Behavioral and Reproductive Plasticity in Eastern Mosquitofish (Gambusia Holbrooki) in Response to Predatory Cues

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BEHAVIORAL AND REPRODUCTIVE PLASTICITY IN EASTERN MOSQUITOFISH (*GAMBUSIA HOLBROOKI*) IN RESPONSE TO PREDATORY CUES

by

CAROLYN VANZWOLL

(Under the Direction of Stephen P. Vives and Edward B. Mondor)

ABSTRACT

Olfaction in fishes can be an important mechanism in determining the level of predation risk. Many fishes possess alarm signaling systems, wherein an individual can detect injured conspecifics via olfaction and respond behaviorally to the presence of a predator. The superorder Ostariophysi exhibits a fright reaction to injured conspecifics, characterized by specialized cells and alarm substances. I tested if a live-bearing non-ostariophysan, *Gambusia holbrooki*, exhibited a similar behavioral response to its injured conspecifics. I also examined the effects of exposure to this and other predatory cues during a single pregnancy cycle. I hypothesized that immediate exposure to the cues would provoke a clear behavioral reaction, and long-term exposure would cause *G. holbrooki* to alter nutrient provisioning to developing embryos, resulting in altered offspring morphology and performance. *Gambusia holbrooki* exposed to skin extract (an alarm substance from injured conspecifics) schooled significantly closer than fish not exposed to skin extract. Mosquitofish did not display any discernible life-history plasticity in response to predatory cues during a single pregnancy. They did, however, exhibit marked differences in fecundity-mother size relationships between two locations in coastal Georgia. These results confirm the existence of a behavioral alarm reaction in *G. holbrooki*, and different reproductive traits between locations. Many animals
experience morphological and life-history plasticity in response to shifts in abiotic and biotic environmental factors. Additional replication is necessary to determine if this species alters nutrient provisioning to embryos in response to predation risk.

INDEX WORDS: Alarm signal, Brood development, Fright reaction, *Gambusia holbrooki*, Mosquitofish, Predation risk, Reproduction
BEHAVIORAL AND REPRODUCTIVE PLASTICITY IN EASTERN MOSQUITOFISH (GAMBUSIA HOLBROOKI) IN RESPONSE TO PREDATORY CUES

by

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B.S., Michigan State University

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

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BEHAVIORAL AND REPRODUCTIVE PLASTICITY IN EASTERN MOSQUITOFISH (*GAMBUSIA HOLBROOKI*) IN RESPONSE TO PREDATORY CUES

by

CAROLYN VANZWOLL

Major Professors: Stephen P. Vives
Edward B. Mondor

Committee: Daniel F. Gleason

Electronic Version Approved: May 2010
DEDICATION

This thesis is dedicated to my husband Mark, who through his unconditional love and support, minimal tolerance for whining, and his shining example of hard work and integrity inspired me to complete my graduate degree.

This dedication would be incomplete without mention of my parents, George and Cynthia Schwedler. Without their passionate encouragement and commitment to my education, this degree would not be possible.
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CHAPTER I
LITERATURE REVIEW: PREDATORY RISK ASSESSMENT AND AVOIDANCE OF PREDATORS IN AQUATIC ECOSYSTEMS

The risk of predation can have considerable influence over the behavioral decisions of prey animals, and may impact entire ecosystems (Lima 1998, Harvey 1991). Successful predation events lead to the removal of individuals (and their genes) from a population, potentially impacting prey population dynamics. Predation can be an instrumental selective pressure on prey animals’ behavior, morphology, and life history traits (Lima and Dill 1990). In many organisms, a predation event progresses through a cycle of steps: search, encounter, attack, capture, and ingestion (Brönmark and Hansson 2000). A suite of cues may be detectable by prey at each step, allowing them to respond appropriately (Brönmark and Hansson 2000). Prey that detect and respond to these cues are able to disrupt the predation cycle and avoid capture or ingestion. Prey responses to predation risk in the short-term can be behavioral or physiological, and longer-term exposure to predators may produce changes in morphology or life-history patterns.

For an organism to decrease local and immediate predation risk, short-term changes in behavior (evasion, hiding, etc.) may be sufficient. In an environment where predation risk is high, behavioral changes may not be sufficient to avoid being depredated. Some organisms are able to change their morphology or life-history traits to reduce the probability of predation (Lima and Dill 1990). But these modifications involve an energy tradeoff; predator-induced behavioral, morphological, or life-history modifications exploit energy used for foraging, mating, other fitness-related behaviors, and growth (Lima 1998). Because of this tradeoff, it is vital for prey species to be able to
accurately assess the magnitude of predation risk in order to make avoidance-based
decisions (Lima and Dill 1990).

Risk assessment can range from individual detection of a predator to complex
alarm signaling systems and cultural transmission of risk information. Several
mechanisms exist to detect predatory risk: visual, chemical (olfaction and taste), tactile,
auditory, or electrical (Smith 1992). Vision is rapid and direct, but olfactory detection of
predatory and other cues can become particularly important (i.e., fewer signaling errors
are likely) in turbid or complex habitats where vision is reduced (Wisenden 2000).
Aquatic environments are particularly conducive to chemical dispersion (Wisenden
2000). Through forward movement and/or pumping mechanisms, fishes experience a
nearly continuous flow of water and solutes over the olfactory organs (Kleerekoper
1969). Olfaction allows the animal to be keenly aware of its surroundings, regardless of
visual or other sensory deprivation. The ability to detect even a minute amount of a
substance can be especially useful in lotic habitats (characterized by moving water),
where dilution of pheromones and other chemical cues can be rapid. Chemical cues are
particularly useful warning mechanisms in group situations. Prey response to injured
conspecifics is a well-documented phenomenon in many taxa. If a prey animal is injured
or ingested, the group (which presumably includes its kin) can retain fitness by
responding to the predator, even though the fitness of the injured individual may be
sacrificed. The Schreckstoff chemical alarm signaling system in fishes is a classic model
system of chemical communication and response to predation risk (see reviews by Smith:
Fishes of the superorder Ostariophysi (which includes minnows, catfishes, and characins) exhibit a fright reaction when exposed to an alarm substance known as Schreckstoff (von Frisch 1942, Schutz 1956). Schreckstoff, likely hypoxanthine-3 (N) oxide (and/or other nitrogen oxides, Brown et al. 2000), is enclosed in modified epidermal club cells (Fig. 1) and is released only when the skin is mechanically damaged (Reed 1969, Pfeiffer 1960). Ostariophysan fishes detect this chemical stimulus from their injured conspecifics through olfaction (Kleerekoper 1969). There are a wide range of behavioral reactions to Schreckstoff (Smith 1992). For species that live among vegetation, such as the blackspotted topminnow (Fundulus olivaceus) a “freezing” response occurs (Reed 1969). Minnows that “freeze” among vegetation are presumably less likely to be spotted by a predator (Reed 1969). Other species increase swimming activity: skittering, swimming rapidly, and darting (Reed 1969, Smith 1992). Excited movements may serve to confuse, distract, or out-swim a potential predator. Many species that exhibit an increase in activity will form a tight, protective school with conspecifics (Smith 1992, Nordell 1998), further confounding the predator (reducing the chance of individuals being depredated). Similar (though not homologous) alarm reactions are present in non-ostariophysan fishes, such as percid darters, gobiids, sculpins, and some cyprinodontiformes including the family Poeciliidae (live-bearers) (Schutz 1956, Pfeiffer 1977, Nordell 1998, Garcia et al. 1992, Reed 1969, Smith 1992).

Because signal-senders are most often sacrificed (captured or consumed by the predator), direct benefits to the individual possessing a chemical alarm signal are difficult to ascertain. Thus, identifying evolutionary pathways to this type of communication is problematic. Smith (1977) suggested that maintaining these cells is an example of
altruism maintained by kin selection. Mathis et al. (1995) and Chivers et al. (1996) proposed that alarm substances act as attractants to other predators. For example, when a prey individual is captured, its alarm substance attracts other predators to the area which disrupts the primary predation event. Smith and Lemly (1986) described a population of wild fathead minnows, of which 16% had survived damaging encounters with predators. If the possibility of surviving a signaling event is high, individual selection may be preserving the signal (Smith and Lemly 1986).

In many species, prey fishes can detect the diet of predators, particularly if the predator has consumed their conspecifics. Fathead minnows avoid areas marked by feces of pike that have not ingested other fathead minnows (Brown et al. 1995). Type of diet and level of satiation in green sunfish (Lepomis cyanellus) is detectable by western mosquitofish (Gambusia affinis), their potential prey. Mosquitofish avoid green sunfish that are hungry or that have been on a mosquitofish diet (Smith and Belk 2001). Green sunfish that feed on chironomids or are satiated are perceived to be less risky (Smith and Belk 2001).

Behavioral reactions of individuals may affect their conspecifics as well as other species of fish in the area. In the fathead minnow, chemical recognition of predatory risk is culturally transmitted (Chivers and Smith 1995). Novice minnows learn to recognize risky habitats from experienced conspecifics that exhibit a behavioral response (Chivers and Smith 1995). Three-spined sticklebacks (Gasterosteus aculeatus) are not sensitive to the Schreckstoff substance (Krause 1993). Chub (Leuciscus cephalus) do display a fright response to Schreckstoff, and are commonly found in similar habitats as stickleback. When stickleback are exposed to the fright behavior of chub, they imitate the chubs’
reaction: increased vigilance, dashing, and possibly shoaling (Krause 1993). Parental behavior can also influence the fitness of their young. Eastern mosquitofish (Gambusia holbrooki), a livebearing species, alter habitat use when under predation by chain pickerel (Esox niger). Because adult Gambusia seek refuge in habitats with more vegetation, neonates have a higher survival rate (Winkelman and Aho 1993). Vegetation provides the neonates with refuge from the same predators, but also from filial cannibalism, a common behavior in this species (Pyke 2005). Even small behavioral shifts can be enough to impact entire communities. For example, largemouth bass (Micropterus salmoides) induce habitat shifts in their prey, cyprinid minnows (Harvey 1991). These shifts in cyprinid communities increase the survival and abundance of sunfish (Lepomis spp.), whose larvae would otherwise be consumed by cyprinids (Harvey 1991).

Heterospecifics may also react to an injured prey fish. Brook stickleback (Culaea inconstans), a non-ostariophysan species, do not contain Schreckstoff (Pfeiffer 1977). They do, however, exhibit fright responses when exposed to the alarm substance of fathead minnows (Mathis and Smith 1993). Fathead minnows are sympatric with stickleback, and share common predators (Mathis and Smith 1993). Fathead minnows can detect and avoid feces of pike that had been fed stickleback or swordtails (Xiphophorus helleri) (Brown et al. 1995). Several other ostariophysan fishes, including northern squawfish (Ptychocheilus oregonense), redside shiner (Richardsonius balteatus), chubs, dace, and suckers also exhibit fright reactions in response to heterospecific alarm substance (Pfeiffer 1963).

Behavioral reactions to predation risk are well-studied. However, morphological and life-history changes can also be predator-induced. Larvae of the marine intertidal
snail (*Littorina scutulata*) develop rounder shells with smaller openings when reared in the presence of predators, larval *Cancer spp.* (Vaughn 2007). Smaller shell apertures hinder the predators’ attempts to open the shell and extract the snails (Vaughn 2007).

*Daphnia spp.* react to predator odors by producing protective spines, altering sex ratios, and varying maturation rates (Boersma *et al.* 1998). Tadpoles raised in the presence of predators possess narrower bodies and deeper tails with wider and thicker tail muscles as compared to tadpoles in the absence of predators (Kraft *et al.* 2006). These predator-induced tadpoles had higher survival rates than their non-induced counterparts (Kraft *et al.* 2006). Goldfish (*Carassius auratus*) increase body depth and weight in response to chemical predation cues, presumably to reduce the likelihood of being consumed by a gape-limited predator (Chivers *et al.* 2007). Crucian carp (*Carrassius carassius*) also have an inducible morphological defense against gape-limited predators. Carp increase their body depth in response to alarm signals from conspecifics (Stabell and Lwin 1997).

Pumpkinseed sunfish (*Lepomis gibbosus*) respond similarly (Januszkiewicz and Robinson 2007). Pumpkinseeds increase body depth and dorsal spine length when reared with walleye (*Sander vitreus*) versus other sunfish (Januszkiewicz and Robinson 2007).

Langerhans *et al.* (2004) documented a predator-induced morphological divergence in *Gambusia affinis*. Mosquitofish from environments containing piscivorous fish have smaller heads, larger caudal regions, and more elongate bodies than those from predator-free environments (Langerhans *et al.* 2004). These divergent morphologies produce a 20-percent difference in burst-swimming speed (Langerhans *et al.* 2004). Female guppies (*P. reticulata*) exposed to chemical and visual cues from live predators increase their reproductive output at first spawn (Dzikowski *et al.* 2004). When female guppies are
exposed to visual and chemical predator cues, they shorten the duration of brood retention (Evans et al. 2007). Swimming performance and ability to avoid capture is directly affected by brood duration, i.e., females with shortened brood retention produce offspring with impaired swimming and avoidance abilities (Evans et al. 2007).

To conclude, accurate assessment of predation risk in the aquatic environment is essential for survival. Chemical communication is particularly important in aquatic ecosystems, where turbid or complex habitats can lead to visual signaling errors. Prey fishes have a variety of methods to detect and respond to risk, and these responses can have considerable influence over population and community dynamics. The literature on the behavioral reactions to predators is profuse, but there have been relatively few studies on life-history or transgenerational responses to predators in fishes. I address prey responses to predation via laboratory studies of the live-bearing Poeciliid Gambusia holbrooki (Girard). Gambusia holbrooki were used as model prey; they are abundant locally, breed rapidly, and serve as prey for many freshwater piscine predators. In Chapter II, I examine the behavioral (short term) reaction of G. holbrooki to skin extract from conspecifics, to determine if a pheromone and fright reaction (similar to Schreckstoff) exists in this species. Chapter III investigates life-history shifts in G. holbrooki in response to long-term visual and chemical predation cues, and examines variation in life-history between two locations in coastal Georgia.
References


Figure 1. Ostariophysan club cells. Typical configuration of specialized “club cells” (A) and mucus cells (B) within the epidermis of ostariophysan fishes. Club cells contain Schreckstoff and are completely enclosed within the skin. Typical club cells do not have an outlet to either the circulatory system or the surface of the skin; Schreckstoff release is not actively mediated by the fish, but only by mechanical damage. Adapted from Pfeiffer (1960).
CHAPTER II

PILOT STUDY: FRIGHT REACTION IN GAMBUSIA HOLBROOKI IN RESPONSE TO SKIN EXTRACT FROM CONSPECIFICS

Introduction

The ability to reliably assess predation risk is critical to individual survival. There are several mechanisms by which fishes can detect predatory risk: visual, chemical, tactile, auditory, or electrical (Smith 1992). Vision is rapid and direct, but in habitats where vision is limited, other methods of predator detection may be essential. The aquatic environment is conducive to the dispersion of chemical cues, and fishes experience a nearly-continuous flow of water and solutes over the olfactory organs via forward movement and/or pumping mechanisms (Kleerekoper 1969). Olfaction allows the animal to be aware of its surroundings regardless of visual or other sensory deprivation. Many fishes are able to detect the presence of a predator, the diet of the predator, or even a predation event on its conspecifics through olfaction (Smith 1992).

Fishes of the order Ostariophysi exhibit a fright reaction when exposed to an alarm substance known as Schreckstoff (Schutz 1956, see Pfeiffer 1962 for review). Schreckstoff, likely hypoxanthine-3 (N) oxide (and/or other nitrogen oxides, Brown et al. 2000), is enclosed in modified epidermal club cells and is released only when the skin is mechanically damaged (Reed 1969, see review by Smith 1992). Ostariophysan fishes can detect this chemical stimulus from injured conspecifics through olfaction (Kleerekoper 1969). Reactions to Schreckstoff include dashing (bursts of fast swimming), tight schooling, reduced foraging, and reduced overall activity (Smith 1992). Further studies have revealed similar (though not homologous) alarm systems in non-ostariophysan
fishes, such as percid darters, gobiids, sculpins, and some cyprinodontiformes including the family Poeciliidae (live-bearers) (Schutz 1956, Pfeiffer 1977, Nordell 1998, Garcia et al. 1992, Reed 1969, Smith 1992).

Schutz (1956) described reactions in least killifish (Poeciliidae, *Heterandria formosa*) and guppies (Poeciliidae, *Poecilia reticulata*) to alarm substance (“skin extract”) obtained from conspecifics. Only females exhibited this reaction, though both male and female skin extracts produced a response (Schutz 1956). A review by Pfieffer (1977) described fright reactions occurring in several species of Poeciliidae, including mosquitofishes (*Gambusia spp.*), *H. formosa* and *P. reticulata*. None of these species, however, have specialized alarm substance cells homologous to the epidermal club cells of the Schreckstoff system in Ostariophysi (Pfeiffer 1977). The specific fright reaction varies only slightly among poeciliids: guppies increase schooling cohesion (Nordell 1998); both guppies and least killifish seek refuge among aquatic plants (Schutz 1956); and guppies, killifish, and *G. affinis* all swim downward in response to these chemical cues (Schutz 1956, Garcia et al. 1992).

Eastern mosquitofish (*Gambusia holbrooki*) have only recently been considered a genetically distinct species from western mosquitofish (*G. affinis*) (Wooten et al. 1988). The two species are similar in appearance and biology and are often confused (see review by Pyke 2005). Much of the early literature on *Gambusia* does not distinguish between the two species; thus, it is not possible to ascertain from the literature if there is a reaction to conspecific skin extract in *G. holbrooki* similar to that of its close poeciliid cousins. If this species does react to its injured conspecifics, it will represent yet another non-
ostariophysan that possesses the Schreckstoff-analogous fright substance. Therefore, I conducted a study to determine if the alarm reaction exists in *G. holbrooki*.

My objective for this study was to determine if a fright reaction exists in *Gambusia holbrooki*. I hypothesized that *G. holbrooki* would exhibit a behavioral response to skin extract from conspecifics. During the acclimatization period of the study I observed that *G. holbrooki* spend a majority of their time at the bottom of their aquaria, with the exception of swimming to the surface to obtain food. As such, it would not have been possible to determine if *G. holbrooki* swim downward in response to this cue, as do *G. affinis*. I predicted, consequently, that if mosquitofish were exposed to skin extract from conspecifics, then they would react to the fright stimulus in a manner similar to that of *Poecilia reticulata* – by forming a close school.

**Methods**

*Study Population and Maintenance*

Eastern mosquitofish (*Gambusia holbrooki*) were collected in late April 2010. Forty-five females and twenty males were collected from Lake Wells at Georgia Southern University (32° 25' 27.83"N, 81° 46' 59.11"W, Statesboro, GA, USA), and the same number were collected from Ebenezer Creek, at a boat launch along County Rd. 307 in Effingham County (32° 21' 51.61"N, 81° 13' 51.08"W, Springfield, GA, USA). Fish were kept in 75.7L (20-gallon) stock tanks, separated by location.

Treatment aquaria contained a single air stone for aeration, but no filtration system to avoid filtering the chemical treatment (see Nordell 1998), and no substrate to facilitate washing between replicates. Trimmed sheets of opaque black plastic (Sunbelt®
4mil plastic sheeting) were used to cover the sides of aquaria visible to other fish, to prevent any secondary visual alarm responses. All aquaria were housed in a laboratory facility at Georgia Southern University, and maintained between 22-24°C, with 12h:12h light/dark cycle. Fish were fed Tetra Fin® brand dry flake food *ad libitum* daily. Fish were allowed to acclimatize to laboratory conditions for approximately one week before being transferred to experimental aquaria.

*Extraction of Alarm Substance*

Procedures similar to Nordell (1998) and Evans *et al.* (2007) were followed to extract alarm substance from *Gambusia holbrooki*. Fish were humanely euthanized by spinal cord pithing, decapitation, and brain-pithing (Georgia Southern University Institutional Animal Care and Use Committee, IACUC protocol #I08010). Immediately following euthanasia, I made several lacerations along each flank of the fish, to simulate predatory damage to epidermal cells. I then rinsed each fish with 20mL of distilled water (Nordell 1998) to free the alarm substance from the skin. The rinse was filtered through one layer of grade no.1 qualitative filter paper (Whatman ®) to make a clear solution, free of suspended particles, hereafter referred to as skin extract. The skin extract was then poured into a clean 20cc syringe.

*Experimental Procedure*

Two males and four females were haphazardly assigned to one of five 37.9L (10-gallon, 50l x 27w x 25h cm) treatment aquaria, *i.e.*, a school of six fish per tank. Fish were allowed to acclimatize to experimental aquaria overnight (~14h).
A twenty mL aliquot of skin extract was randomly assigned to half of the treatment tanks, and the remaining tanks received 20 mL distilled water (control). Treatments were injected from a distance through airline tubing that ran parallel to the air supply and terminated just above the air stone. I performed a dispersal test with food coloring (McCormick® blue) to determine how quickly the treatments would become uniformly mixed into the aquaria. The air flow through the stone provided a sufficient mixing mechanism for the treatment, and the treatment became homogenously mixed into the aquarium water approximately 2 min after injection. Five replicates were performed for each treatment and location (a total of 20 trials). Between replicates, aquaria and air stones were washed thoroughly with Dawn® dish soap and rinsed several times with hot water to remove any chemical residues.

Each trial was photographed using a tripod-mounted Panasonic® DMC-FZ20 digital camera. Photographs of the distribution of fish within the tanks were taken every 30 seconds for 2 minutes with no treatment (“baseline”). At the 2-minute mark, the treatment was injected, and I continued to photograph the tanks every 30 seconds for an additional 8 minutes. To avoid exposing the fish to visual or auditory cues, I sat very still behind a large screen during the injection procedure, and used a remote shutter-release cable to take the photographs. To measure school cohesiveness, a white paper background with a grid of 5x5cm squares was placed on the back of each treatment aquarium. I recorded the index of cohesion (the maximum number of fish present in the same square; Nordell 1998) from each photograph.

Baseline scan measurements were averaged (photographs taken from time 0:30-2:00), as were the scans for the remaining four post-treatment two-minute intervals (2:30-
Repeated measures multivariate analysis of variance (MANOVA) was used to analyze differences in the index of cohesion between treatment groups, locations, and the change over time (baseline versus the four post-treatment 2-minute intervals). Before statistical analyses, cohesion data underwent tangent (x) transformation to better satisfy assumptions of normality and homogeneity of variances. Data were analyzed using JMP version 8.0 software (SAS Institute 2008).

**Results**

Mosquitofish receiving skin extract had significantly higher indices of cohesion than controls (F<sub>1,16</sub>=39.49, p<0.01). There was no effect of location (F<sub>1,16</sub>=0.04, p=0.84) or interaction between treatment and location (F<sub>1,16</sub>=0.47, p=0.50). Overall, index of cohesion was significantly different over time (F<sub>4,13</sub>=5.19, p=0.01) and by treatment over time (F<sub>4,13</sub>=5.82, p<0.01) (Fig. 2). Baseline measurements between treatments were significantly different from the 2:30-4:00 interval (F<sub>1,16</sub>=10.82, p<0.01). Baseline was also significantly different by treatment from the 4:30-6:00 interval (F<sub>1,16</sub>=16.64, p<0.01), the 6:30-8:00 interval (F<sub>1,16</sub>=22.37, p<0.01), and the 8:30-10:00 interval (F<sub>1,16</sub>=25.58, p<0.01). Index of cohesion was not significantly different by location over time (F<sub>4,13</sub>=1.63, p=0.23) and there was no significant interaction between location and treatment over time (F<sub>4,13</sub>=1.25, p=0.34).
Discussion

A fright reaction clearly exists in *Gambusia holbrooki*. Mosquitofish form tight schools in response to skin extract from conspecifics, which supports my hypothesis. The reaction in *G. holbrooki* was similar to that of *Poecilia reticulata* (Nordell 1998). Western mosquitofish (*G. affinis*) also exhibit a behavioral response to injured conspecifics; they flee to the bottom of their holding tanks in response to a homogenization of their conspecifics (Garcia *et al.* 1992). This suggests that the chemical information transmitted via injured fish may serve as a reliable indicator of predation risk to their conspecifics. Because these chemical cues and associated behaviors occur in fishes that are not members of the well-studied superorder Ostariophysi, these predatory risk detection techniques may be more pervasive than was previously believed.

I also observed that *Gambusia holbrooki* began to react to skin extract much sooner (30s after injection, at minimum) than when the extract would have been completely homogeneously mixed into the tank (~2 minutes, as suggested by the food coloring dispersal test). This indicates that *G. holbrooki* may be able to detect and respond to a very small concentration of this chemical cue, which suggests that the active space of *Gambusia holbrooki* skin extract in the wild may be quite large. Large active spaces also occur with cyprinid alarm pheromones (Wisenden 2008). Just 2 cm² of skin removed from red belly dace (*Phoxinus eos*) or fathead minnows (*Pimephales promelas*) was sufficient to cause avoidance of traps by conspecifics within a 2-meter radius (Wisenden 2008). A large active space for this type of alarm pheromone would be beneficial to any species; because the cue is only released by chemical damage and not...
actively mediated by the individuals (i.e., the chance of a “misfire” occurring is small), even a small amount can be perceived as an accurate and reliable indicator of risk.

Fishes exhibit a wide range of short-term responses to predation risk, and these responses have been well studied. This study further demonstrates the reaction of fishes to skin extract, but only assessed the immediate behavioral response. Long-term effects of exposure to predatory cues have only been recently and minimally studied. Evans et al. (2007) and Dzikowski et al. (2004) have documented life-history and reproductive changes in *P. reticulata* in response to conspecific skin extract. In the next chapter, I further investigate the *Gambusia* alarm reaction. I assess the reproductive response of *G. holbrooki* to long-term exposure to chemical and visual predatory cues during pregnancy.
References


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Figure 2. Mean index of cohesion of *Gambusia holbrooki* schools receiving a test substance (conspecific skin extract) or a control substance (deionized water). Schools of six fish were photographed every 30 s and Nordell’s (1998) index of cohesion was recorded from each photograph. Treatments were administered at 2:00 min. Mosquitofish receiving skin extract had a higher index of cohesion than those receiving deionized water ($F_{1,16} = 39.49, p < 0.01$). Schools receiving skin extract had greater indices of cohesion, compared to the baseline, for all post-treatment time intervals. There was no difference in the behavioral responses of fish from the two locations.
CHAPTER III

TRANSGENERATIONAL RESPONSES OF EASTERN MOSQUITOFISH

*(GAMBUSIA HOLBROOKI)* TO PREDATORY CUES

Introduction

Adaptive responses to stressful conditions are ubiquitous among organisms. Many organisms exhibit phenotypic plasticity; where an individual may express alternate phenotypes in response to its environment (Price *et al.* 2003). Biotic and abiotic selective pressures can all influence the behavior, physiology, or morphology of an organism or population. The ability to express alternate phenotypes can be vital for individual survival, and may have significant implications for evolution (Price *et al.* 2003). Organisms that possess plastic phenotypes may be more likely to exhibit novel genetic modifications in response to a novel environment (West-Eberhard 2005).

*Inducible Responses to Predation*

Inducible responses are defined by Alder and Harvell (1990) as phenotypic changes that are environmentally triggered and serve as defenses against biotic selective forces. Inducible defenses against predation occur broadly across taxa and may include changes in behavior, physiology, morphology, or life history, and may also be trans-generational.

Larvae of the snail *Littorina scutulata* develop rounder shells with smaller openings when reared in the presence of predators, larval *Cancer* spp. (Vaughn 2007). *Daphnia* spp. react to predator odors by producing protective spines, altering sex ratios, and varying maturation rates (Boersma *et al.* 1998). Tadpoles raised in the presence of
predators possess narrower bodies and deeper tails with wider and thicker tail muscles as compared to tadpoles in the absence of predators (Kraft et al. 2006). Goldfish (Carassius auratus) increase body depth and weight in response to chemical predation cues, presumably to reduce the likelihood of being consumed by a gape-limited predator (Chivers et al. 2007). Pumpkinseed sunfish (Lepomis gibbosus) increase body depth and dorsal spine length when reared with walleye (Sander vitreus) (Januszkiewicz and Robinson 2007). Mosquitofish (Gambusia affinis) from environments containing piscivorous fish have smaller heads, larger caudal regions, and more elongate bodies than those from predator-free environments. This distinct morphology produces a 20% faster burst-swimming speed than fish that have not been exposed to predators (Langerhans et al. 2004). Changes in morphology can reduce the risk of predation, and the ability to modify morphology may thus act as a selective force, favoring plasticity in high-predation environments.

Organisms may also display life-history plasticity as a defense against predators. Tree frogs (Agalychnis callidryas) exhibit plasticity in hatching age in response to different types of predators (Warkentin 1995). Tadpoles within egg masses that are attacked by snakes hatch immediately and escape the snake by entering the water below (Warkentin 1995). In the absence of snakes, eggs hatch later, and the larger tadpoles have higher survivorship against aquatic predators (Warkentin 1995). Female guppies (P. reticulata) exposed to cues from live predators significantly increase their reproductive output at first spawn (Dzikowski et al. 2004). When female guppies are exposed to visual and chemical predator cues (including skin extract), they shorten the duration of brood retention (Evans et al. 2007). Swimming performance and ability to avoid capture is
directly affected by brood duration – females with shortened brood retention produce offspring with reduced swimming and avoidance abilities (Evans et al. 2007). Shortened brood time may be due to females perceiving themselves at risk; pregnant livebearers have reduced swimming abilities and as a result may be more prone to predation (Plaut 2002). Evans et al. (2007) suggest that if guppies perceive their offspring—rather than themselves—to be at risk (for example, encountering a gape-limited predator that could not consume the mother), it might have the opposite effect on brood retention and consequently offspring performance.

A parent’s phenotype can directly affect the phenotype of its offspring, i.e., a maternal effect (Bernardo 1996). In Canadian populations of red squirrels (Tamiasciurus hudsonicus), maternal effects may account for the majority (as much as 80%) of phenotypic variation in growth in body size and body mass, versus simple heritability (McAdam et al. 2002). Seed beetles (Callosobruchus maculatus) from older parents have shorter life spans than beetles born to younger parents (Fox et al. 2003). Reznick et al. (1996) observed maternal effects in three poeciliids. Poecilia reticulata and Priapichthys festae are considered lecithotrophic, in which all nourishment to embryos is from a yolk formed prior to fertilization (Reznick et al. 1996). The third poeciliid, Heterandria formosa, is considered matrotrophic, in which nourishment to embryos is continually provided throughout embryonic development (Reznick et al. 1996). The lecithotrophic species respond to low food availability by producing larger young with greater fat reserves (Reznick et al. 1996). Heterandria formosa produce smaller and fewer young in response to low food availability. The authors suggest that the response of H. formosa may be due to constraints on the mother (Reznick et al. 1996). Because in some species
the parent phenotype is able to affect the phenotype of its young, maternal effects may significantly influence the rate and direction of natural selection when offspring fitness is altered (Bernardo 1996, Mousseau and Fox 1998).

A Transgenerational Study

*Gambusia holbrooki* are locally abundant in the coastal Savannah and Ogeechee-Canoochee River watersheds (Lydeard *et al.* 1991, Bochung and Mayden 2004). Unlike guppies, which are entirely lecithotrophic, mosquitofish are facultative matrotrophs (DeMarais and Oldis 2005). Mosquitofish have some post-zygotic nutrient transfer ability, but this is variable, even within broods (DeMarais and Oldis 2005). Female mosquitofish are quite prolific, producing up to six broods per season, with up to 40 young per brood (Ross 2001). Young mosquitofish become sexually mature at approximately 45 days (Turner 1942).

Because of these life-history characteristics, I propose that maternal resource allocation in *Gambusia holbrooki* may be plastic, *i.e.*, *G. holbrooki* (facultative matrotrophs) have greater control over nutrient provisioning to embryos than *Poecilia reticulata* (lecithotrophs). Guppies have no maternal option for increasing the fitness of their offspring, post-fertilization (Evans *et al.* 2007), but *G. holbrooki* may have that ability. It is therefore possible that *G. holbrooki* increases offspring fitness in response to predation risk. I address these potential transgenerational responses via a laboratory study, in which this species is exposed to predatory cues during pregnancy.

My objective is to address whether exposure to conspecific skin extract plus visual cues during pregnancy affects resource allocation from females to offspring. I
assessed *G. holbrooki* from two locations in coastal Georgia. Transgenerational effects were evaluated by quantifying neonate size, swimming endurance and the ability to escape capture. I hypothesized that pregnant *G. holbrooki* exposed to predatory cues would alter maternal resource allocation to embryos. I predicted that *G. holbrooki* receiving predator cues would retain their embryos for a longer period of time than controls, thereby producing larger young. I also predicted that offspring from these females would have enhanced swimming performance compared to controls, and an enhanced ability to escape capture.

**Methods**

*Study Population and Maintenance*

Eastern mosquitofish (*Gambusia holbrooki*) were collected multiple times between February and June, 2009. Mosquitofish were collected from a drainage pond located at the Savannah-Hilton Head International Airport (32° 8' 22.79" N, 81° 12' 57.81" W; Savannah, GA, USA) and from the Mill Creek drainage on Fort Stewart Army Installation (31° 54' 36.30"N, 81° 35' 28.53"W; Fort Stewart, GA, USA). Approximately 50 females and 30 males were collected at each location. Males were distinguished from females by the presence of a gonopodium, a modified anal fin used for internal fertilization (Boschung and Mayden 2004).

Stock populations were housed in 75.7L (20-gallon) stock aquaria with 4cm of gravel and a sponge filter (Lustar® “Hydro III”). Water temperature was maintained at 22-24°C with Marineland® Stealth Visi-Therm submersible aquarium heaters. Fish were maintained separately, according to location of origin. Diet included TetraMin® brand
tropical fish flakes daily, supplemented weekly by chopped frozen bloodworms (San Francisco Bay Brand™). All fish were fed *ad libitum*. Light:dark cycle was maintained at 12h:12h.

Females were allowed to acclimatize to laboratory conditions for at least two pregnancy cycles before being transferred to experimental aquaria. Experimental aquaria consisted of 3.78 liter (1-gallon, 22.9x18.10x11.65cm) polypropylene tubs (Ropak®), 2cm of gravel, a small (~6cm) plastic plant (Penn Plax® “Amazon Sword”), and a small sponge filter (Azoo®). Tubs were opaque white in color, eliminating the possibility of visual contact with other experimental animals. Sheets of clear acrylic served as covers for the enclosures. Sixteen females from each population were eventually transferred to experimental aquaria. Remaining fish were kept for collection of skin extract.

*Experimental Protocol*

Single female mosquitofish were transferred to experimental aquaria on the day of the birth of their preceding brood (“day zero”). Mosquitofish were assigned a predator cue or control treatment using a random number table (GraphPad 2005). Eight replicates from each location received the predator cue treatment, and eight replicates received the control (described below). I waited for a period of one week before beginning treatments on mosquitofish, in an attempt to ensure that the females developed and/or fertilized the subsequent clutch of ova (Pyke 2005; Evans et al. 2007) before being subjected to predatory stress.

Temperature, amount of food, and light regime were held constant (as described previously, but see Discussion for notes regarding temperature) throughout the study, as
all of these conditions influence embryo development (Pyke 2005). Females were not mated with males after their introduction to experimental aquaria. *Gambusia spp.* are able to store sperm and only require one mating event to produce several broods (Pyke 2005).

Each experimental female experienced five predator cue events spaced 1 week apart (± 2 days). Female mosquitofish were moved to Hagen® Marina Multi-Breeders when parturition was imminent (characterized by a swollen abdomen and enlarged anal spot). Offspring, hereafter referred to as F1s, were measured within 12 hours of birth, and the mothers were transferred back to the stock population.

**Predator Cue Treatment**

Chemical treatments consisted of the introduction of skin extract. Skin extract was obtained from stock fish in the manner described in Chapter 2. Because of the small size of the experimental aquaria (one-tenth the size of the aquaria used in Chapter 2), only five mL of skin extract was dispensed. This also minimized the number of females that were euthanized for collection of skin extract. Treatments were injected via a clean syringe and 100cm of airline tubing. I placed the end of the tubing slightly below the surface of the water, clipped it into place against the edge of the container, and stood at a distance of 1 meter during injection to avoid exposing the experimental females to additional visual cues (my presence). I then injected an entire syringe (20mL) of air to ensure that all 5mL of extract were delivered.

In poeciliids, predator avoidance behaviors are stronger in response to visual and chemical cues, than to chemical cues alone (Smith and Belk 2001). Therefore, tanks receiving skin extract also received a visual cue. For this cue, I obtained a soft plastic lure
resembling a large centrarchid predator – an amalgamation of bluegill (*Lepomis macrochirus*), black crappie (*Pomoxis nigromaculatus*) and largemouth bass (*Micropterus salmoides*) (Storm® Kickin’ Slab™ Jerkbait; 8-inch “Bluegill” model with hooks removed; Fig. 3). I attached the predator model to a 50cm-long wooden dowel with 22cm of clear fishing line (Stren® Clear/Blue Fluorescent line, 0.20mm diameter) to deploy the stimulus from a distance, so that I was not visible to the fish. The model was submerged into the experimental aquaria immediately after the introduction of skin extract, and moved around haphazardly for one minute.

Control groups received 5mL of distilled water, injected in the same manner, via a clean syringe. For a visual control, I waved the same wooden dowel, *sans* model, above the un-lidded tank for one minute in the same manner as for the treatment groups.

**Assessment of Effects**

F1s were enumerated and photographed using a Panasonic® DMC-FZ20 digital camera within 12 hours of birth. I noted duration of pregnancy (number of days). Total length (from tip of nose to end of caudal fin) and head width (mm) were measured from these photographs using ArcMap™ v.9.3 (ESRI® ArcGIS). Because female length is correlated with the number of embryos in *Gambusia spp.* (Thibault and Schultz 1978) standard length (SL; tip of snout to end of caudal peduncle) was recorded for all parent females immediately prior to their introduction to the experimental enclosures.

*Gambusia holbrooki* are born live and free-swimming. Neonates have a limited ability to escape filial cannibalism during the first 12 hours after birth, indicating some capacity for swimming. During the first week after F1s were born, I had no way to assess
muscle tone or swimming ability against a steady current. To select an appropriate age for the swimming endurance test, I tested several (non-experimental) F1s at 12 hours, 36 hours, five days, and eight days after birth. I measured flow speed in a continuous-flow raceway (Fig. 4) by floating a small plastic bead in the test chamber and timing how long it took to travel 10cm. I repeated this ten times, and calculated an average for each flow setting on the power head. The slowest flow speed I could achieve with the power head was approximately 4 cm/s. Neonates 12h, 36h, and 5d of age became exhausted almost immediately at this speed. Swimming endurance was easily measured on neonates at 8 days (>5s and variable). I waited a period of one week from the first raceway test to allow the F1s to recover. The F1s, now 15 days old, were tested in the raceway a second time. F1s had sufficiently recovered to swim the raceway again, indicating that 15d was an appropriate age for the predator escape trials. Therefore, I tested swimming endurance in all experimental F1s at 8d, and performed predator escape trials at 15d.

To assess swimming performance, F1s 8 days old ±12h were introduced into a steady current (~4cm/s) in the raceway and forced to swim until they became fatigued. Fatigue was defined as a resting period of more than five seconds after more than five tail-beats (most often, the F1s would drift into the protective screen at the rear of the test chamber; Fig. 4). Swimming endurance was measured in seconds.

To test F1 ability to avoid capture by predators, I used five mid-sized (19-25cm SL) largemouth bass (*Micropterus salmoides*). Bass were maintained in individual 37.9L (10-gallon) aquaria with a 4cm layer of gravel, airstone, and a small power filter (AquaTech®). Bass were fed Tetra® JumboMin Cichlid Pellets daily, supplemented weekly with locally caught adult mosquitofish. At 15-days-old (one week after the
raceway test), F1 escape ability was tested. Because the bass associated my presence with being fed, they would almost immediately consume anything that was added to the tank. To obtain a more accurate measure of escape ability, I added pairs of F1s to the predator tanks. The first F1 individual was consumed almost immediately, but the bass was required to hunt and capture the second F1. I assigned F1 pairs to predator tanks using a random number table (GraphPad 2005), and recorded the length of time (seconds) from the addition of the F1s and the consumption of the second F1 by the bass.

Individual mothers (i.e., entire broods; n=32) were treated as replicates. For each variable that consisted of F1 measurements, the mean for each brood was calculated. This avoided pseudoreplication because the F1s of a single brood all received the same treatment (via the mother) and are not truly independent.

To determine if pregnancy duration, brood size, F1 size, swimming endurance, or predator escape ability differed by location or treatment, data were analyzed by two-way analyses of variance (ANOVA). Before the statistical analyses, I checked for homogeneity of variances and if the data were normally distributed. Brood size (offspring count) had unequal variances, so was transformed (tangent[x]) prior to analysis.

To determine if the size of the mother influences her brood size (Thibault and Schultz 1978), I ran a separate regression for each location by brood size. Prior to the regressions, I ascertained that brood size counts for each population were normally distributed and had homogeneous variances. All analyses were conducted using JMP (v. 8.0) statistical software (SAS Institute 2008).
Results

Duration of pregnancy (Fig. 5), number of offspring (Fig. 6), offspring length (Fig. 7), offspring width (Fig. 8), swim performance (Fig. 9), and escape time (Fig. 10) were not significantly different by location or treatment, and there were no significant interactions between the independent variables (Table 1). For power analyses, see Table 2. In the Savannah Airport group, the relationship between the size of the mother and number of offspring was significant ($R^2 = 0.75$, $n=7$, $p=0.01$). Larger mothers produced more offspring (Fig. 11A). In the Fort Stewart group, however, there was no significant relationship between size of the mother and number of offspring ($R^2 < 0.01$, $n=11$, $p=0.95$) (Fig. 11B).

Discussion

*Gambusia holbrooki* do not exhibit phenotypic plasticity in response to predatory cues during a single pregnancy. Mosquitofish did not alter brood duration or brood size, and their young did not differ in size, swimming performance or ability to escape largemouth bass predators (Table 1). These data do not support my hypothesis; the traits I measured do not indicate variation in maternal nutrient provisioning in response to predatory cues.

Fecundity is dependent upon female length in the Savannah Airport population of *Gambusia holbrooki*. There is a positive, linear relationship between female length and number of offspring (Fig. 11A). This corresponds with previous research on this species (Zane *et al.* 1999) and for *G. affinis* (Wu *et al.* 1974). This also corresponds with trends in other poeciliid species, including *Poecilia reticulata, P. monacha*, and *Poeciliopsis*
lucida (Thibault and Schultz 1978). The Fort Stewart population, however, showed no relationship between size of the mother and number of offspring (Fig. 11B). However, the females used for the experiment had different ranges in size; the Fort Stewart females were generally larger (32.5-40mm) than the Savannah Airport females (26-38mm).

Genetic differences between the two populations may be responsible for these variations in life history patterns. Smith et al. (1983) found significant variation in allele frequencies among populations of mosquitofish within the Savannah River drainage. This variation was attributable to water flow regimes, elevation, and water temperature (Smith et al. 1983). The Mill Creek drainage at Fort Stewart is adjacent to a non-putrescible landfill, which is designed to collect sediment from storm water runoff (Lambert 2008). The creek sits at approximately 27 meters above sea level, and has a slight flow. The Savannah Airport pond is part of a larger wetland designed to collect runoff from the airport’s runways (A. Singhas, pers. comm.). There is no connection to larger bodies of water, and little or no flow within the system. Its elevation is approximately 15 meters. Differences in elevation, chemical composition, flow, and other abiotic habitat factors may have influenced the genetic composition of the two populations, and thus may explain the life-history variation between the two populations.

Biotic factors may also play a role in these divergent life-history patterns. While collecting my experimental mosquitofish, I noted other fishes that occurred at each location. Species composition appeared to be similar at both locations; observed predators for mosquitofish consisted mainly of sunfish and other centrarchids. I was not able to conduct an extensive survey of fish populations at either location, however, so large differences in predator composition may be present between these locations.
Predator composition in the aquatic community can play a major role in the divergence of life-history traits and presence of phenotypic plasticity. Because there can be fitness costs associated with life-history plasticity (Relyea 2002), predator-induced traits can be reversible, especially early in ontogeny (Relyea 2003). Change in predator composition or elimination of predators is likely to cause a shift in the frequency of these traits, but quantifying how quickly such shifts occur is difficult.

Duration of pregnancy was not different between locations or treatments. During the experiment, however, extreme temperature fluctuations in the laboratory (ranging from 15.5°C to 32°C) occurred. Because pregnancy duration in Gambusia spp. is highly dependent upon temperature (Pyke 2005), these data may not be accurate indicators of predator-induced variation in pregnancy length. In addition, Evans et al. (2007) found that pregnancy duration in guppies affects swim performance and the ability to escape capture. Additional experiments in a temperature-controlled environment may yield more practicable results. These temperature fluctuations were unlikely to affect brood size or morphology. Clutch size in Gambusia affinis is unaffected by water temperature (Vondracek et al. 1988). Meffe (1992) observed no difference in ovum production in G. holbrooki in response to thermal stress (i.e., extended exposure to a temperature of 32°C).

Power tests indicate that low sample size may be to blame for the lack of significant differences in some variables (Table 2). The least significant numbers (LSN) required for between-treatment comparisons of duration of pregnancy and brood size are 47 and 42, respectively. This suggests that approximately 12 replicates would be required to detect a significant difference between treatments for pregnancy duration and brood size. LSNs for between-location comparisons of F1 length and width were 35 and 30,
respectively, suggesting that 9 replicates would be sufficient to detect differences between locations for these measurements. Eight replicates per group (32 total individuals) were attempted in this study (this was dependent upon the number of stock females that were actively reproducing), and data were only collected for the 4-6 replicates per group that survived and/or successfully reproduced during experiment.

Because life-history components vary by location, and power tests indicate that sample size in this experiment was low, it is possible *Gambusia holbrooki* are phenotypically plastic both geographically and in response to predation. Phenotypic plasticity in response to a particular environment can be a precursor to novel behavioral, physiological, or morphological traits because it permits change without any extensive or harmful alterations to the established genome (West-Eberhard 2005). Such innovation can facilitate adaptation to a novel environment, and eventually result in speciation (Price *et al.* 2003).

Langerhans *et al.* (2007) observed different adaptations in *Gambusia hubbsi* to varying suites of predators. Body shape is different between two (sexually isolated) communities, and females prefer to mate with males from their native populations (Langerhans *et al.* 2007). Though the authors reported no genetic differences between populations, they suggest that the differences in body shape are a precursor to ecological speciation. Langerhans *et al.* (2004) observed morphological divergence in *Gambusia affinis* between populations with different predator composition. Differences in *Gambusia* body shape between predator-rich and predator-free environments results in a significant difference in locomotor performance; fish from the predator-rich population have faster burst-swimming speeds (Langerhans *et al.* 2004). These morphological
differences persist in offspring raised in the laboratory, suggesting a genetic component to this divergence (Langerhans et al. 2004). The authors suggest that they have observed ongoing ecological speciation, though they reported no genetic differences between the populations.

Life-history differences have also been observed in poeciliids. Guppies from environments containing a large predator, the pike cichlid (Crenicichla alta), contribute more resources to each brood and produce a larger number of smaller offspring than guppies from low-predation habitats (Reznick et al. 1997). Dzikowski et al. (2004) observed a similar tendency in the laboratory: guppies exposed to predatory cues have significantly larger broods than those not exposed to predators. Another livebearing species, Brachyraphis rhabdophora, also follows this trend. Fish that occur with piscine predators have more, smaller offspring than those from predator-free environments (Johnson and Belk 2001).

Mosquitofish have been introduced around the world as a mosquito control agent, and have since become one of the world’s most invasive fish. Many characteristics have been identified in Gambusia that contribute to their invasiveness: mosquitofish have short breeding periods and high fecundity (Vila-Gispert et al. 2005), they exhibit higher feeding rates than their non-invasive relatives (Rehage et al. 2005), and also show evidence of plastic responses to salinity-related stress; they produce more offspring in higher salinities (Alcaraz and Garcia-Berthou 2007).

It is possible that Gambusia holbrooki are phenotypically plastic in response to differing abiotic and biotic environments. Mosquitofish species are successfully invasive in many areas of the world, and are highly adaptable to differences in salinity,
temperature, food availability, and predation. I do not have the evidence to draw conclusions about the particular mechanism behind my observed difference in size-fecundity relationships, or conclude from my measurements that mosquitofish alter nutrient provisioning to embryos in response to predation. Additional replication is necessary to determine if reproductive habits vary significantly by location or in response to predatory cues.
References


Table 1. Two-way ANOVA results. Two-way analyses of variance were used to determine any differences in parent reproductive traits or offspring (F1) measurements of *Gambusia holbrooki* between treatments (predator cue versus control) or locations (Savannah, GA versus Fort Stewart, GA). Neither location nor treatment was a significant contributor of variation among these groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>DF</th>
<th>Location n =</th>
<th>Location F</th>
<th>Observation p</th>
<th>Treatment F</th>
<th>Observation p</th>
<th>Interaction F</th>
<th>Observation p</th>
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<td>Duration of pregnancy (days)</td>
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<td>0.681</td>
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<td>Total size of brood (# of offspring)</td>
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<td>0.0014</td>
<td>0.9708</td>
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<tr>
<td>F1 length (mm)</td>
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<td>1,12*</td>
<td>3.1693</td>
<td>0.1003</td>
<td>0.2234</td>
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<tr>
<td>F1 width (mm)</td>
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<tr>
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<td>0.0016</td>
<td>0.9687</td>
<td>0.1109</td>
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</table>

* Differences in sample sizes occurred because some broods were born with some or all F1 premature or dead, and accurate measurements were not possible. In a few broods, all F1s died prior to swim performance and/or predator escape trials.
Table 2. Power test results. Two-way analyses of variance were used to determine differences in several variables between treatments (predator cue versus control) and locations (Savannah Airport and Fort Stewart, GA, USA). None of the measured variables were significantly different between locations or treatments. Power was calculated for each variable, as well as the least significant number (number of replicates required for the test to detect significant differences at $\alpha=0.05$).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Power</th>
<th>Least Significant Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Location</td>
<td>Treatment</td>
</tr>
<tr>
<td>Duration of pregnancy</td>
<td>0.07</td>
<td>0.22</td>
</tr>
<tr>
<td>Brood size</td>
<td>0.13</td>
<td>0.26</td>
</tr>
<tr>
<td>F1 Length</td>
<td>0.26</td>
<td>0.05</td>
</tr>
<tr>
<td>F1 Width</td>
<td>0.27</td>
<td>0.06</td>
</tr>
<tr>
<td>F1 Swim performance</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>F1 Escape time</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure 3. Model predator used as the visual component of the “predator cue” treatment.
Figure 4. Continuous-flow raceway used to measure F1 swimming performance. An aquarium pump is placed at position A, propelling water around the raceway at approximately 4.16cm/s. The water flow is diffused by a stack of drinking straws (B) before it enters the flow chamber (C). Fish are placed in the flow chamber and timed until they become exhausted and drift onto the back screen (D).
Gambusia holbrooki from two locations were subjected to visual and chemical predator cues (or a control of distilled water). Two way analysis of variance determined that there was no significant difference in duration of pregnancy between locations ($F_{1,15}=0.18$, $p=0.68$) or between treatments ($F_{1,15}=1.62$, $p=0.22$).

Figure 5. Mean duration of pregnancy. *Gambusia holbrooki* from two locations were subjected to visual and chemical predator cues (or a control of distilled water). Two way analysis of variance determined that there was no significant difference in duration of pregnancy between locations ($F_{1,15}=0.18$, $p=0.68$) or between treatments ($F_{1,15}=1.62$, $p=0.22$)
Figure 6. Mean number of offspring. *Gambusia holbrooki* from two locations were subjected to visual and chemical predator cues (or a control of distilled water). Two way analysis of variance determined that there was no significant difference in duration of pregnancy between locations ($F_{1,15}=0.77, p=0.39$) or between treatments ($F_{1,15}=1.83, p=0.20$)
Figure 7. Mean length of offspring. *Gambusia holbrooki* from two locations were subjected to visual and chemical predator cues (or a control of distilled water). Two way analysis of variance determined that there was no significant difference in duration of pregnancy between locations ($F_{1,13}=1.99$, $p=0.19$) or between treatments ($F_{1,13}=0.03$, $p=0.87$).
Gambusia holbrooki from two locations were subjected to visual and chemical predator cues (or a control of distilled water). Two way analysis of variance determined that there was no significant difference in duration of pregnancy between locations ($F_{1,12}=2.23, p=0.16$) or between treatments ($F_{1,12}=0.06, p=0.82$).
Figure 9. Mean F1 swimming endurance. *Gambusia holbrooki* from two locations were subjected to visual and chemical predator cues (or a control of distilled water). Two way analysis of variance determined that there was no significant difference in duration of pregnancy between locations ($F_{1,11}=0.65$, $p=0.44$) or between treatments ($F_{1,11}=0.02$, $p=0.89$).
Figure 10. Mean predator escape time. *Gambusia holbrooki* from two locations were subjected to visual and chemical predator cues (or a control of distilled water). Two way analysis of variance determined that there was no significant difference in duration of pregnancy between locations ($F_{1,10} = 0.02, p = 0.90$) or between treatments ($F_{1,10} < 0.01, p = 0.97$).
Figure 11. Size-fecundity regressions by location. Relationship between the size of the mother and the number of offspring produced in the Savannah Airport population (A) and Fort Stewart population (B). In the Savannah Airport population, larger mothers produce more offspring ($p=0.01$). This trend is not present in the Fort Stewart population ($p=0.95$).
CHAPTER IV

SUMMARY & CONCLUSIONS

These studies confirm the existence of a chemical alarm system in *Gambusia holbrooki*, and some variation in reproductive traits between locations in coastal Georgia. Eastern mosquitofish respond to skin extract from conspecifics by forming tighter schools than controls. This behavior, though documented in other poeciliid species, has never been documented in *G. holbrooki*’s closest relative (*G. affinis*).

According to the data I collected, *Gambusia holbrooki* did not alter nutrient provisioning to embryos in response to exposure to predatory cues during a single pregnancy. Power tests, however, suggest that additional replication may be sufficient to detect significant differences between locations (for F1 length and width) and between treatments (for pregnancy duration and brood size). There were trends suggesting that *G. holbrooki* from different locations (even within coastal Georgia) have divergent reproductive characteristics. Fecundity was dependent upon maternal size in Savannah Airport females, but this trend is not present in the population from Fort Stewart. This suggests a difference in selective pressure between the two populations.

Though I do not have evidence to draw any conclusions about the specific selective forces driving this variation, I can propose some areas of focus for future study. Quantification of the abiotic and biotic differences among *Gambusia* population locations in coastal Georgia may shed light on this divergence. Because differences in life-history traits were evident between locations, and genetic studies have revealed some amount of divergence, it is possible that *Gambusia* also have varying behavioral traits (specifically the reaction to skin extract) among locations. Finally, predator-induced life-history or
morphological change may require longer exposure to predatory cues. It may be of some worth, therefore, to test responses to predatory cues applied throughout ontogeny and/or the duration of multiple pregnancies.
APPENDIX A

ATTEMPTED METHODS

Inclusion of *Heterandria formosa*

Least killifish (*Heterandria formosa*) are abundant in coastal Georgia waterways, and often found in the same locations as *Gambusia holbrooki*. They are obligate matrotrophs, whose embryos are entirely dependent upon post-zygotic maternal nutrient transfer through a placenta-like structure (Scrimshaw 1944). Superfetation (clutch overlap) occurs in least killifish, providing a mechanism for large reproductive output despite their diminutive size (Fraser and Renton 1940). Broods usually consist of 1-3 offspring (sometimes more in large females), and may occur as often as every 3-9 days during the summer months (Boschung and Mayden 2004). Females mature at approximately 4 weeks; males at 8 weeks (Fraser and Renton 1940). Schutz (1956) described a fright reaction in *H. formosa* females in response to skin extract from conspecific males and females.

Reznick *et al.* (1996) observed maternal effects in *Heterandria formosa*. Killifish produced smaller and fewer young in response to low food availability. *Heterandria formosa* may have greater control over nutrient provisioning to embryos than *Gambusia holbrooki* (a facultative matrotroph) or *Poecilia reticulata* (a lecithotroph). Lecithotrophic guppies decrease the fitness of their offspring in response to predation risk (Evans *et al.* 2007). I proposed that killifish, as matrotrophs, may have the ability to increase the fitness of their offspring by altering nutrient provisioning during embryonic development. I hypothesized that pregnant female *Heterandria formosa* exposed to predatory cues would alter maternal resource allocation to embryos. I predicted that these
females would produce larger young. I also predicted that offspring from these females would have enhanced swimming performance compared to controls, and an improved ability to escape capture.

I attempted to address these potential transgenerational responses via a laboratory study, following the same methods outlined in Chapter III. Least killifish (*Heterandria formosa*) were collected several times between February and June, 2009. Killifish were collected at Savannah-Hilton Head International Airport (32° 8' 22.79" N, 81° 12' 57.81" W; Savannah, GA, USA) and a small pond on Williams Road (32° 27' 42.85"N, 81° 48' 17.59"W, Statesboro, GA, USA). Approximately 50 females and 30 males were collected from each location. Males were distinguished from females by the presence of a gonopodium (Boschung and Mayden 2004).

*Heterandria formosa* experience superfetation, *i.e.*, multiple embryos develop within the mother at different stages (Turner 1937, Fraser and Renton 1940). Because of this phenomenon, a “day zero” of pregnancy was not possible to establish for adult females of this species. Killifish embryos have an average development time of 40 days (Fraser and Renton 1940). I therefore elected to transfer female killifish to the experimental aquaria at my discretion and immediately begin treatments. Offspring were to be collected for measurements 40-75 days after the first treatment was performed. This scheme would ensure that any offspring born during the collection period would have been developing during at least one treatment event. Forty days after the first treatment date, *Heterandria* were transferred to Lee’s® Multipurpose Three-Way Breeders (a design slightly different than Hagen® breeders, with a lower chamber to facilitate the collection of smaller larvae).
I experienced several problems with the *Heterandria formosa* females. Female behavior indicated unusually high stress levels while being contained within the experimental aquaria. This species normally moves in schools among heavy vegetation; I believe the lack of cover and low conspecific densities contributed to this stress. Females also decreased fertilization rates, though this species can store enough sperm from one mating event to produce multiple broods (Fraser and Renton 1940). During the course of the study, I collected only one *H. formosa* neonate, which died within 12 hours of birth. I also had significant mortality among the experimental adults; of 32 females transferred to experimental aquaria, only 14 survived to the final offspring collection date (75 days after the first predator cue treatment). Deaths were even among the groups. It is possible that the stress of living in the experimental aquaria contributed to delayed fertilization and/or abortion of developing embryos.

**Predator Cue Exposure During Ontogeny**

In an effort to control genetic factors associated with life-history traits in *Gambusia holbrooki*, I obtained virgin F1 females from each population. Parent *G. holbrooki* were allowed to breed in their mixed-sex stock tanks, and were removed during parturition to facilitate the collection of F1s. My attempted methods were as follows. As soon as F1 sexes were distinguishable (by a developing gonopodium in males), females would be transferred to experimental aquaria. Treatments would begin immediately and continue throughout ontogeny, and they reached sexual maturity I would mate them with a single adult male from the original stock population. Consequently, all offspring born to
the F1s (hereafter referred to as F2s) would be genetically similar half-siblings. In theory, this would eliminate the possibility of large genetic differences masking any alterations in life-history brought about by the predator cue treatment. Assessment of effects of the predator cue treatment would be as described in Chapter III, but would be performed on the F2 generation.

I was unable to successfully raise F1 *G. holbrooki* to sexual maturity. High mortality rates reduced the sample size. Low average temperatures and temperature fluctuations prevented F1s from maturing at a normal rate. Zero F1s reached sexual maturity after a period of three months, and no F2s were collected. Therefore, I modified my study to exclude the F2 generation, and performed the predator cues during the course of a single pregnancy in the adult populations.
References


