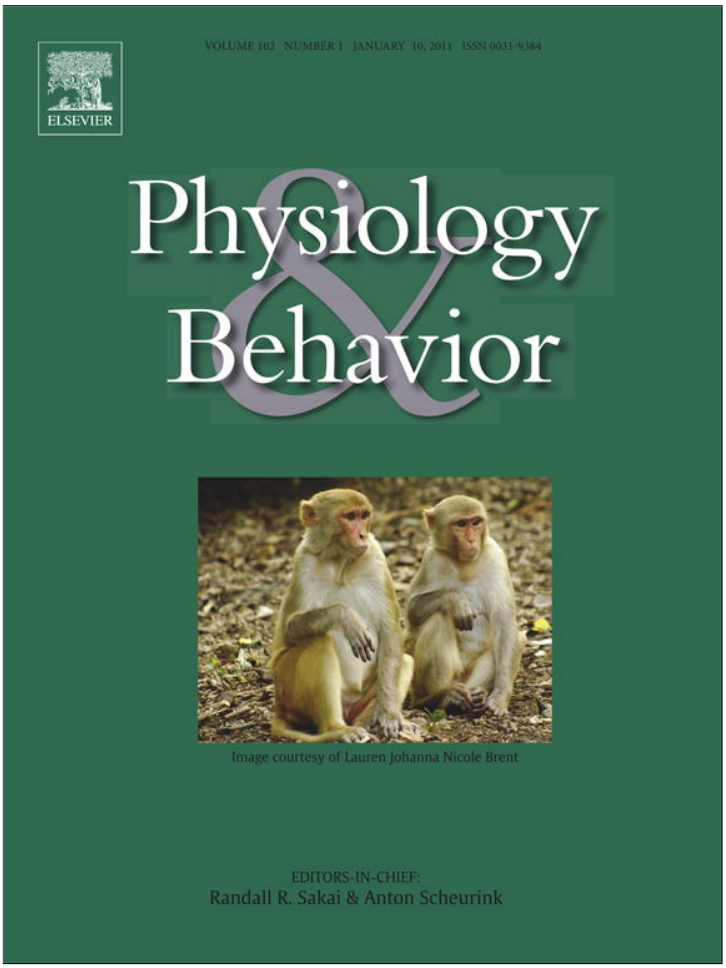

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The role of androgens in species recognition and sperm production in Atlantic mollies (*Poecilia mexicana*)

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ABSTRACT

Much is known about the role of hormones in the regulation of vertebrate mating behavior, including receptivity, and several components of mate choice. Hormones may modulate reproductive behavior in such a way to increase or decrease the individual's motivation, and therefore hormones may be important in mediating behavior associated with reproductive isolation. The mating complex of the all female gynogenetic Amazon mollies, *Poecilia formosa*, and their parental species (sailfin mollies, *P. latipinna,* and Atlantic mollies, *P. mexicana*) is a model system for studying ultimate mechanisms of species recognition. However, proximate mechanisms, such as variation in hormone levels, and the effect of hormones on sperm production have not been extensively examined. We predict that one or more of the sex steroid hormones in teleost fish (11-ketotestosterone (KT), testosterone (T), and estradiol (E)) will play a role in species recognition (during mate choice and/or sperm priming) for Atlantic mollies (the maternal parental species) that are sympatric with Amazon mollies. We sequentially paired male Atlantic mollies with female conspecifics and Amazon mollies and obtained water-borne hormone samples before and after mating for all fish. We measured circulating KT, T, and E from the water samples. Although we did not find an overall KT response to mating with conspecifics as has been found previously in sailfin mollies, male Atlantic mollies that mated more with conspecific females had lower postmating T levels. Additionally, males attempted to mate more with conspecific females that had lower postmating E levels, but attempted to mate more with Amazon mollies that had higher postmating KT levels. We also examined the effect of KT on sperm priming (a mechanism of premating mate choice), and found that KT levels of male Atlantic mollies prior to mating are correlated with the sperm priming response when males were paired with conspecific females, but this correlation was not found when males were paired with Amazon mollies. Our results indicate that male mating behavior is affecting or responding to both male and female hormones, but that the hormones alone are not playing a role in species recognition. Male Atlantic mollies may not discriminate against Amazon mollies as strongly as male sailfin mollies because Amazon mollies resemble their maternal parental species more than their paternal species.

1. Introduction

Mating behaviors and their underlying physiological mechanisms exhibited by one individual can affect the physiology and the behavior of other individuals, leading to facilitation of mating events. Mate choice plays a major role in reproductive isolation between closely related species, and can ultimately lead to speciation [1]. Although the importance of behavior in species recognition and reproductive isolation is clear [1], there are few studies that address the neuroendocrinological basis of species recognition.

Hormones regulate reproductive behavior, and mating behaviors can also affect hormone levels. Circulating hormone levels affect mate choice of both females and males in numerous taxa (birds: reviewed by [2,3]; fish: reviewed by [4]; [5,6]; frogs: [7–9]). However, when males interact with a closely related heterospecific, similar hormonal variation to conspecific interactions is not observed [10–12,5]. The differential hormonal responses when interacting with conspecifics vs. heterospecifics suggests that hormonal feedback between the sexes during mate choice may play a role in species recognition and ultimately in reproductive isolation. In addition to hormones directly affecting mate choice, hormones could also affect species recognition via an increase in sperm production in the presence of female stimuli (sperm priming response: post association sperm count − baseline sperm count) [13]), and might represent a mechanism by which males can conserve energy associated with sperm production [14], and act as a mechanism of species recognition [15,16].

One prior study has found support for hormones playing a role in species recognition. Gabor and Grober [5] found that hormonal feedback during mating interactions play a role in species recognition in the sailfin molly (*Poecilia latipinna*), and the Amazon molly (*P.*

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formosa) bisexual–unisexual mating complex. Male and female sailfin mollies upregulated an endogenous androgen, 11-ketotestosterone (KT), when they mated with each other but neither males nor the closely related gynogenetic Amazon molly upregulated KT when they mated together. However, there was no such relationship for two other steroid hormones, testosterone (T) and estradiol (E) [5]. Moreover, the premating levels of KT, E or T also did not function as mechanism for mate choice or species discrimination as they did not differ significantly between the species. Given that studying closely related species can provide important insights into how brain–hormone–behavior mechanisms have evolved [17] we extended the research performed by Gabor and Grober [5] on sailfin mollies to the maternal parent species of Amazon mollies, the Atlantic molly, *P. mexicana limantouri*. Specifically, we tested the prediction that one or more of the sex steroid hormones in teleost fish (KT, T, and E) play a role in species recognition (during mate choice and/or sperm priming) for Atlantic mollies that are sympatric with the sexually parasitic Amazon mollies. We also predicted that species recognition may be facilitated by differences between the two species in the premating levels of one or more of these steroid hormones.

Amazon mollies are an all female, gynogenetic species that arose via a hybridization event between a male sailfin molly (or another extinct sailfin species) and a female Atlantic molly approximately 120,000 years ago [18,19]. Amazon mollies require sperm, primarily from their parent species (sailfin mollies or Atlantic mollies) to trigger egg development, but inheritance is usually clonal [20]. Because of the lack of genetic benefits to males that mate with the gynogens, Amazon mollies are considered sexual parasites on their parent species. Amazon mollies and their parent species are distributed primarily along coastal Gulf of Mexico. Amazon mollies are sympatric with sailfin mollies from south-eastern Texas into northern Mexico near Rio Tuxpan [21]. Atlantic mollies are sympatric with Amazon mollies from north of Ciudad Tuxpan to Rio San Fernando, Mexico. All three species may still be sympatric south of Tampico [22]. While Amazon mollies generally form mixed shoals with their hosts in nature [23], during dry seasons and periodic droughts pools can conceivably consist of only conspecifics.

Sexually parasitized males in this unique mating system demonstrate two forms of species recognition. Male sailfin mollies that occur in sympatry with Amazon mollies show a stronger preference to mate with conspecifics than do male sailfin mollies from allopatric populations [24,25]. Male sailfin mollies also show mating preference by priming more sperm for conspecific females [15]. Male Atlantic mollies from one population in sympatry also prefer to mate with conspecifics over Amazons [24], and they transfer more sperm to conspecific females than to Amazon mollies [26]. No study has examined whether Atlantic mollies differentially prime sperm for conspecifics as is found in sailfin mollies. Despite these mating preferences exhibit by males of both parent species, males clearly continue to mismate as, paradoxically, Amazon mollies still persist.

The three primary steroid hormones that regulate mating behavior in teleost fish are KT, T, and E. While these steroids are not always necessary for the expression of reproductive behaviors, they modulate neural pathways controlling reproductive behavior, resulting in increases or decreases in motivation [27], suggesting that these sex steroids may play a role in species recognition mechanisms. KT is the primary androgen regulating male mating behavior in teleost fish [reviewed in 28] and is associated with increased sexual displays in bluegill sunfish, *Lepomis macrochirus* [29] and in sticklebacks, *Gasterosteus aculeatus* [30,31] and is also associated with greater responsiveness to ovulating females in some cichlid fish [32]. Additionally, Gabor and Grober [5] found that male sailfin mollies had higher KT levels when they mated with conspecific females suggesting that KT is important in mating behavior in sailfin mollies. Lower whole body T concentrations have been found to decrease male sexual behavior in a poeciliid fish, *Gambusia holbrooki* [33]. Estradiol is another steroid hormone that affects sexual displays in poeciliids [34], and sexual displays of male guppies (*P. reticulata*) are reduced by inhibition of aromatase, which is necessary to aromatize T to E [35]. In female guppies E also affects reproductive behavior, and is highest when females are most receptive after dropping a brood [36]. However, Ramsey et al. [37] did not find support for E affecting receptivity in another livebearing fish, *Xiphophorous nigrensis*, based on water-borne circulating E vs. the localized brain levels studied by Liley [36].

Little is known about how hormonal regulation is involved in the sperm priming response in fish. Androgens affect spermiation (the final stage of spermatogenesis) by binding to receptors on Sertoli cells [38]. KT specifically stimulates spermatogenesis in numerous fish (platyfish, *Xiphophorous maculatus*, [39]; catfish, *Clarias gariepinus*, [40]; Japanese eel, *Anguilla japonica*, [41]). The increase in KT production by male and female sailfin mollies found by Gabor and Grober [5] might represent a proximate mechanism for the rapid spermiation exhibited by male sailfin mollies that were allowed to mate with conspecific females, and that was lacking when males were allowed to mate with Amazon mollies [42]. In this study, we test the prediction that KT levels of male Atlantic mollies are positively correlated with the sperm priming response when males are paired with conspecific females, but that these variables will not be correlated when males are paired with Amazons.

To test our predictions, we first examined male Atlantic molly mate preference when they were sequentially paired with a female Atlantic molly and an Amazon molly and obtained hormone (KT, T, and E) levels before and after each mating trial from both males and females. In a subsequent experiment we examined sperm priming by male Atlantic mollies for conspecific and Amazon mollies and tested for a correlation between preassociation KT levels and baseline sperm levels and the sperm priming response.

2. Materials and methods

2.1. Experiment 1: mate choice and species recognition

We collected Atlantic and Amazon mollies from a sympatric population (Rio Purification: RP), in Mexico (24.04 N, 98.90 W) in July 2008 and returned them to the laboratory. No other potential sperm donor species are sympatric in this population. We maintained the fish on a 14-h light: 10-h dark cycle using UV lighting to simulate daylight, and fed Purina AquaMax 200 twice a day supplemented daily with live brine shrimp. We individually housed males for 20 h prior to testing (in 19 l aquaria) and we housed females in single-sex groups for at least 30 days in 38 l aquaria to control for receptivity and hormonal fluctuations due to mating cycles [43]. We tested fish May–June 2009.

We used the same experimental design as was used in Gabor and Grober [5]. We tested each male Atlantic molly ($N = 20$) with: (1) a female conspecific and (2) an Amazon molly. We paired half of the males with a conspecific on the first day and the other half with an Amazon molly on the first day. The following day, we tested males with the other species. We performed trials from 0900–1300 h each day to control for circadian variation in hormone levels [44] and we tested each male at the same time both days. We matched female size within ± 2 mm standard length (SL). We placed each male and each female in separate sterile 250 ml beakers with 80 ml fresh tank water for one h to collect a premating hormone sample. We then placed each pair of fish (a single male and single female conspecific or Amazon molly) together in a 19 l aquarium and we recorded the number of mating attempts (gonopodial thrusts) directed at the female's gonopore for 25 min. After each mating trial, we put each fish in separate sterile 250 ml beakers with 80 ml fresh tank water for one hour to collect a postmating hormone sample. We defined

hormone (KT, T or E) response as: postmating hormone level/premating hormone level.

2.2. Experiment 2: differential sperm priming and the correlation between KT production and sperm production

We used Atlantic mollies from the same sympatric population as in experiment 1. Because Amazon mollies were scarce in our lab RP population at the time of testing, we used Amazon mollies collected from a nearby population (Rio Cobe: RC) in Tamaulipas, Mexico [23.97 N, 99.11 W] in July 2008. Neither of these populations have other potential sperm donors, and there is very little genetic structure in Amazon mollies among different river systems [19]. We separated males for 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). We performed trials from 0800 to 1100 during August–September 2009.

On the first day of the experiment (day 0), we obtained male (N = 28) pre-association KT by sequestering males in separate 250 ml beakers filled with 100 ml of fresh tank water for 1 h prior to manipulations to create a baseline hormone profile. We then extracted sperm from males to determine baseline sperm counts. Sperm was stripped from the male until no further sperm was extractable (note that sperm are released in bundles, spermatozeugmata, and not individually). Extraction and sperm counting followed methods in [15]. Following sperm extraction, we placed individual males on one side of a 19 l tank that was divided by a clear perforated partition and paired males with either a: (a) female Atlantic molly (N = 14) or (b) Amazon molly (N = 14) for seven days. We then obtained post-association sperm levels from males (day 7). We defined sperm priming as: day 7–day 0 sperm counts. Positive values for sperm priming indicate that males produced more sperm during the association trial than they had prior to the experiment.

2.3. Experiment 3: correlation between free plasma and free water-borne KT release rates

To examine the correlation between water-borne hormone release rates and plasma hormone levels for KT we confined female Atlantic mollies (N = 9) to 250 ml hormone collection beakers with 100 ml of fresh tank water for 30 min. We euthanized the fish and drew blood via dorsal aorta puncture with a 26 G syringe, prepared with 4% sodium citrate as an anti-coagulant. We transferred blood volumes to microcentrifuge tubes, and we stored them at −20 C. All samples were thawed and centrifuged at 3000*g* for 10 min to separate blood from plasma. We transferred 20 μl of plasma into a new microcentrifuge tube and stored at −80 °C until ready for processing free-hormones.

2.4. Hormone assays

Methods for water-borne hormone assays follow Gabor and Grober [5]. We validated EIA kits for water-borne hormones from Amazon mollies previously [5]. To validate hormones from Atlantic mollies, we obtained water samples from 9 non-experimental Atlantic mollies using collection and extraction methods similar to those described above. Evaporated samples were re-suspended in 350 μl EIA buffer and combined in a concentrated pool of 3.15 ml. The pools were diluted to 1:4 for the serial dilutions and the quantitative recovery.

We assessed parallelism of the serial dilution curve, using the pooled (1:4) control for *P. mexicana*. The serial dilutions were run in duplicate. The log-logit transformed dilution curve was constructed using average percent maximum binding and pg/ml concentrations for the eight dilution samples (from 1:8 to 1:256 dilution). The dilution curves were parallel to the standard curve for each of the three hormones (comparison of slopes: KT: $t_{11} = 0.16$, $P = 0.88$; E: $t_{11} = 0.95$, $P = 0.36$; T: $t_{11} = 0.03$, $P = 0.98$).

To determine the quantitative recovery of the water-extracted hormones, we spiked the pooled control samples for Atlantic mollies with each of the eight standards and ran an unmanipulated pooled control sample. Expected recovery concentrations were based on the known amount of hormone (KT, E or T) in control samples. Minimum observed recovery for Atlantic mollies were: 58% (KT), 75% (E), and 86% (T). The slopes of the observed vs. expected curves for Atlantic mollies were: 0.81 (KT), 1.08 (E), and1.06 (T), indicating a linear relationship between observed and expected for all hormones.

2.5. Statistical analyses

Because we did not measure the mass of the fish, we normalized the hormone data by dividing by the standard length (SL) of the fish. In Atlantic mollies, there is a strong positive relationship between SL and mass (Linear regression: $r^2 = 0.91$, N = 15; $P < 0.0001$). The normalized hormone data met the assumptions of parametric analyses when Ln transformed and were analyzed using parametric statistics (linear regression, ANOVA and paired and unpaired Student's *t*-test). A few samples were lost due to spills while extracting hormones. This resulted in a loss of values for hormone responses since pre and post values are needed for these measurements. We used nonparametric analyses when we examined the number of mating attempts (gonopodial thrusts), and sperm counts, as these data did not meet the assumptions of parametric analyses and could not be transformed successfully (Kendall's tau and Wilcoxon signed ranks test). All *P* values were two-tailed and alpha was set at 0.05 and analyses were performed with JMP v9.0.2 (SAS Institute). We did not perform an experiment-wide reduction of alpha because each of our statistical analyses tested a different hypothesis.

3. Results

3.1. Experiment 1: mate choice and species recognition

3.1.1. Baseline hormone relationships

Male premating KT, E and T levels did not significantly differ across both days (for both females of the different species) (Paired *t*-test; KT: $t = 0.29$, N = 17, $P = 0.77$; E: $t = 0.17$, N = 17, $P = 0.87$; T: $t = 0.49$, N = 15, $P = 0.63$; Table 1).

Males did not significantly differ in their premating KT as compared to female Atlantic mollies (Unpaired *t*-test: $t = -1.57$, $df = 35$, $P = 0.13$; Table 1) and Amazon mollies ($t = -1.29$, $df = 36$, $P = 0.20$; Table 1). Male Atlantic mollies produced significantly less premating E than female Atlantic mollies (Unpaired *t*-test: $t = 6.02$, $df = 32$, $P < 0.0001$; Table 1) and Amazon mollies ($t = 7.09$, $df = 33$, $P < 0.001$; Table 1). Males made significantly less premating T than Atlantic mollies (Unpaired *t*-test: $t = 3.10$; $df = 35$; $P = 0.004$; Table 1) and Amazon mollies ($t = 3.67$, $df = 34$, $P = 0.0009$; Table 1). Male

Table 1
Premating hormone levels for male and female Atlantic mollies, *Poecilia mexicana*, and Amazon mollies, *P. formosa*. KT = 11-ketotestosterone, E = estradiol, and T = testosterone.

Individual	N	Premating hormone	Day 1 ± SE (pg/sl/h)	Day 2 ± SE (pg/sl/h)
Male Atlantic molly	20	KT	3.15 ± 0.54	4.85 ± 1.33
	20	E	3.12 ± 3.89	3.49 ± 1.05
	18	T	8.72 ± 0.94	10.60 ± 1.39
Female Atlantic molly	17	KT	2.21 ± 0.46	
	17	E	11.54 ± 1.95	
	16	T	19.85 ± 2.67	
Amazon molly	19	KT	2.15 ± 0.29	
	18	E	13.83 ± 2.20	
	18	T	23.25 ± 3.72	

premating KT and T levels were not correlated with the number of thrusts (Kendell's τ: KT: $\tau = 0.17$, N = 20, $P = 0.13$; T: $\tau = -0.14$, N = 18, $P = 0.43$). Male premating E levels were negatively correlated with the number of thrusts (Kendell's τ: E: $\tau = -0.25$, N = 20, $P = 0.03$).

The order in which males encountered each female of the different species did not significantly affect male KT, E or T response (postmating hormone/pre-mating hormone) (ANOVA: KT: $F_{1,36} = 0.35$, $P = 0.56$; E: $F_{1,36} = 0.27$, $P = 0.61$; T: $F_{1,34} = 0.435$, $P = 0.51$).

3.1.2. Main effects

Male Atlantic mollies showed no significant preference to mate (gonopodial thrusts) with conspecific females vs. Amazon mollies (Wilcoxon signed ranks: T = −13, N = 20, $P = 0.62$; Fig. 1). Eighteen males attempted to mate with Amazon mollies, two did not. Seventeen of those males mated with Atlantic mollies and three did not. There was also no significant difference in the probability that a male attempted to mate if paired with a conspecific vs. a heterospecific female (Fisher's exact test: $P = 1.0$). Moreover, female Atlantic and Amazon mollies did not differ in their premating KT, E and T levels (Unpaired *t*-test; KT: $t = -0.48$, $df = 34$, $P = 0.63$; E: $t = -1.13$, $df = 33$, $P = 0.27$; T: $t = -0.43$, $df = 33$, $P = 0.66$; Table 1). Additionally, female Amazon and Atlantic molly premating KT, E and T levels were not correlated with the number of thrusts directed at them (Kendell's τ; KT: Amazon molly, $\tau = 0.24$, N = 19, $P = 0.16$; Atlantic molly, $\tau = 0.19$, N = 18, $P = 0.27$; E: Amazon molly, $\tau = -0.17$, N = 18, $P = 0.34$; Atlantic molly, $\tau = -0.28$, N = 18, $P = 0.12$; T: Amazon molly, $\tau = -0.23$, N = 19, $P = 0.18$; Atlantic molly, $\tau = -0.19$, N = 18, $P = 0.28$).

3.1.3. Hormone responses and mating behavior

Males that attempted to mate (as measured by gonopodial thrusts directed at the female) with Amazon and Atlantic mollies did not have significantly different KT, E or T responses compared to those that did not attempt to mate (Unpaired *t*-test: KT: Amazon mollies, $df = 15$, $t = -0.89$, $P = 0.53$; Atlantic mollies, $df = 17$, $t = 0.76$, $P = 0.52$; Fig. 2a; E: Amazon mollies, $df = 15$; $t = 0.80$, $P = 0.44$; Atlantic mollies, $df = 17$; $t = -0.52$, $P = 0.65$; Fig. 2d; T: Amazon mollies, only one male did not mate; Atlantic mollies, $df = 15$; $t = -0.31$, $P = 0.78$; Fig. 2g). There was also no significant difference in the KT, E or T response of males that mated with Amazon mollies versus Atlantic mollies (Unpaired *t*-test: KT: $df = 29$, $t = -1.26$, $P = 0.22$; E: $df = 29$, $t = 0.04$, $P = 0.97$; T: $df = 29$, $t = -0.24$, $P = 0.82$). Nor was there any significant difference in the KT, E or T response of Amazon mollies or female Atlantic mollies whether males attempted to mate with them or not (Unpaired *t*-test: KT: Amazon mollies, $df = 16$, $t = -0.34$, $P = 0.79$; Atlantic mollies, $df = 15$, $t = 1.08$, $P = 0.30$; Fig. 2b; E: Amazon mollies, only one male did not mate with Amazons; Atlantic mollies, $df = 15$; $t = 1.11$, $P = 0.33$; Fig. 2e; T: Amazon molly, only one male did not mate; Atlantic molly, $df = 15$; $t = 0.76$, $P = 0.48$; Fig. 2h). Moreover, males did not significantly differ in their KT, E or T response when they were paired with female Atlantic vs. Amazon mollies (Paired *t*-test: KT: $N = 17$, $t = -1.90$, $P = 0.07$; Fig. 2c; E: N = 17, $t = 0.43$, $P = 0.67$; Fig. 2f; T: N = 17, $t = -0.62$, $P = 0.54$; Fig. 2i).

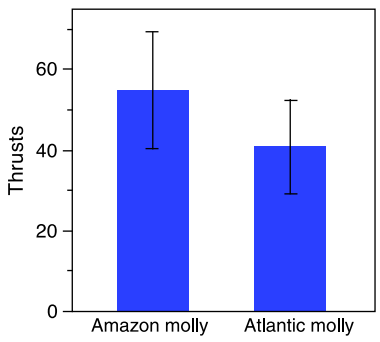

Fig. 1. Mean (±SE) number of mating attempts (thrusts) by male Atlantic mollies (N = 20) directed at Amazon mollies and female Atlantic mollies.

There was no significant correlation between male KT, E or T response and the number of thrusts directed towards Amazon mollies (Kendall's τ: KT: N = 17; $\tau = -0.10$, $P = 0.59$; E: N = 17; $\tau = 0.12$, $P = 0.54$; T: N = 17; $\tau = -0.19$, $P = 0.28$) or Atlantic mollies (Kendall's τ: KT: N = 19; $\tau = 0.02$, $P = 0.92$; E: N = 19; $\tau = -0.07$, $P = 0.70$; T: N = 17; $\tau = -0.12$, $P = 0.51$). There was also no significant correlation between female Amazon molly and Atlantic molly KT and E response with the number of thrusts directed at them by males (Kendall's τ: KT: Amazon molly, N = 18, $\tau = 0.16$, $P = 0.36$; Atlantic molly, N = 17, $\tau = 0.11$, $P = 0.56$; E: Amazon mollies, $N = 17$, $\tau = -0.24$, $P = 0.19$; Atlantic mollies, N = 17, $\tau = -0.18$, $P = 0.32$; T; Amazon mollies N = 17, $\tau = -0.03$, $P = 0.87$; Atlantic mollies, N = 17, $\tau = -0.19$, $P = 0.28$).

3.1.4. Postmating hormones and mating behavior

There was a significant positive correlation between male thrusts towards Amazon mollies and female postmating KT levels (Kendall's τ: N = 19; $\tau = 0.44$, $P = 0.009$; Fig. 3a) but no significant relationship was found for female Atlantic mollies (Kendall's τ: N = 20; $\tau = 0.10$, $P = 0.56$). There was a significant negative correlation between female Atlantic molly postmating E levels and thrusts by males (Kendall's τ: N = 20; $\tau = -0.48$, $P = 0.003$; Fig. 3b) but there was no significant relationship for Amazon mollies (Kendall's τ: N = 19; $\tau = -0.10$, $P = 0.57$). There was a significant negative correlation between male postmating T levels and thrusts towards Atlantic mollies (Kendall's τ: N = 18; $\tau = -0.61$, $P = 0.02$; Fig. 3c). Male post mating T levels were not significantly correlated with thrusting towards Amazon mollies (Kendall's τ: N = 19; $\tau = -0.18$, $P = 0.29$).

3.2. Experiment 2: Differential sperm priming and the correlation between KT production and sperm production

There was no significant correlation between male baseline KT and standard length (SL) (Kendall's τ: N = 25, $\tau = 0.69$, $P = 0.65$). There was also no significant correlation between baseline sperm levels (day 0) and SL (Kendall's τ: N = 25, $\tau = 0.24$, $P = 0.08$), or between baseline KT levels and baseline sperm available in male Atlantic mollies (Kendall's τ: N = 22, $\tau = -0.07$, $P = 0.67$; Fig. 4). There was no correlation between the amount of sperm primed (day 7–day 0 sperm cells; [15,16] and male pre-association KT levels for males that were paired with Amazon mollies (Kendall's τ: N = 9, $\tau = 0.00$, $P = 1.0$; Fig. 5a). There was, however, a significant positive correlation between the amount of sperm primed and male pre-association KT levels when males were paired with conspecific females (Kendall's τ: N = 13, $\tau = 0.44$, $P = 0.04$; Fig. 5b). Males did not significantly differ in their sperm priming response ((Day 7–day 0 sperm cells) when paired with Amazon or Atlantic mollies (Mann Whitney U: N = 12, $Z = 1.77$, $P = 0.08$; Fig. 6). However, males that were paired with conspecific females had significantly less sperm on day 7 than on day 0 (paired *t* test: $t = 2.27$, $df = 12$, $P = 0.04$; Fig. 6), but males that were paired with Amazon mollies did not have a significant difference in day 7 vs. day 0 sperm counts (paired *t* test: $t = 1.00$, $df = 13$, $P = 0.17$; Fig. 6).

3.3. Experiment 3: correlation between free plasma and free water-borne KT release rates

We found a significant positive correlation between free water-borne KT release rates and free plasma KT hormone levels (Pearson correlation adjusted for small samples sizes: N = 9, $r^* = 0.89$, $P = 0.0023$) in Atlantic mollies.

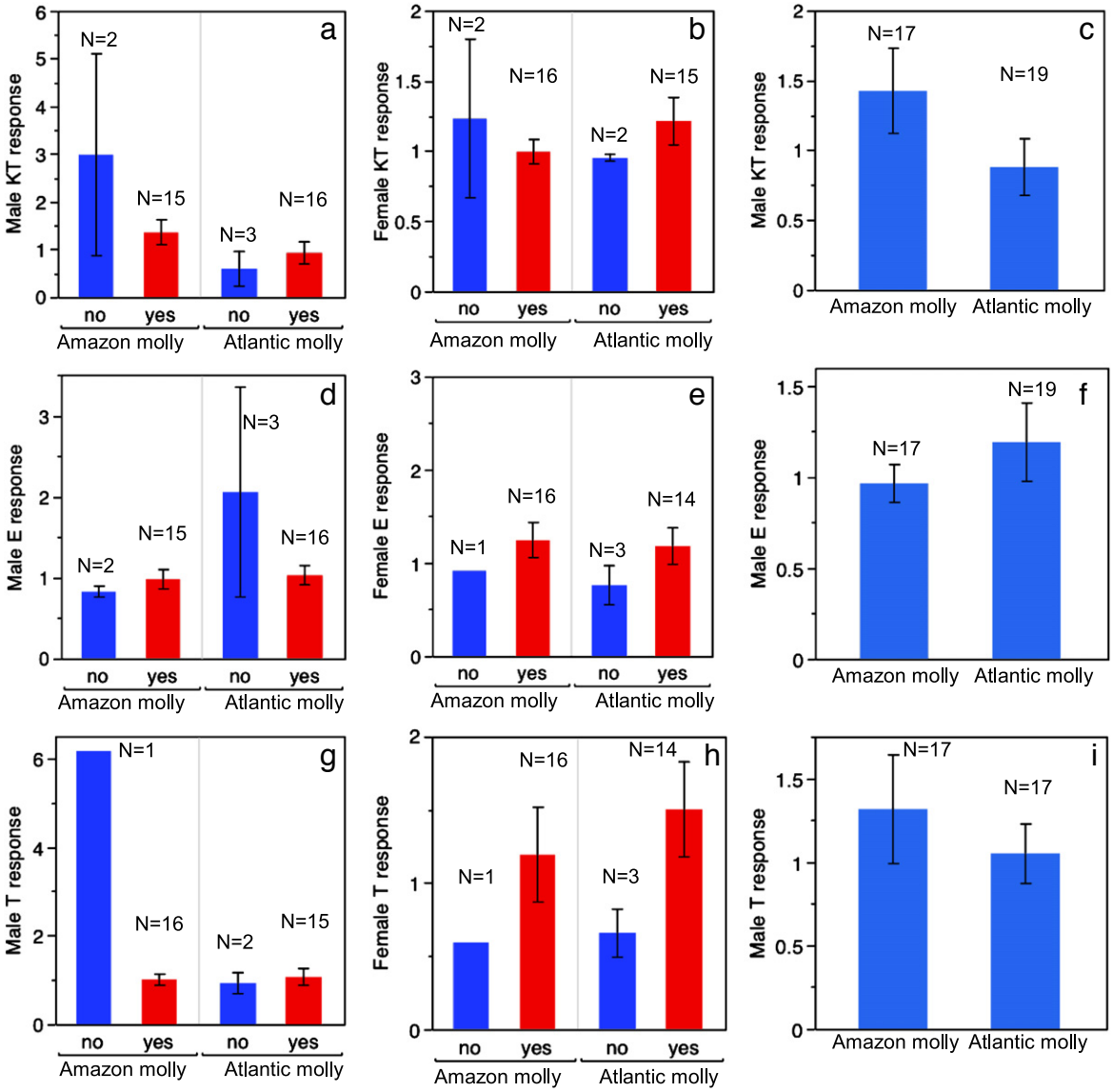

Fig. 2. Mean (±SE) response (postmating/premating hormone) of: 11-ketotestoterone (KT), estradiol (E), and testosterone (T) for male Atlantic mollies that thrusted (Yes) or not (No) when paired with Amazon mollies or female Atlantic mollies (panels A, D, G); KT, E and T for Amazon mollies and female Atlantic mollies when males thrusted towards them or not (panels B, E, H); and KT, E and T for male Atlantic mollies when tested with Amazon mollies or female Atlantic mollies, irrespective of whether males thrusted or not (panels C, F, I). None of the comparisons was significant.

4. Discussion

We predicted that one or more of the primary steroid hormones in teleost fish (KT, T and/or E) would play a role in species recognition for Atlantic mollies that are sympatric with Amazon mollies as KT does for sailfin mollies. We found little evidence to support differences in premating KT, E or T as a mechanism for mate choice or species discrimination in this study. In partial support, however, of this prediction, we found that male mating behavior (gonopodial thrusting) differentially affected hormone levels of each species. Males that directed more mating attempts towards conspecific females had lower postmating T levels. Additionally, males attempted to mate more with conspecific females that had lower postmating E levels, but attempted to mate more with Amazon mollies that had higher postmating KT levels. These results indicate that male mating behavior is affecting or responding to both male and female hormones in these fish, but that the hormones in and of themselves are not playing a role in species recognition.

In this study we tested the prediction that male Atlantic mollies exhibit mate preferences for conspecific females over the gynogenetic Amazon mollies, measured as both mating attempts and differential sperm priming when in the presence of female conspecifics vs. Amazon mollies. These predictions were not met; male Atlantic mollies from this population did not significantly prefer to mate with or prime more sperm for conspecifics. These results are in contrast to prior studies showing both conspecific mating preference [24,26] and increased sperm transfer to conspecifics [26]. One explanation for the differences between our study and prior studies is that there is geographic variation in the mating behaviors and possibly male physiology exhibited by Atlantic mollies. Gabor and Ryan [25] found variation across populations of sailfin mollies in mate preference, and Gumm and Gabor [45] found that this variation might be due to a conflict between species recognition and mate quality cues. In our first experiment, male Atlantic mollies mostly mated indiscriminately and mated more frequently overall than do male sailfin mollies, suggesting that Atlantic mollies also are faced with a conflict in species recognition vs. mate quality recognition [46]. Because male Atlantic mollies mostly mated with any female they encountered, it also decreased our statistical power to differentiate between the hormone responses of males that mated vs. those that did not with each

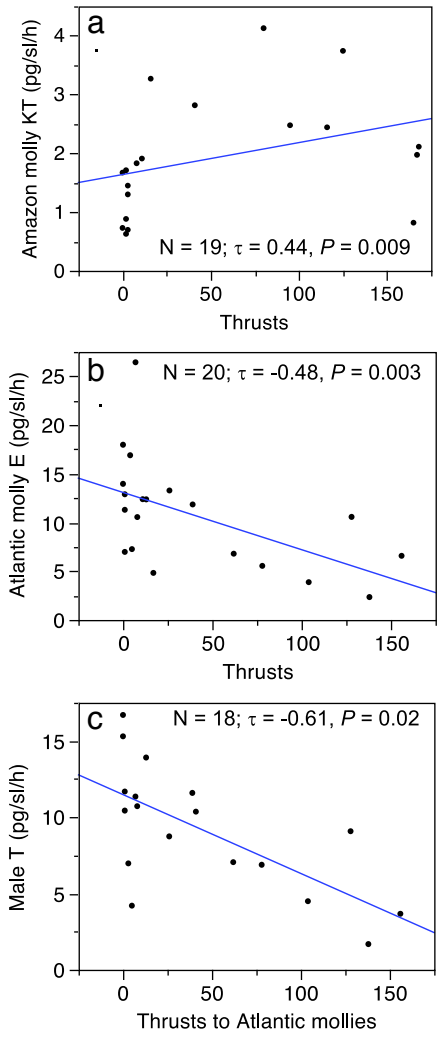

Fig. 3. The correlation between number of thrusts and: (a) postmating 11-ketotestosterone (KT) level (pg/sl/h) of Amazon mollies, (b) postmating estradiol (E) level (pg/sl/h) of female Atlantic mollies, and (c) postmating testosterone (T) level (pg/sl/h) of male Atlantic mollies after thrusting towards female Atlantic mollies.

species. In addition, male Atlantic mollies do not show the increased KT response when mating with conspecifics as found for sailfin mollies [5], which could partially explain the lack of a conspecific mate preference (measured as both mating attempts and sperm production) in this population of Atlantic mollies.

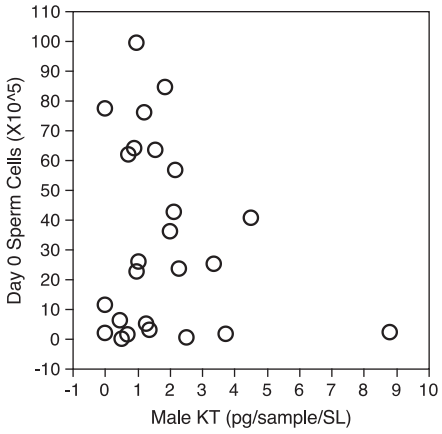

Fig. 4. The correlation between 11-ketotestosterone (KT) level (pg/sl/h) and day 0 sperm cells in male Atlantic mollies.

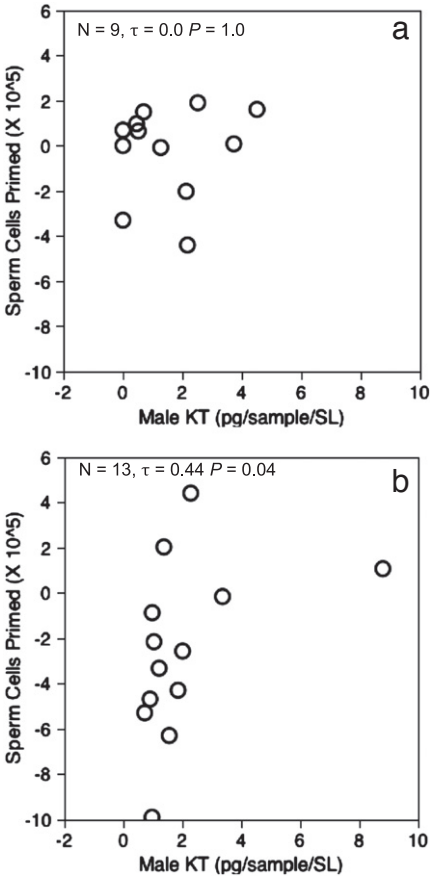

Fig. 5. The correlation between premating 11-ketotestosterone (KT) level (pg/sl/h) in male Atlantic mollies, and sperm cells primed (= post association sperm count − day 0 sperm count) for males paired with (a) Amazon mollies, and (b) for males paired with female Atlantic mollies.

We did not find a significant correlation between baseline sperm counts and pre-association KT levels in male Atlantic mollies. This result is in contrast to another study that administered KT resulting in an increase in spermatogenesis in another livebearing fish *X. maculatus* [39]. Male sailfin mollies rapidly produce sperm in the presence of conspecific females, but not Amazon mollies [42]. We predict that if KT is important in regulating spermatogenesis, then we would find a correlation between KT response and sperm priming. However, for the current study, we did not collect data on KT levels close to the time that males were paired with females. However, we did find support for the prediction that KT levels of male Atlantic mollies prior to mating are correlated with the sperm priming response when males are paired with conspecific females, but this correlation was not found when males are paired

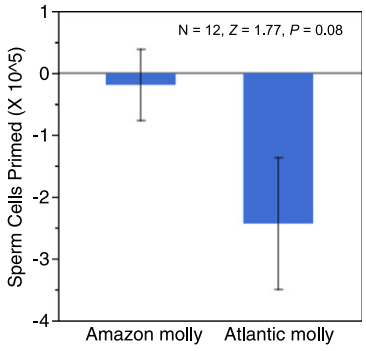

Fig. 6. Mean (±SE) sperm cells primed when male Atlantic mollies were paired with an Amazon molly or a female Atlantic molly.

with Amazons. Male Atlantic mollies did not significantly differ in their sperm priming response when paired with Amazon mollies or conspecific females, unlike in sailfin mollies [15]. In the current study, we used the same experimental design and had similar sample sizes to Aspbury and Gabor [15]. One explanation for the difference between the two species is that male Atlantic mollies from this population are unable to recognize conspecifics. However, we found that males that were paired with conspecific females had significantly less sperm on day 7 than on day 0, but males that were paired with Amazons did not have a significant difference in day 7 vs. day 0 sperm count. These results contradict the prediction that male Atlantic mollies express mating preferences in the form of heightened sperm production, but they do suggest that males are responding differently to conspecific females than to Amazon mollies.

The different relationships between male mating behaviors and hormones may provide insights into differences in male and female behaviors. First, we found that males with lower premating E levels thrusted more. These results are the opposite of what has been found for sailfin mollies [5] but are similar to result for guppies that showed decreased mating behavior with increased E levels [47]. Second, males thrust more at Amazon mollies with higher postmating KT levels. Additionally, males had a marginally greater KT response when paired with Amazon mollies than female conspecifics. This KT response may also be an outcome of the increased postmating KT levels of Amazon mollies that resulted in males also thrusting more towards these females. However, the exact mechanism affecting these differences is not clear from our data. We also found that males thrust more at conspecifics that had lower postmating E levels. Ramsey et al. [37] found that in another livebearing fish species, *X. nigrensis*, females with lower E levels spent more time in association with males and moved less. In our study it is possible that female Atlantic mollies moved less and spent more time with males than did Amazons. We also found that males with lower postmating T levels directed more mating attempts towards conspecific females. Toft et al. [48] found in the livebearing fish, *G. holbrooki*, that when levels of T are higher, males show more sexual behavior. One explanation for the difference between our study and Toft et al. [48] is that male *G. holbrooki* primarily rely on sneak copulations whereas male Atlantic mollies do not.

The differences among the mating and hormonal responses of sailfin and Atlantic mollies parallel intraspecific differences among populations of mollies and suggest rapid evolution of mating behaviors. Differentiation in male mating behavior among sailfin molly populations has occurred faster than differentiation at allozyme loci [49], possibly due to strong selective pressures from parasitic Amazon mollies over approximately 120,000 years [50,18,19]. Behavioral evolution has resulted in reproductive character displacement in mating preferences between sympatric and allopatric populations [25]. This behavioral evolution appears to vary between species as well. One conclusion is that sailfin mollies have evolved the differential KT response when mating with conspecifics, but that Atlantic mollies have not. Alternatively, there could be variation across populations of the same species, as well as across species in the role of steroids in species recognition. Studies of other populations of Atlantic mollies have found evidence of conspecific mate preference in male mate choice [24,26], but we did not in the current study, further suggesting that the mechanisms of conspecific recognition may differ across populations as well as species. We do not, however, think it is possible that Amazon mollies are in an "evolutionary arms race" with their parental species because of their clonal form of reproduction. It is possible that Amazon mollies have inherited traits more similar to their maternal ancestor, the Atlantic molly. Gabor et al. [51] provide support for this hypothesis as the repeatability of the mating preferences of Amazon mollies are more similar to the preferences of female Atlantic mollies than to female sailfin mollies. In sum, these results suggest that Atlantic mollies may not discriminate against Amazon mollies as well as sailfin mollies do because they resemble their maternal parental species more than their paternal species, the sailfin mollies. Examination of additional populations of sympatric Atlantic mollies and sailfin mollies may help further clarify these differences.

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