

PROXIMATE AND ULTIMATE PERSPECTIVES ON SPERM PRODUCTION AND
MATING BEHAVIOR IN A BISEXUAL-UNISEXUAL MATING SYSTEM
BETWEEN SAILFIN (*POECILIA LATIPINNA*) AND ATLANTIC
(*P. MEXICANA*) MOLLIES WITH CLONAL AMAZON
MOLLIES (*P. FORMOSA*)

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ABSTRACT

PROXIMATE AND ULTIMATE PERSPECTIVES ON SPERM PRODUCTION AND
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BETWEEN SAILFIN (*POECILIA LATIPINNA*) AND ATLANTIC
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Hormonal regulation plays an important role in influencing mating behavior in vertebrate species. In mating systems where closely related species are sympatric, hormones may affect species recognition. I examined the potential role of hormones in mediating species recognition in a bisexual-unisexual mating system consisting of bisexual sailfin mollies

(*Poecilia latipinna*) and Atlantic mollies (*P. mexicana*) with clonal Amazon mollies (*P. formosa*). 11-ketotestosterone (KT) is the dominant male androgen in teleosts that influence secondary sex characteristics and sperm production. Male and female sailfin mollies from a population sympatric with Amazon mollies both showed greater KT responsiveness (post-mating KT/pre-mating KT) after a mating with each other whereas this pattern was absent when males mated with Amazon mollies. In this study, I examined whether KT is important in species recognition for sailfin mollies that are from a population allopatric to Amazon mollies. Male sailfin mollies significantly preferred to mate with conspecific females over Amazon mollies. However, I found no significant difference in KT responsiveness of male or female sailfin mollies that mated. Amazon mollies also displayed no change in KT responsiveness. It is possible that the observed KT responsiveness in sailfin mollies from conspecific matings in sympatric populations is a derived trait, which is not present in the allopatric population. However, comparison of multiple sympatric and allopatric populations is needed to test this hypothesis. I also examined the role of KT production in sperm production of male Atlantic mollies when they were associated with either a female Atlantic molly or an Amazon molly for either seven days or for one hour. Pre-association KT levels were not correlated with pre-association sperm levels in male Atlantic mollies. Males that were paired with conspecific females for seven days showed a positive correlation between pre-association KT levels and the amount of sperm primed. There was no correlation between KT responsiveness and post-association sperm levels after one hour of association. Males also did not prime more sperm for conspecific females over Amazon mollies during the

one hour association. Our results suggest KT is not involved with species recognition or sperm priming in Atlantic mollies

Chapter 1

Introduction

Mollies are live bearers in the family Poeciliidae and occupy subterranean water courses, brackish, salt and fresh waters (Miller 1983). The range of sailfin mollies (*Poecilia latipinna*) extend from southern Mexico through North Carolina (Miller 1983) (Fig. 1) where additional populations have been introduced outside of its range in the San Marcos and Comal rivers of central Texas (Brown 1953). Atlantic mollies (*P. mexicana*) occur from the Rio San Fernando, Mexico south at least to Honduras, and possibly into Costa Rica (Miller 1983). The gynogenetic species, Amazon mollies (*P. formosa*), are found in northeastern Mexico and extreme southern Texas (Miller 1983), and were introduced to central Texas in the mid 1990's (Brown 1953; Edwards 2001). The sailfin molly and the Amazon molly are sympatric in northern Mexico, south-eastern Texas and isolated introduced populations in central Texas (Martindale and Comal). Atlantic mollies are sympatric with Amazon mollies spanning north of Laguna de Tampamachoco north to Rio San Fernando, Mexico (Darnell and Abramoff 1968). Amazon mollies are the product of a hybridization event between sailfin and Atlantic mollies approximately 100,000 years ago (Avisé et al. 1991; Scharl et al. 1995; Loewe and Lamatsch 2008; Stöck et al. 2010). Amazon mollies require the sperm from either parental species to induce embryogenesis (Hubbs 1964; Darnell et al. 1967). However, males that mated

with Amazon mollies do not increase their fitness, as offspring only inherit maternal genetic material (Hubbs and Hubbs 1932).

Male mollies reach maturity in 30-60 days (Trexler et al. 1997) when the anal fins fuse to form the male sex organ used to transfer sperm, the gonopodium. There is continuous variation in male sailfin molly size which can also vary by age and coloration (Travis 1994b). Larger males also court females more frequently than smaller males (Travis and Woodward 1989). However, small males are more effective at transferring sperm via forced copulations (Farr et al. 1986). Male sailfin mollies approach females using three main behaviors. Males can assess a female's receptivity by nibbling at the gonopore. If the female is receptive, males will then display towards a female in a sigmoidal curve (Farr and Travis 1986). Males will then follow by attempting to transfer sperm by thrusting their gonopodium towards the female's gonopore, called gonopodial thrusting.

Female sailfin mollies usually display cryptic coloration and brood size is correlated with female size (Trexler et al. 1997). Amazon mollies are usually larger than female sailfin mollies (Gumm and Gabor 2005) and male sailfin mollies prefer to associate with larger females (Gabor 1999). Males may benefit from mating with larger females as body size is an indicator of brood size (Farr and Travis 1986; Travis et al. 1990; Trexler et al. 1997). In populations that are sympatric with Amazon mollies, male mollies are faced with the problem of distinguishing conspecific females (species recognition) and high quality mates (mate recognition), while risking depletion of resources and lowering fitness. However, males from populations sympatric and allopatric to Amazon mollies did not have a preference for Amazon mollies over

conspecific females when only given visual cues (Aspbury et al. 2010). Furthermore, males from allopatry preferred conspecific females over Amazon mollies when given a combination of visual and chemical cues (Aspbury et al. 2010). Thus, other factors other than body size likely influence male mate choice.

Males differentially allocate reproductive resources to higher quality mates and conspecifics. This is not surprising due to the costly nature of producing sperm, where sperm quantity and quality decline after successive mating attempts (Nakatsuru and Kramer 1982; Preston et al. 2001). Male sailfin mollies differentially allocate, prime more sperm, while in the presence of a conspecific female versus than when with an Amazon molly (Aspbury and Gabor 2004). Male sailfin mollies also exhibited faster rates of spermiation, the last stage in spermatogenesis, when mating with conspecific females than when mating with Amazon mollies (Robinson et al. 2008). Male Atlantic mollies preferred to mate and transferred more sperm when mating with conspecific females than with heterospecifics (Schlupp and Plath 2005).

Little is known about how hormonal regulation is involved in the sperm priming response in fish. Androgens affect spermiation by binding to receptors on Sertoli cells and other somatic cells associated with germ cells (Miura et al. 1996). In teleost fish, a derivative of testosterone, 11-ketotestosterone (KT) is the dominant male androgen (Kindler et al. 1991; Miura et al. 1996) and is involved with increases of spermatogenesis (Cavaco et al. 1998) as well as development of secondary sexual characteristics (Miura et al. 1996). In a prior study (Gabor and Grober 2010), male sailfin mollies exhibited an increase in KT, a positive KT response, after mating with a conspecific female, whereas this relationships was absent when males mated with Amazon mollies. Similarly,

conspecific females had a positive KT response after mating, but there was no response in Amazons. Thus, KT may be involved in species recognition in this mating system. I propose to examine the relationship between differential androgen and sperm production in the mating system of the sailfin mollies and Atlantic mollies with the clonal Amazon mollies.

Research direction

In chapter II, I will address whether male sailfin mollies from an allopatric population differ in their KT responsiveness when paired with female conspecifics vs. Amazon mollies. Since males from allopatric populations have not encountered a heterospecific, males may not have developed a mechanism for species recognition via hormonal regulation.

Testing males from an allopatric population will allow me to examine if males from allopatry share the same bi-directional KT response after mating as those in sympatry (Gabor and Grober 2010). If males from an allopatric population demonstrate the same conspecific mate preference, this could suggest hormonal regulation arose prior to isolation of the population. However, if the allopatric population does not share hormonal regulation with sympatric males, then the trait may have only been derived in populations that coexist with Amazon mollies. To fully test this hypothesis, further testing of sympatric and allopatric populations will be required, but these results will be the first step towards examining the involvement of the KT response in species recognition in an allopatric population.

Along with species recognition, sperm production and allocation are important in reproductive isolation. Male sailfin mollies from a population sympatric to Amazon mollies prime more sperm (Aspbury and Gabor 2004) and engage in higher rates of sperm production when paired with conspecific females (Robinson et al. 2008). Male Atlantic mollies also transfer more sperm to conspecifics females than when mating with Amazon mollies (Schlupp and Plath 2005). 11-ketotestosterone plays a dominant role in spermatogenesis in a marine fish, *Solea senegalensis* (García-López et al. 2006) and with sperm maturation in Japanese eels (*Anguilla japonica*) (Miura et al. 1996). In chapter III, I will examine whether baseline KT levels correspond with baseline sperm levels and the amount of sperm primed in male Atlantic mollies from a sympatric population after association with either a conspecific female or an Amazon molly for seven days. I will also exam whether KT responsiveness is correlated with the amount of sperm primed after association with either female species after one hour to determine the role of KT in sperm production during a brief association period.

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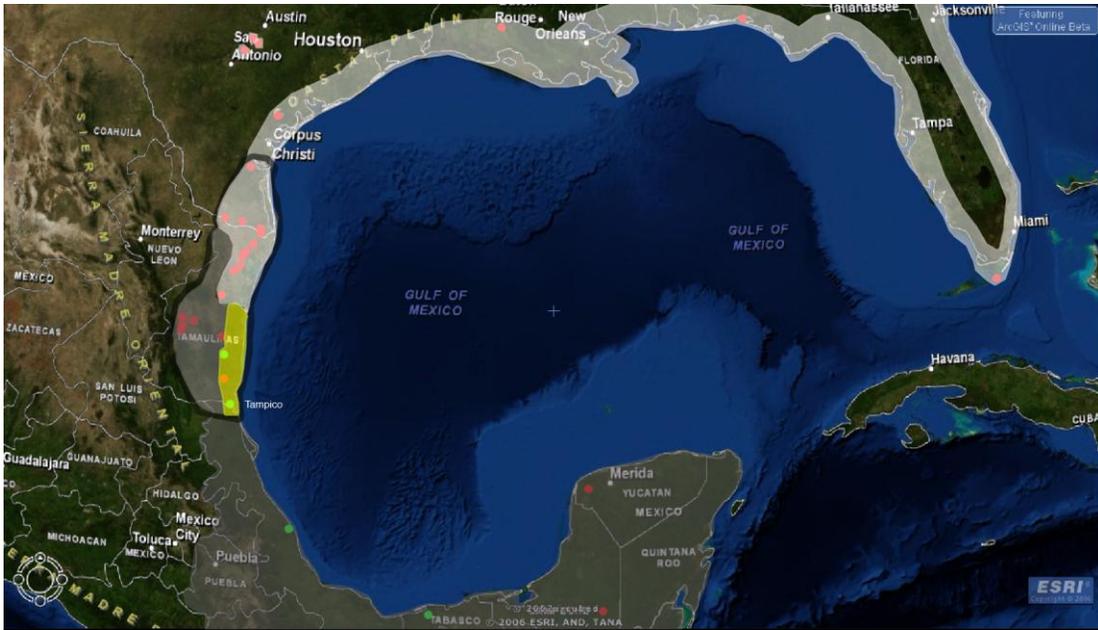


Fig. 1.1 Distribution of sailfin mollies (*Poecilia latipinna*), Amazon mollies (*P. formosa*), Atlantic mollies (*P. mexicana*) along the Gulf coast of Mexico. Sailfin molly range is indicated in white. Atlantic molly range is indicated in grey. The region of sympatry for sailfin and Amazon mollies is white outlined with black. The region of sympatry for Atlantic and Amazon mollies is grey outlined with black. All three species are sympatric in yellow. Dots represent population sites.

Chapter 2

Male sailfin mollies (*Poecilia latipinna*) from a population allopatric to Amazon mollies do not exhibit differential hormone response after mating with conspecifics or gynogenetic Amazon mollies (*P. formosa*)

Abstract

Hormonal regulation has an important role in influencing mating behavior in vertebrate species. In mating systems where closely related species are sympatric, hormones can affect species recognition. The unisexual-bisexual mating system between the clonal Amazon mollies (*P. formosa*) and sexually reproducing sailfin mollies (*Poecilia latipinna*) represents a model system for examining proximate mechanisms of species recognition. Previous work has shown that the androgen 11-ketotestosterone (KT) may play a role in species recognition in this mating system. Male and female sailfin mollies from a population sympatric with Amazon mollies both increased production of KT after mating, whereas this pattern was not found after male sailfin mollies mated with Amazon mollies. In this experiment, I investigated whether this bidirectional increase in KT was also exhibited in male and female sailfin mollies from a population that is allopatric to Amazon mollies. Male sailfin mollies significantly preferred to mate with conspecific females than with Amazon mollies. However, neither male nor female sailfin mollies showed a KT response after mating. Amazon mollies also displayed no KT response to

mating. One possible explanation is that the increase in KT during conspecific mating in sympatric populations is a derived trait.

Introduction

Hormones play a role in sexual selection in a variety of taxa (birds: Wingfield et al. 1990; fish: Kobayashi et al. 2002; Hirschenhauser et al. 2004; frogs: Lynch et al. 2006; lizards: Kabelik et al. 2008). In birds, reproductive hormones (e.g. testosterone in males and progesterone in females) affect reproductive allotment and mating behaviors of individuals of the opposite sex (Erickson and Lehrman 1964; Enstrom et al. 1997; Kobayashi et al. 2002). In doves (*Streptopelia risoria*), females that were paired with castrated male doves maintained smaller reproductive organs than females that were paired with intact males (Erickson and Lehrman 1964). In addition, female dark-eyed juncos (*Junco hyemalis*) exhibited mating preferences for males with high testosterone levels (Enstrom et al. 1997).

Hormonal interactions between individuals can escalate subsequent behaviors (e.g. mating or aggression). The “challenge hypothesis” proposes that increases of testosterone are correlated with increases of aggression in males when responding to social challenges during the breeding season (Wingfield et al. 1990). This hypothesis has been supported by a number of studies from various taxa (birds: Wingfield et al. 1990; Goymann and Wingfield 2004; fishes: Oliveira et al. 2001; Oliveira et al. 2002; Hirschenhauser et al. 2004; Gonçalves et al. 2007; lizards: Rubenstein and Wikelski 2005). Social interactions between individuals of closely related species may also affect

mating behavior and mate choice that are influenced by hormones (Håkan Olsén et al. 2000; McLennan 2004; Wong et al. 2005; Fisher and Rosenthal 2006). Several studies have studied the effects chemical cues have on mate choice between individuals from populations sympatric or allopatric with closely related heterospecifics (Wong et al. 2005; Lewis et al. 2007; McElfresh and Millar 2008; Pureswaran et al. 2008; Aspbury et al. 2010). However, a relatively unexplored topic is the potential difference of hormonal responses to mating with conspecific or heterospecific individuals when populations are sympatric or allopatric with the heterospecifics.

Mate choice consists of two parts, species recognition and mate quality recognition. Males that live in populations that are sympatric with closely related heterospecifics might emphasize species recognition more than mate quality (Pfennig 1998). Female swordtail fish (*Xiphophorus pygmaeus*) preferred to mate with allopatric *X. nigrensis* over conspecific males, which are larger than conspecific *X. pygmaeus* (Ryan and Wagner 1987). Hormones can be excreted and act as chemical cues. In some systems, these cues played a role in species recognition, where excreted hormones and their derivatives could be used to differentiate conspecific individuals from heterospecifics (Mathis and Vincent 2000; McLennan 2003; Wong et al. 2005; Greene and Gordon 2007; Aspbury et al. 2010; Gabor and Grober 2010). However, such cues alone were often not capable of detecting species recognition when not presented with other cues (Cardwell et al. 1992; Håkan Olsén et al. 2000; Aspbury et al. 2010). However, few studies have examined the role androgens played on mate choice in a mating system with closely related heterospecifics where there was conflict between species and mate-quality recognition (see: Gabor and Grober 2010).

One system where there is a potential for conflict between species recognition and mate quality recognition in the bisexual-unisexual mating system of mollies: sailfin mollies, *Poecilia latipinna*, are sexually parasitized by the all-female, clonal species, Amazon mollies (*P. formosa*). Amazon mollies are livebearing fish that arose from a hybridization event between *P. latipinna* and *P. mexicana* approximately 100,000 years ago (Awise et al. 1991; Scharl et al. 1995; Stöck et al. 2010) and require mating with either parental species to induce embryogenesis but inheritance is strictly maternal (Hubbs 1964). Male sailfin mollies from sympatry showed a greater strength of preference for conspecific females over Amazon mollies than did males from allopatric populations (Hubbs 1964; Ryan et al. 1996; Gabor and Ryan 2001). When confronted with a large Amazon molly and a smaller conspecific female, male sailfin mollies from sympatric and allopatric populations lost their preference for conspecific females (Gumm and Gabor 2005) indicating conflict between species and mate quality recognition. Male sailfin mollies from a population sympatric with Amazon mollies were unable to differentiate conspecific females from Amazon using both chemical and visual cues; whereas, males from an allopatric population were able to differentiate between the female species when presented with both cues (Aspbury et al. 2010). Males preferred to mate with conspecific females only when presented with a combination of visual and chemical cues, but the specific hormone involved in species recognition has not yet been identified (see: Gabor and Grober 2010).

In teleost fish, androgens are released in the water via urine and feces or passive diffusion over the gills (Oliveira et al. 1996; Ellis et al. 2005) and may function as pheromones (Scott and Sorensen 1994; Sorensen et al. 2005). Thus, water-borne

hormones and their metabolites may serve as a cue for species recognition in this system. 11-ketotestosterone (KT) is the primary androgen involved in mate choice, spermiation, and development of secondary sex characteristics in fishes (Miura et al. 1996; García-López et al. 2006). Higher levels of KT production have also been associated with parenting behavior found in monogamous species of fish (Hirschenhauser et al. 2004), male mating behavior and increased sexual displays (Kindler et al. 1991). Gabor and Grober (2010) found an increase in KT production in male sailfin mollies and conspecific females after they were mated together, whereas this pattern was absent when males mated with Amazon mollies. In addition, males directed more mating attempts towards conspecific females than to Amazon mollies. Gabor and Grober (2010) also found that there was no difference in estrogen (E) or testosterone (T) responsiveness in male sailfin mollies after mating with either a conspecific female or an Amazon molly. These results suggest that KT is important in promoting species recognition via a bidirectional feedback mechanism in a sympatric population. I propose to examine if a similar hormone response is found in sailfin mollies that are allopatric to Amazon mollies. Males from allopatry may not share the same KT response as males from sympatry due to the lack of heterospecifics that resemble high quality mates. I predict a differential KT response by males from an allopatric population towards conspecific females than towards Amazon mollies.

Materials and methods

The sailfin mollies used in this experiment were collected from an allopatric population in Port Aransas, Nueces County, Texas [28.33N, 97.24W], and Amazon mollies used in this experiment were collected south of Tamaulipas, Mexico [22.92N, 98.07W]. I maintained the fish on a 14-h light/10-h dark cycle using UV lighting to simulate daylight, and fed them Ocean Star International Inc. Spirulina Flake mixed with Ocean Star International Inc. Freshwater Flake food twice daily supplemented daily with live brine shrimp. I housed males in single-sex groups for at least seven days prior to testing (in 37 L aquaria) and females in single-sex groups for at least 30 days in 38 L aquaria to control for receptivity (Liley 1966). I performed trials from 0800-1100 each day during the breeding season (August 2010) to control for the effects of circadian variation on hormonal fluctuation (Lorenzi et al. 2008).

Male test fish ($n = 24$; mean \pm s.e.m standard length (SL), length from snout to caudal peduncle = 31.57 ± 0.64 mm; range of 23.3 - 49.74 mm) were randomly paired with a conspecific female (mean SL \pm s.e.m = 35.64 ± 0.35 mm) or an Amazon molly (mean SL \pm s.e.m = 35.67 ± 0.37 mm). Females were sized matched ± 2 mm. Half of the males were paired with a conspecific on the first day and the other half were paired with an Amazon molly on the first day. The following day, I tested males with the other species of female. Males were tested during the same time during both days. Males and females were sequestered in separate 250ml beakers filled with 100ml of treated water for 1 hour prior to manipulations to create a baseline hormone profile (*pre mating* sample). Each pair of fish (a single male and single female conspecific or Amazon molly) was placed in a 19 L aquarium for 25 min (to potentially provide enough time for

hormone levels to increase in response to the trial). The number of mating attempts (gonopodial thrusts) directed at the female and the amount of time males took to first thrust were recorded. After the mating, males and females were again sequestered in separate 250ml beakers filled with 100ml of treated water for 1 hour to establish their hormonal response (*postmating* sample).

Hormone extraction

Water samples were kept at -20C until the hormone assays were performed (Ellis et al. 2004). All water samples were eluted through solid phase extraction c18 (SPE) columns via a vacuum manifold. Hormones were eluted from the columns with methanol into vials. The elutant was then evaporated with nitrogen gas and resuspended in assay buffer. Enzyme-immunoassay (EIA) kits were used to assay KT (Cayman Chemical). All samples were run in duplicate on 96 well plates and read by a fluorescent plate reader (BioTek Powerwave XS). The EIA kits were previously validated for sailfin and Amazon mollies (Gabor and Grober 2010).

Statistical Analyses

I used Pearson's correlation test to analyze the relationship between KT levels and SL. The hormone data met the assumptions of parametric analyses when ln transformed. I used an unpaired Student's t-test to detect differences in premating and KT responses. I also used a paired t-test to analyze differences in male premating and KT responses. I

used nonparametric analyses when examining behavioral data of mating attempts (gonopodial thrusts) and time to first thrust, since they did not meet all of the assumptions of parametric analyses. I used Wilcoxon Sign-Rank Test, to determine if there are differences in mating attempts and latency to thrust towards conspecific females or Amazon mollies. I also used Fisher's exact test to determine if there was a difference in the probability that a male would attempt to mate with either a conspecific females or with an Amazon molly. I used Kendall's τ to analyze the correlations between mating attempts vs. KT levels, premating vs. postmating KT levels, and KT responsiveness vs. latency to thrust. All p values were two-tailed and alpha was set at 0.05 and analyses were performed with JMP v8 (SAS Institute). I detected one outlier with the Grubb's outlier test. One Amazon molly did not have its pre hormone collected, while two Amazon mollies and one female sailfin molly did not have their post hormone collected as these fish jumped out of their containers while I was collecting hormone samples.

Results

Methodological consideration

Male SL was not significantly correlated with premating KT levels (Pearson correlation: $r = 0.05$, $n = 23$, $p = 0.81$). There was no significant difference in premating KT levels across both days (mean \pm s.e.m pg/sample: Day 1: 5.36 ± 0.18 ; Day 2: 5.50 ± 0.23 ; paired t-test; $n = 23$, $t = 0.57$, $p = 0.58$; Table 2.1). The SL of Amazon mollies was not correlated with their premating KT levels (Pearson correlation: $r = 0.03$, $n = 23$, $p = 0.88$). Premating KT levels were not correlated with the SL of female sailfin molly

(Pearson correlation: $r = -0.39$, $n = 24$, $p = 0.06$). Premating KT levels were not significantly different between Amazon and female sailfin mollies (unpaired t-test: $df = 40$, $t = 1.10$, $p = 0.28$; Table 2.1). Males produced more premating KT than female sailfin mollies and Amazon mollies (unpaired t-test: sailfin mollies: $df = 33$, $t = 9.26$, $p < 0.0001$; Amazon mollies: $n = 23$, $Z = 5.80$, $p < 0.0001$; Table 2.1). Male premating KT levels were not correlated with the amount of mating attempts (gonopodial thrusts: Kendall's τ : $n = 23$, $\tau = -0.05$, $p = 0.76$). Male premating KT did not significantly affect whether they mated or not with either female species (unpaired t-test; Amazon mollies, $df = 21$, $t = 0.43$, $p = 0.68$; sailfin mollies, $df = 21$, $t = 0.60$, $p = 0.57$). Female premating KT did not significantly affect whether males mated or not (unpaired t-test; Amazon mollies, $df = 21$, $t = 0.35$, $p = 0.73$; sailfin mollies, $df = 22$, $t = -1.16$, $p = 0.27$).

Main effects

Males exhibited more mating attempts to conspecific females than to Amazon mollies (Wilcoxon Sign Rank: $n = 23$, $T = 2.16$, $p = 0.04$; Fig 2.1). To assess strength of preference (SOP) for conspecific females, I focused on the number of thrusts directed at a conspecific female relative to the total number of thrusts that was demonstrated for that particular male (Gabor and Ryan 2001). Strength of preference significantly greater from 0.50 indicates that males preferred conspecific females. The SOP for conspecifics was not significantly greater than 0.50 (mean \pm s.e.m = 0.63 ± 0.08 ; Wilcoxon Signed-Rank: $t = 39.50$, $p = 0.11$). Additionally, there was no significant difference in the probability that

a male would attempt to mate with a conspecific female or with an Amazon molly (Fisher's exact test: $p = 0.53$).

To assess KT production during the trials, I focused on KT responsiveness (postmating sample/ pre mating sample) as an indication of the relative changes in KT production (Wingfield et al. 1997; Hirschenhauser et al. 2004). The order in which males encountered females had no effect on KT responsiveness (ANOVA: $F_{1,43} = 0.44$, $p = 0.51$). Males that attempted to mate with either female species did not show a significant difference in KT responsiveness than males that did not attempt to mate (unpaired t-test: Amazon molly: $df = 21$, $t = -0.57$, $p = 0.58$; sailfin molly: $df = 20$, $t = 1.21$, $p = 0.26$; Fig 2.2). Similarly, females that mated did not have a significantly different KT response compared to those that were not mated (unpaired t-test: Amazon mollies, $df = 18$, $t = -1.45$, $p = 0.17$; sailfin mollies, $df = 21$, $t = -0.40$, $p = 0.70$; Fig 2.3). Male KT responsiveness also did not differ as a result of being paired with either female species (paired t-test: $n = 22$, $t = -0.58$, $p = 0.57$; Fig 2.4). There was no difference in female sailfin molly and Amazon molly KT responsiveness when mating with males (unpaired t-test: $df = 41$, $t = 1.25$, $p = 0.22$).

There was no difference in time to first thrust of males that were paired with conspecific females or Amazon mollies (Wilcoxon Sign Rank: $n = 24$, $t = -7.00$, $p = 0.80$). There was no correlation between male KT responsiveness and the time to first thrust with Amazon mollies (Kendall's τ : $n = 23$, $\tau = 0.09$, $p = 0.57$) or female sailfin mollies (Kendall's τ : $n = 22$, $\tau = -0.03$, $p = 0.86$). There was also no correlation between male KT responsiveness and the number of thrusts directed at either female species (Kendall's τ : Amazon mollies, $n = 23$, $\tau = 0.07$, $p = 0.68$; sailfin mollies, $n = 22$, $\tau = 0.85$,

$p = 0.59$). There was no correlation between female KT responsiveness and time to first thrust for Amazons (Kendall's τ : $n = 20$, $\tau = -0.01$, $p = 0.97$) or female sailfin mollies (Kendall's τ : $n = 23$, $\tau = 0.03$, $p = 0.83$). There was also no correlation between female KT responsiveness and the number of thrusts directed at them by males (Kendall's τ : Amazon mollies, $n = 20$, $\tau = -0.21$, $p = 0.22$, sailfin mollies, $n = 23$, $\tau = -0.06$, $p = 0.71$).

Discussion

In this study, I examined whether male sailfin mollies from a population allopatric to Amazon mollies exhibit a similar KT response and mate choice of male sailfin mollies from a sympatric population. While males from allopatric populations on average had weaker preference for conspecific females than males from sympatric populations (Ryan et al. 1996; Gabor and Ryan 2001; Gumm and Gabor 2005), I found that male sailfin mollies in this experiment preferred to mate with conspecific females over Amazon mollies. However, males did not have a significant SOP for conspecific females. Additionally, there was no significant difference in the probability that a male would attempt to mate with either a conspecific female or an Amazon molly. There was also no difference in male KT response after mating with either species. Amazon mollies also did not display a change in KT response after mating with males similar to Gabor and Grober (2010), indicating a KT response is not present after Amazon mollies mate with male sailfin mollies regardless of the context of the population. There was also no difference in KT responsiveness between both males and females that mated and did not mate. 11-ketotestosterone may not affect the latency to thrust, since KT responsiveness was not

correlated with time to first thrust to either Amazon mollies or female sailfin mollies. There was also no correlation between KT responsiveness and mating attempts for both males and females, indicating KT does not affect the frequency or response of mating attempts. These results suggest that KT may not be a mechanism used by males for species recognition in this allopatric population. The lack of change in KT production when mating with conspecifics in this allopatric population suggests that the KT signaling in the sympatric population studied by Gabor and Grober (2010) may not be an ancestral trait. However, further testing of sympatric and allopatric groups is necessary to determine this on a finer scale.

Differences of mate choice may be correlated with the differences of KT regulation between allopatric and sympatric populations. One prediction based on the challenge hypothesis would be that males from sympatry would have more elevated baseline KT levels than males from allopatry due to the added stress of avoiding matings with closely related heterospecifics. However, when comparing data from our current data set with data from Gabor and Grober (2010), males from the sympatric population tested in Gabor and Grober (2010) had significantly lower baseline KT compared to males from our allopatric population (Unpaired t-test: $df = 40$, $t = 2.98$, $p = 0.005$; mean \pm s.e.m: sympatry: 152.77 ± 54.14 KT pg/sample; allopatry: 311.99 ± 65.97 KT pg/sample). One hypothesis that could explain the difference between the allopatric and sympatric populations is that males in sympatric populations may maintain overall lower KT levels due to the chronic stress of closely related heterospecifics within the population. Several studies have shown an increase of a corticosteroid, cortisol, in response to chronic and acute stress, which had inhibitory effects on steroid sex

hormones production (Kubokawa et al. 1999; reviewed in: Goos and Consten 2002). 11β -hydroxysteroid dehydrogenase type 2 (HSD) is an enzyme in teleosts that metabolizes glucocorticoids to inert product and KT precursors to KT (Ozaki et al. 2006). Thus, there may be a relationship between cortisol and KT levels during a mating event. Differences in baseline KT levels may indicate that KT responsiveness would also differ among populations as well. However, further testing of allopatric and sympatric populations is needed to determine the robustness of KT regulation, as well as KT regulation in response to a cortisol response.

Along with differences in magnitude of hormonal response between allopatric and sympatric populations, the latency to a KT response may not be reflected in the data when using sampling methods that do not capture the peak of KT production. Water-borne hormone collection does not provide information about rapid fluctuations of hormones. Thus, KT upregulation may not always be synchronized within the timeframe of when hormones are collected. The KT peak seen in Gabor and Grober (2010) may have occurred prior to or while collecting the postmating hormones. Similarly, male sailfin mollies increase sperm production after mating with conspecific females (Robinson et al. 2008), which is stimulated by an increase of KT (Schulz and Miura 2002; Ozaki et al. 2006). Males from allopatric populations may have started KT production earlier in the mating trial in comparison to males from sympatry. Thus, KT levels may have slowly decreased during the remainder of the mating trial. Further testing is necessary to determine the latency of KT upregulation from this specific population.

Males from this allopatric population preferred to mate with conspecific females, similarly males from this population showed close to no significant preference ($p=0.05$)

for conspecifics over Amazon mollies in a prior study (Gabor and Ryan 2001). However, when I removed the behavioral data for a male whose hormonal data was removed due to being outlying data, males lost their preference for mating with conspecific females ($p = 0.06$). Additionally, males did not have a significant SOP for conspecific females or have a significant probability of mating with conspecific females. All three results suggest that male sailfin mollies from this allopatric population did not prefer to mate with conspecific females over Amazon mollies. The males from the present allopatric population also exhibited a similar level of SOPs compared to the mean of all of the allopatric populations that were tested by Gabor and Ryan (2001). Males from the other allopatric populations also had a weaker SOP than males from sympatric populations. Additionally, the sympatric population that exhibited a positive KT response when mating with conspecific females (Gabor and Grober 2010), also had a significant SOP for conspecific females. These results indicate that further populations need to be tested to better understand the origin of the differential KT response seen in one sympatric population.

In conclusion, sailfin mollies from an allopatric population mated more with conspecifics though they did not exhibit greater KT responsiveness after mating with conspecifics as seen in Gabor and Grober (2010), indicating that the lack of KT response may explain the lack of species recognition in this allopatric population. The differences between allopatric and sympatric populations may affect different characteristics of hormonal regulation (magnitude and latency), which requires further evaluation in multiple populations.

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Table 2.1 Premating levels of 11-Ketotestosterone (pg/sample) of male and female sailfin mollies, *P. latipinna*, and Amazon mollies, *P. formosa*. Males were tested on separate days with either a conspecific female or an Amazon molly.

| Individual | n | Day 1±SE (pg/sample) | Day 2±SE (pg/sample) |
|-------------------------|----|-------------------------|-------------------------|
| Male sailfin molly | 23 | 311.99 ± 65.97 | 410.12 ± 88.64 |
| Female sailfin molly | 24 | 17.84 ± 1.65 | |
| Amazon molly | 23 | 15.69 ± 1.66 | |

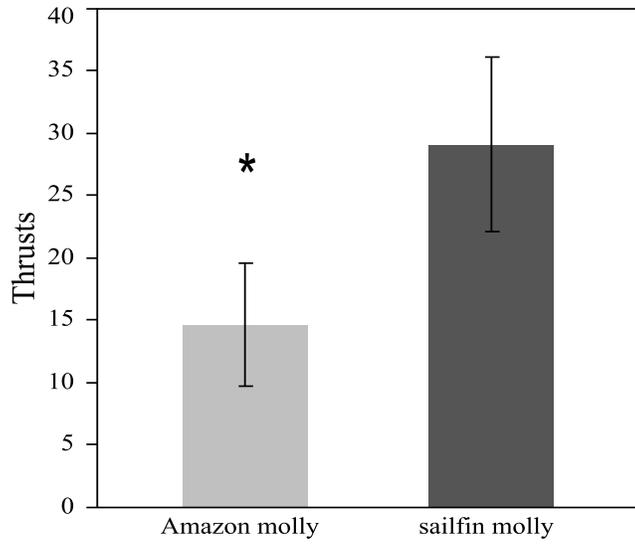


Fig 2.1 Bar graph representing the number of gonopodial thrusts (thrusts) directed at either Amazon or sailfin mollies ($p < 0.05$).

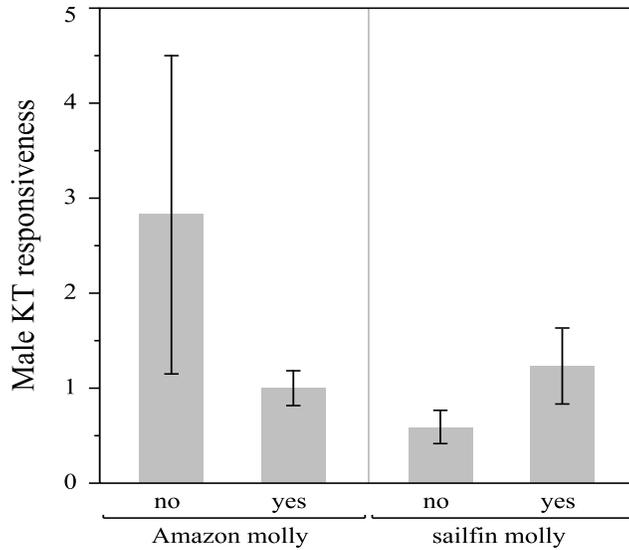


Fig 2.2 Bar graph representing male KT responsiveness after mating (yes) or not mating (no) with both female species. There was no difference in KT responsiveness of males that mated compared with males that did not mate (unpaired t-test: Amazon molly: $p = 0.579$; sailfin molly: $p = 0.261$).

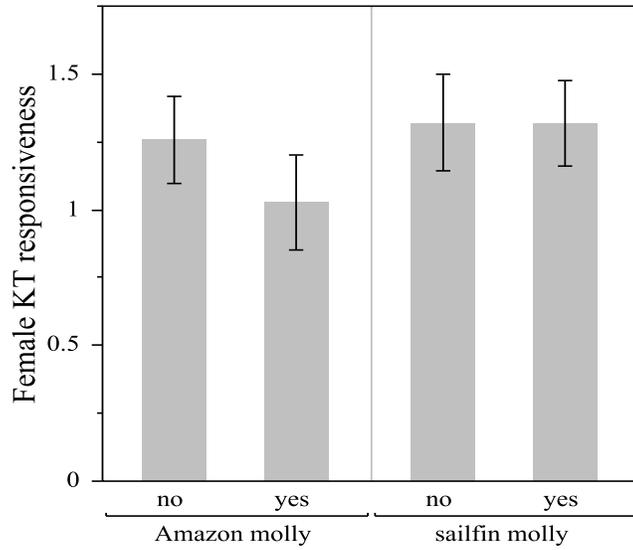


Fig 2.3 Bar graph representing female KT responsiveness after mating (yes) or not mating (no) with a male for each female species. There was no difference in KT responsiveness of females that mated compared with females that did not mate in either female species (unpaired t-test: Amazon mollies, $p = 0.183$; sailfin mollies, $p = 0.743$).

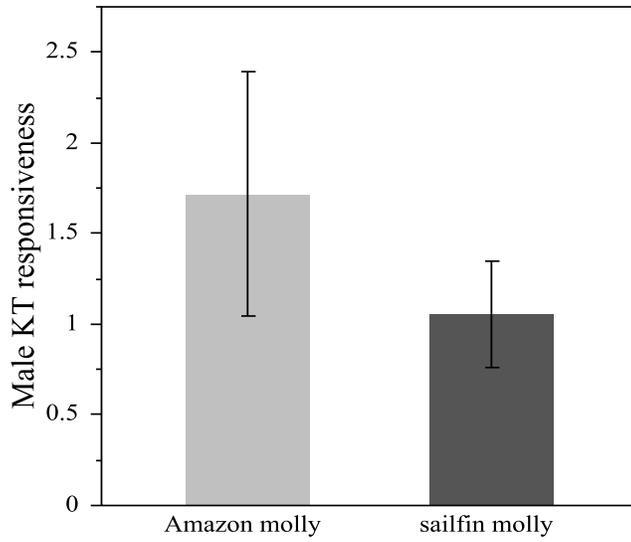


Fig 2.4 Bar graph of male KT responsiveness from mating with both species of female.

Male KT responsiveness did not differ as a result of being paired with either female species (paired t-test: $p = 0.570$).

Chapter 3

Relationship between 11-ketotestosterone and sperm production in Atlantic mollies (*Poecilia mexicana*) when exposed to either conspecific females or clonal Amazon mollies (*P. formosa*) during brief and prolonged association periods

Abstract

In the unisexual-bisexual mating system consisting of bisexual Atlantic mollies (*Poecilia mexicana*) and unisexual gynogenetic Amazon mollies (*P. formosa*), male Atlantic mollies prefer to mate with and transfer more sperm to conspecific females over Amazon mollies. I examined if Atlantic mollies produce more sperm when associated with conspecific females over Amazon mollies. I also examined if 11-ketotestosterone (KT) was correlated with baseline sperm levels and sperm priming. In a second experiment, I investigated whether males showed signs of rapidly priming sperm or a KT response after being associated with either a conspecific female or an Amazon molly for one hour. I did not find any correlation between baseline KT and sperm levels in male Atlantic mollies. Interestingly, male Atlantic mollies also did not show any preference, in the form of sperm priming or KT production, for conspecific females over Amazon mollies. These results suggest KT does not regulate the last stage of sperm production and is not involved in species recognition in male Atlantic mollies.

Introduction

When closely related species occur in sympatry, distinguishing heterospecifics (species recognition) from high quality mates (mate recognition) is crucial in maintaining fitness. Differential sperm production (sperm priming) and transfer (Aspbury and Gabor 2004b; Schlupp and Plath 2005) may be a mechanism of species recognition as this limits gene exchange between species. Males that mate with Amazon mollies risk depletion of sperm and the opportunity of mating with conspecific females (Robinson et al. 2008).

Differential sperm transfer has been demonstrated in the bisexual-unisexual mating system of Atlantic mollies (*P. mexicana*) that are sexually parasitized by the gynogenetic Amazon molly (*P. formosa*) (Schlupp and Plath 2005). Mollies are livebearing fish, and Amazon mollies were derived from a hybridization event between a sailfin and an Atlantic molly about 100,000 years ago (Awise et al. 1991; Scharl et al. 1995; Loewe and Lamatsch 2008; Stöck et al. 2010). This all-female species requires sperm from either of its parental species to induce embryogenesis but inheritance is strictly maternal (Hubbs 1964). Males of the parental species do not increase their fitness and also risk depletion of sperm and the opportunity of mating with conspecific females from these matings (Robinson et al. 2008).

Male sailfin mollies from sympatry showed a greater strength of preference for conspecifics over Amazon mollies than males from populations allopatric to Amazon mollies (Gabor and Ryan 2001). Male sailfin mollies also produced more sperm (priming response) when in the presence of a conspecific female than when in the presence of an Amazon molly (Aspbury and Gabor 2004b). Male sailfin mollies from a sympatric

population also showed higher rates of spermiation when in the presence of conspecific females (Robinson et al. 2008), which may allow males to differentially allocate reproductive resources to higher quality mates and conspecifics. Male Atlantic mollies also preferred to mate with conspecifics (Ryan et al. 1996), and more sperm cells are recovered from conspecific females than from Amazon mollies after mating with male Atlantic mollies (Schlupp and Plath 2005). However, there are no studies that have investigated whether male Atlantic mollies prime more sperm for conspecifics. Males in the presence of conspecifics increased hormone production, which may influence differential sperm production. 11-ketotestosterone (KT) is a primary androgen involved with mate choice, spermiation, and development of secondary sex characteristics in fishes (Miura et al. 1996; García-López et al. 2006). 11-ketotestosterone is also a direct stimulator for spermatogenesis (Miura et al. 1991; Borg 1994; Cavaco et al. 1998). 11-ketotestosterone is present in all stages of spermatogenesis but is present in lower levels in regressing testis where all stages of spermatogenesis is present (Weltzien et al. 2002), which suggests that KT may only be involved in the earlier stages of spermatogenesis. Gabor and Grober (2010) found that male sailfin mollies had higher KT responsiveness after mating with conspecific females than after mating with Amazon mollies. These results suggest that it is important to look at KT production in both males and females because KT production may serve as a bi-directional signal in species recognition. As of yet, there are no studies that have addressed the importance of a KT response and its effect on sperm production in male Atlantic mollies or any livebearing fish.

To determine if circulating KT levels have an effect on spermiation, I utilized a recently developed non-invasive collection technique that allows for the extraction of

water-borne hormones and allows for repeated sampling (Sebire et al. 2007). This method has been validated by Gabor and Grober (2010) for *P. formosa* and Gabor et al. (unpublished data) validated this method for *P. mexicana*. First, I determined if there was a correlation between pre-association KT levels of male Atlantic mollies with baseline sperm levels and also the amount of sperm primed when associated with a conspecific female or Amazon molly for seven days. Secondly, I determined if there was a correlation between KT responsiveness and the amount of sperm primed during a shorter association period of one hour when paired with either a female Atlantic molly or an Amazon molly. I hypothesize that (i) KT is involved in spermatogenesis if baseline KT levels are correlated with baseline sperm levels, (ii) males will have a preference for conspecific female by producing more sperm during association, and (iii) males utilized KT in species recognition if males had a differential KT response when associated with conspecific females over Amazon mollies. I predict that there would be a correlation between baseline KT and baseline sperm levels in male Atlantic mollies. I also predict that males would produce more sperm and exhibit a greater KT response when associated with a conspecific female than when associated with an Amazon molly for both prolonged and brief association periods.

Materials and methods

I separated males at least seven days prior to testing (in 37 L aquaria) and females for at least 30 days prior to the experiment (in 37 L aquaria) to control for receptivity and hormonal fluctuations due to mating cycles (Liley 1966). I maintained fish on a 14-h

light/10-h dark cycle using UV lighting to simulate daylight, and fed them Ocean Star International Inc. Spirulina Flake mixed with Ocean Star International Inc. Freshwater Flake food twice daily and supplemented daily with live brine shrimp.

Experiment 1: Baseline KT vs baseline sperm and differential sperm priming

Atlantic mollies were collected from a sympatric population in Tamaulipas, Mexico [24.04N, 98.90 W]. Amazon mollies were collected from a different population in Tamaulipas, Mexico [23.97N, 99.11W]. I performed trials from 0800-1100 each day during August - September 2009 to control for the effects of circadian variation on hormonal fluctuation (Lorenzi et al. 2008).

Male Atlantic mollies ($n = 25$; mean \pm s.e.m standard length (SL), length from snout to caudal peduncle = 38.68 ± 0.92 mm; range of 30.38 to 49.3 mm) were separated for at least seven days prior to testing (in a 37 L aquarium). On day 0, I obtained male *pre-association* KT by sequestering males in separate 250ml beakers filled with 100ml of fresh tank water for 1 hr prior to manipulations to create a baseline hormone profile. I also extracted sperm from males to determine pre-association sperm counts. (Extraction and sperm counting followed methods of (Aspbury and Gabor 2004b) see brief methods below). Following sperm extraction, individual males were placed on one side of a 19 L tank that was divided by a clear perforated partition and were paired with either a: (a) female Atlantic molly ($n = 13$; mean \pm s.e.m SL= 46.62 ± 1.35 mm; range of 36.72 to 55.07 mm) or (b) Amazon molly ($n = 12$; mean \pm s.e.m SL= 46.36 ± 1.28 mm; range of 39.13 to 51.12 mm) for seven days. On day seven, I obtained post-association sperm

levels from males. Three days were found sufficient for complete sperm recovery (Aspbury and Gabor 2004b). The difference between day seven and day zero sperm levels was defined as the amount of sperm primed during the four days males were associated with a female.

Experiment 2: Sperm priming and KT response during brief exposure to females

To determine if there was a difference in KT response and sperm priming as a result of a brief exposure to a female, I used the same non-invasive methods to collect hormone samples from water as in experiment 1. Trials were performed from 0800-1100 each day during the breeding season (August 2009 and June 2010) to control for the effects of circadian variation on hormonal fluctuation (Lorenzi et al. 2008). Atlantic mollies and Amazon mollies that were tested in August 2009 were collected from a sympatric population in Tamaulipas, Mexico [24.04N, 98.90 W]. Due to a lack of sufficient number of males I also tested Atlantic mollies and Amazon mollies that were collected from a second sympatric population in Tamaulipas, Mexico [23.58N, 99.06 W] in June 2010.

On day 0, sperm was extracted from males ($n = 30$; mean \pm s.e.m SL = 39.72 ± 0.89 mm; range of 30.63 to 48.98 mm). Three days are sufficient for complete sperm recovery (Aspbury and Gabor 2004b). On day three, I obtained *pre-association* KT levels from both males and females by sequestering both the male and his paired female in separate 250ml beakers filled with 100ml of fresh tank water for 1 hour prior to manipulations to create a baseline hormone profile. Males were then placed on one side of a 19 L tank that was divided by a clear perforated partition and paired with either a: (a)

female Atlantic molly ($n = 15$; mean \pm s.e.m SL= 45.50 ± 0.59 mm; range of 39.04 to 49.21 mm) or (b) Amazon molly ($n = 14$; mean \pm s.e.m SL= 46.74 ± 0.70 mm; range of 42.74 to 51.15 mm) for 1 hr in the divided tank. At the end of 1 hr, male and female *post-association* KT levels were collected. Sperm was extracted from males following the post-association KT collection. I measured the KT responsiveness (post-association KT/pre-association KT) for both males and females. Sperm was extracted and counted following methods of Aspbury and Gabor (2004b). See sperm extraction methods below.

Hormone extraction and sperm counting

Water samples were kept at -20C until the hormone assays were performed (Ellis et al. 2004). All water samples were eluted through solid phase extraction c18 columns placed on a vacuum manifold. Hormones were eluted from the columns with methanol into vials. The elutant was evaporated over nitrogen gas and resuspended in assay buffer. Enzyme-immunoassy (EIA) kits (Cayman Chemicals) were used to assay KT. All samples were run in duplicate on 96 well plates and read by a fluorescent plate reader (BioTek Powerwave XS).

Sperm cells were collected using an aspirator consisting of a 200 μ l gel-loading pipette tip connected to a (5-cm) glass tube and airline tubing. The spermatozeugmata were placed into a microcentrifuge tube with 100 μ l of 0.9% saline solution (0.9 g of NaCl per 100 ml of water), and repeatedly drawn up and expelled from a pipette (to distribute sperm cells evenly). Sperm cells were counted five times on an improved Neubauer chamber hemocytometer (Reichert, Buffalo, NY) under 400x magnification.

The total number of sperm cells was determined by multiplying the mean cell count by the sample's initial volume (100 μ l) and dividing by the volume of the hemocytometer (0.1 μ l).

Statistical Analyses

Experiment 1: Baseline KT vs baseline sperm and differential sperm priming

I used a Pearson's correlation test between KT levels and SL. The hormone data met the assumptions of parametric analyses when ln transformed. I used an unpaired Student's t-test to examine differences in pre-mating and KT responses. I also used a paired t-test to analyze differences in male pre-mating and KT responses. I used non-parametric analyses when examining the correlation between sperm levels and KT levels.

Experiment 2: Sperm priming and KT response during brief exposure to females

In experiment 2, I controlled for variation in male size on sperm availability by using residuals plotted from SL and sperm count by treatment, order and sex. I used a Pearson's correlation test KT levels and SL. The hormone data met the assumptions of parametric analyses when ln transformed. I used an unpaired Student's t-test to detect differences in pre-mating and KT responses. I also used a paired t-test to analyze differences in male pre-mating and KT responses. I used non-parametric analyses Mann Whitney U test to analyze differences in sperm levels I also used Kendall's τ to detect correlations between

sperm levels and SL and KT responsiveness. All p values were two-tailed and alpha was set at 0.05 and analyses were performed with JMP v8 (SAS Institute). The Grubb's outlier test detected one outlier from the data set. Six females jumped from their hormone collection chambers so these data were not used.

Results

Experiment 1: Baseline KT vs baseline sperm and differential sperm priming

There was no correlation between male baseline KT, SL, and baseline sperm levels (Fig 3.1; Table 3.1). To assess sperm production during the trials, I focused on sperm priming (post-association sperm – pre-association sperm) as an indicator of relative changes in production (Aspbury and Gabor 2004a). There was no correlation between the amount of sperm primed and male pre-association KT levels for males that were paired with Amazon mollies (Kendall's τ : $n = 9$, $\tau = 0.00$, $p = 1.00$), but there was a significant correlation when males were paired with conspecific females (Kendall's τ : $n = 13$, $\tau = 0.44$, $p = 0.04$; Fig 3.2). Males did not significantly differ in their sperm priming response when paired with Amazon or female Atlantic mollies (Mann Whitney U: $n = 12$, $Z = 1.77$, $p = 0.08$). However, males that were paired with conspecific females primed a significant negative amount compared to zero (Mean sperm cells \pm s.e.m = -2434231 ± 1070210.60 ; One sample t-test: $df = 12$, $t = -2.27$, $p = 0.02$; Fig 3.3). Males that were paired with Amazon mollies did not prime a significant amount of sperm different from zero (Mean \pm s.e.m = -190624 ± 578904.98 ; One sample t-test: $df = 11$, $t = -0.33$, $p = 0.37$; Fig 3.3).

Experiment 2: Sperm priming and KT response during brief exposure to females

There was no significant correlation between male pre-association KT and SL (Pearson's correlation: $r = -0.18$, $n = 28$, $p = 0.36$). There was a correlation between male pre-association sperm and SL (Kendall's τ : $n = 28$, $\tau = 0.55$, $p < 0.001$). Males produced significantly more pre-association KT than females (mean KT \pm s.e.m: males = 4.40 ± 0.14 pg/sample, female Atlantic mollies: 3.44 ± 0.10 pg/sample, Amazon mollies = 3.36 ± 0.13 pg/sample; Unpaired t-test: paired with Amazon mollies: $df = 25$, $t = 3.39$, $p = 0.003$; paired with female Atlantic mollies: $df = 28$, $t = 5.79$, $p < 0.0001$). Males that were paired with female Atlantic mollies did not have a significantly different baseline sperm than males paired with Amazon mollies (Mann Whitney U: $df = 26$, $Z = 0.60$, $p = 0.55$; Table 3.2). There was no significant difference between male pre-association KT for males paired with Amazon or Atlantic mollies (Mann Whitney U: $df = 26$, $Z = -0.69$, $p = 0.49$; Table 3.3).

There was no relationship between female pre-association KT and SL for female Atlantic mollies (Pearson's correlation: $r = -0.09$, $n = 15$, $p = 0.75$) and Amazon mollies (Pearson's correlation: $r = -0.08$, $n = 14$, $p = 0.79$). There was no difference between female pre-association KT between Atlantic and Amazon mollies (Unpaired t-test: $df = 27$, $t = 0.50$, $p = 0.62$; Table 3.4).

To assess KT production during the trials, I focused on KT responsiveness (postmating sample/ premating sample) as an indication of the relative changes in KT production (Wingfield et al. 1997; Hirschenhauser et al. 2004). There was no difference

in male KT responsiveness when males were tested with Amazon or female Atlantic mollies (Mean \pm s.e.m ln pg/sample: Amazon mollies: -0.12 ± 0.18 ; female Atlantic mollies: -0.15 ± 0.14 ; Unpaired t-test: $df = 24$, $t = -0.17$, $p = 0.87$; Fig 3.4).

There was no correlation between post-association sperm levels and male SL (Kendall's τ : $n = 26$, $\tau = -0.09$, $p = 0.53$). There was also no difference in post-association sperm levels among males that were paired with Amazon or Atlantic mollies (Mann Whitney U: $df = 24$, $Z = -0.23$, $p = 0.82$; Fig 5; Table 3.2). There was no relationship between male KT responsiveness and post-association sperm levels when males were paired with either female species (Kendall's τ : Amazon mollies: $n = 12$, $\tau = -0.24$, $p = 0.27$; Atlantic mollies: $n = 14$, $\tau = 0.01$, $p = 0.96$; Fig 3.6).

There was no difference in female KT responsiveness between Amazon and Atlantic mollies (Mean KT responsiveness \pm s.e.m: Amazon mollies: 0.02 ± 0.21 ; female Atlantic mollies: -0.23 ± 0.59 ; Unpaired t-test: $df = 23$, $t = -0.91$, $p = 0.38$). There was no correlation between female KT responsiveness in either species and post-association sperm levels (Kendall's τ : Amazon mollies: $n = 12$, $\tau = -0.20$, $p = 0.39$; female Atlantic mollies: $n = 13$, $\tau = -0.21$, $p = 0.33$).

Discussion

Male Atlantic mollies in both studies did not show signs of KT regulation involved in sperm production. Males also did not differ in sperm production or show differential KT response when paired with a conspecific female over an Amazon molly. There was no

correlation between male pre-association KT levels with pre-association sperm levels for males in both prolonged and brief association periods, indicating that KT does not influence baseline sperm levels in Atlantic mollies. There was a correlation between pre-association KT and sperm priming when males associated with conspecific females over a prolonged period, but this relationship was absent when males were paired with Amazon mollies. Moreover, this pattern was not seen in males that were paired with conspecific or heterospecific females for a short period of time. The correlation with pre-association KT and the amount of sperm primed seen after prolonged periods of time when paired with conspecifics suggests that KT may have more involvement in sperm priming after longer periods of time. However, males did not significantly prime more sperm for either conspecific females or Amazon mollies during prolonged periods of association. Males that were paired with females for brief periods of association did not differ in post-association sperm or KT responsiveness. The lack of change of KT responsiveness or sperm levels suggests that KT may not aid in species recognition as previously found for sailfin mollies that mated with conspecifics (Gabor and Grober 2010). Gabor et al. (unpublished data) have also found no difference in the KT response of male Atlantic mollies after mating with Atlantic or Amazon mollies, further indicating that KT may not be involved in species recognition for Atlantic mollies.

Male Atlantic mollies are capable of differentiating conspecific females from Amazon mollies (Ryan et al. 1996; Schlupp and Plath 2005), but may not prime more sperm or change KT responsiveness due to the inability to recognize conspecific chemical cues. Genes involved in odor production in mammals have been found to covary with genetic similarities to closely related heterospecifics (Heth and Todrank

2000). Male olfactory receptors did not discriminate between sex hormone and metabolites from sympatric suckers (*Catostomus catostomus* and *Catostomus commersoni*) (Cardwell et al. 1992). Additionally, males of two sympatric species, Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) did not differ in KT, testosterone, progesterin, or sperm levels when treated with stimulants from closely related heterospecifics or conspecific females (Häkan Olsén et al. 2000). Thus, male sailfin mollies may distinguish between hormonal cues excreted by conspecific females and Amazon mollies due to differences in hormonal cues that are reflective of this differential KT response found in this population.

Interactions between sex hormones and stress response hormones may influence physiological and behavioral responses. Glucocorticoids, a type of corticosteroid that are produced from the adrenal cortex, are commonly made in response to stress but recently have been identified as a hormone that is highly variable and influenced by sexual selection (reviewed in: Husak and Moore 2008). A negative relationship found between body condition and high baseline cortisol level in males (Moore and Jessop 2003) may have negative effects on sexually selected traits. High stress levels are even capable of suppressing reproduction in birds (reviewed in: Wingfield et al. 1998) and secondary characteristics (reviewed in: Husak and Moore 2008). $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one ($17\alpha,20\beta$), a progesterin that increases the expression of the enzyme homolog of 11β -hydroxysteroid dehydrogenase type 2 (HSD) in teleosts metabolizes glucocorticoids to inert product and KT precursors to KT (Ozaki et al. 2006). Furthermore, HSD activity is restricted to the testis of vocal male midshipman fish (*Porichthys notatus*) (Arterbery et al. 2010). Thus, cortisol may affect KT and indirectly sperm levels by competing for the

same enzyme substrate. Acute cortisol treatments decreased KT conversion but had no effect on lowering KT levels in carp (*Cyprinus carpio*) (Consten et al. 2001). However, long term cortisol treatments decreased plasma KT levels and retarded spermatogenesis (Consten et al. 2001). Additionally, steroid hormone production in Leydig cells is retarded in mammals once a glucocorticoid is bound to a glucocorticoid receptor (Schultz et al. 1993). Mammals have shown increases of cortisol immediately after mating (Levis et al. 1995; Strier et al. 2003), although little work has been performed on teleosts and the relationship between cortisol and KT is still unknown in livebearing fish. Male Atlantic mollies may have a different cortisol response when associated with conspecific females or Amazon mollies. Furthermore, sperm priming and KT responsiveness may be negatively influenced by a higher cortisol levels. Thus, further work is necessary to determine other hormones that can affect KT response in this mating system.

11-ketotestosterone was found in lower levels during the final stages of spermatogenesis in male Atlantic halibut (Weltzien et al. 2002), which suggests another hormone may have a stronger influence on sperm priming. Furthermore, male sailfin mollies have lower levels of KT in testis than males of other fish species (Borg 1994). There was correlation between pre-association KT and the amount of sperm primed in male Atlantic mollies that were paired with conspecific females for seven days. The absence of a correlation between post-association KT and the amount of sperm primed supports the hypothesis that KT may not be involved in sperm priming. Male Senegalese sole (*Solea senegalensis*) associated with females exhibited a decrease in KT seven days after exposure to a female (Cabrita et al. 2011), suggesting a switch of hormone production from KT to $17\alpha,20\beta\text{P}$. This progestin is known to affect spermatozoa, sperm

hydration and spawning behavior in male fish (Asturiano et al. 2002; Schulz et al. 2010). Thus, I hypothesize that post-association $17\alpha,20\beta\text{P}$ may be correlated with the amount of sperm primed in males and may even be involved in species recognition.

To better understand the involvement KT has on sperm priming, further testing is required to understand the regulation of hormonal and physiological processes that differ between males that express a preference for conspecific females over Amazon mollies. 11-ketotestosterone may have more influence on sperm priming in male sailfin mollies, where KT is utilized as mechanism of species recognition (Gabor and Grober 2010). However, Atlantic mollies from a sympatric population did not exhibit a change in spermiation or KT responsiveness when in the presence of either female species. Several factors (e.g. genetic similarities, stress response, or KT latency) may influence differences seen in the strength of preference for conspecific females and the differential KT responsiveness for conspecific over heterospecific females.

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Table 3.1. Correlations between male baseline KT, standard length (SL), and baseline sperm levels of male Atlantic mollies that were associated with a female for 7 days. Baseline KT and SL were analyzed using a Pearson's correlation test. Baseline sperm was compared with baseline KT levels and SL using Kendall's τ .

| | Baseline KT (pg/sample) | Standard length (mm) | Baseline sperm ($\times 10^5$) |
|--|------------------------------------|----------------------------------|--|
| Baseline KT (pg/sample) | | r = 0.13, n = 22, p = 0.56 | $\tau = -0.05$, n = 22, p = 0.76 |
| Standard length (mm) | | | $\tau = 0.27$, n = 25, p = 0.06 |
| Baseline sperm ($\times 10^5$) | | | |

Table 3.2 Pre- and post- association size controlled sperm from male Atlantic mollies when in the presence of conspecifics or heterospecifics for 1 hour. Sperm samples were controlled for male standard length (SL) [sperm sample ($\times 10^5$)]

| | Paired with Amazon molly | Paired with Atlantic molly | df | t | p |
|---------------------------------------|-----------------------------------|-------------------------------------|----|-------|------|
| Pre-association sperm \pm s.e.m | 50.28 \pm 13.19 | 40.15 \pm 10.45 | 26 | -0.62 | 0.54 |
| Post-association sperm \pm s.e.m | 22.09 \pm 6.51 | 18.36 \pm 2.96 | 24 | -0.48 | 0.64 |

Table 3.3 Pre- and post- KT levels from male Atlantic mollies when in the presence of conspecific or heterospecific females for 1 hour.

| | Paired with Female Atlantic molly | Paired with Amazon molly | df | t | p |
|---|---|-----------------------------------|----|-------|------|
| Pre-association KT \pm s.e.m (pg/sample) | 93.79 \pm 12.39 | 144.96 \pm 69.41 | 26 | -0.73 | 0.48 |
| Post-association KT \pm s.e.m (pg/sample) | 88.81 \pm 22.71 | 106.00 \pm 36.24 | 24 | 0.40 | 0.69 |

Table 3.4 Pre- and post- association 11-ketotestosterone of female Atlantic and Amazon mollies when paired with male Atlantic mollies for 1 hour.

| | Female Atlantic molly | Amazon molly | df | t | p |
|--|-----------------------------|-----------------|----|-------|------|
| Pre-association KT ± s.e.m (pg/sample) | 33.08 ± 3.01 | 29.83 ± 4.47 | 28 | 0.60 | 0.55 |
| Post-association KT± s.e.m (pg/sample) | 26.58 ± 4.18 | 31.48 ± 3.41 | 25 | -0.91 | 0.37 |

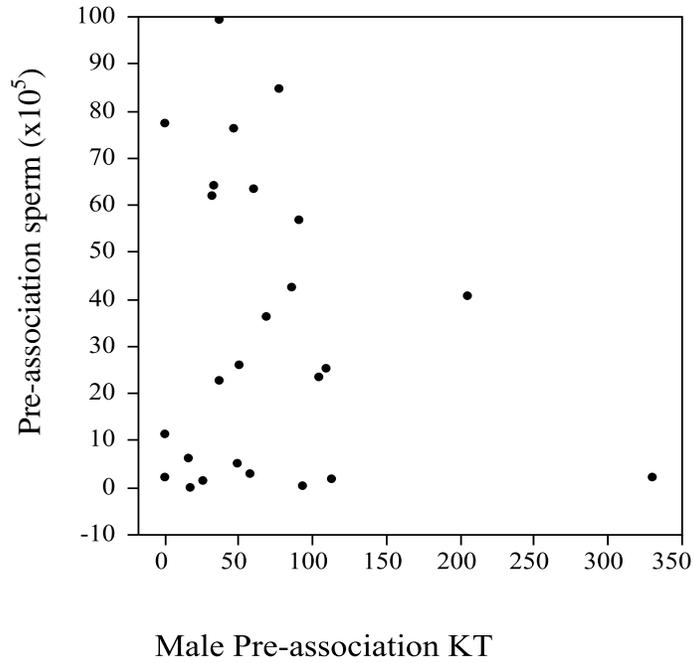


Fig 3.1 The correlation between pre-association 11-ketotestosterone (KT) and pre-association sperm from male Atlantic mollies ($n = 22$) that were paired with a female Atlantic molly or Amazon molly for seven days.

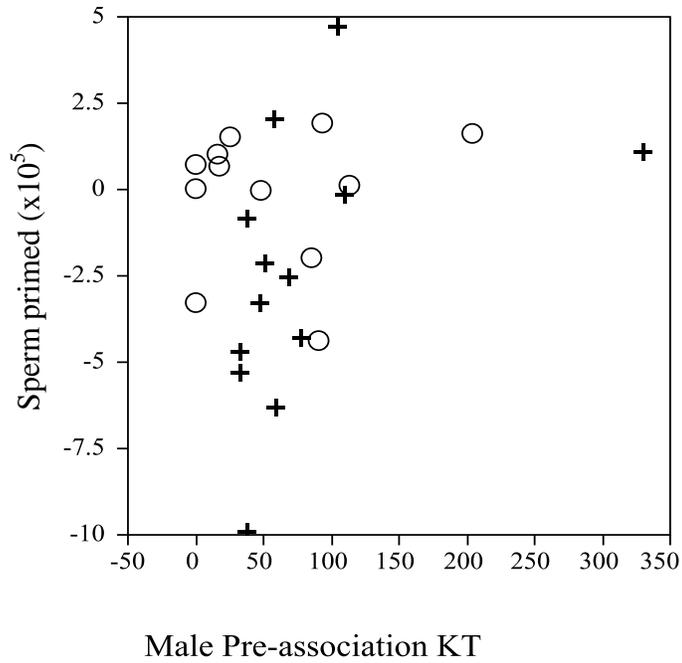


Fig 3.2 The correlation between pre-association 11-ketotestosterone (KT) and sperm primed (post-association – pre-association sperm) from male Atlantic mollies that were paired with a female Atlantic molly (plus symbol) or Amazon molly (circle symbol) for seven days.

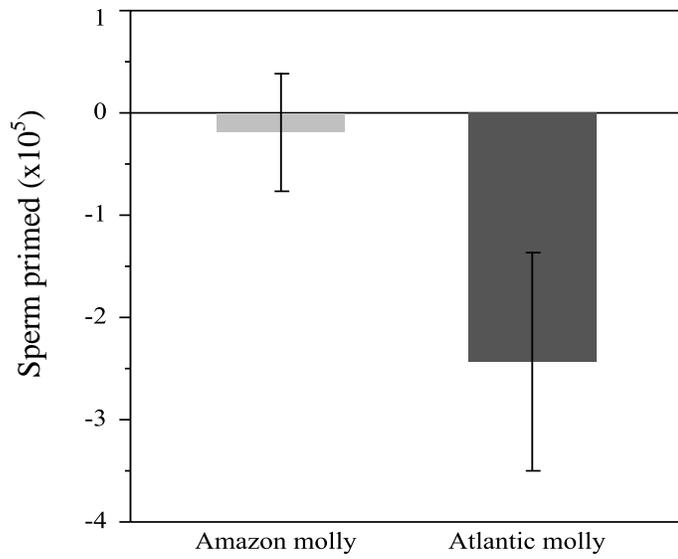


Fig 3.3 Mean \pm SE sperm primed (post-association – pre-association) from male Atlantic mollies that were associated with a female Atlantic molly or an Amazon molly for seven days.

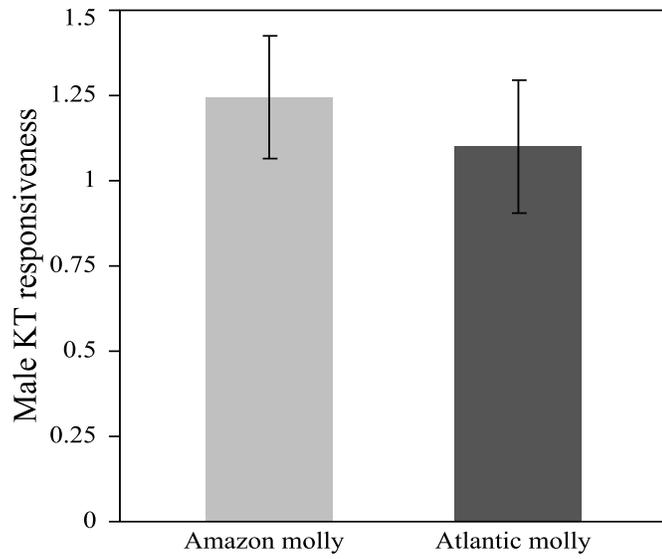


Fig 3.4 Mean \pm SE KT responsiveness (post-association /pre-association) of male Atlantic mollies that were associated with a female Atlantic molly ($n = 14$) or an Amazon molly ($n = 12$) for one hour.

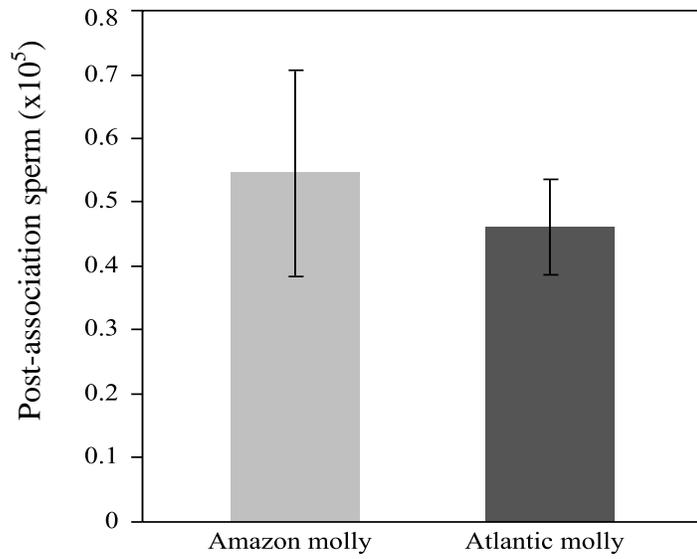


Fig 3.5 Mean \pm SE post- association sperm levels of male Atlantic mollies that were associated with a female Atlantic molly or an Amazon molly for one hour.

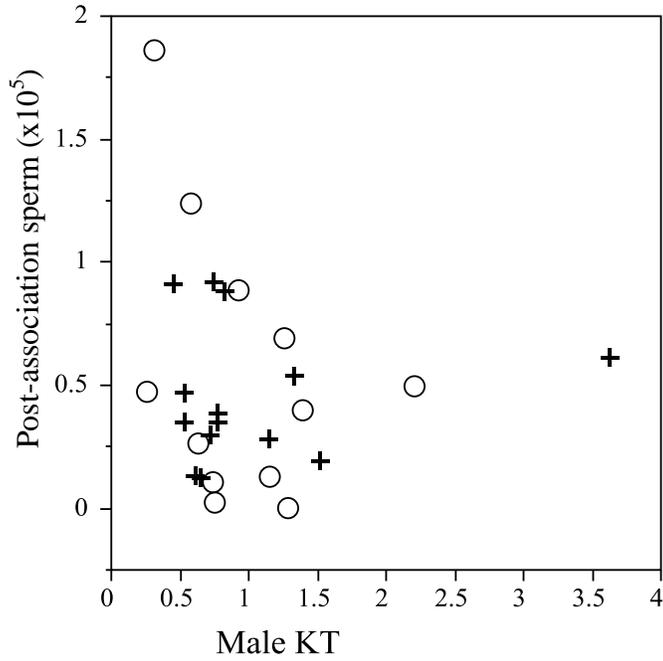


Fig 3.6 The correlation between post-association sperm and KT responsiveness (post-association / pre-association) from male Atlantic mollies that were paired with a female Atlantic molly (plus symbol) or Amazon molly (circle symbol) for one hour.

VITA

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