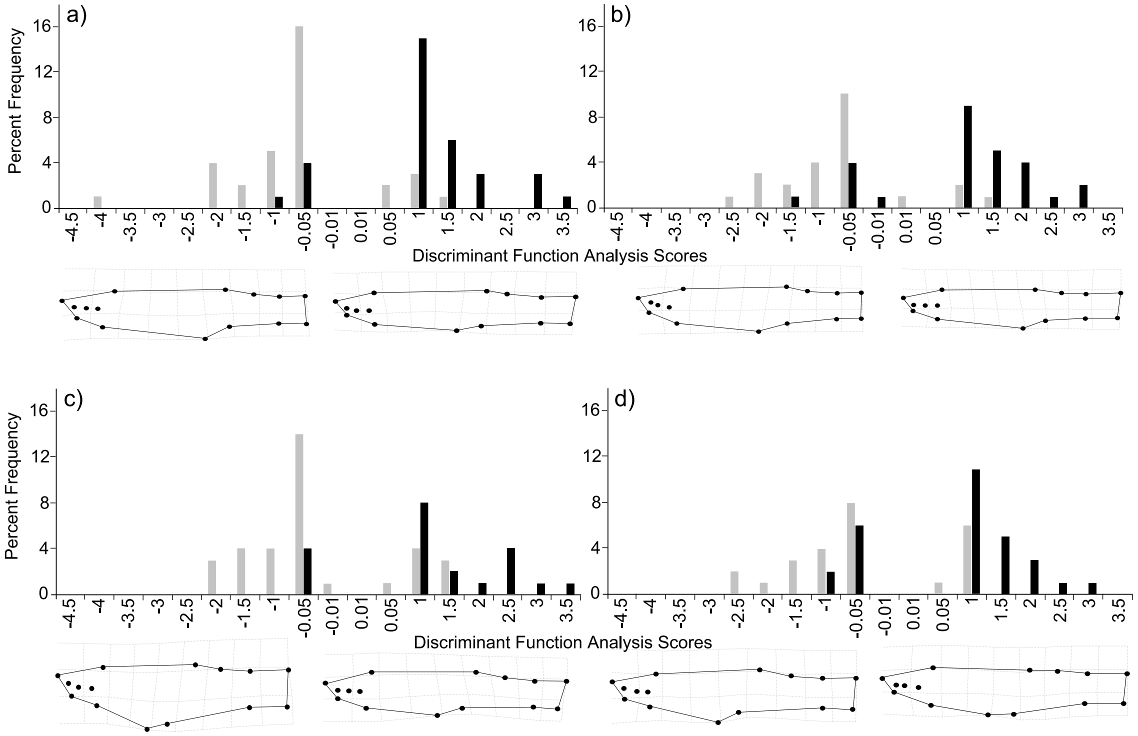

Figure 2.6: Frequency distributions of a series of discriminant function analyses on the 26 relative warps. Below are the appropriate morphological divergence visualizations. Female mosquitofish are represented in a) *G. affinis* and b) *G. holbrooki*. Male mosquitofish are represented in c) *G. affinis* and d) *G. holbrooki*. Grey bars are non-exposure populations and black bars are the exposed populations.

from non-exposure treatments. Caudal regions in predator-exposed fish was 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 showed evidence of predator exposure effects in the region of their anal fins not seen in females (Figure 2.6). Exposure ($_{F1,108}$=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 non-exposed males (Figure 2.7). 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 trended 16% more flat in exposed populations compared to non-exposed individuals (Figure 2.7). Given that exposure groups did not differ in any body size measurements (see above), these shape differences are not associated with differences in body size and analyses of DFA scores revealed no significant correlations with size measures.

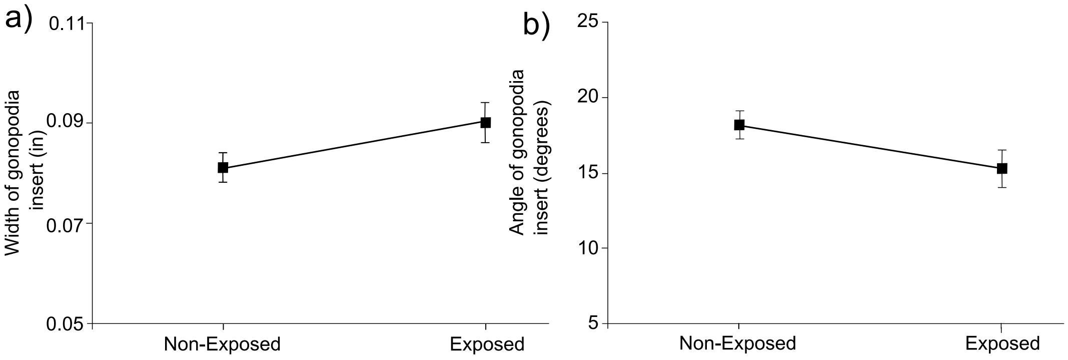

Figure 2.7: Male gonopodia fin insertion metrics among predator-exposed groups. a) The width of the insertion points and b) the angle depth for the insertion in the dorsal direction. All error bars are ±1 standard error.

Discussion

Our study clearly demonstrates that mosquitofishes show marked phenotypic plasticity in response to predator and depredation 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 of the two species becoming bolder after rearing under a predator cue. Body shape 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. We now discuss these patterns of plasticity with regards to what they might tell us about conditions that favor relatively parallel or unique plasticity within the overall integrated phenotypic responses of prey to presence or absence of predators.

Parallel and Unique Plasticity of Behavior

Exposure to predation can elicit bold behaviors in fish (Bell and Sih 2007), so it seems reasonable that such exposure can also plastically exaggerate bold tendency, as seems to be the case for the non-parallel behavioral plasticity of *G. holbrooki* and *G. affinis*. The two 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 shyer, albeit to a lesser degree. Indeed, *G. holbrooki* increased seemingly risky behaviors by 20-29% under predator exposure whereas *G. affinis* reduced such behaviors by 8-37%. Prior studies 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 under alternating food conditions (Thomson et al. 2012). Indeed, our findings support this pattern for the plastic responses of *G. holbrooki* and *G. affinis*, 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. First, both species evolved with fish predators and so it is unlikely that one or the other would express maladaptive plasticity by chance alone. Indeed, it might not be accurate to associate these bolder behaviors with increased risk of depredation. Such bold behaviors may actually help manage risk for those already prone to bolder behavior overall. Studies of boldness in fishes, including poeciliids, often suggest that shorter lag times, greater exploration, and approaching predators are associated with predator vigilance and ability to more efficiently gauge risk (O'Steen et al. 2002). Indeed, 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). A similar line of reasoning might thus explain why two species with somewhat different innate tendencies toward boldness or shyness might evolve plasticity to predators that exaggerates those tendencies with adaptive peaks constrained within the overall behavioral syndrome (Sih et al. 2004; Briffa et al. 2008).

In contrast to comparison of the two species, the two 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 fishes, the larger sex can experience increased depredation (Britton and Moser 1982) and present shyer traits (Wilson et al. 2010; Harris et al. 2010) than the smaller sex. That said, males and females may be somewhat locked together in their plastic responses to predators through both their social and genetic associations. Socially, mature males almost continuously pursue females and because of this the attention of predators might depend on the joint behavior of interacting males and females. Even so, males and females of many species show different reaction norms to similar environmental cues (Øverli et al. 2006; Rodríguez-Prieto et al. 2011), suggesting that this is not a universal constraint on parallelism.

Lack of Plasticity of Size

It would seem intuitive that size might influence relative risk of predation, but 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 might be offset if predators target the larger prey for their greater energetic value (Brooks 1968; Britton and Moser 1982). Similarly, nonlethal predation cues often drive responses of reduced body size (Fraser et al. 2004; Bell et al. 2011). While some prey may be able to grow large enough to surpass a certain predator's gape limitation (Cowan et al. 1996), if the predator has no functional gape limitation for that prey (reviewed in Sogard 1997) or if the prey are small enough that the cost of capture and consumption varies little with size (Gill and Hart 1994), larger size may only make an individual a more attractive target. Mosquitofish are exceedingly small relative to their predators and the largest mosquitofish in most populations are unlikely to ever exceed the gape limitations of bass.

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), even if smaller individuals oftentimes show bolder behaviors (Wilson et al. 2010; Harris et al. 2010). Hence, smaller body types are common in prey populations facing predation risk (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 limitations on the opportunity for plasticity favoring even smaller size and earlier maturation in response to predator cues. That said studies have shown that mosquitofish and other poeciliid populations can evolve population differences in life histories in association with predators (Reznick and Endler 1982) or environmental predictability (Stearns 1989), so this constraint does not seem likely for plasticity.

Parallel Plasticity of Shape

Parallelism in divergence or plasticity might be predicted to be greatest where populations or species with similar genetic and phenotypic makeup are subjected to ecologically comparable gradients. The two 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). Hence, it is not unexpected that we found a strong degree of parallelism of shape plasticity in these two species, much the way that prior studies have provided evidence for a significant degree of parallelism in trait divergence (as opposed to plasticity) across predation regimes in several poeciliid fishes (Langerhans and DeWitt 2004; Scharnweber et al. 2013). We also found evidence for parallel patterns of plasticity across sexes. Our analyses clearly substantiate this sexual dimorphism. But aside from some differences associated with male reproductive anatomy, discussed below, gross morphological plasticity associated with streamlining and cranial proportions were markedly similar for the two sexes.

Interestingly, the broad parallelism of morphologically plasticity for the sexes and two species in this study may have relevance for parallelism at an even broader scale. Specifically, plastic morphological responses to presence or absence of predator cues in this study appear remarkably analogous to patterns of putatively adaptive morphological divergence among wild populations of mosquitofish and other species that live with or without fish predators. Multiple prey fish studies support that a more slender body shape, a longitudinal expansion of the caudal regions and a shift in eye position are geographically associated with presence of piscivorous fish predators (Langerhans and DeWitt 2004; González and Gianoli 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). These population differences in body shape in prior studies show substantial parallelism within and among poeciliid species and sexes, and have been broadly interpreted as adaptive evolutionary divergence (Langerhans et al. 2004; Hendry et al. 2006; Langerhans and Gifford 2009), although that has been contested (Downhower et al. 2000; Downhower et al. 2009; Langerhans and Gifford 2009). Morphological plasticity of females in the present study showed particularly similar predator-associated patterns of overall streamlining and dorsal-side longitudinal expansion of the caudal peduncle to patterns in divergent wild populations of G. affinis (Langerhans et al. 2004)(Figure 2.8). Males showed a broadly similar, but weaker, extent of morphological change (Figure 2.8). This similarity of plasticity pattern with that identified for adaptive population divergence could come about two ways that have implications for past and future studies.

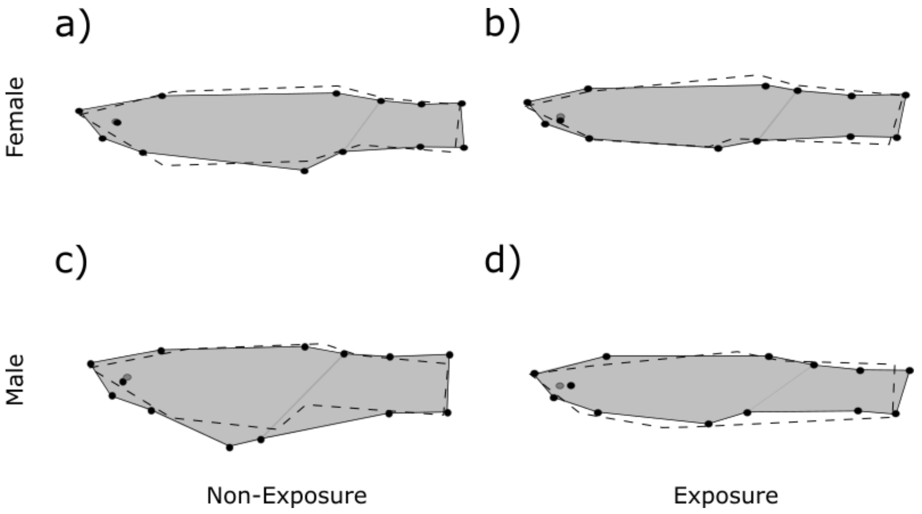

Figure 2.8: Superimposition of morphological deformations due to plasticity (this study) and due to population divergence (Langerhans et al. 2004). All panels are of *G. affinis*. Thin plate spine images of plasticity from the present study are exaggerated by 4 units (shaded, black points). Images are paired with corresponding sex and predator exposure outlines from Langerhans et al. (2004) with 2 unit exaggeration (dashed, grey points). Different point sets from the two studies contribute to some differences in shape resolution, but overall deformation patterns are broadly analogous.

First, it is possible that previous work examining population divergence did not adequately control for plasticity when examining fish collected in the wild or even under common garden rearing. Although a degree of captive rearing has been employed in a few prior studies to reduce potential environmental effects on trait variation (Stockwell and Weeks 1999; Langerhans et al. 2004), others have not (Hendry et al. 2006; Berner et al. 2010; Ingley et al. 2014). Even for studies employing a degree of captive rearing, environmental cues experienced in early life of fishes may persist for extended periods in captivity and mosquitofish are live bearers that may be particularly prone to pass cues on to developing offspring (maternal effects). Conversely, our experimental design precludes the possibility that we would have confused adaptive divergence for plasticity. However, we did not examine multiple populations from different predation conditions and wish to make clear that proof of plasticity in our study is not proof that adaptive divergence does not also contribute substantially to previously observed patterns in nature. Indeed, the second explanation for similarity of divergence and plasticity is the two might be theoretically predicted to be similar and serve complimentary adaptive roles. 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). Simply stated, plasticity and adaptation are not mutually exclusive explanations for geographic patterns of parallelism.

Although sexual parallelism of plasticity predominated, it was not complete. The primary exception in this case, however, was a trait strongly associated with the different reproductive anatomy of males and females. Anal 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. This suggests potential plasticity in male reproductive anatomy in response to predation risk. 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. Pre-copulatory behavior in males often involves “thrusting” the gonopodia in a display for the female from a “tucked” position (Peden 1972). An increased angle would increase the visual effect from “tucked” to “thrusted.” However, presence of a predator reduces pre-copulatory displays, increasing sneak matings (Godin 1995), and reducing female choosiness (Kelly and Godin 2001). The expansion of width in the gonopodia may in part compensate for shortening of the gonopodia, as smaller gonopodia are less likely to succeed in mating and are less effective in sperm competition (Langerhans et al. 2005; Heinen-Kay and Langerhans 2013). Recently, Anderson and Langerhans (2015) suggested females show divergence patterns in genital aperture that compliments male gonopodial divergence, but our study was not designed to measure possible plasticity in this reproductive trait. Overall, 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

Phenotypic plasticity is presumed to serve, at least in part, as an adaptation to the challenges of a heterogeneous landscape, including both the temporal and spatial components of environmental variation (Via et al. 1995). The prevalence of phenotypic plasticity in key adaptive traits, as found in this study, is consistent with an important role in organism performance and fitness. Plasticity may be especially important to colonization capacity (Thibert-Plante and Hendry 2011; Torres-Dowdal et al. 2012) and contribute to the invasiveness of species such as mosquitofish. Given its important adaptive role, we should thus expect plasticity, like other adaptive traits often show parallel, or even convergent expression across repeated adaptive gradients experienced by both sexes and ecologically similar species. Parallel evolution of plasticity is perhaps particularly likely under many of the conditions of the current study, involving the two sexes within a species and two closely-related species, all of which face a very similar ecological gradient of predation in nature and share similar genetic and phenotypic backgrounds. In the current study, parallelism of plasticity occurred for nearly all plastic traits of the two sexes, with the possible exception of actual reproductive anatomy. In contrast, the two species showed much less integrated parallelism, wherein they showed parallel patterns of morphological plasticity but non-parallel patterns of behavioral plasticity. These findings thus indicate the combined effects of shared selection and constraints impeding divergent patterns of plasticity are a potentially more potent pairing for producing parallelism of plasticity than the combination of shared selection and contingency. However, we only considered one species pairing and the two sexes in our study, and while our approach is unique in combining both multiple traits and group contrasts, the generality of our inference must await additional future studies that adopt such broadly comparative approaches.

Of course, the other comparative question that can be asked within a dataset such as ours concerns potential differences among trait types. In our study, morphological plasticity was more conserved than behavioral plasticity, showing parallel patterns for both sexes and both species when behavioral plasticity only showed parallelism for the sexes. There may be theoretical reasons to expect such a pattern for many fishes based on the relative roles that morphology and behavior play in mediating predator-prey outcomes and how that influences opportunities for alternative adaptive outcomes of plasticity. The morphological attributes associated with environments where predators so vastly outsize their prey are strongly associated with the ability to escape the predator attack with changes in speed and direction that commonly approach the physical and hydrodynamic limitations of fishes (Bainbridge 1957; Domenici and Blake 1997). This implies strong adaptive constraints and little opportunity for alternative body conformations that would similarly aid escape. As such both adaptive divergence and plasticity would be expected to follow very similar patterns within and among a wide diversity of fishes (Baylis 1983; Burns et al. 2009; Fu et al. 2013; Scharnweber et al. 2013).

In contrast, behavioral attributes associated with predation environments involve mechanisms to reduce the initial risk of eliciting a potential predator attack in the first place which might more variable and contingent on the particular predator-prey pairing and environment (Martin et al. 2010; Pascual and Senar 2014). These mechanisms could involve either shy behaviors that reduce odds of encounter or detection, or bold behaviors that improve surveillance or signal awareness to predators (Bergstrom and Lachmann 2001; Sinn et al. 2008; Smith et al. 2009; Cooper 2010). Hence, behavioral antipredator traits and plasticity might be hypothesized to vary more widely based on the innate behavioral tendencies and sensory capabilities of the specific prey and predators (Fuiman and Magurran 1994; Brown et al. 2006), as well as the physical and sensory environments in which they interact (Olla et al. 1998; Domenici et al. 2007).

Again, support for the hypothesis that morphological plasticity to predators is more prone to parallelism than behavioral plasticity will of course ultimately depend on evidence from a larger pool of studies that incorporate more species and contexts. Certainly, most trait shifts in shape and behavior investigated in our study would be relevant to other fishes (Langerhans et al. 2004; Hendry et al. 2006; Dingemanse et al. 2009; Scharnweber et al. 2013) or even larval amphibians (Dayton et al. 2005; Kishida et al. 2009). Beyond aquatic systems, many behavioral traits such as vigilance and alarm calling are pervasive across taxa (Fichtel and Kappeler 2002; O'Steen et al. 2002; Brown 2003; Morosinotto et al. 2013), and escape performance in terrestrial systems is probably equally constrained against the biophysical limitations of predators and prey (Jones 1990, reviewed in Emerson et al. 1994, Lenz and Marajó dos Reis 2011). Hence, our study could serve as a model for others in showing the value of a comparative approach to studying plasticity that considers the degree of parallel and unique patterns across multiple modes of plasticity in the same groups of individuals.

CHAPTER 3: TRANSGENERATIONAL EFFECTS OF PREDATOR CUES ON RISK BEHAVIORS OF EASTERN MOSQUITOFISH (*Gambusia holbrooki*)

Introduction

Different environmental conditions or cues often induce differential expression of phenotypic traits. This phenotypic plasticity is common and is usually an adaptation to spatially and temporally heterogeneous habitats (Via et al. 1995, Alpert and Simms 2002, Baythavong 2011). Most research on environmentally-induced trait plasticity has focused on how phenotypes respond to environments experienced directly by those individuals, within a single generation. However, a growing body of literature suggests that phenotypes of individuals can also be influenced by the environments experienced by their parent, or even grandparents, through non-allelic transmission processes in a form of transgenerational plasticity (Jablonka et al. 1992, Agrawal et al. 1999, Jablonka and Raz 2009, Salinas and Munch 2012, Shama et al. 2014). Like environmental plasticity within generations, transgenerational plasticity is thought to be a potential adaptation providing offspring with better phenotypic match to local environmental conditions (Mondor et al. 2005, Storm and Lima 2010, Giesing et al. 2011). Although transgenerational plasticity might at times represent an alternative to intra-generational plasticity, both types of plasticity could often interact in determining phenotypes (Sultan et al. 2009, Salinas and Munch 2012). However, while many studies have demonstrated plastic effects within and among a single generation, comparatively few have assessed the strength and nature of their interactions across multiple generations. In this study I consider the multigenerational effects of non-lethal predator exposure on the exploratory behavior of both sexes of a live-bearing fish.

Transgenerational plasticity subsumes a complex suite of multigenerational, non-allelic inheritance of past environmental effects on trait expression. The earliest work on transgenerational environmental effects surrounded detection of maternal effects, and to a lesser extent non-genetic paternal effects, within quantitative genetic analyses (Yamanaka 1928, Haldane 1932, Park 1935). These parental effects, which can represent indirect genetic effects of either parent's genotype or indirect effects of the parent's environment, were often treated as a statistical nuisance that could bias estimations of trait heritability and genetic correlations. Maternal effects are often seen as particularly weighty because of the large role of mothers in provisioning eggs, embryos, and post-parturition offspring (Rossiter et al. 1993, Reznick et al. 1996b, Storm and Lima 2010, Bestion et al. 2014). More recent work has emphasized the likely adaptive significance of such parental effects, and maternal effects in particular (Mousseau and Fox 1998a, 1998b, Giesing et al. 2011, Bestion et al. 2014). For this study I am primarily concerned with non-allelic carry-over effects of a parent's environment, as opposed to indirect genetic effects of parents, because the former reflects true transgenerational plasticity in the form of cross-generation norms of reaction.

In more recent years there has been increasing attention to transgenerational effects that span even more generations, including studies providing evidence that phenotypes of a wide variety of species can be influenced by the environments of not just their parents, but of grandparents (Reznick 1981, Yazbek et al. 2010, Hafer et al. 2011, Herman et al. 2012). As with maternal and paternal effects, any such grandparental effects are attributed to various forms of epigenetic inheritance that modify the expression of DNA across multiple generations without changing its allelic sequence structure (Rando and Verstrepen 2007, Jablonka and Raz 2009). However, epigenetic inheritance is not the only mechanism that can produce transgenerational plasticity. For example, serial effects of maternal provisioning could permit grandmothers experiencing exceptionally good or bad environments to produce exceptionally high or low quality daughters that are themselves at a lifetime advantage or disadvantage for producing similar quality offspring (Mitchell and Read 2005, Alonso-Alvarez et al. 2007, reviewed in Schmidt 2013). Regardless of the mechanism, however, these multigenerational effects again reflect a form of phenotypic plasticity as captured in cross-generational norms of reaction relating the environments experienced in a given generation to phenotypes expressed one, two, or even more generations later. One would predict that the reliability of environmental cues should become less and less with more generations and so grandparental plasticity effects might be generally weaker than parental or intra-generational plasticity, but that prediction has not been widely tested.

Prey responses to predator cues represent a particularly compelling form of plasticity to examine for intra-generational and transgenerational effects because predators have large effects on prey fitness (Abrams 2000, Peacor et al. 2013), are often patchy in space and time (Bernstein et al. 1988), and provide robust cues that prey can readily detect (McIntosh and Peckarsky 2004, Lehtiniemi 2005). Phenotypically plastic responses to predators are very common in nature (Trussell et al. 2003, Relyea and Auld 2005, Peacor et al. 2012) and respond to a wide variety of nonlethal predator-related cues, including depredation attempts (Hammill et al. 2010), predator chemical and visual cues (Sih et al. 2011, Brossman et al. 2014), and visual, auditory, or chemical alarm cues from conspecifics (Suboski et al. 1990, Brossman et al. 2014). One of the most overt responses of prey to cues of depredation risk are adjustments to behavior, such as might occur through environmental modulation of correlated personality traits associated with stereotypically bold or shy personality attributes within a broader behavioral syndrome (Sih et al. 2004, Bell and Sih 2007). For example, prey species experiencing predator cues might seek out relatively safer habitats that afford less risk of encountering predators or improve the odds of escaping attacks (Sih et al. 2012). Prey could also adjust their activity types and levels to avoid drawing predator attention (Smith et al. 2009), improve their surveillance of predator presence (Pitcher et al. 1988, Pellegrini et al. 2009), or prioritize short-term fitness gains over longer-term benefits (Eggers 1978, Scrimgeour and Culp 1994). Although predator cues might elicit novel behaviors or loss of behaviors, the most common responses to predators may revolve around allocations to bold or shy behaviors within an overall time budget, with a trade-off between relatively safe refuge and shoaling or group behaviors, versus riskier behaviors such as foraging or courtship (Johnson and Basolo 2003, Sih et al. 2003, Chalfoun and Martin 2010). That said, not all seemingly bold behaviors are associated with greater risk of depredation. For example, some prey exhibit seemingly bold predator inspection or alertness signals that draw predator attention but nonetheless reduce risk of predator attack (Fichtel and Kappeler 2002, O'Steen et al. 2002, Pellegrini et al. 2009, Wisenden et al. 2011, Pascual and Senar 2014). Hence, whether seemingly bold or shy behaviors are adaptive in the face of predators could be highly context specific.

While most behavioral responses to predator cues have been studied within the context of intra-generational responses, anti-predator behaviors have become an important area of study for transgenerational effects. Offspring from predator stressed mothers tend to exhibit greater risk avoidance behaviors, such as increased predator sensitivity (Storm and Lima 2010), increased socializing or grouping (Giesing et al. 2011), or increased dispersal (Mondor et al. 2005, Bestion et al. 2014). This is sometimes referred to as "pre-adaptation" (Dyer et al. 2010) and can also make offspring more competitive and invasive, even in light of these biological stressors. Indeed, in some cases the maternal environment is the most important factor in determining the phenotype or success of the offspring (Mousseau and Fox 1998b). However, aside from a limited number of studies using clonally reproducing species (e.g. Walsh et al.

2014), most studies of predator exposure effects in vertebrates have been limited to single-generation parental effects, and it is unclear how much predator exposure effects can link the phenotypes of individuals to the predator environments of their grandparents.

A further downside of most multigenerational plasticity studies relying on clonal species or life stages is that they obviate any ability to assess whether males and females show the same plastic responses to predator cues within or among generations. Males and females within species often show markedly different behavioral tendencies towards boldness or shyness and allocate their time budgets quite differently, irrespective of predators, likely as an expression of their differing energetic (Santos et al. 2007), reproductive (Genner et al. 2008, Pauers and McKinnon 2012), and social (Dadda et al. 2005, Hunt et al. 2009) requirements. In these cases, males often invest large portions of their time into behaviors associated with attracting or competing for more mates (Bisazza et al. 2001, Dadda et al. 2005), whereas females invest more time in feeding and growth to appreciate higher fecundity (Magurran and Seghers 1994, Fryxell et al. 2015). Males often possess more elaborate colors or display structures (Ruell et al. 2013) and spend more time out of cover in exploration (King et al. 2013). Indeed in some cases sex has been shown to be one of the strongest driving factors in stress response to predation (Mommer and Bell 2013). These types of differences in reproductive strategies may incur different tradeoffs with respect to predation risk and thus favor different types or magnitudes of transgenerational plastic responses to predator cues.

Hence, whereas most vertebrate transgenerational plasticity studies are performed over a single generation and use both sexes as a single population type unless sex is used to trace a specific epigenetic (cellular) mechanism (e.g. mitochondrial epigenetics, neuroendocrinology), I sought to examine intra-generational and transgenerational plasticity across three generations, and for both sexes to more fully understand the interplay between sex, current environment, and inherited environment. My study species, the Eastern Mosquitofish (*Gambusia holbrooki*) provides an interesting and powerful study system since the sexes are highly sexually dimorphic and because unlike most fish, mosquitofish are viviparous so substantial intergenerational environmental effects might be transmitted through a broad suite of mechanisms, including epigenetically, in ova and during gestation.

Objectives

In this study I examined the transgenerational behavioral effects of nonlethal depredation cues on Eastern Mosquitofish (*Gambusia holbrooki*) over three generations. Specifically I assessed 1) the presence and strength of predator-driven behavioral responses passed along generational lineages at both the parental and grandparental scales, 2) the degree to which the presence and strength of transgenerational responses are shared or unique across sexes, and 3) the potential for transgenerational effects to constrain or reinforce intra-generational phenotypic plasticity.

Methods

Study System

Eastern Mosquitofish (*Gambusia holbrooki*) are an abundant and widespread poeciliid fish species native to the southeastern United States (Lee and Burgess 1980). However, this species, and the closely-related Western Mosquitofish (*G. affinis*), have been introduced to freshwater and estuarine habitats at a near global scale (Brown 1987, Cote et al. 2011) for biocontrol of invertebrate vectors of human disease (Meffe 1985). A significant part of the perceived value of these species for biocontrol is their natural ability to quickly colonize new habitats (Rehage et al. 2005, Grapputo et al. 2006, Cote et al. 2011), including highly disturbance prone habitats that might be otherwise marginal or inhospitable for many other species. This colonization success is generally attribute to the species' high fecundity, short generations times, and viviparous reproduction (Krumholz 1948, Vondracek et al. 1988, Haynes and Cashner 1995). However, these same traits that have made mosquitofish a favored species for biocontrol are also the reason they are considered an IUCN Red List invasive species (Pyke and White 2000). Indeed, these fishes are voracious consumers from multiple trophic levels, including insects, zooplankton, and algae (Goodsell and Kats 1999, Matveev et al. 2000), and aggressive competitors that can displace native species (Carmona-Catot et al. 2013).

Important for this study, the colonizing capacity and broad habitat tolerances of mosquitofish have likely long caused populations and individuals to encounter habitats with or without predators. Mosquitofish are a common prey item both for piscivorous fish and aquatic feeding birds (Britton and Moser 1982, Meffe and Snelson 1989). The Eastern Mosquitofish coevolved in a region with a diversity of voracious centrarchid predators, including Largemouth Bass (*Micropterus salmoides*) and Bluegill Sunfish (*Lepomis macrochirus*). Prior studies show that mosquitofish from habitats with and without fish predators show divergent morphology and behaviors (Langerhans et al. 2004, Heinen et al. 2013). These differences in morphology and behavior have often been attributed to local adaptation, but there are good reasons to believe that mosquitofish might also respond to different predator environments via adaptive phenotypic plasticity and transgenerational environmental effects. Indeed, I have previously shown evidence for phenotypic plasticity in mosquitofish behavior and morphology in response to predator cues (Arnett and Kinnison In Review, Chapter 3). As live bearing fishes, mosquitofish maintain prolonged bodily contact with their developing embryos and prior studies have provided evidence that maternal genotypes can have strong influences on offspring size (Reznick 1981) and that mothers transfer nutrients to their offspring throughout pregnancy

(Marsh-Matthews et al. 2005). Hence, mosquitofish may have greater capacity to pass environmental cues across generations than some egg-laying fishes, although even egg-laying fishes can show transgenerational plasticity (Salinas and Munch 2012). Nonetheless, while it is tantalizing to hypothesize that adaptive transgenerational environmental effects might even contribute to the renowned colonizing capacity and invasiveness of mosquitofish, it is by no means certain that such effects exist or are adaptive.

Finally, mosquitofish are also sexually dimorphic and may face different tradeoffs in how they allocate time and risk in response to predators. Male mosquitofish reproductive success is thought to be highly tied to numbers of mating opportunities and males spend a very large portion of their short lifetimes pursuing and displaying to mates (Bisazza et al. 2001, Dadda et al. 2005, 2008), to the point that they experience deterministic growth once they reach sexual maturity. Conversely, female reproductive success is strongly tied to fecundity and females tend to spend relatively more time foraging (Blanco et al. 2004) and have indeterminate growth. These differences between males and females could in principle lead to different adaptive responses to predator cues within and among generations, something that has not been addressed with prior studies of multigenerational responses to predator cues that have focused largely on clonally reproducing species (e.g., Walsh et al. 2014).

Collection and Breeding

Wild Eastern Mosquitofish were collected using long pole dip nets from a pond in Croatan National Park Forest in North Carolina that coexisted at the time of capture with an established population of centrarchid predators (Lilly Pond 34.79°N, -76.86°W). Wild fish were bred and reared in a laboratory setting for a minimum of one year to ensure all laboratory produced offspring were sired under non-predator cue conditions (Turner 1937, Constanz 2012).

Laboratory acclimated wild fish produced offspring (F1) that were laboratory reared and produced the laboratory stock used for experimentation and breeding of further generations (Figure 3.2). Laboratory conditions of 26.5°C water temperature and 15L:9D photoperiod were used to emulate ideal breeding conditions and best mimic a temperate zone breeding season (Krumholz 1948, Haynes and Cashner 1995). All aquaria, pools, and the assay arena were filled with conditioned well water and maintained with deionized water. All fish were fed *ad libitum* 1-2 times daily. Fish diet consisted of livebearer flake food and a supplement of dried tubifex worms, dried krill, and algae flakes.

The full experimental breeding and exposure design was replicated three times, beginning with random allocation of laboratory-bred offspring from a shared laboratory stock. Breeding and offspring production were done in small shoal groups to mimic natural mating systems and using a 60-70% female-biased sex ratio. Breeding individuals were placed in a 295 liter pool by treatment group. Each breeding pool contained a central refuge with artificial floating plant mats and a 5 mm diamond mesh barrier to allow fry passage but block cannibalistic adults (Figure 3.1a). Breeding pools were checked daily for fry, which were removed for allocation to predator cue treatments. Fry measuring >12 mm were discarded to ensure similar age and developmental stage of fry at the time of experimental exposure.

The sibling group(s) of fry removed from breeding pools associated with the first generation of exposures were evenly divided and allocated to a predator-exposure or predator-free cue environment to create the second exposure generation (Figure 3.2). Offspring from the second generation were not divided and were instead allocated to the same cue environment as their parents to create the 3rd exposure generation (Figure 3.2). The exposure treatment pools were again 295 liter wading pools with a 1 mm polyurethane mesh central chamber (predator enclosure). In predator-free treatments, this chamber remained empty, while predator-exposure treatments contained one live Largemouth Bass (Figure 3.1b). The live predator was fed surplus live mosquitofish throughout the study to ensure a constant and cohesive supply of cues including auditory, visual, and chemical associated with both predator presence and conspecific depredation. Treatment pools were further subdivided into two halves with the same 1 mm mesh (Figure 3.1b). One side of the pool was dedicated to second or third generation fry derived from the first generation exposed parents/grandparents and the other side housed fry derived the first generation non-exposed parents/grandparents. (i.e. Figure 3.2 boxes 4 & 6). This design ensured that all fry within a given replicate, generation, and exposure treatment experienced the exact same environmental cues.

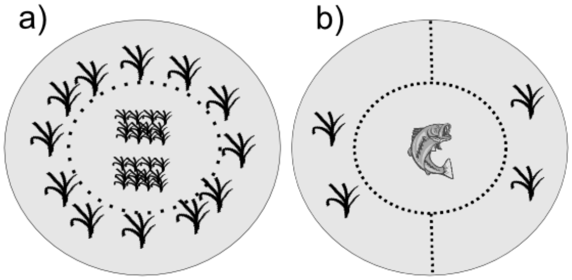

Figure 3.1: Experimental design for replicated breeding and exposure treatments. Panel a) is the breeding system with a central fry refugium and outer ring of plants to reduce aggression and increase post-parturition survival. Panel b) is the treatment pool with an inner ring either containing or not containing a predator for cues , and two subdivisions for rearing and exposing 2nd and 3rd generation treatment lineages (from the exposed and non-exposed 1st generation shoals). The full design of breeding and exposure treatments was independently replicated three times. Replicated from image 2.1 and placed here for ease and convenience.

Fry remained in treatment pools for 4-12 weeks until the clear presence of secondary sexual characteristics at which time they were assayed for behavior. Recently matured adults were also taken from each first and second generation exposure treatment to establish the next generation of breeding and exposure pools. Parturition took place in pools without predator cues, but females did become pregnant in the presence or absence of predator cues depending on the experimental treatment within which they themselves were reared.

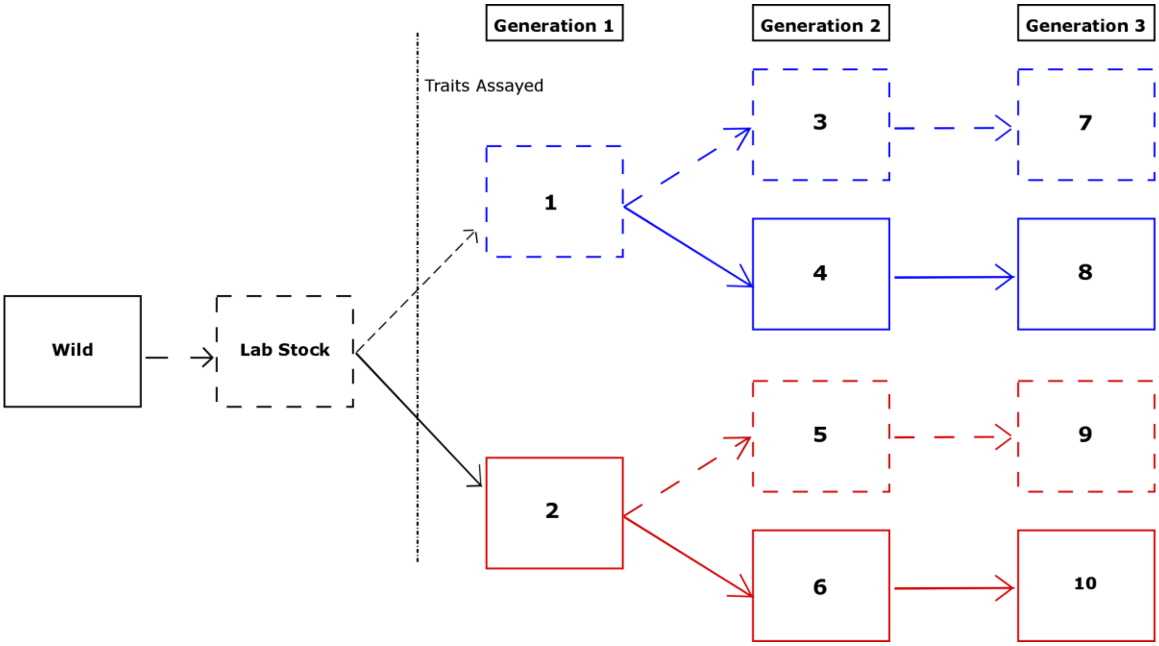

Figure 3.2: Lineage model for nonlethal predator cues over three generations. Boxes represent groups of mosquitofish reared under a given set of predator cues. Boxes with solid borders and connecting lines represent predator-exposed treatment groups and boxes with dashed boarders and lines represent predator-free treatments. The blue colored boxes and arrows reflect the lineages of fish deriving from the non-predator-exposed fish in the first generation (box 1). The red colored boxes and arrows reflect the lineages derived from the predator-exposed fish in generation one (box 2). The group numbers, line colors and line style format in this figure are used as a convention in all subsequent figures and tables. Behavioral traits were assayed for all groups to the right of the vertical dashed line.

Behavioral Assays

Sexually mature fish were fasted for 72 hours before behavioral assays were performed. From each treatment, a minimum of twenty individuals (ten of each sex) were assayed for risk and foraging behaviors. Behavioral assays were performed in a risk-reward test arena (Figure 3.3). One end of the arena contained a Largemouth Bass in an enclosure, while the opposite end enclosed three to five conspecifics. Both enclosures were made of 5 mm polyurethane coated diamond mesh. The central region of the arena was subdivided into a 2X5 grid to quantify movement and contained a food reward to encourage exploration. Each pair of squares had one provided with freeze-dried krill and one provided with spirulina algae flakes, these were randomly distributed along the grid in these pairs. The two grid squares closest to the bass enclosure represented the “danger zone” during risk assessment. Closest to the conspecific shoal group was an open space before the initiation of the grid. This space represented the “safe zone” during risk assessment and was also the location of acclimation and release. Individual fish were placed in the acclimation tube for two minutes before the tube was removed and the assay began. Behaviors were recorded continuously for ten minutes using the JWatcher software (ver. 1.0, Macquarie University and UCLA). Individuals were scored for

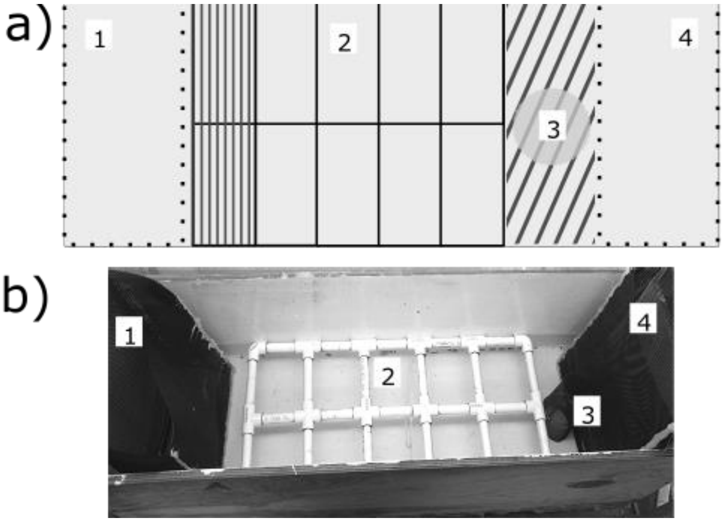

Figure 3.3: Risk-reward test arena. Panel a) is a diagrammatic representation of the actual assay tank in panel b). Area 1) is the bass enclosure with the associated “danger zone” represented by vertical striping. Area 2) notes the movement grid. Area 3) is the acclimation tube and the associated “safe zone” is represented by diagonal striping. Area 4) is the conspecific shoal group enclosure. Replicated from Figure 2.2 for ease and convenience.

activity (swimming), location (distances from shoal group), and time to first exit the “safe zone” after release.

Because different behaviors can be strongly positively or negatively correlated within a finite time budget, and because the various behavioral traits may relate to different expressions of an underlying behavioral syndrome (Sih et al. 2004, Bell and Sih 2007), I first sought to determine if among-individual variation in the suite of assayed behaviors could be reasonably summarized as a single bold-shy trade-off variable. A principle components analysis (PCA) with Varimax rotation was conducted to produce a single major component factor for group comparisons within and among generations. Based on eigenvalues from the PCA, the resulting component explained a total of 73% of trait variation in behavior among all individuals in the study design, and showed strong trait loadings fully consistent with a bold-shy behavioral syndrome (Table 3.1). Specifically, positive scores on this factor are associated with strong positive loadings of exploration time and time in the danger zone and strong negative loadings of lag time and time in the safe zone (Table 3.1). Although I used scores derived from this universal PC for subsequent analyses, to allow behavioral comparison across generations, nearly identical PC solutions are obtained with sub-PCAs run within each generation, demonstrating this bold-shy trade-off factor is very robust and not weighted by a single generation group (Table 3.1). To help support and interpret the analyses based on the bold-shy PC scores, I also conducted multivariate and univariate analyses of variance (MANOVA) on the four separate behavior types; lag time, exploration time, percent time in the safe zone, and percent time in the danger zone.

Table 3.1: Varimax rotation behavior loadings on a PCA with all mosquitofish individuals. Loadings for each of the four behavioral types on a single Varimax rotated component. Under the double line are the variances for each principle components. The Varimax PCA includes all three generations and both sexes of mosquitofish.

	Full Model Component 1	Generation 1 Varimax PC 1	Generation 2 Varimax PC 1	Generation 3 Varimax PC 1
Lag Time	-0.78	-0.76	-0.79	-0.75
Exploration Time	0.95	0.93	0.96	0.95
Time in Safe Zone	-0.91	-0.89	-0.92	-0.89
Time in Danger Zone	0.75	0.76	0.77	0.71
Proportion of Variance	0.73	0.71	0.75	0.71

Bold-shy PC scores and the separate behavior traits were analyzed with linear mixed-effect models (LME) that initially included Sex, the exposure conditions of the individual (hereafter "Exposure"), and the exposure condition of the individual's ancestors from the first generation (hereafter "Lineage") as fixed effects where appropriate (e.g., no lineage effect when comparing the two generation 1 groups of fish). Experimental replicate was treated as a random effect and models were initially constructed with two-way interaction terms among the fixed factors and reduce where interaction terms were non-significant (p>0.05). Many initial analyses showed strong interactions between males and females in full models, so subsequent analyses and figures are presented separately for females and males for tractability. Because of the challenges in raising large numbers of fish through multiple generations and the great variability in individual behaviors, p-value less than 0.1 were considered evidence of significant effects and p-value less than 0.05 were considered highly-significant. Effect sizes were quantified using a Cohen's d.

Results

Intra-generational Plasticity

The first generation plastic effects of exposure environment was one of the few cases where there was not a significant interaction between Sex and other model terms for the bold-shy PC. However, there were significant fixed-effects of Sex ($F_{1,105}$=17.24; p<0.001), and Exposure ($F_{1,105}$=8.52; p<0.004), with males being much bolder (higher PC scores) than females and predator-exposed fish being bolder than non-exposed fish. However, failure to reject an interaction between Sex and Exposure in this initial analyses may have been artefactual, since further analyses suggest that any such plastic effect for females was weak if present it all (Table 3.2, Table 3.3, Figure 3.4a, Figure 3.5a-d). Specifically, no plastic effect of predator exposure was detected for generation one females when they were analyzed separately from males, for either PC or univariate analyses of behavior. Moreover, in contrast to the case for males, paired comparisons of exposed and unexposed females at later generations (within respective lineages: groups 3 v 4; 5 v 6; 7 v 8; 9 v 10) did not support analogous increases of boldness with exposure to predators (Table 3.3, Figure 3.4a).

Table 3.2: Reduced complete MANOVA model on PC behavior for all three generations of both sexes of mosquitofish. Lineage refers to the exposure treatment of the first generation relative. All two-way interactions were included and interactions with non-significant terms (p>0.05) were removed. Model was reduced using backwards selection. Significance (p<0.10) is represented with an asterisk (*), and highly significant values (p<0.05) are represented with a double asterisk (**). Effect size values are Cohen's d, using non-exposed treatment values as the baseline values.

FEMALES		Isolated Effect	df	F-value	p-value	Significance	Effect Size
Gen 1	Plasticity	Exposure	1,54	1.37	0.25		0.41
Gen 2	Parental Effects	Exposure	1,115	0.25	0.62		-0.09
		Lineage	1,115	6.17	0.01	**	-0.46
Gen 3	Grandparental Effects	Exposure	1,86	1.12	0.29		-0.22
		Lineage	1,86	4.07	0.05	*	-0.46
MALES		Isolated Effect	dF	F-value	p-value	Significance	Effect Size
Gen 1	Plasticity	Exposure	1,48	8.54	0.005	**	0.81
Gen 2	Parental Effects	Exposure	1,90	0.01	0.91		-0.02
		Lineage	1,90	0.12	0.73		0.07
Gen 3	Grandparental Effects	Exposure	1,81	3.13	0.08	*	0.36
		Lineage	1,81	0.00	0.97		0.05

Table 3.3: ANOVA model on PC behavior within generations for three generations of both sexes of mosquitofish. Numbered group comparisons within generations references breeding schematic Figure 3.2. Model was reduced using backwards selection. Significance (p<0.10) is represented with an asterisk (*), and highly significant values (p<0.05) are represented with a double asterisk (**). Effect size values are Cohen's d, using non-exposed treatment values as the baseline values.

FEMALE		Isolated Effect	dF	F-value	p-value	Significance	Effect Size
Gen 1	1 vs 2	Exposure	1,54	1.37	0.25		0.41
Gen 2	3 vs 4	Exposure	1,66	0.32	0.57		-0.14
	5 vs 6	Exposure	1,47	0.01	0.93		-0.03
	3 vs 5	Lineage	1,58	4.12	0.05	*	-0.52
	4 vs 6	Lineage	1,54	2.15	0.15		-0.39
	3 vs 6	Both	1,56	4.29	0.04	**	-0.55
Gen 3	7 vs 8	Exposure	1,40	2.72	0.10		-0.61
	9 vs 10	Exposure	1,44	0.07	0.80		0.02
	7 vs 9	Lineage	1,36	8.20	0.007	**	0.93
	8 vs 10	Lineage	1,48	0.97	0.33		-0.21
	7 vs 10	Both	1,39	5.91	0.02	**	-0.85
MALE		Isolated Effect	dF	F-value	p-value	Significance	Effect Size
Gen 1	1 vs 2	Exposure	1,48	8.54	0.005	**	0.81
Gen 2	3 vs 4	Exposure	1,54	1.22	0.27		-0.26
	5 vs 6	Exposure	1,34	0.75	0.39		0.29
	3 vs 5	Lineage	1,46	0.40	0.54		-0.18
	4 vs 6	Lineage	1,41	1.47	0.23		0.39
	3 vs 6	Both	1,45	0.14	0.71		0.11
Gen 3	7 vs 8	Exposure	1,42	0.12	0.73		0.11
	9 vs 10	Exposure	1,37	3.18	0.08	*	0.64
	7 vs 9	Lineage	1,45	0.28	0.60		0.17
	8 vs 10	Lineage	1,34	0.85	0.36		0.30
	7 vs 10	Both	1,45	2.76	0.10		0.47

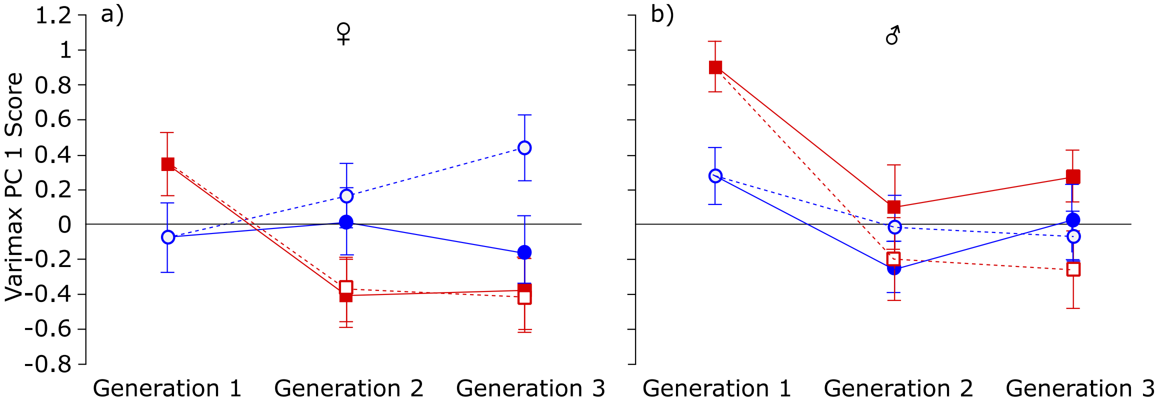

Figure 3.4: PC behavioral response trajectories across three generations of two sexes of mosquitofish. Panel a) shows female fish and Panel b) show male fish. Red colors (square points) are fish with exposed lineages (generation 1 exposure treatment). Blue colors (round points) are fish with non-exposed lineages. Filled markers with solid lines are groups that were themselves exposed to predator cues in that generation, while open markers with dashed lines are groups from non-exposure treatments. Color and line scheme follows the pattern established in the breeding schematic Figure 3.2.

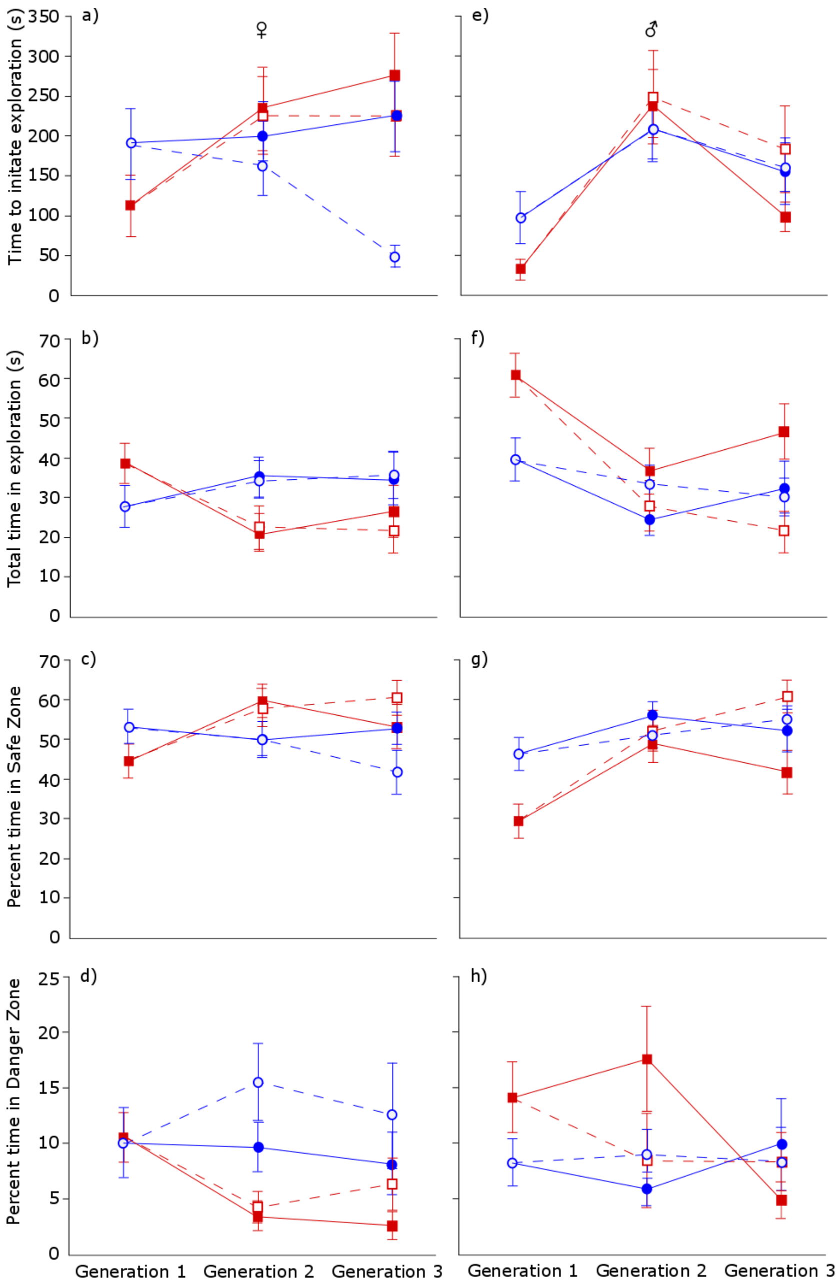

Figure 3.5: Individual behavioral response trajectories across all three generations for mosquitofish. Panels a-d) are female mosquitofish and panels e-h) are male mosquitofish. Panels a) and e) are total lag time, panels b) and f) are total time exploring the arena, panels c) and g) are percent time spent in the zone closest to the shoal group, and panels d) and h) are percent time spent in the zone close to the Largemouth Bass. Red colors (square points) are fish with exposed lineages (generation 1 exposure treatment). Blue colors (round points) are fish with non-exposed lineages. Filled markers with solid lines are groups that were themselves exposed to predator cues in that generation, while open markers with dashed lines are groups from non-exposure treatments. Color and line scheme follows the pattern established in the breeding schematic Figure 3.2.

Looking at the univariate behaviors suggests that the overall plastic increases in boldness in response to predator cues, again primarily apparent in males, were most associated with reductions of time in the safe zone (16% females, 36% males), reduced lag time (41% females, 36% males), and an increase in exploration time (30% females, 36% males) (Figure 3.4b, Figure 3.5 a-c, Table B.1, Table B.2).

Female Transgenerational Plasticity

Generation two "daughters" of the generation one treatment groups showed significant evidence of a generation one parental environment (Lineage) effect (Table 3.2, Figure 3.4a). Daughters from the first-generation group that had experienced predator cues (groups 5 & 6) became much shyer than females in their parent's generation (group 2), regardless of their own predator exposure (Table 3.4). This was not only true for overall bold-shy PC scores (Table 3.4a) but for all separate behavioral traits (Table B.3). For example, these female offspring spent 60-67% less time in the danger zone of the arena than the females of their parent's generation. At the same time, daughters from non-exposed parents (groups 3 & 4) maintained nearly the same boldness as their parental generation counterparts (group 2), such that the non-exposed offspring of non-exposed parents (group 3) ended up significantly bolder than their counterparts from exposed parents (group 5) (Figure 3.4a). Generally, generation two females expressed overall trends of individuals with exposed parents, having stronger shy behaviors while those with non-exposed parent expressed stronger bold behaviors (Figure 3.5a-d), though most cases the trend for individual behaviors is non-significant (Table 3.3, Table B.1).

Table 3.4: ANOVA model on PC behavior among generations for three generations of both sexes of mosquitofish. Model compares generation one to generation two and three, and generation two to generation three PC behavior. Numbered group comparisons across generations references breeding schematic Figure 3.2. Model was reduced using backwards selection. Significance (p<0.10) is represented with an asterisk (*), and highly significant values (p<0.05) are represented with a double asterisk (**). Effect size values are Cohen's d, using generation one values as the baseline values.

FEMALE		dF	F-value	p-value	Significance	Effect Size
Gen 1 and Gen 2	1 vs 3	1,62	0.81	0.37		0.22
	1 vs 4	1,60	0.07	0.79		0.08
	2 vs 5	1,50	7.58	0.008	**	-0.75
	2 vs 6	1,48	7.84	0.007	**	-0.78
Gen 1 and Gen 3	1 vs 7	1,43	0.32	0.57		0.51
	1 vs 8	1,52	2.52	0.12		-0.08
	2 vs 9	1,46	6.89	0.01	**	-0.77
	2 vs 10	1,49	7.59	0.008	**	-0.72
Gen 2 and Gen 3	3 vs 7	1,49	0.85	0.36		0.27
	4 vs 8	1,56	0.31	0.58		-0.16
	5 vs 9	1,45	0.00	0.96		-0.03
	6 vs 10	1,46	0.00	0.98		0.01
MALE		dF	F-value	p-value	Significance	Effect Size
Gen 1 and Gen 2	1 vs 3	1,52	1.34	0.25		-0.31
	1 vs 4	1,48	6.78	0.01	**	-0.66
	2 vs 5	1,42	15.83	0.0003	**	-1.24
	2 vs 6	1,41	9.10	0.004	**	-0.92
Gen 1 and Gen 3	1 vs 7	1,49	5.35	0.03	**	-0.44
	1 vs 8	1,38	2.00	0.17		-0.28
	2 vs 9	1,43	18.63	0.0001	**	-1.35
	2 vs 10	1,43	8.67	0.005	**	-0.86
Gen 2 and Gen 3	3 vs 7	1,55	0.05	0.82		-0.06
	4 vs 8	1,40	0.52	0.48		0.31
	5 vs 9	1,36	0.10	0.76		-0.06
	6 vs 10	1,35	0.41	0.53		0.21

Moving on to the third generation, ANOVAs with Exposure and Lineage terms indicated that the "granddaughters" of the first generation fish again showed evidence for enduring influences of their generation one grandparent's environments (Lineage), such that fish with predator-exposed grandparents were significantly shyer than those with grandparents that had not experienced predators (Table 3.4, Figure 3.4a). Much of this transgenerational plasticity owed to a seeming enduring effect of grandparent exposure favoring reduced boldness (increased shyness) in granddaughters, as it had in daughters, irrespective of the environment experienced by those subsequent generations (i.e., groups: [9 & 10] = [5 & 6] < 2). This enduring shyness (groups [9&10] vs 2) was again apparent in all of the associated univariate behavioral traits (Table B.3). As with the parental effects for daughters, granddaughters with unexposed grandparents (and parents) remained the boldest of all generation three treatment groups (Figure 3.4a, Figure 3.5a-d). Indeed, this never-exposed lineage (group 7) was significantly bolder than both of the lineages deriving from exposed grandparents (groups 9 and 10) (Table 3.3, Table B.1). By generation three, any lineages that were ever exposed to predators, via grandparents, parents, or directly, showed 78-83% longer lag times than the never-exposed lineage fish (Figure 3.4a, Figure 3.5a, Table B.1), as well as some lesser shifts toward shyness on other traits (e.g., 35% increase in time in safe zone and 27% less time exploring for the unexposed granddaughters of exposed grandparents).

Male Transgenerational Plasticity

Generation two male mosquitofish showed no overall effects of exposure treatment or lineage (Table 3.2). Indeed, none of the groups in generation two groups significantly differed from each other in overall boldness-shyness or most univariate trait comparisons (Table 3.3, Figure 3.4b, Figure 3.5e-h); the only exception being some evidence that predator-exposed sons with predator-exposed parents might have spent slightly more time in the danger zone than other groups (4 & 5). However, given the large differences in boldness of first generation males, this meant that the sons of exposed parents (groups 5 and 6) showed substantial reductions in boldness compared to males in their parent's generation (group 2) whereas the sons of unexposed parents showed either no significant reduction (group 3) or a much smaller reduction (group 4) relative to their already shy parent generation (group 1) (Table 3.4, Figure 3.4b). Reductions in boldness from generation one to two were most commonly associated with increased lag time by 53-87% (Figure 3.5e, Table B.4). Additionally, groups that experienced either parental- or direct-exposure to predators increased time in the safe zone by 18-43% and decreased total exploration time by 39-54% (Figure 3.5f-h, Table B.4).

Generation three male mosquitofish showed a significant overall effect of recent (generation 2 + generation 3) predator exposure but no effect of the grandparent's predator environment in ANOVAs with lineage and exposure effects (Table 3.2). In this model the third generation effect of exposure to predator cues (generation 2 + generation 3) was analogous to that in the first generation, with the exposed groups being generally bolder than their unexposed counterparts (Figure 3.4a). At a pairwise comparison level, this effect of exposure was most apparent in contrasting individuals from the group with predator exposure in three generations (group 10) against the groups that had not experienced predators in either their own and their parent's generation or their grandparent's (9 vs 10 and 7 vs 10), with latter marginal for PC and significant for two individual traits (Table 3.3, Table B.3). The greater boldness of the exposed fish was driven primarily by significantly longer exploration times (34-55%) and significantly shorter times in the safe zone (24-32%) (Figure 3.5f-g). There was no evidence of changes in boldness between the second and third generations of males within their respective lineages (Table 3.4, Table B.4). Hence, like the second generation, nearly all generation three grandsons continued to express significantly shyer traits than the males from their grandparent's generation (generation 1). The only exception being for exposed generation three males from the non-exposed lineage (Table 3.4).

Discussion

Mosquitofish clearly show behavioral plasticity to predators and depredation cues along an overt bold-shy continuum. However, males and females differed substantially in the extent or patterns of their intra-generational and transgenerational plasticity. Females showed little if any intra-generational plasticity but relatively strong plastic responses to parental and grandparental exposure. In contrast, males showed stronger intra-generational plasticity in the first generation, and evidence of some analogous plasticity in subsequent generations (third), but much less evidence of transgenerational plasticity. I discuss these patterns of behavior with regards to what they might tell us about the relative important of direct versus inherited predator cues for potentially adaptive plasticity of the two sexes of this live bearing fish.

Females Respond to Ancestral Cues

Theory suggests that transgenerational effects are expected to be most adaptive where the inherited environmental cues from parents or grandparents are dependable (Moret 2006, Uller 2008, Storm and Lima 2010, Hoyle and Ezard 2012) and provide the offspring with a form of "pre-adaptation," or head-start in performance (Moret 2006, Dyer et al. 2010, Salinas and Munch 2012). In the current study female mosquitofish showed overt plastic responses to the environments of their parents and grandparents that often spanned a whole integrated suite of bold-shy behaviors. Specifically, the daughters and granddaughters of fish that were exposed to predator cues became substantially shyer than both their predecessors and the daughters and granddaughters of fish that had not experienced predator cues. Other studies of offspring responses to parent's exposure to predator cues often show a similar shift toward antipredator behaviors (Mondor et al. 2005, Giesing et al. 2011, Bestion et al. 2014) or selection of safer habitats (Storm and Lima 2010, Keiser and Mondor 2013). The current study extends these transgenerational responses to predator cues through even more generations. Hence, based on the above adaptive theory, we might thus presume that past exposure of parents or grandparents to predators is a fairly reliable cue. Female mosquitofish face a risk of similar exposure and should thus adopt behaviors that mitigate this risk, even if they don't directly experience predator cues themselves.

It is interesting that this transgenerational plasticity of females appears to be even stronger than intra-generational plasticity, such that daughters and granddaughters may rely more on the cues faced by their parents or grandparents than those they experience directly. This might seem somewhat counterintuitive if one presumes that an individual's own sensory experience in the present should be more reliable than cues passed from other individuals that experienced the environment longer ago. However, that presumption might not be true if one considers reliability in terms of an environmental sampling scale problem (Templeton and Franklin 1992, Koops 2004). Many predators are transitory, have large ranges, or migrations which make their presence patchy in both space and time (Bernstein et al. 1988, Folt and Schulze 1993). Wild predators also often go to great lengths to conceal their presence from prey (Quinn and Cresswell 2004, Cresswell et al. 2010). Prey on the other hand are only able to sample environmental conditions across a finite period of time and space, and must ultimately use that acquired information and remaining uncertainty to make decisions about predator presence (Sih 1992). This uncertainty may also be greater or lesser, depending on the predator-prey relationship and the extent of the risk the predator may pose (Ferrari et al. 2010). While prey are often sensitive to cues (McIntosh et al. 1999), prey individuals do not respond as strongly or even in the same direction for all cue types (e.g. olfaction vs vision) (Martin et al.

2010), likely due to differences in cue reliability (Magurran et al. 1996, Brown 2003, Wisenden et al. 2004). Ultimately, individuals make predator detection and response decisions with a significant degree of error, balancing between the energetic costs of overestimating predator abundance with the fatal costs of underestimation (Bouskila and Blumstein 1992, Abrams 1994). Hence, an individual that does not perceive predator cues itself could face dire consequences from behaving in a risky fashion if it made an error in assessing its environment, or in the event that predators do eventually show up after a period of local absence. The swampy lakes in Croatan National Forest where the focal population from this study originates average just four feet deep (USDA and Forest Service 2014), and are prone to drying and flooding. Moreover, many of the lakes have an acidic pH, ranging from 4.5-5.0 (USDA and Forest Service 2014). Acidic pH and low water levels reduce the condition of Largemouth Bass and reduce reproduction and recruitment (Miranda et al. 1984, Leino and McCormick 1993), potentially making these predators relatively sparse and patchy in the environment. During some seasons or years, mosquitofish might even be isolated in marginal pools or habitats for extended periods, equating with one or more generations, until rains and flooding reconnect habitats and provide access by predators or distribute offspring in isolated habitats back into areas with predators. Therefore, predator cues passed from prior generations could thus provide a more comprehensive and reliable sampling of predation risk.

A tantalizing trend in my data suggests an exception that could prove the rule. At the same time that females in lineages where parents experienced predators dropped to a low level of boldness in generation two and stayed at that bottomed-out level through generation three, fish from the lineage that had never experienced predators in any generation showed a trend towards increasing boldness generation-to-generation, or at least greater divergence from other groups (Figure 3.4a). This would fit with transgenerational plasticity as a solution to an environmental sampling scale problem, in that more generations without predators would, in principle, be a more reliable cue of predator absence. This could theoretically function much like an individual increasing sampling time or sites (Bouskila and Blumstein 1992, Sih 1992, Abrams 1994) or increase signal evaluation capacity (Wiley 2009, Ferrari et al. 2010). In this case, ancestral information may increase cue reliability, in which case greater boldness might be favored for greater competitive ability.

Males Respond to Current Environmental Experience

In contrast to females, the bold-shy behaviors of males were much more influenced by the environments they themselves experienced and less clearly influenced by the environments of their parents or grandparents. In both the first and third generations, exposure to predator cues was associated with significantly greater boldness. In other words, the effects of intra-generational exposure to predators in males is opposite that of transgenerational plasticity in females. On the other hand, the only suggestions of a possible transgenerational effect for male mosquitofish was some evidence for a greater decrease in boldness for males from the predator-exposed lineages than the non-exposed lineages between the first and second generations. Given an overall trend toward reduction in boldness across all treatments from the first to second generation (Figure 3.4b), and the absence of any group differences in this second generation, I am hesitant to interpret this pattern as more than equivocal evidence of a parental effect. So then, why might male mosquitofish show such a different reliance on intra-generational versus transgenerational plasticity than their female counterparts?

The aforementioned theories would suggest that transgenerational cues might be somehow less reliable for males than they are females. It is not immediately clear why this should be the case since both sexes coexist in the same environment and thus experience the same histories and odds of predator occurrence at particular points in time. A mother's, father's, grandmother's, or grandfather's exposure would thus presumably have the same information value to their male or female descendants. All individuals had both parents exposed to a given set of predator-presence or absence cues, so this difference also cannot be explained by differential capacity for information transmission through maternal or paternal inheritance mechanisms. With this in mind, it may make sense to hypothesize that the difference in male versus female reliance on within- versus between- generation predator cues might depend less on cue reliability and more on the relative utility of the two forms of information to individuals of different sex. Indeed, a few other works have found that intra-generational and transgenerational effects are negatively correlated, so individuals may express one or the other but not both (Hoyle and Ezard 2012, Walsh et al. 2014), perhaps providing additional support for segregation of responses in males and females.

Female mosquitofish and other female poeciliids have much longer reproductive life spans compared to their period of juvenile rearing than males. Development from parturition to maturity in mosquitofish takes roughly 4-6 weeks (Krumholz 1948). Many mature females may have post-maturation lifespans of equal or greater duration, whereas males may rarely live so long (Krumholz 1948), females living up to three times longer. Because of their greater longevity, female mosquitofish are more likely than males to experience a change in predator conditions from absence of predators to presence sometime after their period of their early rearing, increasing the value of transgenerational cues of past predator presence. Males on the other hand live such short times on average that their adult experience is unlikely to be different than their juvenile experience, even if their parents or grandparents experienced something similar.

This difference in reliance on transgenerational versus intra-generational plasticity might be thought to imply that females are more developmentally locked into their behavioral tendencies than males. However, that inference is not clearly warranted. The current study was not designed to test for reversibility of behavioral tendencies within generations, which would have required systematically switching individual exposures partway through their juvenile rearing. Moreover, studies of behavioral syndromes in fishes and many other organisms suggest that most individuals can 1) up or down regulate their boldness to some degree, but 2) tend to remain relatively bold or shy with respect to other individuals across a broad spectrum of contexts (Sinn et al. 2008). Indeed, this cross-context consistency has often been used as a defining feature of such a behavioral syndrome (Sih et al. 2004, Sinn et al. 2008), suggesting that relative boldness may be developmentally determined early in life. Whether individuals are developmentally set on a relatively bold or shy pathway could occur whether that pathway is initiated by cues transmitted from prior generations, or by the individual's own early environmental exposure. Indeed, these two alternatives are not always distinct for mosquitofish and other viviparous species where internally developing embryos might not only be indirectly programed through epigenetic mechanisms, but also directly experience some cues transmitted passively through their mother's blood supply.

Sexual differences in Direction of Plastic Responses

Setting aside generational distinctions, prior studies suggest that females of many species are generally shyer and more conservative in their behavioral strategies than their male counterparts, spending greater time foraging and increasing their fecundity through size and gestational success (Klimley 1987, Shine 1988, Prenter et al. 1999, Kamilar and Pokempner 2008). On the other hand, variation in male reproductive success of many species is more often associated with competition for numbers and quality of mates. Boldness in males can aid them in locating or defending mates or mating territories, successful courtship, or coercive matings (Wells 1977, Vehrencamp et al. 1989, Luyten and Liley 1991, Matthews et al. 1997, Hack 1998). Although these activities often increase risk of predator encounter or make males more conspicuous to predators (Endler 1987, Candolin 1997, Sih et al. 2003), such risks could actually favor greater boldness by placing a selective premium on males to mate early and often. These differences in roles within mating systems, and initial levels of boldness, could predispose males and females to different risk tradeoffs and divergent plastic responses to predator cues. Indeed, in clonal comparisons of intra- versus trans-generational effects, increased early reproductive investment was commonly associated with within-generation plasticity (Walsh et al. 2014), such as seen in male mosquitofish in the present study. Conversely, clonal individuals that expressed transgenerational responses favored slower growth and later reproductive timing (Walsh et al. 2014), more similar to the general life history of a female mosquitofish (Krumholz 1948).

In the present study, males were notably bolder than females in generation one. This general sex difference was obscured for most groups by generations two and three; however, the lineage that experienced predator cues in all generations was the top ranked treatment group for boldness in males, but the bottom-ranked (shyest) treatment for females. Hence, there is some empirical reason to believe that male and female mosquitofish do differ in their underlying boldness to begin with, and that this fits with the natural history of this species.

The vast majority of female mosquitofish are mated almost immediately upon sexual maturation, provided males are present. However, the live-bearing nature of this species means that mated female mosquitofish face a prolonged window where depredation risks loss of all reproductive success. Internal development of embryos also means females may often have more to lose than gain from repeated courtships or mating attempts during pregnancy (Arnqvist

1989, Kawagoe et al. 2001, reviewed in Kokko et al. 2003). Pregnancy itself can increase risks of depredation and prior work has shown that gestating females shift toward shyer behaviors to reduce their predation risk (Bauwens and Thoen 1981, Stankowich and Blumstein 2005, Ibáñez et al. 2015). As noted above, females that are bolder in seeking food resources might benefit competitively when predators are absent and conspecifics are abundant, but behaviors that reduce risk and aid longevity may be strongly favored for mature females when predators are present and conspecifics competitors are less abundant.

In contrast to females, the almost scramble nature of male reproductive success in mosquitofish places less of a premium on male size and more on male activity, as evidenced by their determinant growth at maturation (Krumholz 1948). Sex ratios in wild populations without predators are often strongly skewed toward females (Fryxell et al. 2015), suggesting that males experience higher mortality rates and have a shorter average time window to reproduce, but could experience very high reproductive success by mating quickly, even coercively, with the larger pool of available females. In contrast, it's almost more difficult to envision cases where less boldness could be favored. However, in the presence of predators sex ratios are more even (Chapter 5); thus males must compete more directly among each other for mates, and females are able to engage in more mate choice. These conditions could favor males that are less apt to expend energy in restless exploration for short term mating payoffs in favor of reserving energy and time for a greater period of likely mating competitiveness.

Interestingly, the very different mating system roles and tradeoffs of males and females suggests a final reason why they might show opposing plastic responses to predators – their response might in part be responses to demographic and behavioral responses of the opposite sex. When piscivores are present, mosquitofish are often less abundant and our study suggests females adopt shy behaviors that could even include avoidance of males since male courtship could draw predator attention (Agrillo et al. 2007), but males are also very rarely ever limiting for fertilizations when needed and females are able to store sperm from past matings (Houde 1997), resulting in a form of sexual conflict that has been studied in related poeciliid fishes (Magurran and Seghers 1990, Magurran and Nowak 1991). Hence, in the presence of predator cues, males might adopt greater boldness, particularly in the form of increased exploratory restlessness, to increase their odds of encountering the relatively smaller number of more cautious mates. Males that wander more might also be more aware of predator risks in the vicinity of different potential mates. Conversely, females might become shyer under predator cues in part to offset some of the predation risks and lost foraging opportunities associated with being pursued by bolder males.

Concluding Remarks

Overall, this study supports the hypothesis that transgenerational environmental plasticity is passed through lineages and, at least for some individuals within a population, is likely to be adaptive. Adaptive behavioral transgenerational plasticity is relevant in other fishes (Giesing et al. 2011) and other aquatic invertebrates (Marsh-Matthews et al. 2005), and some terrestrial organisms (Reedy et al. 2012). Across many study systems females are often considered to be under higher depredation risk largely due to their size and gestation constraints (Britton and Moser 1982, Magurran and Nowak 1991, Trexler et al. 1994, Pocklington and Dill 1995, Götmark et al. 1997) and this is often suggested as the basis for their relative shyness (Bauwens and Thoen 1981, Stankowich and Blumstein 2005, Ibáñez et al. 2015). Some other studies have also shown that females appear more likely to plastically inherit parental behaviors (Brown and Shine 2007, Benton et al. 2008). However, this explanation based on female depredation risk is probably not sufficient on its own to explain all differences between males and females in their relative reliance on transgenerational versus current environmental cues. In mosquitofishes, and related poeciliids, empirical evidence suggests males often face higher mortality rates and have much shorter life spans, particularly in the presence of predators. This often leads to highly biased sex ratios, as I discuss in Chapter 5. As I noted above, the high mortality risks and extremely short reproductive life spans of males might be just as important as an explanation for why males don't rely on transgenerational plasticity as any arguments for why depredation risk somehow favors female reliance on transgenerational cues and shyness in females. I think this raises an interesting question about how we treat transgenerational plasticity when it comes to theory and empirical inference.

Specifically, is it appropriate that we often treat transgenerational plasticity (e.g., in female mosquitofish) as an exceptional process that requires special explanation, when it could turn out to be the general expectation? Our current tendency to treat cases of transgenerational plasticity as novelties likely derives from the current paucity of examples, but that paucity could owe more to the recent emergence of the field more than prevalence of transgenerational plasticity itself. If it turns out that both transgenerational and intra-generational plasticity are very common, then cases where transgenerational plasticity is absent might be more equally or more informative to testing and building theory. Unfortunately, the vast majority of published studies of transgenerational plasticity test for such effects in single species, sexes and traits, and as a result are prone to putting forth adaptive explanations that are rarely rigorously testable (Salinas et al. 2013, Burgess and Marshall 2014). Worse still, studies that don't find transgenerational effects might often face publication bias. Hence, comparative studies, like the one I present here, where transgenerational plasticity is tested in different sexes, species, environments, or traits, and where non-detections are reported along with detections, are greatly needed.

CHAPTER 4: TRANSGENERATIONAL MORPHOLOGICAL RESPONSES TO NONLETHAL PREDATOR CUES IN FEMALE EASTERN MOSQUITOFISH (*Gambusia holbrooki*)

Introduction

Phenotypic plasticity is the expression of a reaction norm, the differential expression of phenotypic trait values for a given genotype experiencing different environmental conditions (Via and Lande 1987, Via et al. 1995, Baythavong 2011). Much of the research on environmentally induced phenotypic plasticity is centered on the adaptive potential of reaction norms expressed by individuals during their own lifetime (Chapter 2, Arnett and Kinnison In Review). While such adaptive phenotypic plasticity is common, a more recent body of research indicates that environmental cues non-genetically inherited by individuals from their parents (Hoyle and Ezard 2012) or grandparents (Yazbek et al. 2010, Hafer et al. 2011) can also have a significant effect on their trait expression. Such multigenerational environmental transmission, termed “transgenerational plasticity”, is similarly theorized to provide individuals with the ability to produce phenotypes that are advantageous in their current environments (Galloway and Etterson 2007, Herman et al. 2012), assuming that environments experienced by parents, or more distant ancestors, are informative of risks or opportunities in the current generation. In this chapter I consider the multigenerational effects of non-lethal predator exposure on the size and shape of females of a live-bearing fish.

Transgenerational plasticity encompasses a suite of mechanisms providing for non-genetic transmission of environmental influences from generation to generation in a fashion that influences the trait expression of offspring or other descendants (Jablonka et al. 1992, Day and Bonduriansky 2011). It represents a form of phenotypic plasticity in the sense that this transmission represents the potential for a given genotype to produce alternate phenotypes based on a particularly environmental experience, albeit in this case the environment was experienced by parents or other progenitors, resulting in essentially a cross-generation norm of reaction. The earliest research attributed to transgenerational plasticity revolved around the detection of maternal environment effects that were often seen as confounding many quantitative genetic estimates of trait heritability (Yamanaka 1928, Park 1935). Both maternal and paternal environmental experiences can be conveyed to offspring by a diversity of non-allelic inheritance mechanisms (Alexander 1974, Augspurger 1984, Wulff 1986, Westneat and Sargent 1996), but maternal effects are considered particularly strong due to the greater opportunity for mothers to influence offspring traits via nutrient provisioning of eggs or embryos, cytoplasmic transfer, and post-parturition provisioning or parental care (Metcalfe and Monaghan 2001, Marsh-Matthews et al. 2005, White et al. 2008, Wolff and Gemmell 2013). Although “epigenetic inheritance”, “parental effects” and “transgenerational plasticity” have sometimes been used synonymously with one another, this is an artifact of the most recent decade or so (Kirkpatrick and Lande 1989, Wolff and Gemmell 2013). In the present study I follow a more formal framework wherein the former (epigenetic inheritance) represents a specific set of molecular mechanisms that essentially program gene expression or other cellular functions, including DNA methylation. In contrast, I consider transgenerational plasticity to include epigenetic inheritance along with many non-epigenetic processes that can occur during gestation, parturition, parental status, or parental investment (Yamanaka 1928, Lévy et al. 1993, Travis et al. 1999, Pryke and Rollins 2012). For example, transgenerational effects can even include aspects of parental investment that increase offspring survival, success, and fecundity (Alexander 1974, Feldman and Laland 1996, Westneat and Sargent 1996).

Recently, more attention has been focused on not just the potential of transgenerational plasticity between parents and offspring (i.e. maternal and paternal effects), but on the possibility of cues that are transmitted across multiple generations. Indeed, a growing body of work provides support that environments of grandparents can influence a diversity of traits of their grand-offspring (Alonso-Alvarez et al. 2007, Herman et al. 2012). Because of the multi-generation nature of these transgenerational effects they are often considered indicative of epigenetic inheritance mechanisms, but that is not an absolute requirement. For example, sequential effects of maternal investment or site choice could permit grandmothers with exceptionally high investment to produce higher quality daughters, themselves at an increased advantage for offspring investment and similar quality offspring (Plaistow et al. 2007, Monteith et al. 2009, Plaistow and Benton 2009). As with single generation parental effects, these transgenerational effects encompass a wide variety of mechanisms that can be mathematically expressed as reaction norms linking phenotypes in the current generation to environments experienced by progenitors.

Risk of depredation provides a strong basis for within- and among- generation plasticity. Plastic responses to predator cues are common in nature (Relyea and Auld 2005, Hossie and Murray 2012). One of the best documented responses to risk of depredation is physical alterations to body size and shape. Adjustments to body size are usually represented in two ways, individuals may increase their body size in order to surpass an easily consumed or palatable life history stage (Semlitsch 1990, Januszkiewicz and Robinson 2007). Alternatively, some species may decrease or limit overall body size in the presence of predator cues, making their capture relatively unrewarding to a predator (Smith et al. 2014, Rodgers et al. 2015). This latter pattern is frequently associated with a trade-off in the form of an associated tendency to mature at a smaller size or earlier age (Torres-Dowdal et al. 2012, Handelsman et al. 2013). Thus, the form of plasticity a group may take could depend upon both the nature of the predator-prey relationship and the nature of specific reproductive trade-offs.

In additional to changes in size, populations of many species express body shape alternations that increase evasion and escape from potential predators (Dahl and Peckarsky 2003, Burns et al. 2009, Fu et al. 2013). Commonly, these body shape alternations are manifested in a fashion that is thought to aid individuals in aspects of their aerodynamic or hydrodynamic performance for escaping depredation (McCollum and Leimberger 1997, Swaddle and Lockwood 1998, Fu et al. 2013). These alterations can include aspects of shape that enhance maneuverability, burst escapes or sustained flight, such as wing tip shape in birds (Swaddle and Lockwood 1998) or caudal region dimensions in fishes (Langerhans et al. 2004, Scharnweber et al. 2013). Again, we might suspect that the form of the actual plasticity expressed would be dependent on the nature of the predator-prey relationship. For example, morphological arrangements that aid burst speed and maneuverability might be favored by prey that evade stalking or lie-in-wait predators in complex habitats; whereas, arrangements that aid sustained speed or endurance might aid prey that must outpace predators in the open.

These types of plastic phenotypic responses to depredation risk, both in size and shape, are well established for cases of intra-generational, but what of size and shape responses to predator cues experienced by parents or grandparents? Examples of such transgenerational predator effects do exist, particularly between parents and offspring. For example, female aphids exposed to predator-cues produce more offspring with wings, presumably to aid in predator evasion and dispersal (Mondor et al. 2005). Likewise, snail offspring from exposed parents develop thicker shells and smaller apertures to dissuade depredation attempts (Salice and Plautz 2011). Most transgenerational plasticity studies in vertebrates have been focused at this single generation scale (Marsh-Matthews et al. 2005, Salinas and Munch 2012, Roche et al. 2012, Mommer and Bell 2013, but see Monteith et al. 2009). By comparison, many fewer studies have assessed the presence of transgenerational plasticity that might span more than a single generation, and where such work has been done, it has often been conducted in clonal organisms such as daphnia (Walsh et al. 2014) or springtails (Hafer et al. 2011). In theory, such multigenerational plasticity effects have the potential to be adaptive if predator conditions are maintained across generations (Bestion et al. 2014), or if encounters with predators are rare enough that a multigenerational sampling window provides a more integrative assessment of depredation risk than can be assayed by an individual's own limited environmental sampling abilities (as described in Chapter 3). However, just because such transgenerational effects might be adaptive in some contexts does not mean they necessarily exist for all species and traits. However, if such parental or grandparental effects are common or strong, this would be important to appreciate for studies that seek to understand how complex responses to predators shape prey evolution, ecology and their interactions.

Objectives

In this chapter I examine the capacity of nonlethal predator cues to induce transgenerational size and morphological effects in female Eastern Mosquitofish (*Gambusia holbrooki*). Specifically, I assessed 1) the degree that predator cues influence the size of offspring or grand-offspring, 2) the degree that predator cues influence the body shapes of offspring or grand-offspring and 3) whether such patterns of transgenerational plasticity are similar or different from those of intra-generational plasticity.

Methods

Study Species

Eastern Mosquitofish (*Gambusia holbrooki*) are an abundant species of poeciliids with a native range in Eastern North America (Lee and Burgess 1980). However, along with the closely related Western Mosquitofish (*Gambusia affinis*) these fishes are found nearly worldwide

(Brown 1987, Cote et al. 2011). These species are sexually dimorphic, with females larger than males. The greatly expanded modern day distribution of mosquitofish owes in large part to their extensive anthropogenic use for biological control of pest invertebrates (Meffe 1985). However, the traits of mosquitofish that make them so successful in biocontrol, such as high fecundity, short generation times, and viviparous reproduction (Krumholz 1948, Haynes and Cashner 1995) increase their ability to colonize new habitats as an IUCN Red List of invasive species (Pyke and White 2000). Likewise, mosquitofish have a wide diet niche and feed from many trophic levels (Goodsell and Kats 1999, Matveev et al. 2000) and they are aggressive, efficient feeders that can displace or extirpate native species (Carmona-Catot et al. 2013).

Central to this study, mosquitofish are often not the top predators in their communities, but are instead common prey for both piscivorous fishes and aquatic birds (Britton and Moser 1982, Meffe and Snelson 1989). The population used in this study coevolved with two centrarchid predators, Largemouth Bass (*Micropterus salmoides*) and bluegill sunfish (*Lepomis macrochirus*). Prior studies have shown that mosquitofish populations experiencing presence or absence of predators often differ in aspects of body shape, behavior and color (Horth 2004, Langerhans et al. 2004). Morphologically, mosquitofish populations that have coevolved with predators have streamline body shapes combined with an extended caudal peduncle to aid in velocity and burst escapes (Langerhans et al. 2004, 2005). Although past studies have tended to emphasize the role of predators in genetic population divergence, I have shown evidence for intra-generational phenotypic plasticity in mosquitofish morphology and behavior (Chapter 2, Arnett and Kinnison In Review). However, I hypothesize that Eastern Mosquitofish are also a good candidates for detecting transgenerational plasticity of body size and shape. As Poeciliid live-bearers, mother mosquitofish maintain prolonged contact with their embryos and transfer nutrients to offspring throughout the gestational period (Marsh-Matthews et al. 2005). Hence, mosquitofish may have more opportunity and a greater capacity to pass on non-genetic cues than many oviparous fishes (but see Salinas and Munch 2012). Along these lines, I showed in chapter 4 that female mosquitofish, but not males, are indeed subject to parental and grandparental effects on bold-shy behavior, and so one might hypothesize that females might show an integrated transgenerational plasticity response that also includes responses in size and body shape.

Experimental Procedures

All aspects for initial wild fish collection, laboratory acclimation, breeding of experimental lineages, and exposure to predator cues are described in the preceding chapter (Chapter 3). As with the previously described experimental design, the full experiment was replicated three times from the same common lab-acclimated stock, beginning at generation one. The resulting lineages are displayed in Figure 3.2, which was reproduced here in Figure 4.1 for convenience.

Assaying Trait Variation and Analyses

Upon reaching sexual maturity (based on development of secondary sexual traits), experimental individuals were euthanized with a lethal dose of MS-222 (>250 mgL^{-1}). Individuals were weighed (±4 mg) and photographed on their left side against a grid with a ruler for scale. Linear measures of body size dimensions were obtained using Image J (ver. 1.6.0_20, Rasband and NIH). These size measures included total length (snout to tip of caudal fin), total body depth (dorsal ventral measure at the deepest portion of the body), and caudal peduncle (shallowest portion of caudal region). Size measurements were analyzed with a multivariate analysis of variance (MANOVA) on a linear mixed effects model (LME). In the model, exposure treatment, treatment lineage, and sex were fixed effects while replicate served as a random effect. Models were constructed using backwards model selections and terms were removed if not significant ($p<0.05$). Subsequent univariate comparisons within fixed effect groups were performed using an ANOVA with the same model parameters for each size metrics.

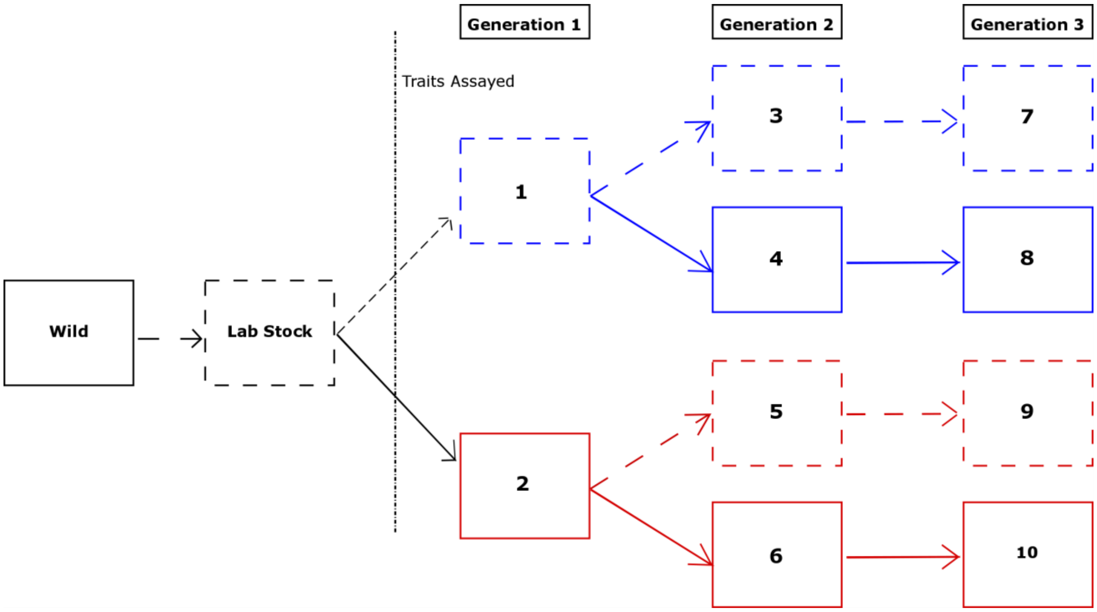

Figure 4.1: Lineage model for breeding and assay groups over three generations. Boxes represent groups of mosquitofish reared under a given set of predator cues. Boxes with solid borders and connecting lines represent predator-exposed treatment groups and boxes with dashed boarders and lines represent predator-free treatments. The blue colored boxes and arrows reflect the lineages of fish deriving from the non-predator-exposed fish in the first generation (box 1). The red colored boxes and arrows reflect the lineages derived from the predator-exposed fish in generation one (box 2). The group numbers, line colors and line style format in this figure are used as a convention in all subsequent figures and tables. Behavioral traits were assayed for all groups to the right of the vertical dashed line.

Using the fish photographs, data were collected using tpsDig2 (ver. 2.22, Rohlf and SUNY) for fifteen fixed landmarks (Figure 4.2, Appendix A) adapted from a series of landmarks established in Palkovacs et al. (2011). Geometric morphometric analyses were then performed on these landmarks to summarize overall fish body shape variation. Due to sexual dimorphism (Krumholz 1948, Chapters 2&5), relative warps were obtained for females separately from males. Twenty-six relative warps were obtained from a principle component analysis (PCA)

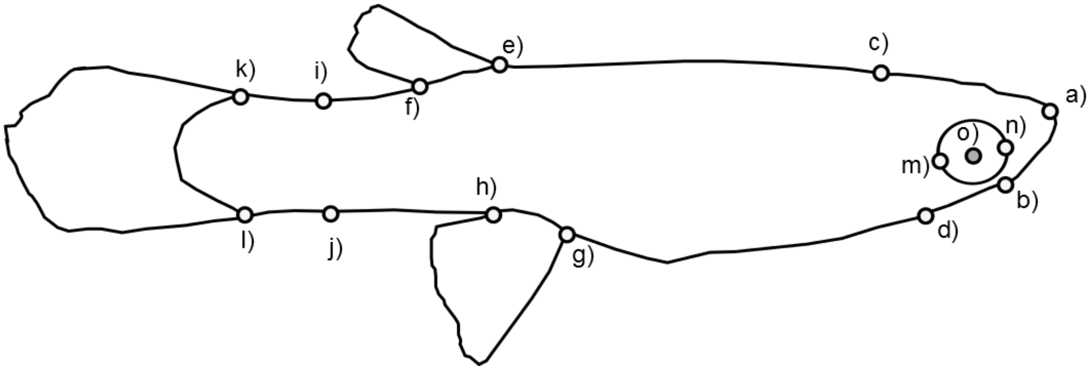

Figure 4.2: Morphometric landmarks for body shape comparisons. a)-d) are cranial markers, e)-h) and k)-l) are fin insertions and positions, i)-j) identify caudal peduncle, m)-n) are eye marks, and o) served as the eye centroid. Complete landmark descriptions may be found in Appendix A. Figure is duplicate from Figure 2.1 and included here for easy reference.

performed on the thin-plate spline variation using tpsRelw (ver. 1.62, Rohlf and SUNY). The first fifteen (clearly interpretable) relative warps were visualized to the extremes of actual sample variation, and interpreted for females (Figure C.1). A MANOVA on an LME performed on the full 26 relative warps using Exposure and Lineage factors (along with their interaction), and replicate as a random term, was used to initially test for intra-generational and transgenerational plasticity effects and established relative warps of particular significance to body shape under these conditions (Table C.1). Pairwise ANOVAs between treatment groups with each of these four significant relative warps were subsequently performed to further interpret the overall MANOVA models. To help visualize shape trends associated with current Exposure to predators or transgenerational Lineage effects, a discriminant function analysis (DFA) was performed on the relative warps pooling fish by either Exposure type or Lineage type within a given generation. The associated Exposure or Lineage DFA scores were then visualized by regressing scores back on landmark deformations using tpsRegr (ver. 1.42, Rohlf and SUNY).

All statistical analyses, beyond the summarization of shape using geometric morphometrics, were performed using the R Programming Environment (Ver. 3.1.1, R Core Team 2014) 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 *effsize* (Torchiano Ver. 0.54). For the same reasons noted in the preceding chapter (used the same breeding design), I considered p-values less than 0.1 as significant and values less than 0.05 as highly significant.

Results

Body Size: Intra-generational Plasticity

Generation one females did not show a significant overall effect of exposure treatment on body size in MANOVA (Table 4.1), indicating little if any effect of intra-generational plasticity (Figure 4.3). Likewise, there were no indications of significant exposure effects on individual size traits (Table D.1). This absence of an intra-generational exposure effect was consistent for the two remaining pairwise comparisons of offspring split between predator exposed and non-exposed conditions in generation two (groups 3 v 4; 5 v 6) (Table 4.2, Figure 4.3). There was some evidence of an overall Exposure effect for generation three. Specifically, fish that reared in the presence of predators tended to be somewhat larger than those that had not. However, this third generation exposure effect cannot be uniquely attributed to intra-generational plasticity alone, because these third generation females came from parents with the same exposure and not from splitting of broods like the first two generations. As such, Exposure effects at generation three could in principle include potential parental effects transmitted from the second generation.

Table 4.1: Reduced complete MANOVA on size metrics for all three generations of female mosquitofish. Model includes total length, body depth, tail peduncle, and weight. Lineage refers to the exposure treatment of the first generation relative (parent or grandparent). All two-way interactions were included, interactions with non-significant terms (p>0.10) were removed. Model was reduced using backwards selection. Significance (p<0.10) is represented with an asterisk (*), and highly significant values (p<0.05) are represented with a double asterisk (**). Effect size values are Eta Squared, using exposed treatment values as the reference values.

Females	Effect	dF	F-value	p-value	Significance	Effect Size
Gen 1	Exposure	1,55	0.19	0.67		0.23
Gen 2	Exposure	1,120	0.40	0.53		0.03
	Lineage	1,120	0.04	0.85		0.07
Gen 3	Exposure	1,101	5.32	0.03	**	0.05
	Lineage	1,101	0.79	0.38		0.22

Table 4.2: MANOVA pairwise comparisons on size metrics within generations for female mosquitofish. Model compares groups within each generation using the four size metrics, total length, body depth, tail peduncle, and weight. Numbered group comparisons within generations reference breeding schematic Figure 5.1. Model was reduced using backwards selection. Significance (p<0.10) is represented with an asterisk (*), and highly significant values (p<0.05) are represented with a double asterisk (**). Effect size values are Eta Squared, using exposed treatment values as the reference values.

Females		Isolated Effect	dF	F-value	p-value	Significance	Effect Size
Gen 1	1 vs 2	Exposure	1,55	0.19	0.67		0.23
Gen 2	3 vs 4	Exposure	1,72	0.59	0.45		0.01
	5 vs 6	Exposure	1,47	0.02	0.88		0.29
	3 vs 5	Lineage	1,61	0.07	0.79		0.13
	4 vs 6	Lineage	1,57	0.04	0.83		0.10
	3 vs 6	Both	1,47	2.59	0.11		0.32
Gen 3	7 vs 8	Exposure	1,50	2.12	0.15		0.03
	9 vs 10	Exposure	1,49	3.38	0.07	*	0.15
	7 vs 9	Lineage	1,49	0.49	0.49		0.10
	8 vs 10	Lineage	1,50	0.45	0.51		0.40
	7 vs 10	Bothz	1,49	5.16	0.03	**	0.40

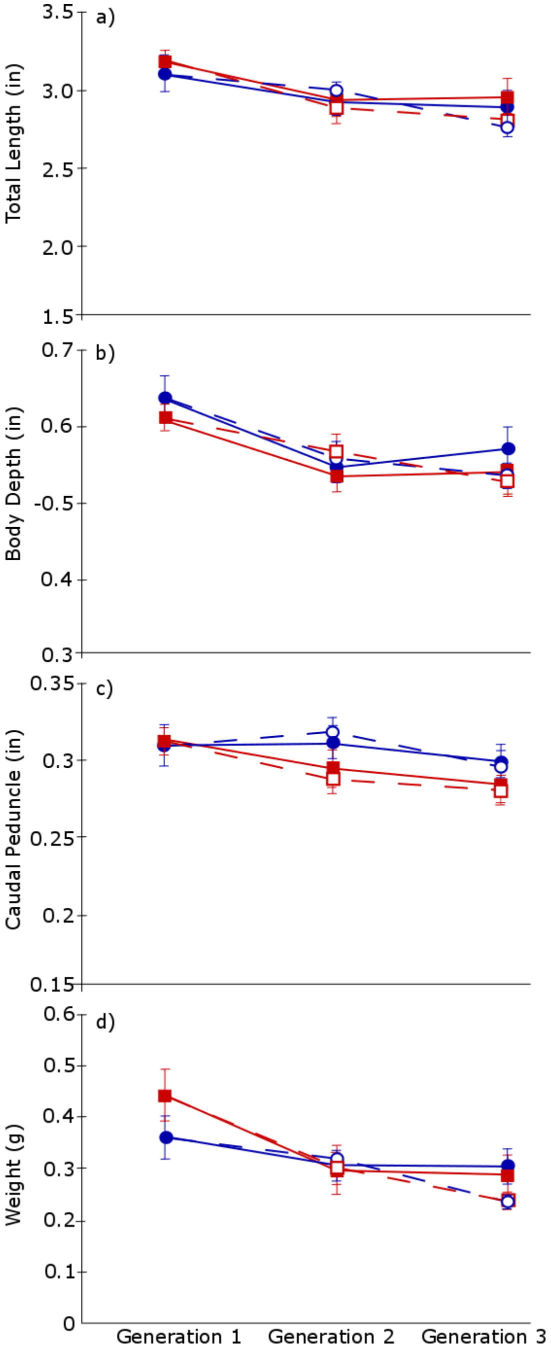

Figure 4.3: Individual size metric response trajectories across all generations for female mosquitofish. Panel a) is total length (snout to tip of tail), panel b) is body depth (deepest portion of body), panel c) is caudal peduncle depth (slimmest portion of caudal region), and panels d) is weight. Red colors (square points) are fish with exposed lineages (generation 1 exposure treatment). Blue colors (round points) are fish with non-exposed lineages. Filled markers with solid lines are groups that were themselves exposed to predator cues in that generation, while open markers with dashed lines are groups from non-exposure treatments. Color and line scheme follows the pattern established in the breeding schematic Figure 4.1.

Body Size: Transgenerational Plasticity

Generation two "daughters" of the generation one groups showed no significant interactions or fixed effects of Lineage (generation one parental exposure effect) or current Exposure on overall body size when assessed at a multivariate scale (Table 4.1). That said, there was some modest evidence of a univariate Lineage effect for caudal peduncle depth (p=0.06), with non-exposed individuals from exposed parents having more slender caudal regions than those with non-exposed parents (groups 3 v 5; 4 v 6) (Figure 4.3c, Table D.1), though some comparisons were non-significant. Females from generation two were significantly smaller than generation one females overall (Table 4.3), regardless of the individual's exposure or lineage treatment.

Table 4.3: MANOVA pairwise comparisons on size metrics across generations for female mosquitofish. Model compares groups within each generation using the four size metrics, total length, body depth, tail peduncle, and weight. Numbered group comparisons within generations references breeding schematic Figure 4.1. Model was reduced using backwards selection. Significance (p<0.10) is represented with an asterisk (*), and highly significant values (p<0.05) are represented with a double asterisk (**). Effect size values are Eta Squared, using exposed treatment values as the reference values.

		dF	F-value	p-value	Significance	Effect Size
Gen 1 and Gen 2	1 vs 3	1,66	1.20	0.28		0.18
	1 vs 4	1,64	3.19	0.08	*	0.30
	2 vs 5	1,50	9.95	0.003	**	0.21
	2 vs 6	1,48	15.44	<0.0001	**	0.21
Gen 1 and Gen 3	1 vs 7	1,53	18.75	<0.0001	**	0.19
	1 vs 8	1,54	8.67	0.005	**	0.10
	2 vs 9	1,50	33.51	<0.0001	**	0.35
	2 vs 10	1,50	6.86	0.01	**	0.02
Gen 2 and Gen 3	3 vs 7	1,61	3.86	0.05	*	0.17
	4 vs 8	1,60	0.08	0.78		0.11
	5 vs 9	1,49	1.01	0.32		0.10
	6 vs 10	1,47	1.00	0.32		0.05

Generation three “granddaughters” of generation one grandparents again did not show multivariate support for a Lineage effect (Table 4.1). As with generation two, generation three females were smaller overall than generation one females but were not significantly different in size at age than generation two fish for most paired comparisons (Figure 4.3, Appendix I). By this final generation, the cumulative effects of combined grandparent, parent, and individual exposure or non-exposure (groups 7 vs 10) was significant, suggesting that weak intra-generational and transgenerational effects might accumulate into overall larger group differences in size (particularly total length) across generations where exposures are consistent (Table 4.2; Appendix H).

Body Shape: Intra-generational Plasticity

Generation one females did not show significant overall effects of the current Exposure treatment on body shape in MANOVA when all relative warps were combined in a single analysis (Table 4.4). However, examination of individual relative warps indicated that relative warp three (RW 3) did differ between exposed and non-exposed lineages (Figure 4.4b, Table D.3) with exposed individuals showing streamlining of body form in having a reduction in cranial features, and a degree of narrowing of the caudal peduncle with the narrowest caudal region moved more anterior (Figure 4.5). For generation two, the overall MANOVA a significant interaction between Exposure and Lineage (Table 4.4). Based on subsequent pairwise MANOVA comparisons for exposed and non-exposed fish, that interaction was associated with a strong effect of current Exposure within the predator-exposed Lineage (groups 5 v 6) but no current Exposure effect in the Lineage with non-exposed parents (groups 3 v 4).

Table 4.4: MANOVA of 26 relative warps for three generations of female mosquitofish. Lineage refers to the exposure treatment of the first generation relative (parent or grandparent). All two-way interactions were included, interactions with non-significant terms ($p>0.10$) were removed. Model was reduced using backwards selection. Significance ($p<0.10$) is represented with an asterisk (*), and highly significant values ($p<0.05$) are represented with a double asterisk (**). Effect size values are Eta Squared, using exposed treatment values as the reference values.

Females	Effect	dF	F-value	p-value	Significance	Effect Size
Gen 1	Exposure	1,47	0.006	0.94		0.12
Gen 2	Exposure	1,119	11.95	0.0008	**	0.11
	Lineage	1,119	0.37	0.55		0.10
	Exposure: Lineage Interaction	1,119	5.54	0.02	**	0.21
Gen 3	Exposure	1,101	0.31	0.58		0.03
	Lineage	1,101	0.06	0.81		0.03

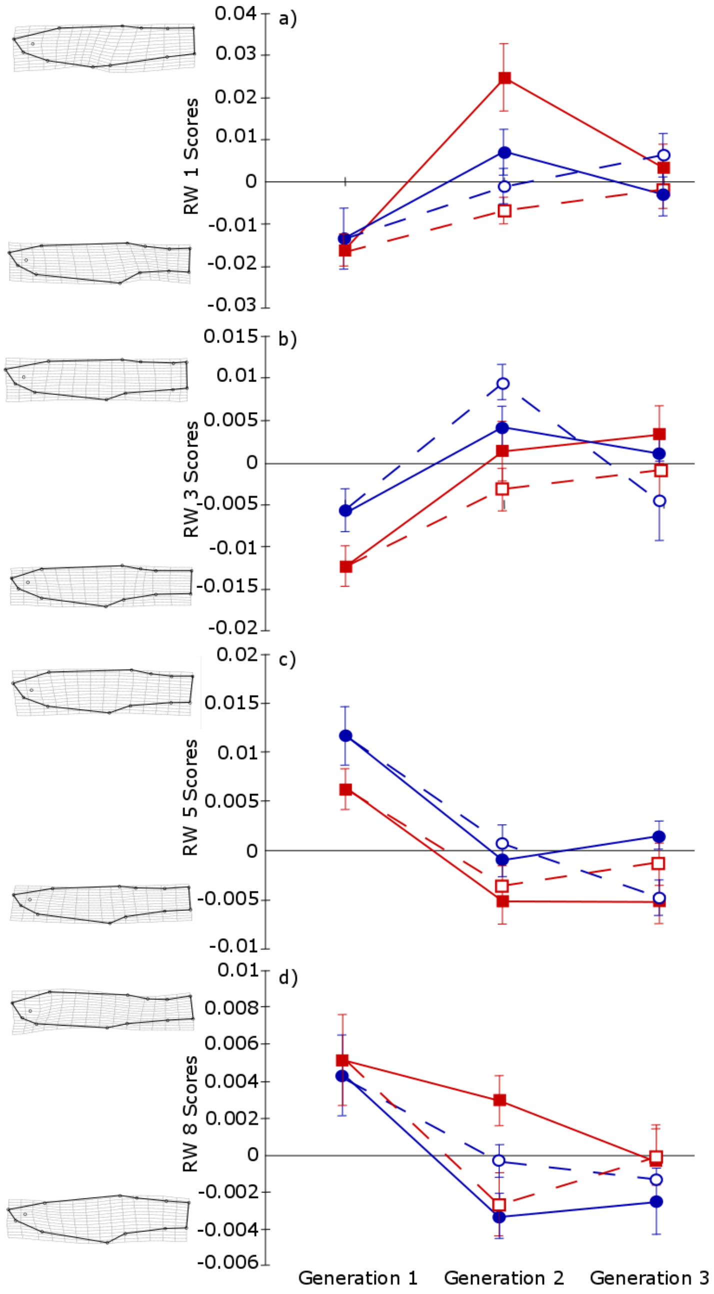

Figure 4.4: Individual significant relative warp score trajectories for three generations of female mosquitofish. Individual panels refer to descriptions from table 5.1. Red colors (square points) are fish with exposed lineages (generation 1 exposure treatment). Blue colors (round points) are fish with non-exposed lineages. Filled markers with solid lines are groups that were themselves exposed to predator cues in that generation, while open markers with dashed lines are groups from non-exposure treatments. Color and line scheme follows the pattern established in the breeding schematic Figure 4.1.

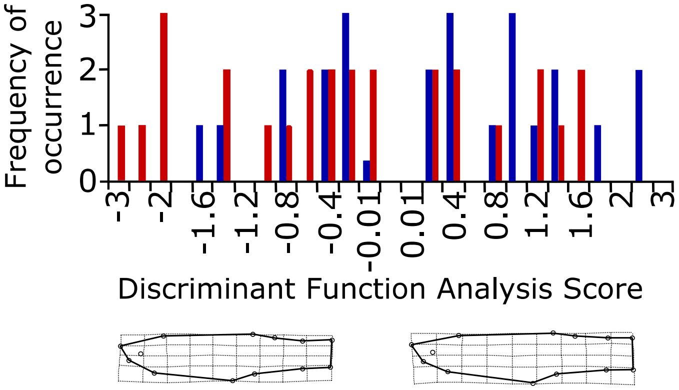

Figure 4.5: Morphometric visualizations based on DFA scores for generation one female mosquitofish. Exposure DFA scores were based on the full RW set for all three generations. Red bars indicate exposed fish while blue bars indicate fish that were non-exposed.

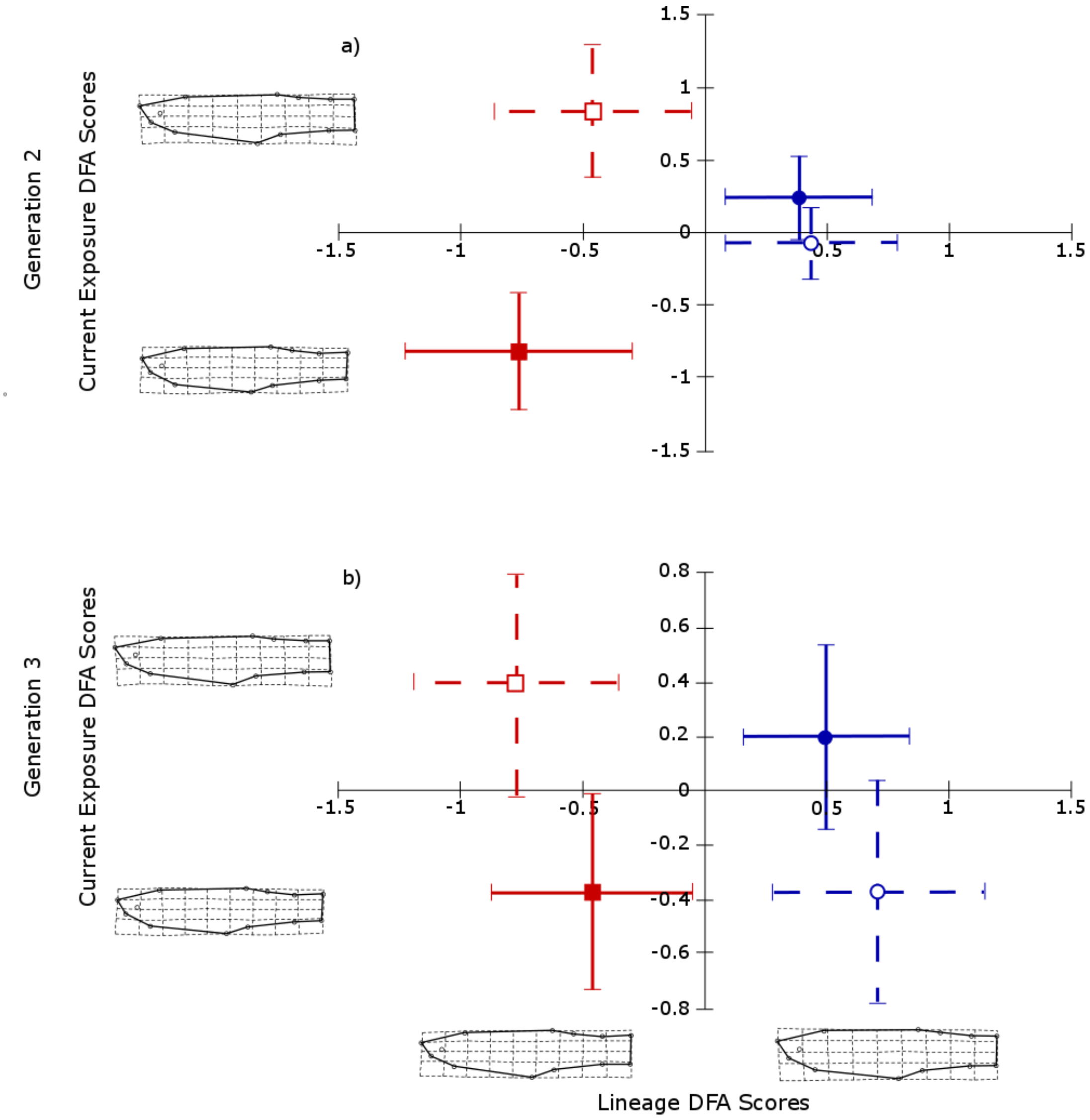

Figure 4.6: Regression of relative warp visualizations and DFA biplots for generations two and three of female mosquitofish. Visualizations were regressed against current Exposure treatment DFA scores (y-axis) and Lineage DFA scores (x-axis). Biplots represent the average and 95% confidence intervals for each treatment group based on the Exposure and Lineage DFA scores. Red colors (square points) are fish with exposed lineages (generation 1 exposure treatment). Blue colors (round points) are fish with non-exposed lineages. Filled markers with solid lines are groups that were themselves exposed to predator cues in that generation, while open markers with dashed lines are groups from non-exposure treatments. Color and line scheme follows the pattern established in the breeding schematic Figure 4.1.

Body Shape: Transgenerational Plasticity

As noted above, MANOVA of overall body shape of generation two daughters showed a significant interaction between Lineage and current Exposure (Table 4.4), suggesting that Lineage influences how Exposure effects are expressed and vice versa. DFA visualization of this Lineage pattern suggests that the shape trend was significant ($F_{1,120}$=26.60, p<0.0001) and was broadly toward greater streamlining in the predator exposed lineage, particularly in the region of the abdomen and posterior aspects of the caudal peduncle, with some reduction in head proportions (largely length)(Figure 5.6a). Examination of pairwise MANOVAs within generation two supported that parental exposure to predators had a strong effect on their daughters that reared in the absence of predators (groups 3 v 5), but showed less of a difference for daughters that reared in the presence of predators (groups 4 v 6) (Table 4.5, Figure 4.4, Figure 4.6a). Examination of pairwise comparison for individual relative warps suggested most Lineage effects were tied to warps 1, 3, 5 and 8, which again show broad patterns consistent with streamlining in various portions of the body (Figure 4.5; Appendix K). This is also consistent with the narrower caudal (tail) depth detected in our assessment of fish size.

Generation three females did not show overall MANOVA support for either Exposure (carried through from generation 2) or Lineage effects in the full model (Table 4.4). Likewise, none of the pairwise MANOVA comparisons within generation three (7 v 9, 8 v 10) supported a Lineage effect. However, testing of Lineage and Exposure effects with DFAs that pooled within factor levels did provide some support of a Lineage effect ($F_{1,101}$=39.87, p<0.001) (Figure 5.6b). Specifically, females again showed a significant effect of Lineage with predator-exposed grandparents streamlining via dorsal-ventral flattening of the thoracic and abdominal regions, cranium, and snout (Figure 5.6b).

Table 4.5: Within generation pairwise MANOVA comparisons for 26 relative warps for three generations of female mosquitofish. Lineage refers to generation one exposure (parent or grandparent) and Exposure refers to current environmental conditions. Significance (p<0.10) is represented with an asterisk (*), and highly significant values (p<0.05) are represented with a double asterisk (**). Effect size values are Eta Squared, using exposed treatment values as the reference values.

Females		Isolated Effect	dF	F-value	p-value	Significance	Effect Size
Gen 1	1 vs 2	Exposure	1,47	0.006	0.94		0.12
Gen 2	3 vs 4	Exposure	1,71	1.55	0.22		0.13
	5 vs 6	Exposure	1,47	14.39	0.0004	**	0.50
	3 vs 5	Lineage	1,61	4.84	0.03	**	0.32
	4 vs 6	Lineage	1,56	0.92	0.34		0.26
	3 vs 6	Both	1,59	5.20	0.03	**	0.30
Gen 3	7 vs 8	Exposure	1,50	2.91	0.09	*	0.20
	9 vs 10	Exposure	1,49	0.47	0.50		0.07
	7 vs 9	Lineage	1,49	1.46	0.23		0.06
	8 vs 10	Lineage	1,50	0.69	0.41		0.14
	7 vs 10	Both	1,49	0.40	0.53		0.07

Discussion

Female body size showed relatively little evidence of plasticity in response to predator cues at either intra- or inter-generational scales. In contrast, there was broad evidence that predator exposure does influence body shapes of female mosquitofish in a fashion that generally favors streamlining of the body of the including reductions in head and body depth and a re-proportioning of the caudal peduncle to favor a somewhat longer and narrower profile. Individuals showed a mixture of intra-generational (current Exposure) and transgenerational (generation one Lineage) responses to parent and grandparent exposure, particularly in body shape showing reductions in cranial and body depths while longitudinally expanding the caudal region. These patterns are interesting in suggesting that shape at age may be more responsive to predators than size at age. I discuss these patterns of morphology with regards to what they might tell us about the relative importance of current versus ancestrally inherited predator cues for adaptive phenotypic plasticity in female mosquitofish.

Body Size Does Not Express Plasticity

While it may be intuitive that size would influence predation risk, we did not detect significant intra-generational or transgenerational plasticity in size. This outcome is consistent with my previous analysis of intra-generational plasticity for two species and both sexes of mosquitofish (Chapter 2, Arnett and Kinnison In Review). Intra-generational size plasticity does occur in other fishes (Januszkiewicz and Robinson 2007), commonly to increase overall body size and surpass predator gapes (Cowan et al. 1996, Januszkiewicz and Robinson 2007). However, even the largest mosquitofish are much smaller than the average gapes of their major predators, such as Largemouth Bass, so plastic increases in size are unlikely to yield much in the way of gape limitation benefits. Conversely, modest increases in size could make individual mosquitofish more energetically valuable and thus attractive as food items for predators (Britton and Moser 1982, Schumann et al. 2012). Along these lines, a plastic reduction in body size occurs in some prey taxa facing predator cues (Blanckenhorn 2000) and this has been attributed as a potential adaptation to reduce energetic transfer and foraging value of the prey relative to the effort expended by predators (Werner and Hall 1974, Goss-Custard 1977).

However, in theory, plastic changes in size at age might also arise as a correlated response to plastic alterations in life history traits. Individuals that mature at younger ages are expected to also generally mature at smaller sizes. My experiments were not expressly designed to examine plastic responses in life history traits, and relatively little is known of the potential for such responses in poeciliid fishes in general (but see Stearns 1983a, Torres-Dowdal et al. 2012) because most work on life history variation on this group of fishes has emphasized genetic trait divergence (Reznick and Endler 1982, Stearns 1983a, 1983b, Ghalambor et al. 2004). Nonetheless, the lack of evidence for plastic effects of predators on size at maturity in my study might be considered indirect evidence that predator cues did not induce dramatic shifts in age at maturity. However, this again begs the question of why not, particularly given that studies of population divergence in the wild have often emphasized the importance of such life history traits for fitness (Reznick and Endler 1982, Ghalambor et al. 2004).

One possibility is there may be constraints on the capacity for smaller (and earlier) size at maturation in the populations I studied. Mosquitofish courtship and mating occurs almost immediately upon sexual maturation (Bisazza et al. 1989) and females are commonly harassed or coerced into mating (Bisazza et al. 2001, Dadda et al. 2008), resulting in individuals that are pregnant throughout most of their short sexually mature adulthood. Given that fecundity is also strongly correlated with female size in fishes (Milton and Arthington 1983, Berglund et al. 1986, Herdman et al. 2004, Farley et al. 2015), and strongly capped by the number of embryos that a female can carry to term in live bearers, this could place selective restrictions on how small females can be and still produce sufficient numbers of offspring. This might seem at odds with other studies of poeciliids that show interpopulation variation in life history traits like age at maturity and offspring number (Reznick and Endler 1982, Stearns 1983a, 1983b), but might not be when taking into account the population source of my fish which derived from a high-predation environment containing bass and other predators. Such a population might already be genetically predisposed to mature at the near limits for small size and early age, leaving less opportunity for intra- or transgenerational plasticity to further modulate these traits.

Multigenerational Plastic Responses of Body Shape

Contrary to body size, I did detect evidence for body shape plasticity in mosquitofish toward more streamlined body configurations for fish experiencing direct or ancestral transmitted predator cues. A streamlining pattern was most apparent for parental effects (generation 2) of predator exposure, but analogous morphological patterns were apparent in pooled DFA comparisons of third generation females from different lineages and in examination of individual relative warps for intra-generational plasticity. Relative streamlining of body shape is a common phenotypic difference found in comparisons of wild populations living in the presence versus absence of predators (Langerhans et al. 2004, González and Gianoli 2004, Hendry et al. 2006, Fu et al. 2013). The most common pattern noted for poeciliid fishes is a reduction in the area of the head, a reduction in the depth of the body in the region of the abdomen and anal fin, and an expansion in the relative area of the caudal region compared to the rest of the body. These alterations are generally ascribed to predators driving selection and population evolution that favors hydrodynamic body shapes conducive to rapid escape from actual predator attacks (Langerhans et al. 2004, Dayton et al. 2005, Hendry et al. 2006, Borazjani 2013), via increases in force generation and decreases in drag (Blob et al. 2008, Fu et al. 2013). Conversely, populations without fish predators have larger populations that experience more competition. Under such conditions larger heads and deeper bodies might benefit individuals by permitting individuals to consume larger prey, including insects entering from terrestrial habitats (Chapter 5, Rowland et al. 2015) and store more energy reserves (Einen et al. 1998).

I will note that there was a subtle difference between the plastic shape effects noted in the present study and these in prior investigations focusing on population divergence. Specifically, while other studies have often emphasized an increase in the depth or overall area of the caudal peduncle region in populations with predators, my findings often pointed toward a reduction in the vertical depth of this region, with some mixed evidence for increases in other proportions. Indeed, this was true for not just geometric morphometric assays, but also for some comparisons involving simple univariate comparisons of caudal peduncle depth. Nonetheless, the overall streamlining pattern associated with predators, that appears to be conserved across population comparisons, species and sexes, also appears to apply to plasticity both within (Chapter 2) and now among generations. Indeed, this finding again supports my prior assertion (Arnett and Kinnison in Review, Chapter 2) that the severe hydrodynamic constraints (Domenici and Blake 1997) associated with effective escape from a predator attack strongly constrains the morphological axis along which phenotypes might be molded by the joint effects of adaptive divergence or plasticity in response to current or ancestral environments (Arnett and Kinnison In Review, Chapter 2).

<u>Integration of Multigenerational Plasticity Effects</u>

In this chapter I provide evidence that transgenerational plasticity occurs in morphology in response to predator cues, and in Chapter 4 I found that female mosquitofish also became less bold in the presence of parental and grandparental predator cues. Indeed, for both types of traits, there was less support for intra-generational plasticity than transgenerational plasticity. The presence of such a pattern for two types of traits would in theory suggest that transgenerational cues of predator presence might often be more reliable indicators of predation risks for female mosquitofish than even an individual's own narrower range of experiences. Transgenerational cue reliability is not itself expected to vary with different traits, and so an integrated plastic response might be generally expected for behavior and morphology, and even other traits that I did not directly study (e.g., life history). There is also reason to suspect integrated transgenerational plasticity on that grounds that mismatched behavioral and morphological responses could nullify one another. For example, the morphological benefits of a relatively streamlined and inconspicuous body shape could be undone if paired with the wrong types of bold behaviors that draw the attention of predators. Similarly, in the absence of predators, individuals that are too shy might not appreciate the potential competitive advantages of a large head and deep body.

However, I do not think it is entirely clear from my analyses that such integration of transgenerational plasticity exists at the level of the phenotypes and genotypes of individuals. There were nuances across analyses to suggest a potentially more complex picture. For example, transgenerational plasticity of morphology showed more of an interaction with an individual's current exposure than behavior. Also, transgenerational plasticity of behavior was more apparent at the scale of grandparent effects for behavior than for morphology. Hence, while I think there is reason to expect possible integration of transgenerational plasticity across trait types, this remains to be rigorously tested. My dataset includes assays of behavior and morphology for most of the same individuals, and thus might be used as a first-cut analysis of such integration in the form of phenotypic correlations. However, a more rigorous analysis would look for genetic correlations, which is not possible with my dataset because I was unable to track siblings when they were split among exposure treatments.

CHAPTER 5: ECOLOGICAL IMPLICATIONS OF SEX RATIO, DENSITY, AND POPULATION SOURCE IN EASTERN MOSQUITOFISH (*Gambusia holbrooki*)

Context Statement

This chapter of my dissertation reflects a novel idea for how an often-neglected form of intraspecific trait variation might be important in shaping aquatic communities and ecosystems. Prior to the work described in this chapter, no studies had considered the combined ecological significance of sexual dimorphism and sex ratio variation. It also represented the first time I, and the broader research team with which I collaborated, conducted a mesocosm experiment with wild mosquitofish. The goal of the experiment was very ambitious, to consider not just sex ratio effects but to also contrast these with effects of population divergence and densities. Ultimately, we likely integrated more pieces than we should have, and there were some logistical shortcomings in this initial experiment that likely limited our inferences. Nonetheless, this work provided insights and impetus to conduct a subsequent mosquitofish experiment, with a modified design, that proved very successful and indeed showed that sex ratio variation, combined with sexual dimorphism, can strongly shape community and ecosystem conditions in aquatic mesocosms. That subsequent study was recently published in the *Proceedings of the Royal Society B* and is included as Appendix L. While this chapter is largely written as a standalone study, I think it is useful for readers to also be aware of how the study rationale, design, and findings I present here were important to facilitating the broader team experiment, and also that some of my conclusions here are based on hindsight and my knowledge of those subsequent experimental outcomes.

Introduction

A growing body of literature suggests ways in which trait variation among populations can shape local ecological outcomes at population, community, and ecosystem scales (Kylafis and Loreau 2008, Pelletier et al. 2009, Bell and Gonzalez 2011, Palkovacs et al. 2011, Carlson et al. 2011). In general, these studies have focused on trait variation associated with natural selection driving adaptations to local abiotic and biotic conditions. But what about trait variation shaped by other modes of selection, such as sexual selection? Is such variation ecologically irrelevant?

As I review below, sexual selection often gives rise to sexual dimorphism, which is in turn an overt component of phenotypic variation within populations of many organisms. Sexual dimorphism is commonly studied with respect to the different reproductive roles of male and females, but such trait differences do not exist in a vacuum and there are good reasons to suspect that many of the overt differences between males and females, including differences in size and behavior, might influence their ecological roles in communities and ecosystems. One consequence of such ecological difference is that males and females may experience different mortality risks and thus the sex ratios of wild populations can become markedly skewed in favor of one sex or the other depending on local conditions. In combination, highly skewed sex ratios and ecological differences between males and females could lead to marked differences in communities and ecosystems. In this chapter, I provide background to support components of this theory and then experimentally consider how sexual dimorphism, through its potential combined effects on sex ratios and the ecological role of males and females, might be important in shaping community and ecosystem conditions.

Sexual Dimorphism and Sexual Segregation

Sexual dimorphism takes many forms. It can be overt in the case of morphological differences between the sexes (Walker and Rypstra 2002, Drovetski et al. 2006, Stein et al. 2008), or more cryptic in the case of physiological and behavioral differences (Walker and Rypstra 2002, Stein et al. 2008). Size dimorphism is common in many species, with either males or females being the larger sex, depending on the importance of size for fecundity in females or for mate competition in males (Krüger 2005, Morbey and Guglielmo 2006, Santos et al. 2007, Lailvaux and Irschick 2007). Such size dimorphism has been associated in several cases with different energetic needs and associated foraging strategies (Walker and Rypstra 2002, Drovetski et al. 2006, Kamilar and Pokempner 2008). Although the most common perspective on such dimorphism is that it reflects the product of sexual selection on males and females, sexual dimorphism may also reflect some degree of specialization to alternate niches driven by natural selection. Akin to models of sympatric speciation, competition for limited resources may drive disruptive selection on the sexes and favor the development of alternate phenotypes that differ genetically in their niche preference and adaption (Slatkin 1984, Bolnick and Lau 2008). This ecological differentiation of the sexes based on competitive release is often referred to as “sexual segregation” (Bleich et al. 1997, Phillips et al. 2011).

For example, when females are larger they often require a larger pool of resources (Kamilar and Pokempner 2008) and higher quality food (Wilkinson and Barclay 1997) to support their size and associated greater egg number or quality. Such females tend to spend greater time foraging than males (Weimerskirch et al. 1997, Barclay et al. 2000). In this case, males can be outcompeted for similar resources and this may act as a driver towards behavioral divergence and diet segregation (Phillips et al. 2011). In this fashion, sexual dimorphism can lead to sexual niche segregation and character displacement akin to processes that have been more extensively studied in the context of ecological speciation (Abrams 1986, Kie and Bowyer 1999, Schluter 2000). It is not a large conceptual leap to suggest that sexual divergence in the intensity and the targets of consumption could in turn lead to different effects of males and females on their community and ecosystems.

In fishes, sexual dimorphism is often witnessed as differences in size, with females often larger than males (Berglund et al. 1986, Parker and Parker 1992, Santos et al. 2007, Herler et al. 2010), but differences in ornamentation, tissue allocation, life histories, and behavior are also very common. Males tend to have longer fins, colored bodies with ornamented fins or scales, larger hearts, and a higher ratio of somatic to reproductive body mass (Hopkins et al. 1990, Santos et al. 2007), most commonly traits that facilitate intrasexual aggression and competition for females. Females conversely have large livers and intestines (Santos et al. 2007), lending to increased nutrient partitioning efficiency from food to support a larger ratio of reproductive to somatic mass (Slatkin 1984). From a life history perspective, males often mature at a younger size and age than females, largely due to the substantial increase in potential fecundity females appreciate from a longer period of growth and larger size (Bell 1980, Stearns 1992). Behaviorally, females of some species spend more time foraging in the relative safety of benthic and littoral habitats (Willacker et al. 2013), therefore accessing a different set of resources and habitat than their male counterparts.

Sex Ratio Variation in Nature

While it is interesting that males and females may have different ecological effects within their communities and ecosystems, those differential effects are not expected to be very important to geographic variation in community or ecosystem conditions where sex ratios are nearly always the same wherever the species occurs. However, sex ratios of many ecologically dominant species can vary widely among geographic locations (Table 2.1). Such sex ratio differences might derive from sexual differences in habitat associations (Komdeur et al. 1997), dispersal or migratory patterns (Morbey 2002, Miller and Scarnecchia 2011, Nelson and Greeff 2011), or intersexual competition (Byholm et al. 2002, Stein et al. 2008). However, in many cases such differences in sex ratios are likely the result of differential mortality (Britton and Moser 1982), itself a potential consequence of niche dimorphism, that can lead to extremely skewed sex ratios depending on local environmental conditions. In particular, local variability in predators and competitors, or the various food or other resources that males or females depend upon for survival, are expected to shape whether sex ratios strongly favor males or females (Table 5.1).

A good example of this is many prey fish species. Many prey fishes are differentially affected by fish versus avian predators. Piscivorous fishes often target smaller prey individuals to overcome gape limitations and escape abilities tied to size (Nilsson and Brönmark 2000), whereas avian predators can be positively size selective due to the different mechanisms by which they capture prey (Britton and Moser 1982). Hence, geographic variation in the relative prevalence of piscivorous fishes and birds could lead to very different patterns of net size selectively on prey fish and in turn drive skewed sex ratios in species with sexual size dimorphism (Britton and Moser 1982, Godin 1995). Regardless of the exact mechanism, once sex ratios differ among sites, sexual dimorphism could, in principle, take on more importance as a factor shaping community and ecosystem conditions.

Table 5.1: Common variation in sex-ratio across taxa. Included studies are field or natural populations studies finding variation in sex ratio across populations of a given taxa.

Class	***Sex Ratio (%Female)***	***Sex Ratio Driver***	***Species***	***Source***
Fishes	79-97%	Fishing	*Mycteroperca*	McGovern et al. 1998
	30-93%	pH	*Apistogamma*	Roemer and Beisenherz 1996
	3-60%	Predation	*Gambusia*	Britton and Moser 1982
	0-50%	Regional Reproductive Strategies	*Plectropomus*	Adams et al. 2000
	5-70%	Temperature	*Apistogamma*	Roemer and Beisenherz 1996
	15-90%	Temporal Variation	*Rhinomugil*	Mortuza and Rahman 2007
	20-99%		*Salmo*	Consuegra and García De Leániz 2007
Herps	20-75%	Regional Mating Strategy	*Dendrobates*	Prohl 2002
	32-57%	Temperature	*Niveoscincus*	Wapstra et al. 2009
	0-100%	Temporal Variation	*Chrysemys*	Bowden et al. 2000
Aves	45-53%	Density	*Parus*	Michler et al. 2012
	45-82%	Maternal Condition	*Larus*	Nager et al. 1999
	20-70%	Migration Distance	*Junco*	Ketterson and Nolan Jr. 1976
	0-100%	Regional Mating Strategy	*Ficedula*	Ellegren et al. 1996
	32-58%	Temporal Variation	*Falco*	Dijkstra et al. 1990
	20-100%	Territory Quality	*Acrocephalus*	Komdeur et al. 1997
Mammals	24-55%	Competition	*Galago*	Clark 1978
	35-51%	Maternal Quality	*Cercus*	Kruuk et al. 1999
	50-85%		*Didelphis*	Austad and Sunquist 1986
	20-96%		*Equus*	Cameron and Linklater 2007
	30-67%	Resource Allocation	*Microtus*	McShea and Madison 1986

Trait Variation and Ecology

A substantial body of literature on "community genetics" (reviewed in Whitham et al. 2006) and "eco-evolutionary dynamics" (Pelletier et al. 2009, Schoener 2011) has emerged over the last decade demonstrating how intraspecific trait variation can have wide reaching effects on communities and ecosystems. The majority of studies of such ecological effects in animals have centered on predator avoidance, life history, and trophic traits (Yoshida et al. 2003, Hairston Jr et al. 2005, Fussmann et al. 2007, Bailey et al. 2009, Palkovacs et al. 2009, 2011, Post and Palkovacs 2009). Indeed, these various dimensions of divergence often co-vary and may be linked through a tradeoff between ability to successfully evade and reproduce in the presence of intensive predation versus the ability to compete with high densities of conspecifics where predation is less intense (Reznick and Endler 1982, Palkovacs et al. 2011). For example, in poeciliid fish, like guppies (*Poecilia reticulata*), the intensity with which fish forage and the relative proportions of cranial features contributing their foraging efficiency varies inversely with streamlining of their caudal anatomy and associated predator escape performance (O'Steen et al. 2002, Langerhans and DeWitt 2004, Langerhans et al. 2004, Hendry et al. 2006, Palkovacs et al. 2011, Torres-Dowdal et al. 2012). Experiments with guppies in stream mesocosms in turn show that such divergence has significant implications for community and ecosystem conditions, including algal biomass. Experiments and models suggest this is driven in large part by a tendency for omnivorous guppies from low predation systems to consume more algae, with mixed support that guppies from different populations also recycle nutrients at different rates and ratios (Palkovacs et al. 2009, 2011, Bassar et al. 2010, 2012).

An Experimental Approach

But how important are such sex ratio and sexual dimorphism effects? Addressing this question in fully wild systems may be very difficult due to the challenge of isolating the indirect sex-ratio effects of environmental factors, like predators, from other direct or indirect effects of those environmental factors. However, one way to gauge such importance would be to manipulate sex ratios independently of other environmental factors to assess sex ratio effects directly. Such an experimental approach can also be used to compare and quantify the scope of sex-ratio effects against other factors, like density or presence/absence of a species, that are already known to have important effects on ecosystems (Kinnison et al. 2008, Palkovacs et al. 2009, Post and Palkovacs 2009).

Objectives

Given the preceding arguments, the relative importance of these characteristics in eco-evolutionary dynamics is expected to be dependent, at least in part, on the degree to which the sexes differ in their foraging or metabolizing tendencies and associated per capita ecological effects on a system. In order to understand the potential ecological significance of sex ratio variation, both the variation in sex ratio among wild populations and the magnitude of the sex ratio effect needs to be explored. The objectives of this study were to: 1) evaluate potential trophic trait divergence between sexes or predation regimes that might contribute to community and ecosystem divergence, 2) assess whether the sex, predation regime, or density of mosquitofish contribute to ecological conditions in pond communities and ecosystems, and 3) quantify variation in sex ratios among natural pond systems to compare with mesocosm responses. These objectives were accomplished through a combination of field surveys (natural sex ratio variation), a geometric morphometric analysis of body shape variation, and a factorial mesocosm experiment.

Study Species

Gambusia holbrooki, and the very closely related species *Gambusia affinis*, are abundant and widespread throughout North America. Collectively, these live-bearing poeciliid fishes are referred to as “mosquitofish”, and in combination they range from California east to North Carolina and from Mexico north to New Jersey (Lee and Burgess 1980). Notably for the current study*, Gambusia* species are sexually size dimorphic, with females generally larger than males (Lee and Burgess 1980, Haynes and Cashner 1995). In natural populations, sexes are born in roughly equivalent ratios (Krumholz 1948); however, females live longer and are hardier, with males spending substantial proportions of their energy on mating displays and attempted copulations (Giesel 1972). As a result, stable populations of mosquitofish often tend to be skewed toward the female sex. Alternatively, in systems with voracious predators, females may experience greater selective predation because they are larger and more nutritious prey, leaving such populations heavily male skewed (Britton and Moser 1982).

Adaptive population divergence is well known in populations of mosquitofish, particularly in association with predation (Horth 2004, Langerhans and DeWitt 2004, Langerhans et al. 2004, Heinen-Kay and Langerhans 2013). Phenotypic divergence between populations of mosquitofish and other closely related poeciliids includes morphology (Langerhans et al. 2004, Heinen-Kay and Langerhans 2013), physiology (Johnson and Belk 2001), and behavior (Endler 1987, Abrahams and Dill 1989, Langerhans 2009). Additionally, while mosquitofish are known to shoal in large groups (Magurran and Pitcher 1987, Dadda et al. 2009), there is substantial variation in the relative densities of these shoals (Agrillo et al. 2008, Dadda et al. 2009, Cote et al. 2012), oftentimes in association with probable predation risks (Magurran and Pitcher 1987, Winkelman and Aho 1993, Ward and Mehner 2010).

Also highly relevant for this study, mosquitofish are major ecological players in aquatic communities (Goodsell and Kats 1999, Matveev et al. 2000). *Gambusia* species are voracious, generalist feeders that can very effectively deplete aquatic insect larvae and thus influence ecosystem pathways tied to these consumers (Goodsell and Kats 1999, Matveev et al. 2000). As a result of this effect, mosquitofish have been used extensively for stocking and biocontrol, leading (inadvertently) to a near global spread in the range of this fish (Krumholz 1948, Lee and Burgess 1980, Vondracek et al. 1988, Haynes and Cashner 1995).

Methods

Natural Population Survey

Nine pond sites containing mosquitofish populations were surveyed within a thirty mile radius from Morehead City, North Carolina (34.7278° N, 76.7467° W). The sites were chosen to maximize the range of water quality, aquatic plant community (open to weedy), predation regime (presence or absence of predatory fishes), and other dominant environmental factors (e.g., human influence) that might jointly promote local adaptation and variation in ecological roles. Photographs were taken of each study site to record local habitat conditions. It was also recorded whether predators were visible in the system or are known indirectly (angler reports). Overt behavior of the mosquitofish at that site was also noted, including observations of habitat associations (pelagic or littoral), boldness during water disturbance, and swimming pattern (skittish or nonchalant).

A minimum of 100 fish were captured and sorted by sex for each site using landing nets, excluding immature fish that did not display secondary sexual characters (i.e. anal fin, brood pouch). After the fish were counted, they were released back into their source pond. In addition to the sex-ratio survey, one liter of water was collected at each site for chlorophyll *a* analysis to assess phytoplankton standing stocks.

Experimental Groups

Eastern mosquitofish for mesocosm experiments were obtained from the wild in the regions around Morehead City, North Carolina. Two populations of mosquitofish were used in the mesocosm experiment. One population was obtained from an uninhabited gated community, Bogue Watch (34°42’55”N, 76°58’13”W). This population had likely evolved in the absence of predators for at least three decades. The second population was collected from a heavily trafficked and disturbed drainage pond near the Morehead City Walmart (34°44’03”N, 76°48’49”W). This population is thought to have existed for >10 years and is under high predation pressure from locally stocked largemouth bass (*Micropterus salmoides*) and bluegill sunfish (*Lepomis microchirus*). This population also receives runoff from a large parking lot. These two populations were chosen to represent two extremes on the relative predation regime and mortality rate spectrum. Individuals used in the experiment were size limited. Male individuals were selected based on expression of gonopodia. Females were chosen to closely match the smaller sized males so all individuals were similar in size.

Experimental Design

Experiments were performed between June 23rd and July 14th, 2011 at the Nicholas School for the Environment, a satellite school of Duke University that is located in the same region of North Carolina as our study populations. The experimental layout was based on a three-factor design with sex ratio, source predation regime, and population density as the three factors. Hence, mesocosm experiments were performed in forty-four Sterilite® containers filled with thirty-five gallons of water. The containers were arranged in a nine-by-five grid on a cement pad. Each container received four liters of sandy substrate and four liters of lake benthos collected from one of the mosquitofish inhabited ponds used as a population source (Bogue Watch). Following settling of the substrate, one liter of water filled with concentrated pelagic zooplankton from the same pond was added to each container. Zooplankton were collected by sixty-five tows at zero to one meter in depth in a twelve centimeter plankton net. Lastly, two ceramic, unglazed tiles were added to the bottom of each tub as a standardized substrate to quantify periphyton growth (Bassar et al. 2010).

The mesocosms were allowed to establish for one week before fish additions. Fish were allocated based on population source, three densities, and seven sex ratios. Given a mesocosm pool size, the final densities were a total of 4, 8, or 16 fish. These densities were crossed with seven sex ratios, 0% female, 20% female, 40% female, 50% female, 60% female, 80% female, and 100% female. Actual sex densities were rounded to the nearest whole fish using the round half-up method, with a minimum of one fish in all non-zero percent ratios (Table 5.2). All resulting combinations of sex ratio and density were created for each of the two population sources. After the fish introductions, all containers were covered with screens to help reduce extreme heat and drought conditions as well as potential bird predation.

Sampling Methods

Data were collected from each container on a weekly basis, including one sample before fish stocking. Following the protocol from Harmon et al. (2009), dissolved oxygen was measured at different times of day and night using a YSI Model 55 meter to estimate primary production via respiration. One liter of water was then collected and filtered through Whatman GF/F filters for each tank. Each filter was wrapped in aluminum foil and immediately frozen until

Table 5.2: Mesocosm factorial design. The design crossed three densities with seven sex-ratios and was replicated for the two source populations that derived from either a low predation or high predation habitat.

LOW PREDATION SOURCE	**0% Males**	**20% Males**	**40% Males**	**50% Males**	**60% Males**	**80% Males**	**100% Males**
Low Density (4)	4 Females	3 Females 1 Male	3 Females 1 Male	2 Females 2 Males	1 Female 3 Males	1 Female 3 Males	4 Males
Moderate Density (8)	8 Females	7 Females 1 Male	5 Females 3 Males	4 Females 4 Males	3 Females 5 Males	1 Female 7 Males	8 Males
High Density (16)	16 Females	13 Females 3 Males	10 Females 6 Males	8 Females 8 Males	6 Females 10 Males	3 Females 13 Males	16 Males
HIGH PREDATION SOURCE	**0% Males**	**20% Males**	**40% Males**	**50% Males**	**60% Males**	**80% Males**	**100% Males**
Low Density (4)	4 Females	3 Females 1 Male	3 Females 1 Male	2 Females 2 Males	1 Female 3 Males	1 Female 3 Males	4 Males
Moderate Density (8)	8 Females	7 Females 1 Male	5 Females 3 Males	4 Females 4 Males	3 Females 5 Males	1 Female 7 Males	8 Males
High Density (16)	16 Females	13 Females 3 Males	10 Females 6 Males	8 Females 8 Males	6 Females 10 Males	3 Females 13 Males	16 Males

chlorophyll analysis could be performed to estimate phytoplankton standing stocks (Rice et al. 2012). At the final collection time point, the unglazed tiles at the bottom of each mesocosm were also collected. Tiles were scrubbed and rinsed with deionized water that was then filtered through Whatman GF/F filters and preserved in the same fashion as chlorophyll samples from the water column (Hauer and Lamberti 2007).

One liter of water was filtered through forty-two micron mesh to isolate zooplankton before fish stocking and at each chlorophyll collection time point. Zooplankton samples were preserved in 80% ethanol for later quantification of zooplankton abundance, size, and community structure. After the final time point, the fish were removed from the mesocosms and recounted to determine any mortality. All fish were euthanized with a lethal dose of MS-

222 (>250mg L^{-1}). Individual fish were then photographed on their left side, with a size standard, before being frozen.

Laboratory Methods

Pelagic and periphyton chlorophyll filters were processed after four weeks of freezing. Chlorophyll concentrations were measured by extracting filters with 100% methanol in the dark for twelve to fifteen hours (Rice et al. 2012). Samples were centrifuged and fluorescence was obtained on a Turner Designs 10-AU fluorometer that was calibrated against a standard chlorophyll solution. Chlorophyll fluorescence was corrected for volume with the equation $chla = (F_o * V_e)/V_f$, where F_o is the fluorometer reading, V_e is the volume of the extract, and V_f is the original filtered volume (Rice et al. 2012). Periphyton chlorophyll was computed by modifying the V_f term to reflect the area of each tile instead of volume of water filtered.

Zooplankton samples were washed with deionized water and loaded on a Ward acrylic counting wheel. However, zooplankton samples contained few to no specimens in all treatments, including one fishless mesocosm created as a back-up. This likely reflected population crashes from high temperature extremes associated with the small mesocosm sizes. As a result we could not further analyze these samples and they are not discussed. However, mosquitofish survival was high in all treatments (96% overall) and offspring were present in many mesocosms by the end of the study, suggesting environmental conditions were acceptable for this species and they were able to consume pelagic, benthic and algae resources, even though pelagic resources were likely at very low densities.

A single longitudinal section was cut across stomach of each frozen fish for dissection and diet analysis. Contents were analyzed under a dissecting microscope and all invertebrates or other food items (e.g., juvenile mosquitofish) were classified and counted. Photographs of fish were used to examine sizes and shape variation of males and females from the two different source populations. Linear measurements of photographed fish traits were made using the program Image J (ver. 1.45s, Wayne Rasband). Three measurements were taken on each fish, total length (snout to tip of the tail), body depth (straight vertical measurement from the deepest portion of the body), and tail peduncle (straight vertical measurement from the thinnest portion of the tail). Geometric morphometric analysis of overall fish body shape was performed using the programs tpsUtil (ver. 1.55, F. James Rohlf and SUNY at Stony Brook) and tpsDig2 (ver. 2.17, F. James Rohlf and SUNY at Stony Brook). Twelve fixed landmarks were used to assess fish shape (Figure 5.1, Appendix B), with landmarks focused around the cranium, the caudal area, and the dorsal and ventral fin insertions. Landmarks were adapted from Palkovacs et al. (2011).

Statistical Analysis

In order to better understand population differences we looked at morphological divergence between two populations. Morphometric data were analyzed using the tpsDig2 landmark and centroid data points with tpsRelw (ver. 1.9, F. James Rohlf and SUNY at Stony Brook), allowing data to be rotated along major axes and scaled. Principle component and partial warps are based on bending energy transformations of the thin plate spline grid. A principle components analysis (PCA) was then performed on these partial warps in order to obtain the relative warps. In order to visualize the shape differences captured by these relative warps, a discriminant function analysis (DFA) was performed on the relative warp data from TPS RELW. The DFA scores were input in tpsRegr (ver. 1.40, F. James Rohlf and SUNY at Stony Brook), performing a linear regression analysis on the points using sex and population source as variable components and size as a covariate to obtain a depiction of morphological variation as a deformation grid. Resulting deformations were exaggerated by four units to aid visualization.

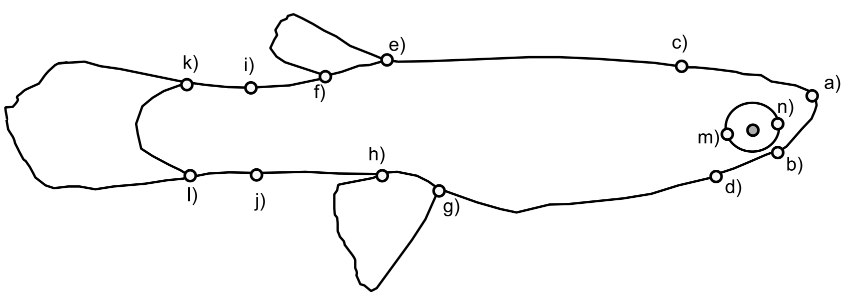

Figure 5.1: Landmarks for mosquitofish body shape. Landmarks a-d are cranial landmarks, e-h represent body depth and shape, and i-l are landmarks for caudal peduncle and tail shape. Landmarks m-n were used for eye size and position while the greyed marker was used as the eye centroid (non-landmark).

Subsequently, we used the mesocosm experiment to explore the potential divergence in dietary preferences between the two populations. Diet was analyzed using a Pearson's Chi-Square contingency table. Additional Person's Chi-Square goodness-of-fit tests were used on total dietary item counts across both sex and source predation regime, using size as a covariate, to isolate within population preferences and habitat bins.

Lastly, mesocosm limnological variables were used to estimate differences in trophic interactions between the populations and compared these values to the surveyed natural populations. Variables including pelagic chlorophyll, benthic chlorophyll, and dissolved oxygen were validated for normality and homoscedasticity. Variables with ranges over an order of magnitude or more were log10 transformed. Response variables were then analyzed using a mixed-model Multivariate Analysis of Covariance (MANCOVA) with population type as a fixed factor and both sex ratio and density as continuous covariates. Size was used as a covariate, represented as biomass in the mesocosm models. Interaction terms were included in initial models but were removed when not significant ($p>0.05$). Individual treatment comparisons were made using an analysis of variance (ANOVA) on a reduced linear model.

All statistics on mesocosm and fish data, including geometric morphometric scores for individuals, were performed on the R Programming Platform (Ver. 30101, R Core Team 2014), using the libraries *vegan* (Oksanen Ver. 2.0), *car* (Fox and Weisberg Ver. 2.0), *MASS* (Ripley Ver. 7.3), and *heplots* (Fox, Friendly, and Monette Ver. 1.0-1.6).

Results

Fish Morphology

Individual fish body shape was heavily dominated by sexual dimorphism. Given the overwhelming effects of sex on shape, I further examined shape within each sex to better characterize the effects of population source and density. Density did not have a significant effect on body shape for either sex (Table 5.3). Population source had a significant effect on body shape for both sexes, accounting for 34% of body shape variation in males and 65% of body shape variation in females (Table 5.3). Size had some additional shape effects, but explained 51% to 63% less than population source (Table 5.3). Visualizations of discriminant functions of shape show that, regardless of sex, individuals from high predation environments consistently have more slender bodies and narrower caudal peduncles (Figure 5.2). Specifically, in the high predation source population the dorsal-ventral depth at the abdomen and head was reduced (Figure 5.2a-b), particularly in males (Figure 5.2a), creating a more fusiform shaped body. Additionally, the caudal peduncle was thinned dorsal-ventrally and lengthened longitudinally relative to other body regions. Conversely, the low predation source fish showed more dorsally oriented mouths and eye position than the high predation source fish, consistent with modest trophic divergence.

Table 5.3: MANOVA analysis on linear mixed models for body shape DFA scores. Models are subset by sex to correct for overt sexual dimorphism. Size covariate is centroid size.

	Females				**Males**			
Population Characteristic	**F-value**	**p-value**	**Sig.**	**Proportion Variance**	**F-value**	**p-value**	**Sig.**	**Proportion Variance**
Source	601	<0.001	***	0.65	237	<0.001	***	0.34
Density	0.71	0.53		0.01	2.75	0.07		0.03
Size	20.9	<0.001	***	0.11	18.8	<0.001	***	0.11
Source: Size Interaction					5.23	0.02	*	0.04

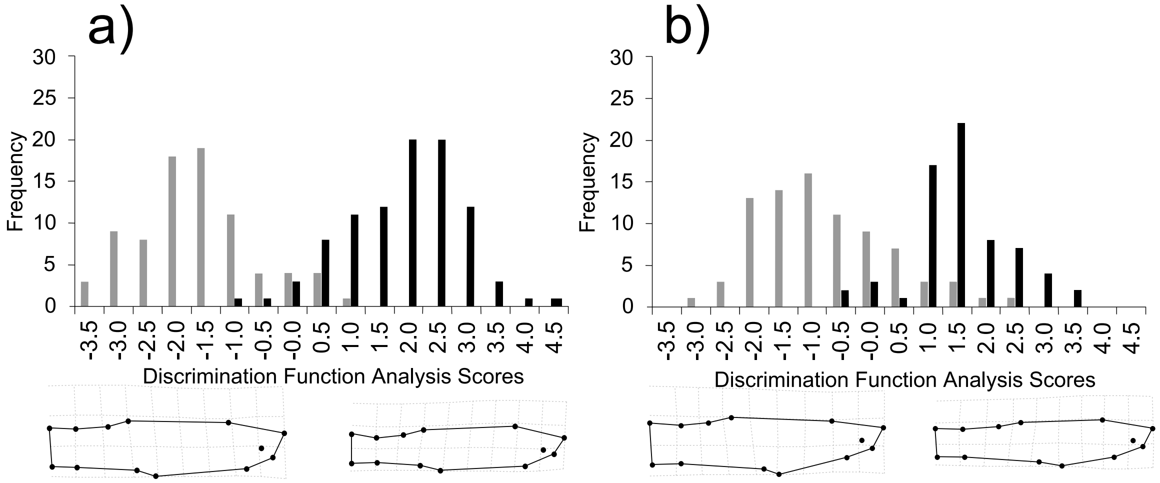

Figure 5.2: Relative warp analysis of experimental fishes. Scores are based on predation regime. Shape deformations of 4 times the y axis (for visualization) are paired with individual scores from a discriminant function analysis. Panels are grouped into females (a) and males (b). Left side of each axis depicts the low predation deformation and the right side represents the high predation deformation (4X exaggerated).

However, as suggested by the prior analyses including all fish, males and females showed some sex-specific differences with respect to their predation source. In particular, males from predator-exposed populations have a decreased anal fin insertion angle with a shorter insertion surface. Source differences in the anal fin insertion region were not apparent in female mosquitofish that do not use the anal fin as a reproductive organ in the same way as males.

Fish Diets within Mesocosms

Examination of the diets of individual fish from the different mesocosm treatments indicated that population source did have significant interacting effects with both the sex of the individual fish and the mesocosm sex ratio on individual fish diets (Table 5.4). While male and female fish from the high predation source did not consume significantly different dietary items (χ^2=0.694, p=0.40), male and female fish from the low predation source did (χ^2=3.89, p=0.05) (Figure 5.3a, Figure 5.4). Detailed analysis of diet by taxonomic group did not reveal any differences by sex ($F_{1,9}$=1.31, p=0.24) or source population ($F_{1,9}$=1.10, p=0.37) (Figure 5.4).

The joint effect of sex ratio and source population diet appears non-linear. Chi-Squared analysis reveals an interaction between diet and population source (χ^2=17677, p<0.001). Splitting the analysis into the two population sources reveal source predation as a driver. High predation source mesocosms with extreme sex ratios (mostly male or mostly female) tended to consume less benthic resources (fry omitted) than balanced sex ratios experiencing a more drastic change in diet (χ^2=5.00, p=0.03), reducing benthic species up to 30% in the extreme sex ratios. Conversely, low predation source mesocosms did not have a significant shift in diet when considered in isolation (χ^2=0.00, p=1) (Figure 5.3b).

Table 5.4: Reduced MANOVA model expressing the effect of sex, predation regime, and density on fish diets. Individual invertebrate diet items were grouped by broad classification (rotifers, arthropods, small crustaceans <0.5mm, large crustaceans >0.5mm, and snails) and expressed as total count. Mosquitofish fry were excluded from analysis to avoid bias in mesocosms with a higher density of pregnant females. Model was reduced using backwards model selection.

Population Characteristic	**DF**	**F-value**	**p-value**	**Sig.**
Sex	105	0.86	0.49	
Sex Ratio	105	0.37	0.83	
Source (predation regime)	105	0.34	0.83	
Density	105	1.47	0.22	
Sex: Source Interaction	105	3.81	0.006	**
Sex Ratio: Source Interaction	105	2.81	0.03	*

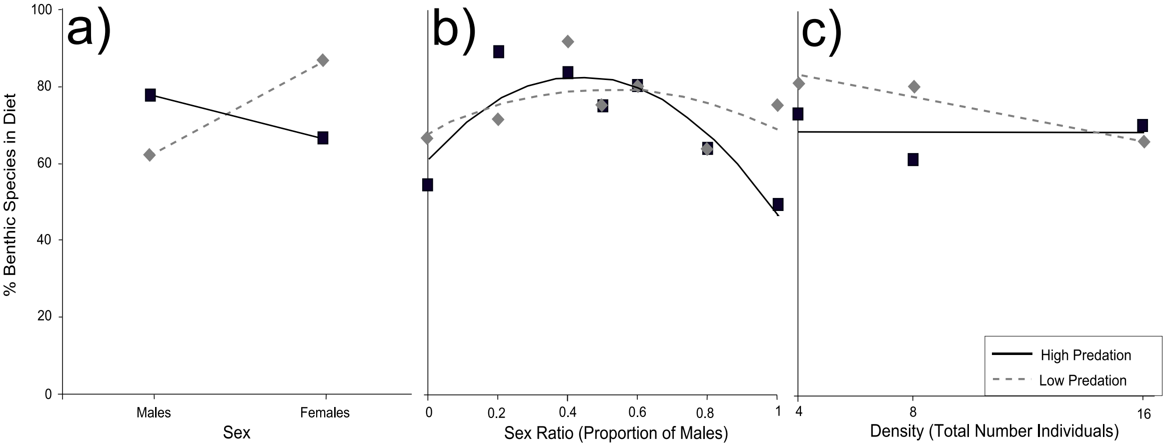

Figure 5.3: Percent of benthic items in stomach content of all individual mesocosm mosquitofish. Percent is based on total number of fish with identifiable stomach content items, not including cannibalism of young fry. Figures show percent benthic species for a) sex, b) sex ratio, and c) density.

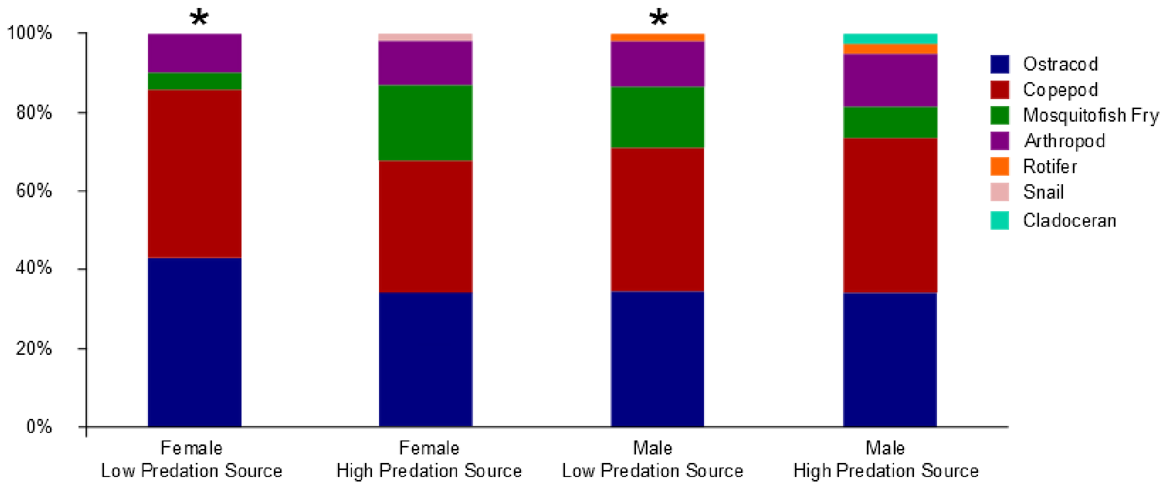

Figure 5.4: Diet analysis of individual fish from the mesocosm experiment. Stomach contents were identified to class or lower. Diet is broken down by sex and predation regime, showing content as a percent of the whole diet for the group. Asterisks represent significant comparisons.

Unlike sex and sex-ratio, that influenced fish diets through interactions with population source, density did not have a significant effect on dietary composition for either high predation (χ^2=1.20, p=0.55) or low predation (χ^2=1.87, p=0.39) sources (Figure 5.3c).

Mesocosm Conditions

Mesocosm data provided evidence for effects of both population source and overall densities of individuals (Table 5.5). In particular, the high predation treatments had marginally higher respiration rates ($F_{1,12}$=3.93, p=0.07) at higher densities, but not at the lowest densities ($F_{1,11}$=0.078, p=0.78), while water column ($F_{1,40}$=0.014, p=0.90) and periphyton ($F_{1,40}$=0.16, p=0.69) chlorophyll did not significantly differ by population treatment (Figure 5.5b, d, f). Sex ratio had no significant effects on any mesocosm response variable (Figure 5.5a, c, e).

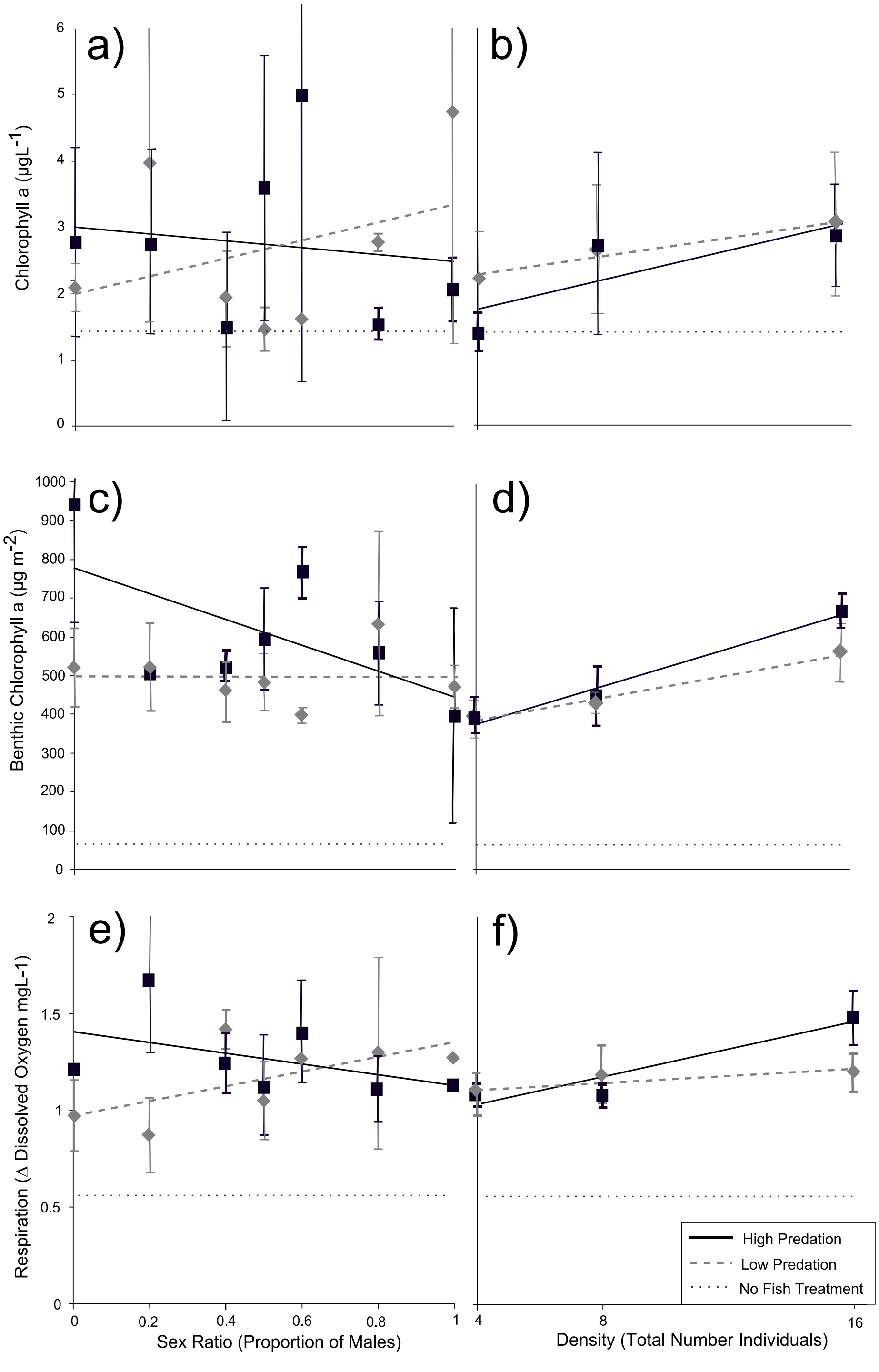

Figure 5.5: Response variable results from the mesocosm experiment for high predation (solid line) and low predation (dashed line) groups. Relationships of water column chlorophyll *a* to sex ratio (a) and density (b). Similarly, the relationships of benthic chlorophyll *a* to sex ratio (c) and density (d). Finally, the relationship of mesocosm respiration to sex ratio (e) and density (f). Points refer to sample average and error bars are standard error.

Table 5.5: Reduced MANOVA model expressing the effect of sex, source predation regime, and density on mesocosm environment. Density was measured by count and the size covariate was estimated biomass. Environmental conditions include respiration, water column chlorophyll *a*, and periphyton chlorophyll *a*. Model was reduced using backwards model selection.

Population Characteristic	**DF**	**F-value**	**p-value**	**Sig.**
Sex Ratio	33	0.53	0.73	
Source (predator regime)	33	3.82	0.02	*
Density	33	14.74	<0.001	***
Biomass	33	1.66	0.27	

Natural Population Survey

Sex ratios varied from 14% to 49% males and differed significantly between ponds with and without strong piscivory risk ($F_{1,7}$=46.09, $p<0.001$). Specifically, ponds with fish predators tended to have more even sex ratios, whereas those without predators were biased in favor of females. The MANOVA indicated that pond limnological conditions were correlated with the presence of mosquitofish predators and to a further marginal degree with sex ratios (Table 5.6). Pelagic chlorophyll *a* showed the strongest response to predators and sex ratios, by three orders of magnitude (Table 5.7). Higher levels of chlorophyll *a* were associated with low predation ponds and tended to increase with more-balanced sex ratios for both population types (Figure 5.6). However, there was little overlap in sex ratio for high and low predation ponds and so the relative effects of sex ratio and predation risk may not be fully independent.

Table 5.6: Full MANOVA model expressing the effect of sex ratio and predation regime on natural pond environmental conditions. Model includes pelagic chlorophyll *a*, general water quality, and amount of plant material.

Population Characteristic	**DF**	**F-value**	**p-value**	**Sig.**
Sex Ratio	3	6.76	0.08	.
Predation	3	10.43	0.04	*
Ratio: Exposure Interaction	3	1.98	0.30	

Table 5.7: Effect sizes from the full MANOVA model of sex ratio and predation regime on natural pond environmental conditions.

Environmental Parameter	**Sex Ratio**	**Predation**	**Sex Ratio : Predation Interaction**
Chlorophyll *a*	1979	8388	4883
Water Quality	3.1	0.77	0.12
Plant Material	1.6	2.9	1.1

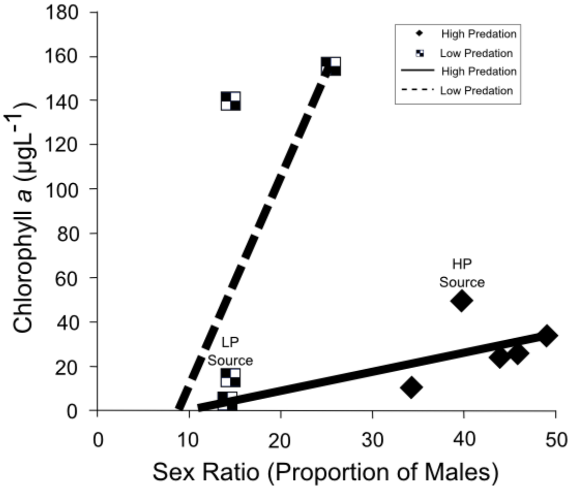

Figure 5.6: Pelagic chlorophyll *a* concentrations of the nine natural population survey sites. Sites were categorized by count estimated sex ratio and the clear presence/absence of predation either by observation or known stocking.

Discussion

Our study shows that sex ratios do vary in the wild and that variation is, at least in part, associated with local predator regime. Moreover, predator regime and, to a lesser extent, sex ratio are correlated with pond limnology in natural systems, particularly chlorophyll *a* concentrations. However, experimentally we found little indication that sex ratio directly affects communities, although there was evidence that male and female mosquitofish do display a degree of dietary niche divergence. By comparison, population source had some effects on ecosystem conditions that varied with fish density. However, the population source effects on mesocosm conditions are not likely to be attributable merely to source effects of individuals, because diet often depended on source population, sex, and sex ratios that interacted in a complex fashion. Nonetheless, fish from different predator environments did differ appreciably in body morphology, and did so in a fashion that was most consistent with specialization to predator regime, and perhaps to some degree with trophic specialization.

Population Source Shapes Morphology

Adaptation to predator regime has the potential to alter a wide variety of physical and behavioral traits in prey organisms (Reznick et al. 1996a, Langerhans et al. 2004, Pruitt et al. 2012). Evolution of size and shape can mitigate predation risk (Välimäki et al. 2012) through effects on overall speed (Chapman et al. 2007), or facilitate burst speeds during predator evasion (Langerhans et al. 2004). Both males and females from the high predation source population showed an anterior-posterior expansion and a dorsal-ventral streamlining in shape, consistent with adaptation to predator evasion; whereas low predation males and females showed more robust cranial features with higher eye and mouth positons consistent with investment in traits that may aid prey consumption and competitiveness, particularly of pelagic or surface prey. Hence, morphological differences between the populations were consistent with theoretical expectations along a predator evasion versus competitiveness tradeoff (Walker 1997, Blob et al. 2010, Palkovacs et al. 2011). Such morphological adaptations to predator regime are particularly widely supported for poeciliid fishes (Langerhans et al. 2004, Hendry et al. 2006). Such body shapes in poeciliids and other fishes may support a wide variety of anti-predator defenses including increased burst speed (Langerhans and DeWitt 2004, Langerhans et al. 2004), increased escape velocity (Ingley et al. 2014), and reduced predator consumption preference (Dingemanse et al. 2009). A very sizeable literature also supports the importance of cranial anatomy and eye position for trophic specializations across a large diversity of taxa (Walls et al. 1993, Motta et al. 1995, Pierce et al. 2009, Cooper et al. 2010, Herrel et al. 2010) with a generally conserved pattern of more dorsal position of the mouth and eyes being associated with greater pelagic or surface-oriented feeding (Wikramanayake 1990, Pouilly et al. 2003).

Effects of Sexual Segregation and Sex Ratios on Ecosystems

Although the most common perspective on sexual dimorphism surrounds its role in sexual selection and mating systems (Berglund et al. 1986, Székely et al. 2004), sexual dimorphism may also take the form of sexual segregation and reflect some degree of specialization to alternate ecological niches to reduce competition via natural selection and character displacement (Erlinge 1979, Wolf et al. 2005). For example, here females are the larger sex and in many systems this has been associated with different reproductive and energetic needs and associated foraging strategies (Walker and Rypstra 2002, Drovetski et al. 2006, Kamilar and Pokempner 2008). Where sexual niche dimorphism is tied to differences in diet and foraging behavior, it may not only reduce competition between the sexes but expand foraging effectiveness and species niche breadth overall (Selander 1966, Krüger 2005, Santiago-Alarcon and Parker 2007, Stein et al. 2008, Breed et al. 2009, Nebel and Thompson 2011, Cooper et al. 2011). Moreover, such sexual segregation is not limited to feeding behaviors and can extend to habitat use and preference as well (Drovetski et al. 2006, Pincheira-Donoso et al. 2009) with a whole suite of additional effects to ecosystems.

It is not a large conceptual leap to suggest that differences in the intensity and patterns of consumption by males and females might influence community and ecosystem conditions where sex ratios vary. That said, this finding merits some critique. The mesocosms in this study may not have been well-suited to detecting such effects given the depauperate zooplankton communities. Such a die-off could drastically limit the degree that sexual dimorphism and its ties to sexual niche segregation could elicit a cascading response under varying sex ratios. Indeed, a subsequent mesocosm experiment based on a modification of this study's design did find strong sex ratio effects on trophic cascades in mesocosms with healthy zooplankton populations (Appendix A). Studies that are focused on the cascade potential of mosquitofish indicate that the effects of mosquitofish are primarily in the pelagic cascade (Hargrave 2006, Castro and Gonçalves 2011, Fryxell et al. 2015). Although the benthic algae standing stock showed no response to mosquitofish treatments, the predominantly benthic diets of mosquitofish in the present study suggests they may have been very constrained in their foraging and the results of the current study should be weighed in light of those constraints.

Top-Down Pressures: Predation Exaggerates Sex Diet Preferences

Regardless of population source, male and female mosquitofish have separate diet preferences and these preferences are affected by population predation history. Males from the low predation source consumed significantly more pelagic species, while high predation pressure source males consume relatively more benthic species. Female mosquitofish however, showed the opposite trend, with high predation sources consuming proportionately more pelagic items than their low predation source counterparts. Such differences in diet choices related to sex could again be attributed to a degree of sexual segregation. Differences in dietary needs (Klimley 1987), energetic needs (Sims et al. 2001), uneven predation risk (Croft et al. 2009), and sexually biased aggression (Darden and Croft 2008) have been shown to lead the preference divergence in other fishes.

Pelagic food items are often of higher nutritional value and better energy transfer efficiency (Rowland et al. 2015) with more items such as copepods, cladocerans, and insects filling the diet. With higher nutritional needs, perhaps it is not surprising that females from a high predation source would increase the relative amount of these high quality prey to offset the reduced foraging time that often accompany predation risk (Roberts 1996), while at the same time meeting the nutritional needs of their indeterminate growth and internally developing offspring. In addition, females will often avoid locations with males to avoid sexual aggression and increased courtship (Dadda et al. 2005, Darden and Croft 2008), that reduce the female's ability to feed (Dadda et al. 2008). Conversely, males have determinate growth, suggesting their reproductive success is less dependent on size and so they may opt for safer benthic resources in the presence of predators, particularly if females out-compete them for pelagic resources.

However, these explanations do not make clear why the two sexes should switch their relative preferences so completely when derived from a low predation source. Perhaps males from low predation environments are more competitive for pelagic prey resources, or females from such highly competitive environments are more apt to take advantage of a greater diet breadth. Diet is unlikely to be associated with individual size. Males and females were approximately size matched at the beginning of the experiment. Post-experimental sizes were used as a covariate in diet analysis and was not a significant contributing factor.

Balanced sex ratios (40-60% female) result in more benthic resource use than extreme sex ratios (0-20% and 80-100% female). This effect is significantly exaggerated for the high predation source. Once again, it seems likely that some degree of intraspecific competition may be a driving force. Following that there is potential for high quality and better energy transfer in pelagic food items, it seems sensible that fish might generally be biased towards the higher quality food source (Edwards 1983, Grant and Scholes 2006) and perhaps more so in extreme ratios where intersexual competition is greatly reduced. However, at balanced sex ratios intersexual competition is strongest and sexual segregation most strongly favored, pushing some individuals toward more benthic diets to reduce competition or avoid sexual harassment (Klimley 1987, Sims et al. 2001, Phillips et al. 2011). However, the overall high use of benthic resources in this study suggests some constraints on diet so the present results should be interpreted cautiously in light of such limitations.

Density Shapes Mesocosms but not Diets

Mosquitofish achieve high population densities and shoal in large numbers in many in systems with few predators (Magurran and Pitcher 1987, Dadda et al. 2009). Moreover, prior studies with mosquitofish, like many other studies of trophic cascades, show that population densities can have strong cascading effects (Hurlbert and Mulla 1981). As a result, it is not surprising the number of individuals in my treatments had a strong effect on chlorophyll *a* concentrations in my mesocosms. Here, I found a clear correlation between increasing density of mosquitofish and increase in chlorophyll *a* (both benthic and pelagic) and respiration, consistent with trophic cascade. I also found some weak evidence that adaptation to high predation conditions may exaggerate these density effects, though not significantly.

Since all mesocosms experienced a zooplankton community crash within the first week, the effects of fish number may be more mediated in our results by bottom-up mechanisms tied to nutrient cycling. Consistent with this, I did not find any support that treatment densities influenced the diets of mosquitofish. Given the small size of mosquitofish, they tend to metabolize at a relatively high rate, and thus nutrient turnover rates through the mosquitofish pool may be relatively high (Brown et al. 2004). Certainly it is well established that fish excretion contains a high (relative) amount of biologically available nutrients (Allgeier et al. 2013), so mosquitofish act as nutrient converters, quickly and efficiently converting plant and animal material into “new”, biologically available fuel for the ecosystem. As the density of mosquitofish increased in mesocosms, the amount of material recycled and available to algae may have also increased (Vanni et al. 1997).

Natural Systems: Predation Drives Top-Down Cascades and Sex Ratios

Presence or absence of top predators is known to drive top-down trophic cascades (reviewed in Carpenter et al. 2010). In our system, mosquitofish represent an intermediate consumer link between piscivorous top predators and zooplankton. I found that low predation ponds had the capacity for larger standing stocks of chlorophyll *a*, which is expected if absence of top predators permits mosquitofish to more effectively graze zooplankton that would otherwise limit algal biomass. This could happen if absence of predators permits larger densities of mosquitofish (reviewed in Heithaus et al. 2008) or greater unabated foraging (Cerri and Fraser 1983, Roberts 1996). Additionally, larger numbers of mosquitofish could also recycle more nutrients (primarily phosphorus) (Vanni et al. 1997), contributing to some bottom-up reinforcement. We were not able to assess mosquitofish density in these ponds, however, due to the restricted amount of sampling time at many sites. Nonetheless, most studies of poeciliid fishes support that population densities are notably lower in areas with fish predators (Reznick and Endler 1982, Nowlin et al. 2006, Richardson et al. 2006, Zandonà et al. 2011) and that was consistent with my anecdotal observations.

In addition, predation also appears to influence sex-ratio, with the main effect of predators appearing to push the strongly female-biased sex ratios of low predation ponds towards a more even sex ratio in systems with fish predators. This is consistent with prior studies suggesting a predominance of females in populations without predators, due to greater energy investment and riskier behavior of males leading to shorter lifespans (Giesel 1972, Reznick et al. 1996a, Reznick and Bryant 2007). With a sexually dimorphic system such as mosquitofish, larger individuals are almost exclusively females (Krumholz 1948) so this outcome suggests potential positive size selection by predators (Britton and Moser 1982). The presence of predators might also weaken the competitive effects of females on the growth and survival of remaining males, through influences on female behavior and overall population densities (Walsh and Reznick 2008, Välimäki and Herczeg 2012). Regardless of the mechanism, the higher ratio of females to males in low predation ponds, combined with greater zooplankton grazing capacity of those large females, is expected to reinforce any density-mediated trophic cascades of predators (Hambright 1994, Layman et al. 2005, Nieoczym and Kloskowski 2014). Larger individuals can also increase the recycled nutrients in a system (McIntyre et al. 2008, El-Sabaawi et al. 2015), again supplementing top-down effects with some bottom-up contributions.

Conclusions

Mosquitofish are a sexually dimorphic species with many documented examples of skewed sex ratios in the wild despite a fifty percent birth ratio (Krumholz 1948, Giesel 1972, Britton and Moser 1982, Fryxell et al. 2015). Although sexual segregation has been shown in a variety of other species with sexes divergent in feeding structures (Berner et al. 2010, Christiansen and Harris 2012), food choice (Negovetic and Jokela 2000, Kamilar and Pokempner 2008), and habitat choice (Kie and Bowyer 1999, Croft et al. 2009), these studies have not examined how sex ratios interact with dimorphism to shape local communities and ecosystems.

The present study provided little if any direct support for community and ecosystem of effect of sex ratio variation. Population source and densities were much more important to variation in ecological conditions. That said, the findings of this study should be taken in light of evidence that pelagic zooplankton resources were very likely limiting and that a subsequent experiment affording of such a resource (Fryxell et al. 2015) showed clear support for cascading effects of sex ratio variation on pelagic food webs. Variation in fish size was also much more constrained at the initiation of the this experiment than in the subsequent experiment, which may have further limited the capacity to detect strong ecological effects of sex ratio variation tied to sexual size dimorphism.

Nonetheless, the current study did provide some support for sexual diet segregation and morphological differences between the sexes dominated all other sources of shape variation. I also found evidence consistent with effects of source population type on diet (interacting with sex and sex ratios) and morphological differences between populations consistent with a hypothesized tradeoff of shape features favoring predator escape and competitive ability. Work is now underway to better understand the functional and evolutionary basis for a general tradeoff between predator escape and competitive ability in the context of the implications of such a tradeoff for evolutionary contributions to trophic cascades in systems with and without mosquitofish predators.

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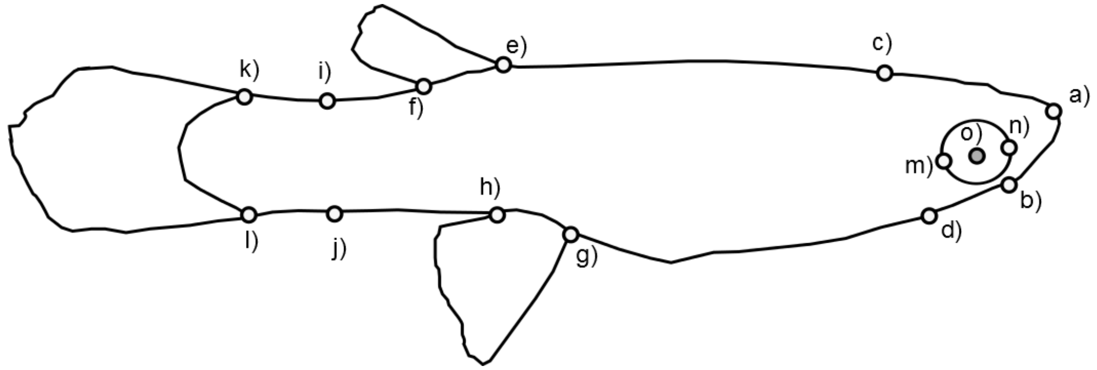
APPENDIX A: GEOMETRIC MORPHOMETRIC LANDMARK DESCRIPTIONS

Adapted from Palkovacs et al. 2011.



<u>Body landmarks used in shape analysis:</u> a) tip of snout, b) premaxilla, c) superior posterior extent of cranium, d) inferior posterior extend of cranium, e) anterior extent of dorsal fin insertion, f) posterior extent of dorsal fin insertion, g) anterior extend 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.

<u>Eye landmarks used in eye size:</u> m) posterior edge of ocular socket and n) anterior edge of ocular socket.

APPENDIX B: UNIVARIATE BEHAVIORAL RESPONSES WITHIN AND AMONG GENERATIONS FOR MALE AND FEMALE MOSQUITOFISH

Table B.1: Female univariate behavioral responses within generational groups. Linear mixed effects model on groups based on Figure 3.2. Effect size is exposed treatments or lineages relative to non-exposed.

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 1	1 vs 2	Lag Time	1,54	1.42	0.24		-0.39
		Exploration	1,54	1.80	0.18		0.45
		Safe%	1,54	1.29	0.26		-0.37
		Danger%	1,54	0.12	0.73		0.18
Gen 2	3 vs 4	Lag Time	1,66	0.60	0.44		0.18
		Exploration	1,66	0.05	0.83		-0.05
		Safe%	1,66	0.003	0.96		0.01
		Danger%	1,66	1.18	0.28		-0.26
	5 vs 6	Lag Time	1,47	0.00	0.99		0.00
		Exploration	1,47	0.18	0.89		0.04
		Safe%	1,47	0.06	0.81		0.07
		Danger%	1,47	0.09	0.77		-0.08
	3 vs 5	Lag Time	1,58	0.46	0.50		0.26
		Exploration	1,58	3.63	0.06	*	-0.49
		Safe%	1,58	2.06	0.16		0.37
		Danger%	1,58	6.81	0.01	**	-0.67
	4 vs 6	Lag Time	1,54	0.08	0.77		0.08
		Exploration	1,54	2.19	0.14		-0.39
		Safe%	1,54	2.65	0.10		0.43
		Danger%	1,54	3.29	0.07	*	-0.53
	3 vs 6	Lag Time	1,56	0.93	0.34		0.25
		Exploration	1,56	3.28	0.08	*	-0.48
		Safe%	1,56	3.28	0.08	*	0.48
		Danger%	1,56	7.77	0.007	**	-0.73

Table B.1 CONTINUED

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 3	7 vs 8	Lag Time	1,40	9.08	0.005	**	1.02
		Exploration	1,40	0.29	0.59		-0.30
		Safe%	1,40	2.12	0.15		0.45
		Danger%	1,40	1.16	0.29		-0.40
	9 vs 10	Lag Time	1,44	0.48	0.49		0.12
		Exploration	1,44	0.00	0.98		0.08
		Safe%	1,44	0.61	0.44		-0.29
		Danger%	1,44	2.27	0.14		-0.38
	7 vs 9	Lag Time	1,36	12.48	0.001	**	1.14
		Exploration	1,36	4.59	0.04	**	-0.77
		Safe%	1,36	7.36	0.01	**	0.87
		Danger%	1,36	2.10	0.16		-0.46
	8 vs 10	Lag Time	1,48	0.52	0.47		0.15
		Exploration	1,48	1.12	0.29		-0.23
		Safe%	1,48	0.01	0.89		0.02
		Danger%	1,48	2.15	0.15		-0.37
	7 vs 10	Lag Time	1,39	10.95	0.002	**	0.53
		Exploration	1,39	2.41	0.13		-0.21
		Safe%	1,39	1.46	0.23		0.04
		Danger%	1,39	7.43	0.01	**	-0.56

Table B.2: Male univariate behavioral responses within generational groups. Linear mixed effects model on groups based on Figure 3.2. Effect size is exposed treatments or lineages relative to non-exposed.

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 1	1 vs 2	Lag Time	1,48	4.82	0.03	**	-0.63
		Exploration	1,48	7.21	0.01	**	0.72
		Safe%	1,48	8.74	0.005	**	-0.78
		Danger%	1,48	1.31	0.26		0.36
Gen 2	3 vs 4	Lag Time	1,46	0.04	0.84		0.15
		Exploration	1,46	0.56	0.46		-0.23
		Safe%	1,46	0.01	0.91		0.03
		Danger%	1,46	0.48	0.49		-0.20
	5 vs 6	Lag Time	1,34	0.27	0.60		0.17
		Exploration	1,34	1.20	0.28		0.36
		Safe%	1,34	0.24	0.63		-0.16
		Danger%	1,34	2.98	0.09	.	0.57
	3 vs 5	Lag Time	1,46	0.04	0.84		0.15
		Exploration	1,46	0.56	0.46		-0.23
		Safe%	1,46	0.01	0.91		0.03
		Danger%	1,46	0.48	0.49		-0.20
	4 vs 6	Lag Time	1,41	0.67	0.42		0.25
		Exploration	1,41	1.47	0.23		0.39
		Safe%	1,41	1.65	0.21		-0.41
		Danger%	1,41	5.15	0.03	**	0.69
	3 vs 6	Lag Time	1,45	0.86	0.36		0.34
		Exploration	1,45	0.27	0.61		0.14
		Safe%	1,45	0.33	0.57		-0.13
		Danger%	1,45	1.97	0.17		0.43
Gen 3	7 vs 8	Lag Time	1,42	0.01	0.95		-0.02
		Exploration	1,42	0.00	0.97		0.01
		Safe%	1,42	0.24	0.63		-0.15
		Danger%	1,42	0.21	0.65		0.16
	9 vs 10	Lag Time	1,37	0.22	0.64		-0.20
		Exploration	1,37	9.01	0.005	**	0.97
		Safe%	1,37	7.05	0.01	**	-0.88
		Danger%	1,37	0.71	0.41		-0.21
	7 vs 9	Lag Time	1,45	0.02	0.88		-0.04
		Exploration	1,45	1.97	0.17		-0.47
		Safe%	1,45	0.55	0.46		0.32
		Danger%	1,45	0.03	0.87		0.02
	8 vs 10	Lag Time	1,34	0.81	0.37		-0.29
		Exploration	1,34	1.88	0.18		0.50
		Safe%	1,34	1.52	0.23		-0.44
		Danger%	1,34	0.97	0.33		-0.37
	7 vs 10	Lag Time	1,45	1.06	0.31		-0.23
		Exploration	1,45	3.91	0.05	*	0.72
		Safe%	1,45	5.59	0.02	**	-0.77
		Danger%	1,45	0.41	0.52		-0.19

Table B.3: Female univariate behavioral responses among generational groups. Linear mixed effects model on groups based on Figure 3.2. Effect size is early generation to relative later generation.

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 1 and Gen 2	1 vs 3	Lag Time	1,62	0.11	0.74		-0.08
		Exploration	1,62	1.30	0.26		0.28
		Safe%	1,62	0.39	0.53		-0.16
		Danger%	1,62	1.04	0.31		0.25
	1 vs 4	Lag Time	1,60	0.26	0.61		0.10
		Exploration	1,60	0.67	0.42		0.22
		Safe%	1,60	0.24	0.62		-0.14
		Danger%	1,60	0.00	0.98		-0.01
	2 vs 5	Lag Time	1,50	4.20	0.05	*	0.58
		Exploration	1,50	6.22	0.02	**	-0.68
		Safe%	1,50	4.42	0.04	**	0.57
		Danger%	1,50	6.92	0.01	**	-0.71
	2 vs 6	Lag Time	1,48	4.07	0.05	*	0.56
		Exploration	1,48	6.00	0.02	**	-0.68
		Safe%	1,48	6.37	0.02	**	0.70
		Danger%	1,48	8.41	0.006	**	-0.81
Gen 1 and Gen 3	1 vs 7	Lag Time	1,43	0.65	0.42		-0.60
		Exploration	1,43	0.29	0.59		0.49
		Safe%	1,43	0.88	0.35		-0.49
		Danger%	1,43	0.01	0.91		0.17
	1 vs 8	Lag Time	1,52	5.54	0.02	**	0.27
		Exploration	1,52	0.19	0.67		0.13
		Safe%	1,52	0.83	0.37		-0.02
		Danger%	1,52	2.54	0.12		-0.23
	2 vs 9	Lag Time	1,46	6.32	0.02	**	0.75
		Exploration	1,46	5.90	0.02	**	-0.70
		Safe%	1,46	6.76	0.02	**	0.74
		Danger%	1,46	2.86	0.09	*	-0.50
	2 vs 10	Lag Time	1,49	9.74	0.003	**	0.82
		Exploration	1,49	4.66	0.04	**	-0.56
		Safe%	1,49	1.43	0.24		0.34
		Danger%	1,49	12.84	<0.0001	**	-0.95
Gen 2 and Gen 3	3 vs 7	Lag Time	1,49	3.53	0.07	*	-0.56
		Exploration	1,49	0.44	0.51		0.19
		Safe%	1,49	1.49	0.23		-0.36
		Danger%	1,49	0.09	0.77		-0.09
	4 vs 8	Lag Time	1,56	0.44	0.51		0.17
		Exploration	1,56	0.07	0.79		-0.07
		Safe%	1,56	0.21	0.65		0.12
		Danger%	1,56	0.25	0.62		-0.23
	5 vs 9	Lag Time	1,45	0.13	0.72		0.11
		Exploration	1,45	0.01	0.93		-0.01
		Safe%	1,45	0.13	0.72		0.10
		Danger%	1,45	0.40	0.53		0.17
	6 vs 10	Lag Time	1,46	0.71	0.41		0.22
		Exploration	1,46	0.00	0.95		0.03
		Safe%	1,46	0.76	0.39		-0.26
		Danger%	1,46	0.21	0.65		-0.13

Table B.4: Male univariate behavioral responses among generational groups. Linear mixed effects model on groups based on Figure 3.2. Effect size is early generation to later generation.

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 1 and Gen 2	1 vs 3	Lag Time	1,52	4.23	0.04	**	0.55
		Exploration	1,52	1.03	0.32		-0.27
		Safe%	1,52	0.87	0.35		0.25
		Danger%	1,52	0.02	0.88		0.04
	1 vs 4	Lag Time	1,48	8.15	0.006	**	0.79
		Exploration	1,48	5.37	0.03	**	-0.55
		Safe%	1,48	4.46	0.04	**	0.52
		Danger%	1,48	0.72	0.40		-0.24
	2 vs 5	Lag Time	1,42	19.31	<0.0001	**	1.37
		Exploration	1,42	15.72	<0.0001	**	-1.20
		Safe%	1,42	11.42	0.002	**	1.01
		Danger%	1,42	1.70	0.20		-0.48
	2 vs 6	Lag Time	1,41	48.93	<0.0001	**	2.11
		Exploration	1,41	7.68	0.008	**	-0.84
		Safe%	1,41	9.02	0.005	**	0.91
		Danger%	1,41	0.15	0.70		0.12
Gen 1 and Gen 3	1 vs 7	Lag Time	1,49	3.52	0.07	*	0.52
		Exploration	1,49	3.62	0.06	*	-0.30
		Safe%	1,49	5.95	0.02	**	0.45
		Danger%	1,49	0.15	0.70		-0.11
	1 vs 8	Lag Time	1,38	2.51	0.12		0.50
		Exploration	1,38	2.56	0.12		-0.27
		Safe%	1,38	2.75	0.12		0.27
		Danger%	1,38	0.04	0.85		0.06
	2 vs 9	Lag Time	1,43	10.65	0.002	**	1.00
		Exploration	1,43	23.86	<0.0001	**	-1.51
		Safe%	1,43	26.48	<0.0001	**	1.52
		Danger%	1,43	1.01	0.32		-0.42
	2 vs 10	Lag Time	1,43	23.29	<0.0001	**	1.27
		Exploration	1,43	2.51	0.12		-0.47
		Safe%	1,43	3.15	0.08	*	0.53
		Danger%	1,43	4.95	0.03	**	-0.65
Gen 2 and Gen 3	3 vs 7	Lag Time	1,55	0.20	0.66		-0.12
		Exploration	1,55	0.002	0.96		0.01
		Safe%	1,55	0.52	0.47		0.19
		Danger%	1,55	0.30	0.58		-0.14
	4 vs 8	Lag Time	1,40	0.97	0.33		-0.30
		Exploration	1,40	0.28	0.60		0.27
		Safe%	1,40	0.23	0.64		-0.21
		Danger%	1,40	0.38	0.54		0.29
	5 vs 9	Lag Time	1,36	0.76	0.39		-0.29
		Exploration	1,36	0.37	0.54		-0.15
		Safe%	1,36	2.11	0.16		0.42
		Danger%	1,36	0.03	0.87		0.08
	6 vs 10	Lag Time	1,35	10.54	0.003	**	-1.06
		Exploration	1,35	1.07	0.31		0.37
		Safe%	1,35	0.82	0.37		-0.32
		Danger%	1,35	5.17	0.03	**	-0.75

APPENDIX C: GEOMETRIC MORPHOMETRIC ANALYSIS OF RELATIVE WARPS FOR THREE GENERATIONS OF FEMALE MOSQUITOFISH.

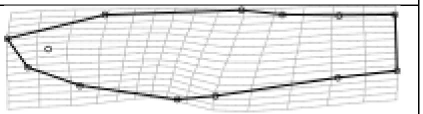
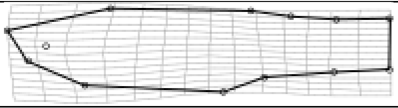
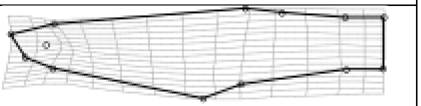
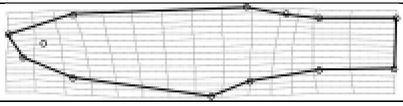
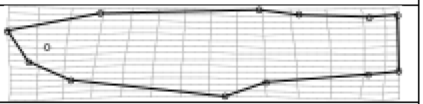
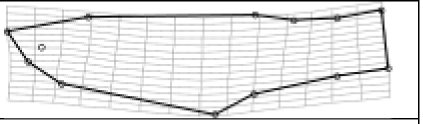
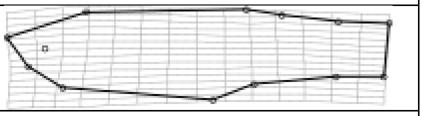
RW	Negative Value Visualization	Positive Value Visualization
1		
	• Longitudinal expansion of the caudal region on the ventral side • Ventral positioning of eyes • Ventral flattening of snout	
2		
	• Longitudinal expansion of the caudal region on the ventral side • Ventral lowering of snout and eyes • Dorsal-ventral flattening of the snout and cranium	
3		
	• Anterior positioning of dorsal fin • Longitudinal reduction in caudal region • Dorsal side expansion of cranium	
4		
	• Dorsal expansion of cranium and thoracic and abdominal region • Artificial bend of individuals	
5		
	• Ventral and dorsal body depth expansion • Ventral and dorsal expansion of cranium • Ventral positioning of eyes	
6		
	• Dorsal-ventral expansion of cranium and caudal peduncle • Ventral expansion of thoracic region and overall body depth	

Figure C.1: Negative and positive visualizations for the first fifteen relative warps of three generations of female mosquitofish. Exaggerations are from the sample extremes to the negative and positive directions. Descriptions are written for the positive visualizations from negative references.

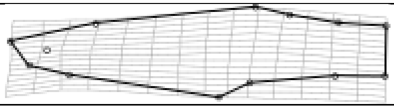
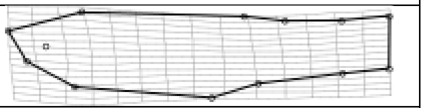
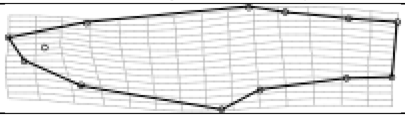
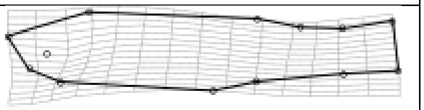
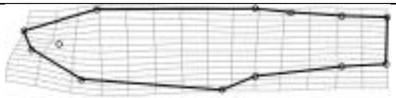
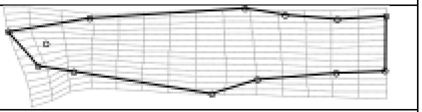
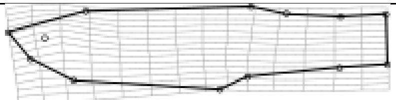
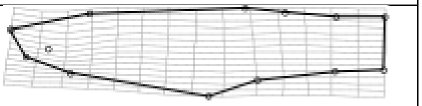
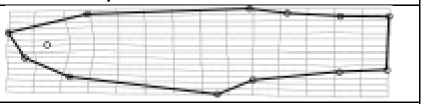
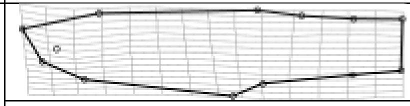
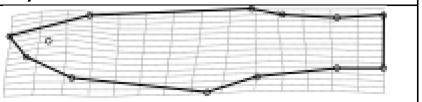
<table>
<tr><th>RW</th><th>Negative Value Visualization</th><th>Positive Value Visualization</th></tr>
<tr><td rowspan="2">7</td><td></td><td></td></tr>
<tr><td colspan="2">• Dorsal-ventral cranial expansion
• Anterior-posterior narrowing of cranium
• Dorsal-ventral flattening of dorsal fin insertion
• Narrowing of caudal peduncle.</td></tr>
<tr><td rowspan="2">8</td><td></td><td></td></tr>
<tr><td colspan="2">• Anterior-posterior narrowing of the ventral cranial edge
• Body depth narrowing
• Dorsal fin insertion posteriorly shifted</td></tr>
<tr><td rowspan="2">9</td><td></td><td></td></tr>
<tr><td colspan="2">• Flattening of dorsal and ventral cranial edges
• Anterior-posterior cranial expansion on dorsal edges
• Dorsal-ventral lower jaw expansion</td></tr>
<tr><td rowspan="2">10</td><td></td><td></td></tr>
<tr><td colspan="2">• Ventral positioning of eyes
• Dorsal-ventral flattening of ventral cranial edge
• Expansion of anal fin anterior-posteriorly</td></tr>
<tr><td rowspan="2">11</td><td></td><td></td></tr>
<tr><td colspan="2">• Anterior-posterior expansion of eye</td></tr>
<tr><td rowspan="2">12</td><td></td><td></td></tr>
<tr><td colspan="2">• Dorsal positioning of eye
• Dorsal-ventral reduction of lower jaw
• Flattening of the snout
• Dorsal-ventral slimming of caudal peduncle</td></tr>
</table>

Figure C.1 CONTINUED

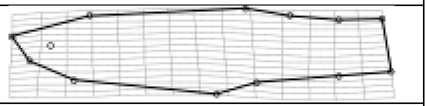
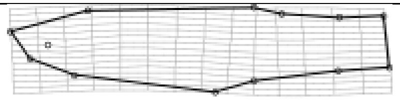
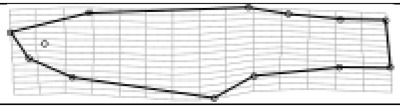
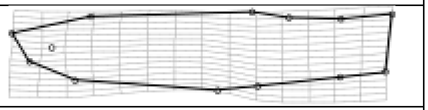
RW	Negative Value Visualization	Positive Value Visualization
13		
	• Ventral flattening snout • Dorsal positioning of eye • Dorsal-ventral expansion of caudal peduncle	
14		
	• Anterior-posterior expansion of dorsal fin • Dorsal positioning of eye	
15		
	• Body depth reduction on the ventral side • Reduction in the angle of the anal fin	

Figure C.1 CONTINUED

Table C.1: ANOVA on the 26 relative warps for three generations of female mosquitofish. Due to capacity of program to detect detailed changes between specimens, significance levels of ≤0.0001 were used.

	dF	F-value	p-value	Significance
RW1	1,252	15.67	0.0001	*
RW2	1,252	4.95	0.03	
RW3	1,252	25.77	<0.0001	*
RW4	1,252	0.88	0.35	
RW5	1,252	15.49	0.0001	*
RW6	1,252	0.04	0.84	
RW7	1,252	5.29	0.02	
RW8	1,252	22.38	<0.0001	*
RW9	1,252	0.08	0.78	
RW10	1,252	14.19	0.0002	
RW11	1,252	0.10	0.76	
RW12	1,252	0.06	0.81	
RW13	1,252	0.03	0.85	
RW14	1,252	13.39	0.0003	
RW15	1,252	0.49	0.48	
RW16	1,252	2.94	0.09	
RW17	1,252	0.13	0.72	
RW18	1,252	2.80	0.10	
RW19	1,252	0.46	0.50	
RW20	1,252	0.40	0.53	
RW21	1,252	4.59	0.03	
RW22	1,252	4.20	0.04	
RW23	1,252	0.01	0.92	
RW24	1,252	4.58	0.03	
RW25	1,252	0.003	0.96	
RW26	1,252	1.04	0.31	

APPENDIX D: UNIVARIATE MORPHOLOGICAL RESPONSES WITHIN AND AMONG GENERATIONS FOR MALE AND FEMALE MOSQUITOFISH.

Table D.1: Within generation pairwise comparisons of size metrics for three generations of female mosquitofish. Effect sizes are Cohen's d with non-exposure treatment or lineage as the baseline value.

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 1	1 vs 2	Total Length	1,55	0.19	0.67		0.15
		Body Depth	1,55	2.53	0.12		-0.18
		Tail Peduncle	1,55	0.02	0.88		0.05
		Weight	1,55	2.54	0.12		0.49
Gen 2	3 vs 4	Total Length	1,72	0.59	0.45		-0.18
		Body Depth	1,72	0.17	0.69		-0.09
		Tail Peduncle	1,72	0.18	0.67		-0.10
		Weight	1,72	1.07	0.31		-0.21
	5 vs 6	Total Length	1,47	0.02	0.88		0.01
		Body Depth	1,47	4.30	0.04	**	-0.42
		Tail Peduncle	1,47	0.03	0.86		0.01
		Weight	1,47	1.95	0.17		-0.22
	3 vs 5	Total Length	1,61	0.07	0.80		-0.23
		Body Depth	1,61	0.41	0.53		0.16
		Tail Peduncle	1,61	3.69	0.06	*	-0.45
		Weight	1,61	0.28	0.60		-0.10
	4 vs 6	Total Length	1,57	0.04	0.83		0.02
		Body Depth	1,57	0.00	0.98		-0.15
		Tail Peduncle	1,57	1.33	0.25		-0.25
		Weight	1,57	0.87	0.36		-0.12
	3 vs 6	Total Length	1,59	0.04	0.83		-0.24
		Body Depth	1,59	0.78	0.38		-0.23
		Tail Peduncle	1,59	4.57	0.04	**	-0.43
		Weight	159	0.37	0.54		-0.37

Table D.1 CONTINUED

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 3	7 vs 8	Total Length	1,50	2.12	0.15		0.30
		Body Depth	1,50	2.81	0.10		0.31
		Tail Peduncle	1,50	0.07	0.80		0.06
		Weight	1,50	2.43	0.13		0.33
	9 vs 10	Total Length	1,49	3.38	0.07	*	0.30
		Body Depth	1,49	0.53	0.47		0.09
		Tail Peduncle	1,49	0.37	0.55		0.06
		Weight	1,49	1.51	0.23		0.19
	7 vs 9	Total Length	1,49	0.49	0.49		0.14
		Body Depth	1,49	0.12	0.73		-0.07
		Tail Peduncle	1,49	1.36	0.25		-0.29
		Weight	1,49	0.01	0.91		-0.02
	8 vs 10	Total Length	1,50	0.45	0.51		0.11
		Body Depth	1,50	0.87	0.36		-0.21
		Tail Peduncle	1,50	0.92	0.34		-0.24
		Weight	1,50	0.23	0.63		-0.13
	7 vs 10	Total Length	1,49	5.16	0.03	**	0.41
		Body Depth	1,49	0.27	0.61		0.05
		Tail Peduncle	1,49	0.38	0.54		-0.18
		Weight	1,49	1.62	0.21		0.18

Table D.2: Among generation pairwise comparisons of size metrics for three generations of female mosquitofish. Effect sizes are Cohen's d with earlier generations as the baseline value.

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 1 and Gen 2	1 vs 3	Total Length	1,66	1.20	0.28		-0.21
		Body Depth	1,66	5.17	0.03	**	-0.52
		Tail Peduncle	1,66	0.19	0.66		0.13
		Weight	1,66	0.21	0.65		-0.07
	1 vs 4	Total Length	1,64	3.19	0.08	*	-0.31
		Body Depth	1,64	10,18	0.002	**	-0.63
		Tail Peduncle	1,64	0.01	0.93		0.04
		Weight	1,64	2.05	0.16		-0.24
	2 vs 5	Total Length	1,50	9.95	0.003	**	-0.65
		Body Depth	1,50	2.53	0.12		-0.28
		Tail Peduncle	1,50	3.05	0.08	*	-0.09
		Weight	1,50	6.70	0.01	**	-0.66
	2 vs 6	Total Length	1,48	15.44	<0.0001	**	-0.70
		Body Depth	1,48	17.97	<0.0001	**	-0.82
		Tail Peduncle	1,48	3.19	0.08	*	-0.36
		Weight	1,48	14.23	<0.0001	**	-0.91
Gen 1 and Gen 3	1 vs 7	Total Length	1,53	18.75	<0.0001	**	-0.64
		Body Depth	1,53	20.26	<0.0001	**	-0.76
		Tail Peduncle	1,53	0.91	0.34		-0.20
		Weight	1,53	18.19	<0.0001	**	-0.58
	1 vs 8	Total Length	1,54	8.67	0.005	**	-0.35
		Body Depth	1,54	9.01	0.004	**	-0.42
		Tail Peduncle	1,54	0.79	0.38		-0.16
		Weight	1,54	4.52	0.04	**	-0.24
	2 vs 9	Total Length	1,50	33.51	<0.0001	**	-1.09
		Body Depth	1,50	20.55	<0.0001	**	-0.90
		Tail Peduncle	1,50	8.76	0.005	**	-0.67
		Weight	1,50	27.01	<0.0001	**	-1.36
	2 vs 10	Total Length	1,50	6.86	0.01	**	-0.48
		Body Depth	1,50	7.96	0.007	**	-0.57
		Tail Peduncle	1,50	4.46	0.04	**	-0.48
		Weight	1,50	13.08	<0.0001	**	-0.92

Table D.2 CONTINUED

		Behavior	df	F-value	p-value	Significance	Effect size
Gen 2 and Gen 3	3 vs 7	Total Length	1,61	3.86	0.05	*	-0.74
		Body Depth	1,61	0.68	0.41		-0.21
		Tail Peduncle	1,61	2.68	0.11		-0.39
		Weight	1,61	1.90	0.17		-0.66
	4 vs 8	Total Length	1,60	0.08	0.78		-0.06
		Body Depth	1,60	2.35	0.13		0.19
		Tail Peduncle	1,60	0.09	0.76		-0.21
		Weight	1,60	3.21	0.08	*	0.02
	5 vs 9	Total Length	1,49	1.01	0.32		-0.24
		Body Depth	1,49	4.28	0.04	**	-0.46
		Tail Peduncle	1,49	0.94	0.34		-0.23
		Weight	1,49	3.95	0.05	*	-0.43
	6 vs 10	Total Length	1,47	1.00	0.32		0.08
		Body Depth	1,47	0.84	0.36		0.08
		Tail Peduncle	1,47	0.00	0.96		-0.13
		Weight	1,47	0.58	0.45		0.02

Table D.3: Within generation pairwise comparisons of significant relative warps for three generations of female mosquitofish. Effect sizes are Cohen's d with non-exposure treatment or lineage as the baseline value.

		RW	df	F-value	p-value	Significance	Effect size
Gen 1	1 vs 2	RW 1	1,47	0.01	0.94		-0.11
		RW 3	1,47	3.66	0.06	*	-0.55
		RW 5	1,47	2.11	0.15		-0.43
		RW 8	1,47	0.01	0.94		0.07
Gen 2	3 vs 4	RW 1	1,71	1.54	0.22		0.8
		RW 3	1,71	3.64	0.06	*	-0.38
		RW 5	1,71	0.61	0.43		-0.16
		RW 8	1,71	4.46	0.04	**	-0.47
	5 vs 6	RW 1	1,47	14.39	0.0004	**	1.07
		RW 3	1,47	1.07	0.31		0.30
		RW 5	1,47	0.20	0.66		-0.13
		RW 8	1,47	6.58	0.01	**	0.73
	3 vs 5	RW 1	1,61	4.84	0.03	**	-0.24
		RW 3	1,61	7.74	0.007	**	-0.99
		RW 5	1,61	6.13	0.02	**	-0.39
		RW 8	1,61	1.84	0.18		-0.34
	4 vs 6	RW 1	1,56	0.92	0.34		0.50
		RW 3	1,56	0.05	0.83		-0.18
		RW 5	1,56	2.07	0.16		-0.38
		RW 8	1,56	0.05	0.02	**	0.89
	3 vs 6	RW 1	1,59	5.20	0.03	**	0.81
		RW 3	1,59	1.65	0.20		-0.56
		RW 5	1,59	7.32	0.009	**	-0.50
		RW 8	1,59	5.27	0.03	**	0.55
Gen 3	7 vs 8	RW 1	1,50	2.91	0.09	*	-0.34
		RW 3	1,50	1.39	0.24		0.30
		RW 5	1,50	7.63	0.008	**	0.76
		RW 8	1,50	0.43	0.51		-0.15
	9 vs 10	RW 1	1,49	0.47	0.50		0.19
		RW 3	1,49	0.88	0.35		0.23
		RW 5	1,49	1.52	0.22		-0.34
		RW 8	1,49	0.10	0.76		-0.03
	7 vs 9	RW 1	1,49	1.46	0.23		-0.33
		RW 3	1,49	1.12	0.30		0.17
		RW 5	1,49	1.41	0.24		0.33
		RW 8	1,49	0.47	0.50		0.18
	8 vs 10	RW 1	1,50	0.69	0.41		0.21
		RW 3	1,50	0.27	0.61		0.15
		RW 5	1,50	6.70	0.01	**	-0.71
		RW 8	1,50	0.91	0.35		0.22
	7 vs 10	RW 1	1,49	0.40	0.53		-0.11
		RW 3	1,49	2.52	0.12		0.38
		RW 5	1,49	0.03	0.87		-0.05
		RW 8	1,49	0.11	0.74		0.12

Table D.4: Among generation pairwise comparisons of significant relative warps for three generations of female mosquitofish. Effect sizes are Cohen's d with earlier generations as the baseline value.

		RW	df	F-value	p-value	Significance	Effect size
Gen 1 and Gen 2	1 vs 3	RW 1	1,59	4.64	0.04	**	0.41
		RW 3	1,59	19.54	<0.0001	**	1.21
		RW 5	1,59	8.23	0.006	**	-0.87
		RW 8	1,59	5.24	0.03	**	-0.59
	1 vs 4	RW 1	1,56	10.10	0.002	**	0.61
		RW 3	1,56	5.28	0.03	**	0.71
		RW 5	1,56	16.37	0.0002	**	-1.07
		RW 8	1,56	9.37	0.003	**	-0.87
	2 vs 5	RW 1	1,49	4.11	0.05	*	0.55
		RW 3	1,49	7.02	0.01	**	0.73
		RW 5	1,49	11.08	0.002	**	-0.91
		RW 8	1,49	7.25	0.01	**	-0.71
	2 vs 6	RW 1	1,47	23.94	<0.0001	**	1.37
		RW 3	1,47	10.86	0.002	**	0.92
		RW 5	1,47	12.51	0.0009	**	-0.99
		RW 8	1,47	0.57	0.45		-0.21
Gen 1 and Gen 3	1 vs 7	RW 1	1,46	12.80	0.0008	**	0.65
		RW 3	1,46	0.46	0.50		0.06
		RW 5	1,46	22.38	<0.0001	**	-1.37
		RW 8	1,46	5.29	0.03	**	-0.65
	1 vs 8	RW 1	1,47	5.16	0.03	**	0.34
		RW 3	1,47	3.99	0.05	*	0.54
		RW 5	1,47	9.76	0.003	**	-0.90
		RW 8	1,47	5.95	0.02	**	-0.69
	2 vs 9	RW 1	1,49	6.56	0.01	**	0.70
		RW 3	1,49	10.75	0.002	**	0.71
		RW 5	1,49	5.65	0.02	**	-0.65
		RW 8	1,49	3.19	0.08	*	-0.49
	2 vs 10	RW 1	1,49	8.91	0.004	**	0.79
		RW 3	1,49	15.94	0.0002	**	1.03
		RW 5	1,49	14.15	0.0005	**	-1.03
		RW 8	1,49	3.00	0.09	*	-0.48

Table D.4 CONTINUED

		RW	df	F-value	p-value	Significance	Effect size
Gen 2 and Gen 3	3 vs 7	RW 1	1,61	0.01	0.91		0.29
		RW 3	1,61	5.64	0.02	**	-0.78
		RW 5	1,61	9.40	0.003	**	-0.53
		RW 8	1,61	0.29	0.59		-0.18
	4 vs 8	RW 1	1,59	6.91	0.01	**	-0.32
		RW 3	1,59	0.13	0.72		-0.22
		RW 5	1,59	1.23	0.27		0.28
		RW 8	1,59	0.33	0.57		0.09
	5 vs 9	RW 1	1,49	0.83	0.37		0.25
		RW 3	1,49	0.31	0.58		0.14
		RW 5	1,49	0.55	0.46		0.21
		RW 8	1,49	1.33	0.26		0.32
	6 vs 10	RW 1	1,47	4.91	0.03	**	-0.63
		RW 3	1,47	0.15	0.70		0.11
		RW 5	1,47	0.01	0.94		-0.02
		RW 8	1,47	3.88	0.05	*	-0.39

APPENDIX E: SEX RATIO VARIATION SHAPES THE COLOGICAL EFFECTS OF A GLOBALLY INTRODUCED FRESHWATER

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Manuscript Abstract

Sex ratio and sexual dimorphism have long been of interest in population and evolutionary ecology, but consequences for communities and ecosystems remain untested. Sex ratio could influence ecological conditions whenever sexual dimorphism is associated with ecological dimorphism in species with strong ecological interactions. We tested for ecological implications of sex ratio variation in the sexually dimorphic western mosquitofish, *Gambusia affinis*. This species causes strong pelagic trophic cascades and exhibits substantial variation in adult sex ratios. We found that female-biased populations induced stronger pelagic trophic cascades compared to male-biased populations, causing larger changes to key community and ecosystem responses including zooplankton abundance, phytoplankton abundance, productivity, pH, and temperature. The magnitude of such effects indicates that sex ratio is important for mediating the ecological role of mosquitofish. Because sex ratio variation and sexual dimorphism are common features of natural populations, our findings should encourage broader consideration of the ecological significance of sex ratio variation in nature, including the relative contributions of various sexually dimorphic traits to these effects.

Keywords

Sexual dimorphism, trophic cascade, mosquitofish, *Gambusia*, freshwater ecology

Introduction

Sex ratio variation is a longstanding theme in evolutionary biology. Fisher [1] famously theorized that natural selection should maintain 1:1 sex ratios by continuously favoring the rare sex, thereby always returning skewed sex ratios to equality. In nature, skewed sex ratios are a common observation across the tree of life, and explanations include differential mortality rates for males and females [2], inbreeding and local competition for mates [3], endocrine-disrupting environmental pollutants [4,5], and adaptive maternal effects that allow differential investment in male or female offspring [6–8]. Despite the attention paid to the causes of sex ratio variation in nature, and in some cases its consequences for population growth [9], theory and tests of its effects on communities and ecosystems are lacking. This lack of attention may in part owe to a presumption that the sexes of most species are ecologically equivalent in their effects on communities and ecosystems.

However, many species show marked sexual dimorphism in body size and other traits related to ecological function. Sexual size dimorphism has the potential to influence resource use because prey capture is size-dependent [10] and body size influences overall feeding rates [11]. Body size and physiology also influence rates of nutrient excretion [12], which have important effects for ecosystem processes [13]. Males and females can also be dimorphic in behavioral or morphological traits, affecting resource use [14]. The widespread observations that sex ratios vary in nature, and that males and females often differ in key ecological traits, suggest that sex ratio variation may have impacts on communities and ecosystems. These effects may be particularly prevalent when sex ratio variation is present in ecologically important species, such as keystone, foundation, dominant, or invasive species.

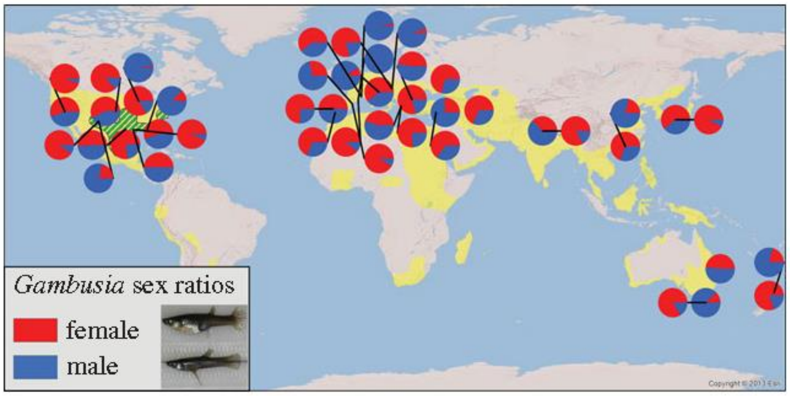

Figure E.1: Sex ratio variation over part of the present day range of mosquitofish (*Gambusia affinis and G. holbrooki*). These species are both widely introduced for mosquito control and were grouped together into a single species until 1988 [55]. Therefore, data for both species are presented. Yellow represents the approximate present day range of mosquitofish, while green hatching represents their native range (modified from [56], with supplemental data from [28,57]). Their range is likely to be larger than presented due to the unreported nature of historical transplantations. For studies reporting more than two sex ratios (within a region or in a single location through time), two pie charts were plotted representing the most female-biased and most male-biased sample taken. Data and sources are reported in Table S1.

We examined the ecological consequences of sex ratio variation in the western mosquitofish (*Gambusia affinis*). Mosquitofish (*G. affinis* and its congener *G. holbrooki*) are small (< 6 cm) livebearing fishes (Poeciliidae), which lack sexual dimorphism in age and size at maturity [15], but show pronounced sexual size dimorphism (Fig. 1) due to differences in post-maturation growth rates. Empirical tests show female mosquitofish display higher feeding rates per unit body size [16,17] and typically show greater niche breadths, with a notable preference for prey of larger body sizes [18,19]. Females also spend relatively more time foraging when in the presence of other females compared to when in the presence of males [20,21]. In addition to these dimorphic foraging characteristics, the relatively large size of females could increase overall nutrient excretion rates for the same density of fish in female-biased populations compared to male-biased populations, which could in-turn affect primary production [13]. As a result of these sex-specific tendencies, we predict that female-biased populations induce stronger pelagic trophic cascades compared to male-biased populations. We expect these effects to be strong because mosquitofish play a major role in aquatic ecosystems by altering invertebrate communities and driving strong trophic cascades that can even change abiotic conditions, including light penetration and nutrient dynamics [22,23].

We performed a pond mesocosm experiment to test this prediction. The experiment was conducted in California (USA), where the western mosquitofish was introduced in 1922 and has since been spread by mosquito vector control districts (MVCD’s) as a means of disease control [24], most notably West Nile Virus [25]. We obtained fish for our experiments from the Sacramento-Yolo MVCD (Elk Grove, CA, USA). This district alone stocks about one million mosquitofish annually over an area of about 5,000 hectares of agricultural fields, wildlife refuges, and private lands in Sacramento and Yolo Counties, California [26]. Mosquitofish were historically introduced for the same purposes elsewhere and are today one of the most widespread and abundant freshwater fishes in the world ([27], Fig. 1). Their strong negative consequences for native fauna across their global range has resulted in their being listed as one of the world’s 100 worst invasive species by the IUCN [28].

Mosquitofish sex ratios vary substantially across their present-day range (Fig. 1). The mechanism of sex determinism is chromosomal, and primary and secondary sex ratios have rarely been found to differ from 1:1 (but see [8]). Therefore, sex ratio variation has primarily been attributed to differential postpartum mortality induced by a variety of physiological and ecological mechanisms, including temperature, salinity, seasonality, and interactions with other species. Predation may be a particularly important driver. For example, avian predators prefer females over males, leading to sex ratio estimates as high as 97% males in some locations [29]. Female-biased populations are more common, and are often attributed to the greater longevity of females (often > 6 months) relative to males (often < 6 months) [30]. Therefore, mosquitofish are a relevant system to understand whether sex ratio variation shapes ecological effects because they are dimorphic, ecologically important, and show substantial sex ratio variation across their global range.

Materials and Methods

Design

We created a 6x6 array of experimental pond mesocosms (1136 L Rubbermaid® stock tanks) on a level field on the grounds of Long Marine Laboratory at the University of California, Santa Cruz. Each mesocosm was layered with 19 L of sand and filled with dechlorinated municipal water. We inoculated each mesocosm with 4 L of thoroughly homogenized sediment from Westlake Pond (Santa Cruz, CA) to introduce nutrients and benthic communities. After the sediment settled (~5 days) a cinderblock (9.3 x 19.0 x 39.3 cm^3) was placed in the center of each mesocosm for habitat cover, and the water was inoculated with a homogenized plankton community comprised of multiple horizontal plankton tows (80 µm mesh) at Westlake Pond.

Experimental fish were obtained from the husbandry facility operated by the Sacramento-Yolo MVCD. Fish at or above the approximate size at maturity were sex-sorted before being assigned to treatments. Fish were identified as adult males if they had evidence of a gonopodium and as adult females if they were larger than the smallest adult male and lacked evidence of a gonopodium [31]. Our experiment thus incorporated the normal range of sexual size dimorphism, as well as other aspects of dimorphism, inherent to the species. We then counted out sex ratio treatments of 0, 25, 50, 75, and 100 percent males at a density of 12 individual fish per mesocosm. We chose this density because it is consistent with stocking densities used by MVCD's and is within the range of densities used in similar experiments [22,23]. Our design included a fishless reference treatment, yielding a grand total of six treatments, each with six replicates. Treatments were assigned to the mesocosm array using a latin-squares random number generator. Fish were introduced to the mesocosms on March 31, 2014, one week after adding plankton to the pond communities. On the same day, we placed two unglazed ceramic tiles (2.8 x 4.3 cm^2) on the cinderblock in each mesocosm to measure periphyton accrual. A 1.27 cm mesh bird-netting was used over all mesocosms throughout the experiment to prevent catastrophic avian and mammalian disturbance, meanwhile allowing for exposure to all other natural physical, chemical, and biological elements, including oviposition by insects and amphibians.

Sampling

Mesocosms were sampled two and four weeks after fish introduction for various pelagic responses including zooplankton abundance, phytoplankton abundance, primary productivity and respiration, pH, temperature at dusk, and water nutrient concentrations. Zooplankton samples were taken from a 1 L depth-integrated water sample filtered through 80 µm mesh and preserved in 80% ethanol. All zooplankters were thereafter counted and identified to the lowest taxonomical distinction possible at 100x magnification. Phytoplankton abundance was estimated as the pelagic chlorophyll *a* concentration in 1 L of water. Water was filtered through a 0.7 µm glass microfiber filter (Whatman® GF/F) and the filters frozen for less than four weeks when we extracted the chlorophyll *a* with 90% acetone for 24 hours at 2° C [32]. The concentration of chlorophyll *a* in the acetone was then measured fluorometrically on a Trilogy

Laboratory Fluorometer (Turner Designs®) using the Turner Designs non-acid module. Ecosystem respiration (ER), net primary productivity (NPP), and gross primary productivity (GPP) were estimated using diel change in dissolved oxygen measured at dawn, dusk, and the following dawn, using a handheld sonde (YSI® Pro 2030) as in [33]. Responses are reported in units of dissolved oxygen concentration (mg/L) change over time. ER is, by convention, reported with negative values which represent the decrease in dissolved oxygen with increasing respiration. Pond temperature and pH were measured with a handheld sonde (Oakton® PTTestr 35). To determine water nutrient concentrations 50 mL water samples were taken from 5 cm below the surface of each mesocosm, filtered through 0.7 µm glass microfiber filters (Whatman® GF/F), and frozen. A week later the water samples were thawed and analyzed for soluble reactive phosphorous (PO4) and nitrate+nitrite (NOx) concentrations on a flow injection analyzer (Lachat® QuickChem) following standard methods [32].

At the end of the experiment (April 28, week 4), we collected samples for benthic community composition, snail abundance, periphyton accrual, and larval amphibian abundance. While mosquitofish primarily feed on pelagic food resources [15], their use of benthic resources has also been observed to cause changes to benthic communities [22]. Benthic community composition was determined from invertebrate counts taken from an 18 cm diameter benthic core in a common central location of each mesocosm. The dry mass of the dominant benthic invertebrate group, Chironomidae (nonbiting midges), was then determined after drying at 60° C for 48 hours. All snails (Planorbidae and Physidae) were then picked from each mesocosm, counted, and then dried and weighed in the same manner as the chironomids above. Periphyton tiles were scrubbed and rinsed into a filter apparatus and then filtered through a 0.7 µm glass microfiber filter (Whatman® GF/F). The filters were frozen and then analyzed for chlorophyll *a* as in the phytoplankton abundance estimation above. Larval Pacific tree frogs (*Pseudacris* *regilla*) were counted in mesocosms where they were present, euthanized using an overdose of tricaine methanesulfonate (MS-222), and dry mass obtained using methods above.

On April 29, we captured all fish from each mesocosm and ran excretion trials to estimate mesocosm-level fish N and P excretion. Fish within a mesocosm were temporarily held in a 10 L floating tub until all fish were captured. Thereafter, all fish from a given mesocosm were introduced to a 2 L Nalgene® bottle filled with 1.5 L of dechlorinated city water, and floated in their respective mesocosm for 30-60 minutes. In fishless treatments, a Nalgene® bottle was floated in the same manner with the same water, however without any fish added. A water sample was then taken from each bottle as in the NOx and PO4 measurements above, and its ammonium (NH4) and PO4 concentrations were determined using the same instrumentation and standard practice [32]. Excretion rate was calculated for all fish treatments as the concentration measured in each tank minus the mean-fishless concentration, all divided by the excretion trial time length. After the excretion assay, we euthanized experimental fish using an overdose of MS-222. Fish were then dried and weighed as above.

Analyses

At the end of the experiment we discovered that four fish had been initially misidentified to sex and three individuals had died (0.8% overall mortality). No fish in the even sex ratio treatment died nor were misidentified to sex, so to avoid dropping replicates, we combined the 0% and 25% male treatments into a single 'female-biased' treatment (N=12 replicates) and the 75% and 100% male treatments into a single 'male-biased' treatment (N=12 replicates). We then ran two separate but identical analyses to determine the differences between 1) the male-biased treatment versus the female-biased treatment, representing the ecological effects of sex ratio variation and 2) the fishless treatment versus the 'even' (50:50 female:male) treatment, representing the ecological effects of addition of mosquitofish as occurs in the context of an introduction or invasion. Mosquitofish introduction is known to have very strong impacts on most of the ecological variables measured, and the main purpose of this latter analysis was to provide a frame of reference effect size to better interpret the relative importance of sex ratio effects.

We employed MANOVA of repeated measures for pelagic (time series) responses. Treatment and Time x Treatment interaction effects were of primary interest. Treatment effects were also tested independently for each time point using student's t-tests, after checking for equality of variances using Levene's test ($\alpha = 0.05$) [34]. If Levene's test was significant for a given response, then Welch's t-test for unequal variances was used [35]. Bonferroni corrections were implemented for interpretation of significance for the two non-independent t-tests performed on time-series data (i.e. those performed at both times separately). Benthic responses, excretion rates, and fish biomass were measured once at the end of the experiment, and thus were analyzed using t-tests as above. Analyses were performed in JMP® Pro (11.2.0) and R Programming Environment (3.1.2) [36]. Zooplankton abundance data were log10(x+1) transformed in order to meet assumptions of normality of residuals. A summary of all the above statistical tests is provided in Table S2.

Calculations of effect size (Cohen's *d*, where $d=(M_1 - M_2)/s_{pooled}$, $s_{pooled} = \sqrt{[(s_1^2 + s_2^2) / 2]}$, M=mean, and s=standard deviation) [37] were used to compare sex ratio effects to the effect of mosquitofish intoduction. For pelagic responses, which were measured twice, we used data from the sampling time point showing the greatest sex ratio effect. *Post hoc* path analyses were used to explore the strength of potential causal linkages among response variables. These analyses were performed on standardized data in R with the package '*lavaan*' [38]. Lastly, we examined whether total fish biomass explained variation in important pelagic responses within grouped sex ratio treatments using simple linear regression.

Results

Female-biased treatments had lower abundances of crustacean zooplankton (dominated by *Daphnia*, *Bosmina*, *Sida*, and calanoid copepods) than male-biased treatments ($F_{1,22}$=11.620, P=0.003). Rotifer abundance (dominated by the family Brachionidae) did not differ between sex ratio treatments ($F_{1,22}$=1.069, P=0.312). Female-biased treatments had higher phytoplankton abundance ($F_{1,22}$=3.953, P=0.059) and higher rates of NPP ($F_{1,22}$=4.819, P=0.039), ER ($F_{1,22}$=12.315, P=0.002), and GPP ($F_{1,22}$=9.441, P=0.006). Although the Sex Ratio effect was nonsignificant for pH ($F_{1,22}$=2.084, P=0.163) and temperature ($F_{1,22}$=2.698, P=0.115), the Time x Sex Ratio interaction effects were significant (pH: $F_{1,22}$=4.400, P=0.048, temperature: $F_{1,22}$=9.333, P=0.006). Overall these results suggest that female-biased populations induced stronger pelagic trophic cascades when compared with male-biased populations by driving a relative decrease in crustacean zooplankton abundance, an increase in phytoplankton abundance, and an increase in ecosystem production and respiration (Fig. 2). Concentrations of NOx and PO4 were not influenced by Sex Ratio (NOx: $F_{1,22}$=0.021, P=0.887, PO4: $F_{1,22}$=1.742, P=0.201) or the Time x Sex Ratio interaction (NOx: $F_{1,22}$=0.078, P=0.783, PO4: $F_{1,22}$=2.046, P=0.167). The t-tests performed to analyze pelagic response trends at each time separately were in general support of results from the MANOVA of repeated measures. Pelagic responses, other than crustacean zooplankton abundance, were significant at week 2 but not at week 4 (Table S2).

Benthic invertebrate samples were dominated by chironomid larvae (98% of all organisms counted). Sex ratio had no effect on their abundance (t_{22}=0.164, P=0.871), total mass (t_{22}=0.499, P=0.622), or mean individual mass (t_{22}=0.154, P=0.879). Mean individual snail mass was larger in female-biased treatments (mean=0.061 g, S.D.=0.025) than male-biased

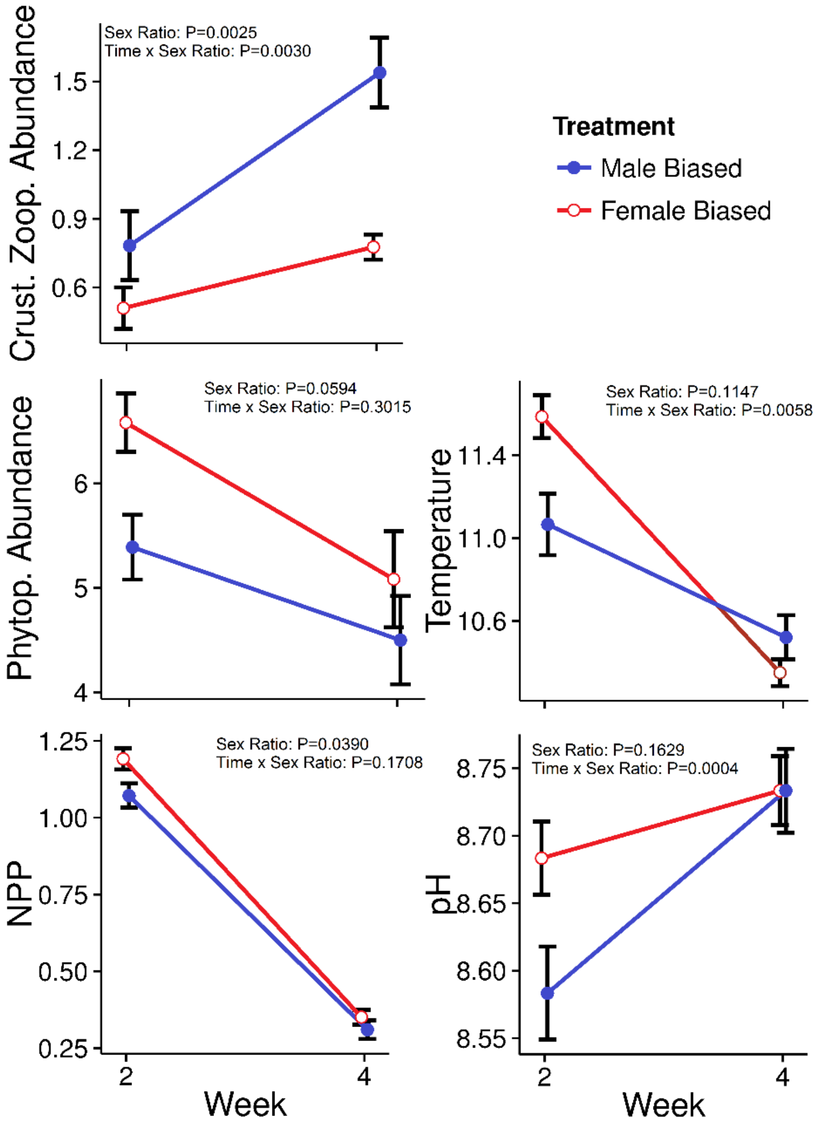

Figure E.2: Impacts of mosquitofish sex ratio on the strength of various components of the pelagic trophic cascade (mean ± SE). Ponds with female-biased sex ratios had relatively lower densities of crustacean zooplankton, higher densities of phytoplankton, higher net primary productivity (NPP), higher pH, and higher temperature. P-values are reported for the Sex Ratio and Time*Sex Ratio effects from the MANOVA of repeated measures.

treatments (mean=0.045 g, S.D.=0.013) (Welch's $t_{14.83}$=1.883, P=0.040), but there were no differences in overall snail abundance (t_{22}=1.189, P=0.247) or biomass (t_{22}=0.242, P=0.811). Periphyton accrual was not affected by sex ratio (t_{22}=1.243, P=0.227).

Female-biased treatments had a mean fish biomass of 1.877 g (S.D.=0.1707) and excretion rates of 4.470 µg N per minute (S.D.=1.0197) and 0.485 µg P per minute (S.D.=0.4430), while male-biased treatments had a mean fish biomass of 0.962 g (S.D.= 0.2454) and excretion rates of 1.807 µg N per minute (S.D.=0.4667) and 0.132 µg P per minute (S.D.=0.3445). These differences were significant for biomass of mosquitofish (t_{22}=10.602, P<0.001) and excretion rates for both N (t_{22}=8.225, P<0.001) and P (t_{22}=2.178, P=0.040).

Amphibian eggs were deposited in at least one replicate of each treatment within the first week of fish introduction. However, larval amphibians were only recovered in three replicates of the fishless treatment at week 4. The biomass of tadpoles in those replicates ranged from 0.051 g to 0.289 g.

Mosquitofish addition (relative to fishless conditions) affected all pelagic responses except for NOx concentration (Table S2, Fig. S1). Mosquitofish addition also had no effect on the number of snails (t_{10}=0.282, P=0.784) and chironomids (t_{10}=0.772, P=0.458), the mean individual snail mass (t_{10}=0.444, P=0.667) and the mean individual chironomid mass (t_{10}=1.031, P=0.327), or the overall snail mass (t_{10}=0.005, P=0.996) and overall chironomid mass (t_{10}=1.367, P=0.202). Periphyton accrual was higher in the mosquitofish addition treatment (mean=0.172 µg/cm^2, S.D.=0.1229) than the fishless (mean=0.049 µg/cm^2, S.D.=0.0158) treatment (t_{10}=2.433, P=0.035).

Upper effect sizes were calculated on week 2 data for all pelagic responses except for zooplankton abundance, which was performed on week 4 data. Sex ratio effect sizes for all significant responses were "large" (i.e. greater than 0.8, [37])(Fig. 3). Interestingly, although sex-ratio effects were less than half those of mosquitofish introduction (presence versus absence) female-biased sex-ratio effects nearly always reinforced introduction effects (Fig. 3). Path analyses were performed to investigate the drivers of phytoplankton abundance, NPP, and snail size across all treatments. We found that phytoplankton abundance at week 2 across all treatments with fish was affected by fish excretion rather than by crustacean zooplankton abundance (Fig. S2). NPP was found to be driven by direct effects of both temperature and phytoplankton abundance (Fig. S3). Snail size was related to the number of females present in a mesocosm, but not significantly related to temperature or the amount of primary production (Fig. S4). Linear regressions of significant pelagic responses on biomass were not significant ($\alpha=0.10$) in any case (Table S3).

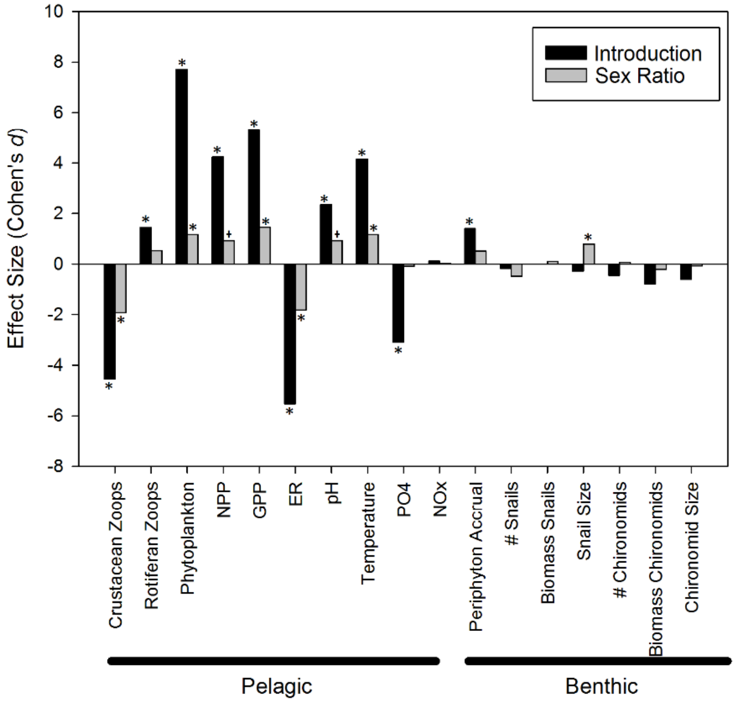

Figure E.3: Comparisons of upper sex ratio effect sizes to effect sizes of mosquitofish introduction at an even sex ratio. Significance symbols from t-tests ($p < 0.10^{+}$, $p < 0.05^{*}$) (Table S2) are based on unadjusted p-values for benthic responses and Bonferroni-adjusted p-values (i.e. 2 x p) for Pelagic responses since they were measured twice. Effects going in the same direction (positive or negative) indicate that female-biased treatments exacerbated the effects of mosquitofish introduction relative to male-biased treatments.

Discussion

Mosquitofish are a globally introduced freshwater fish showing pronounced sexual and ecological dimorphism and widespread variation in adult sex ratios (Fig. 1, [15,31]). Female mosquitofish are larger than males, prefer larger food items [18,19], exhibit higher feeding rates [16,17], and spend more time foraging in the presence of other females [20,21]. We therefore predicted that female-biased populations would induce stronger trophic cascades than male-biased populations. Our results show that female-biased populations also exhibit higher nitrogen and phosphorous excretion rates, as expected given the sexual size dimorphism, which could also lead to an increase in trophic cascade strength.

Consistent with this prediction, we found that experimental ponds with female-biased populations had reduced crustacean zooplankton abundances and increased phytoplankton abundances relative to ponds with male-biased populations (Fig. 2). Female-biased ponds had higher water temperatures, likely caused by the increase in turbidity associated with increased phytoplankton abundance, which can increase the absorption of solar heat energy [39]. Female-biased ponds also had higher NPP and pH compared to male-biased ponds. Increases in NPP can be attributed to both the increase in pond temperature and the increase in phytoplankton abundance (Fig. S3). Pond pH increases when primary production reduces the amount of dissolved carbon dioxide, shifting the equilibrium in the aqueous carbonate system [40]. While the large effect of mosquitofish introduction on pond temperature and pH has been noted in the past [22,41], our results suggest that sex ratio has a surprisingly large role mediating the magnitude of these effects (Fig. 3).

Early on in our experiment (week 2), the effect of sex ratio for trophic cascades was clear, despite relatively modest effects of sex ratio on crustacean zooplankton abundance. Excretion is known to play a large role in contributing to trophic cascades [43], and thus, treatment differences in excretion could have driven this early cascade (Fig. S2). This trend may additionally highlight the importance of behavioral changes induced by perceived threats of predation [42]. In this case, zooplankton could have reduced their foraging rates to a greater extent in female-biased treatments relative to male biased treatments. Later in our experiment (week 4) the cascading effects of sex ratio diminished, while zooplankton effects greatly strengthened. It is possible that proliferation of grazing-resistant forms of phytoplankton led to the observed reduction in trophic cascade strength through time (Fig. 2, Fig. S1, [43]) or that these patterns represent natural cycling of zooplankton and phytoplankton abundances.

Mosquitofish are typically consumers of pelagic and epipelagic resources [15]. However, mosquitofish may also consume benthic food resources, especially in the littoral zone. In our experiment, mosquitofish introduction did not decrease chironomid density, which may be due to greater resources available in the pelagic zone of our artificial pond mesocosms. Indeed, the only significant effect of mosquitofish on benthic resources (P=0.04) was for snails. Snails are not a major diet item, yet there is evidence that they are occasionally consumed, especially by large females [44]. Surprisingly, sex ratio influenced the average individual size of snails while fish introduction had no effect on snail size. Predation is known to plastically induce higher growth rates in snails [45]. Female-biased mesocosms had larger snails than male-biased mesocosms, which could be the result of faster growth rates of snails in response to the presence of snail-consuming females. Alternatively, both the higher temperatures and the relatively high rate of primary production in ponds with female-biased sex ratios could have led to faster snail growth. Path analysis suggests that the number of females present had a larger effect on snail size than did temperature or NPP (Fig. S4); however, the mechanism driving this effect remains unclear.

Mosquitofish have been introduced worldwide throughout the past century for mosquito biocontrol purposes, resulting in unintended consequences for native biodiversity. Their negative impacts on amphibians and native fishes have been the subject of extensive study (reviewed in [15]). In our experiment, amphibians deposited eggs in all treatments, but larval amphibians were found only in mesocosms lacking mosquitofish. The introduction of mosquitofish into historically fishless habitats (e.g. isolated springs) has led to declines in native invertebrates as well [46]. Because of these global impacts, mosquitofish have been nicknamed the ‘plague minnow’ [47] and identified as one of the world’s 100 worst invasive species [48]. Where mosquitofish have invaded, population control efforts are commonly employed to mitigate such negative consequences. Traditional methods of mosquitofish removal such as the use of minnow traps may selectively remove females due to their larger body size [20]. A recently described control method proposes to use ‘Trojan sex chromosomes’ to control mosquitofish abundance by generating females that can only produce male offspring, with the goal of creating male-biased populations in order to lower reproductive output and increase the probability of local extinction [46]. Our results suggest that control measures that reduce the relative abundance of females may have added benefits for aquatic communities and ecosystems since it is the females that cause the strongest ecological effects.

Our experimental design sought to control fish density while allowing for natural variation in biomass associated with sexual size dimorphism. Controlling density was necessary because density strongly influences behavior of these social fish [49,50]. At the same time, including the effects of sexual size dimorphism was important because it is a principal expression of sexual dimorphism in mosquitofish, a widespread form of dimorphism in general, and theoretically important due to its effects on consumption and excretion. Biomass and sex ratio were strongly collinear in our experiment (Fig. S5), suggesting sexual size dimorphism is indeed an important component of our findings, but it is important to recognize that our study design does not preclude effects of other forms of ecological dimorphism. Indeed biomass variation from size dimorphism did not explain a significant amount of variation in pelagic responses within treatments (Table S3). Moreover, one might predict some amount of sex ratio effects tied to other ecological aspects of sexual dimorphism. Empirical evidence suggests females have higher feeding rates than males per unit body size [16,17] and spend more time feeding when in the presence of other females [20,21]. In addition, mosquitofish size dimorphism is largely related to differences in post-maturation growth rates. Since excretion rates are determined partly by instantaneous growth rate [51], it is likely that there exists some size-specific sexual dimorphism in excretion rates. Future investigation of sex-ratio effects might thus profitably employ study designs to isolate and estimate the ecological effects of size- and non-size components of sexual dimorphism.

Conclusions

Our work demonstrates that sex ratio variation in ecologically important species showing sexual dimorphism can lead to marked ecological effects. This study adds to a growing literature suggesting that intraspecific variation may be important for shaping ecology [52–54]. Indeed, sexual dimorphism is one of the most common and well-known forms of intraspecific trait variation in the wild and many populations in nature show marked demographic differences in sex ratios from the commonly assumed expectation of 1:1 [2–5,7]. As such, we suggest that sex ratios may be a common driver of community and ecosystem variation across a wide diversity of organisms and habitat types. We recommend future investigations into other study systems where there is known sexual dimorphism in functional traits, where the focal species is ecologically important (e.g. keystone species, invasive species, dominant species), and where there is substantial sex ratio variation in the wild. Subsequent work with size dimorphic species should aim to isolate biomass-dependent and biomass-independent sex ratio effects. Such work can further inform our understanding of the ecological importance of one of the most

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