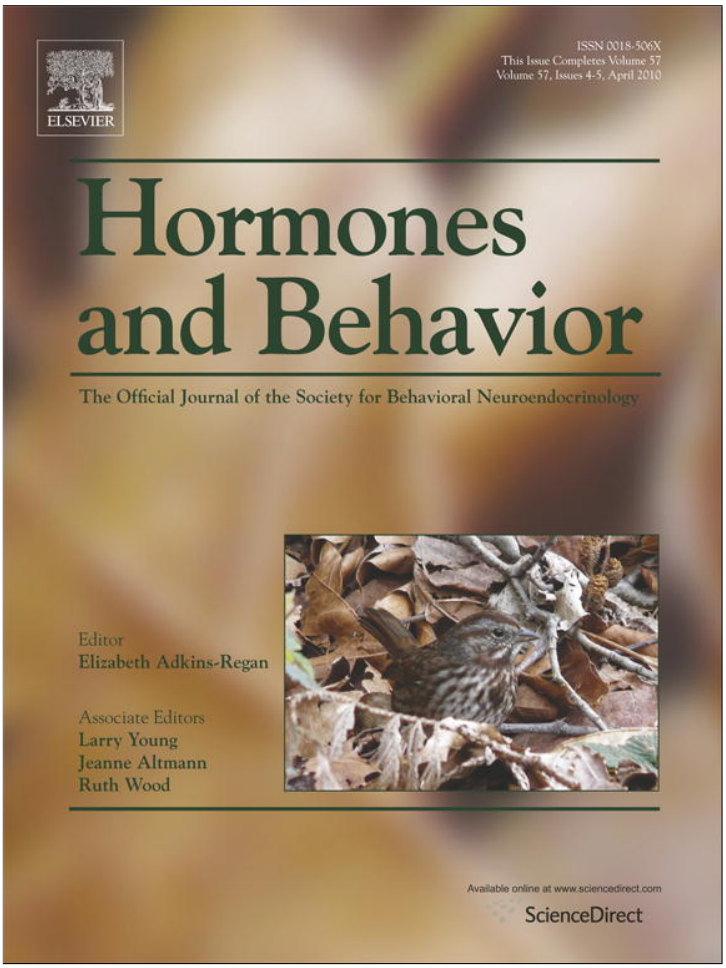

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A potential role of male and female androgen in species recognition in a unisexual–bisexual mating complex

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ABSTRACT

Hormones play a critical role in the regulation of vertebrate mating behavior, including receptivity, and several components of mate choice. However, less is known about the role of these chemical messengers in mediating behavior associated with premating reproductive isolation. The bisexual–unisexual mating complex of sailfin mollies, *Poecilia latipinna*, and Amazon mollies, *Poecilia formosa* (sexual parasites of sailfins) has been a model system for studying ultimate mechanisms of species recognition. However proximate mechanisms, such as variation in hormone levels, have not been examined. We paired male sailfin mollies with either female conspecifics or Amazon mollies and obtained water-borne hormone samples before and after mating for all fish. We measured 11-ketotestosterone, testosterone, and estradiol from the water samples. As expected from previous studies, males mated with conspecifics more frequently than with Amazon mollies. 11-Ketotestosterone production by males increased when they mated with female sailfin mollies who themselves also showed elevated production of 11-ketotestosterone. This increase in male and female 11-ketotestosterone levels was not seen when males mated with Amazon mollies. This unique endocrine interaction represents a potential proximate mechanism for species recognition by male sailfin mollies. We found no significant change in testosterone or estradiol under these conditions suggesting that a single hormone mediates bidirectional interactions between males and females during courtship.

Introduction

Studies of the mechanisms of reproductive isolation have revealed that ecological factors, such as different environments driving divergence in phenotypic traits (reviewed by Schluter, 2001), chemical signaling (reviewed by Smadja and Butlin, 2009) and behavioral factors, such as sexual selection via mate choice (reviewed by Panhuis et al., 2001) have played important roles in speciation. However, little is currently known about how the common neuroendocrine mechanisms that regulate mating behavior might participate in the process of reproductive isolation. Hormones have dramatic and well-established effects on mating behavior and mate choice, and thus could represent an important mechanism with regard to the proximate regulation of premating reproductive isolation.

Hormones are critical for reproductive function; they influence spermatogenesis and regulate reproductive and aggressive behavior. Hormone levels affect a variety of behaviors, including mate selection by both males and females in a range of taxa (birds: reviewed by Wingfield et al., 2001; McGlothlin et al., 2004; fish: reviewed by Hirschenhauser and Oliveira, 2006; Knapp and Neff, 2007; frogs: Leary et al., 2008; Lynch et al., 2005, 2006). For example, McGlothlin et al. (2004) found that female dark-eyed juncos, *Junco hyemalis*, treated with testosterone were less discriminating in their mate choice than were control females. Hormones are also responsive to social interactions, and thus behavior in one sex can influence hormone production in members of the opposite sex, as well as in members of the same sex. For example, in many vertebrates, male androgen levels increase in response to social challenges by other males (review by Hirschenhauser and Oliveira, 2006; birds: Wingfield et al., 1990; review by Goymann et al., 2007; fish: Remage-Healey and Bass, 2004; Earley et al., 2006; frogs: Burmeister and Wilczynski, 2000; lizards: Greenberg and Crews, 1990; Yang and Wilczynski, 2002). Male reproductive behavior can influence female hormone levels and female behavior (birds: Lehrman, 1964; frogs: Lynch and Wilczynski, 2008; rodents: Pfaff, 1980; salamanders: Propper and Moore, 1991). Similarly female presence and behaviors can influence male hormone levels (birds: Sorenson et al., 1997; Pinxten et al., 2003; Goymann et al., 2007; fish: Kobayashi et al., 1986; Hirschenhauser et al., 2004; rodents: Graham and Desjardins, 1980; Bronson and Desjardins, 1982). These studies establish bidirectional interactions wherein hormones regulate reproductive behavior and recent behavioral interactions rapidly regulate androgen levels. In several of these studies (Sorenson et al., 1997; Pinxten et al., 2003; Goymann et al., 2007), the dramatic effect of behavioral interactions on hormone

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levels is not observed when males interact with members of a different, but closely related species. Given the dynamic feedback between hormones and behavior and the species specificity of this feedback, the hormonal responses of two interacting individuals during mate choice may also provide a mechanism for species recognition, and therefore reproductive isolation.

Sailfin mollies, *Poecilia latipinna*, and Amazon mollies, *Poecilia formosa* are part of a well studied bisexual–unisexual species complex, however the underlying hormonal mechanisms for their mating behavior are unknown. Amazon mollies are a clonal, all female species of livebearing fish of hybrid origin. They reproduce via gynogenesis: Amazon mollies need the sperm of their parental species (sailfin mollies or Atlantic mollies, *Poecilia mexicana*) to trigger the development of their eggs, but genetic inheritance is entirely maternal (Hubbs and Hubbs, 1932). Thus, sailfin mollies are essentially sexually parasitized by the all-female Amazon mollies. Male sailfin mollies in sympatry with Amazon mollies show a stronger preference to mate with conspecifics than do male sailfin mollies from allopatric populations (Ryan et al., 1996; Gabor and Ryan, 2001) and prime more sperm for conspecifics relative to Amazon mollies (Aspbury and Gabor, 2004). Yet, mating mistakes still occur as Amazon mollies have persisted for about 100,000 years (Schartl et al., 1995; but see Dries, 2003).

Here we present a study that examines a potential proximate mechanism underlying species recognition/reproductive isolation in sailfin mollies by examining variation in hormones produced by both males and females. To date, no work has been done on the influence of hormone levels on mate choice or the influence of mate choice on hormone levels in mollies or other poeciliid fish. Additionally, in poeciliids there is considerable variation among males in the expression of mating behavior. For example, male sailfin mollies exposed to females in the same experimental treatments exhibit rates of mating attempts that range from five or fewer times to over 100 times in 10 min mating trials (Gabor and Aspbury, 2008). What has not yet been explored is the role, if any, of hormones in generating this variation in male mating intensity. One proximate factor that could affect the expression and intensity of male mating behavior, and hence the evolutionary persistence of Amazon mollies, is differences in the level of hormone production by male sailfin mollies when exposed to Amazon mollies compared to conspecifics. The three steroid hormones that are known to play significant roles in reproduction and mate choice are 11-ketotestosterone (KT), estradiol (E), and testosterone (T). Prior studies in teleost fish, based on both blood plasma (Borg, 1994) and water-borne hormone concentrations (Hirschenhauser et al., 2004; Goncalves et al., 2007; Sebire et al., 2007), found that KT is the primary androgen regulating male mating behavior and increased sexual displays (Kindler et al., 1991). Toft and Guillette (2005) found that male *Gambusia affinis* (another poeciliid) with lower whole body T concentrations showed decreased sexual behavior. Estrogen is also known to affect female reproductive behavior (Liley, 1972) and male courtship behavior in *Poecilia reticulata* (Bayley et al., 1999). Given the role of these three hormones in regulating male mating behavior and the fact that male sailfin mollies still mate with Amazon mollies, one prediction is that there will be a direct relationship, between the species of female being courted, male hormone production, and subsequently mating behavior. If hormones play a role in species recognition, then hormone levels should be higher when male sailfin mollies mate with conspecifics as compared to with Amazon mollies. Also, males that exhibit lower latency to mate and higher courtship intensity might produce more hormones when mating with conspecifics but not when mating with Amazons mollies.

Materials and methods

We collected sailfin and Amazon mollies from a sympatric population in Mexico (25.11N, 97.56W) in July 2008 and returned them to the laboratory. We maintained the fish on a 14-h light/10-h dark cycle using UV lighting to simulate daylight, and fed Ocean Star International Inc. Spirulina Flake mixed with Ocean Star International Inc. Freshwater Flake food twice daily and supplemented daily with live brine shrimp. Males were individually housed for 20 h prior to testing (in 19 l aquaria) and females were housed in single-sex groups for at least 30 days in 38 l aquaria to control for receptivity. Testing was performed in September–October 2008. We tested each male sailfin molly ($n = 19$) in two trials with: (1) a female conspecific and (2) an Amazon molly. 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 we tested males with the other species of female. Trials were performed from 0900 to 1300 h each day to control for circadian variation in hormone levels (Lorenzi et al., 2008) and each male was tested at the exact 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 1 h to collect a premating hormone sample. Each pair of fish (a single male and single female conspecific or Amazon molly) was placed together in a 19 l aquarium and we recorded the number of mating attempts (gonopodial thrusts) directed at the female for 25 min (to potentially provide enough time for hormone levels to increase in response to the trial). After each mating trial, we put each fish in separate sterile 250 ml beakers with 80 ml fresh tank water for 1 h to collect a postmating hormone sample. Thus each trial lasted 2 h and 25 min. Texas State University IACUC approved the collection and research procedures.

Hormone assays

Water-borne hormone samples (Scott and Ellis, 2007) were maintained at −20 °C until the hormone assays were performed (Ellis et al., 2004). Hormones were extracted from the water samples using C18 solid phase extraction (SPE) columns placed on a vacuum manifold. Hormones were eluted into vials from the columns using methyl alcohol. The eluted solvent was evaporated and samples were resuspended in the assay buffer. We used commercially available enzyme-immunoassay (EIA) kits to assay KT, E, and T (Cayman Chemicals). All samples were run in duplicate on 96 well plates and read by a fluorescent plate reader (BioTek Powerwave XS).

To validate the EIA kits for water-borne hormones from sailfin mollies and Amazon mollies, we obtained water samples from 10 non-experimental sailfin mollies and 8 non-experimental Amazon 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 3.5 ml for sailfin mollies and 2.8 ml for Amazon mollies. The pools were diluted to 1:2 for the serial dilutions and the quantitative recovery for each species and all hormones (except 1:4 for sailfin mollies in the KT quantitative recovery).

We assessed parallelism of the serial dilution curve, using the pooled (1:2) control for both species. The serial dilutions were run in duplicate. The log–logit transformed dilution curve was constructed using average % 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 all hormones (comparison of slopes, ANCOVA: KT: sailfin mollies, $F_{1,12} = 0.138$, $p = 0.717$; Amazon mollies, $F_{1,12} = 0.019$, $p = 0.891$; E: sailfin mollies, $F_{1,12} = 0.055$, $p = 0.818$; Amazon mollies, $F_{1,12} = 6.89$, $p = 0.998$; T: sailfin mollies, $F_{1,12} = 0.006$, $p = 0.939$; Amazon mollies, $F_{1,12} = 0.004$, $p = 0.953$).

To determine the quantitative recovery of the water-extracted hormones, we spiked the pooled control samples for sailfin mollies and Amazon 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 the standards and the pooled control sample. Minimum observed

recovery for sailfin mollies were 100% (KT), 71.7% (E), 96.7% (T) and for Amazon mollies were 93.6% (KT), 67.6% (E), 74.3% (T). The slopes of the observed vs. expected curves for sailfin mollies were 1.18 (KT), 1.54 (E), 1.43 (T), and for Amazon mollies were 1.13 (KT), 1.64 (E), 1.48 (T), indicating a linear relationship between observed and expected for all hormones for both species.

Statistical analyses

The 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). We used nonparametric analyses when we examined the number of mating attempts (thrusts) and time to first thrust 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 v7.1 (SAS Institute). We did not perform an experimental wide reduction of alpha because each of our statistical analyses tested a different hypothesis.

Results

Methodological considerations

11-Ketotestosterone

Male size was not significantly correlated with their premating KT level (Linear regression: $r^2 = 0.01$; $n = 19$; $p = 0.70$). Males did not significantly differ in their premating KT level across both days (for both species) (Paired *t*-test: $n = 19$, $t = 0.126$, $p = 0.90$; Table 1). Female sailfin and Amazon mollies did not significantly differ in their premating KT level (Unpaired *t*-test: $df = 35$; $t = -0.31$, $p = 0.76$; Table 1). Males produced significantly more premating KT than female sailfin mollies (Unpaired *t*-test: $df = 36$; $t = 5.68$; $p < 0.0001$; Table 1) and Amazon mollies ($df = 37$; $t = 5.66$; $p < 0.0001$; Table 1). Male premating KT levels were not correlated with the number of mating attempts (Kendall's τ; $n = 38$, $\tau = 0.003$, $p = 0.979$). Male and female (of each species) premating KT levels did not significantly affect whether they mated or not and time to first thrust (we do not present this data). To examine KT, E and T production during the trials we focused on KT, E and T responsiveness (postmating sample/premating sample) as an indication of the relative change of hormone production (sensu Wingfield et al., 1990; Hirschenhauser et al., 2004) in response to the mating trial. The order in which males encountered each species of female did not significantly affect male KT responsiveness (ANOVA: $F_{1,34} = 1.39$, $p = 0.26$).

Estradiol

Male size was not significantly correlated with their premating E level (linear regression: $r^2 = 0.05$; $n = 19$; $p = 0.35$). Males did not significantly differ in their premating E level across both days (for both species) (Paired *t*-test: $n = 18$, $t = -0.266$, $p = 0.79$; Table 1). Female sailfin and Amazon mollies did not significantly differ in their premating E level (Unpaired *t*-test: $df = 35$; $t = -1.79$, $p = 0.08$; Table 1) however the mean level of E for Amazon mollies was double that of sailfin molly females. Males did not produce significantly different levels of premating E than female sailfin mollies (Unpaired *t*-test: $df = 34$; $t = 1.56$; $p = 0.133$; Table 1) and Amazon mollies ($df = 37$; $t = -0.18$; $p = 0.86$; Table 1). Male premating E levels were positively correlated with the number of mating attempts (Kendall's τ; $n = 38$, $\tau = 0.327$, $p = 0.007$). Male and female (of each species) premating E levels did not significantly affect whether they mated or not and time to first thrust (we do not present this data). The order in which males encountered each species of female did not significantly affect male E responsiveness (ANOVA: $F_{1,33} = 1.24$, $p = 0.31$).

Testosterone

Male size was not significantly correlated with their premating T level (linear regression: $r^2 = 0.05$; $n = 14$; $p = 0.43$). Males did not significantly differ in their premating T level across both days (for both species) (Paired *t*-test: $n = 12$, $t = 1.31$, $p = 0.22$; Table 1). Female sailfin and Amazon mollies did not significantly differ in their premating T level (Unpaired *t*-test: $df = 28$; $t = -0.36$, $p = 0.72$; Table 1). Males did not produce significantly different levels of premating T than female sailfin mollies (Unpaired *t*-test: $df = 26$; $t = -0.53$; $p = 0.601$; Table 1) and Amazon mollies ($df = 28$; $t = -1.01$; $p = 0.320$; Table 1). Male premating T levels were not correlated with the number of mating attempts (Kendall's τ; $n = 27$, $\tau = 0.120$, $p = 0.411$). Male and female (of each species) premating T levels did not significantly affect whether they mated or not and time to first thrust (we do not present this data). The order in which males encountered each species of female did not significantly affect male T responsiveness (ANOVA: $F_{1,22} = 1.35$, $p = 0.28$).

Main effects

Male sailfin mollies exhibited significantly more mating attempts towards conspecific females than towards Amazon mollies (Wilcoxon signed ranks: $n = 19$, $t = -31$, $p = 0.01$; Fig. 1). There was no significant difference in the probability that a male would attempt to mate if paired with a conspecific vs. a heterospecific female (Fishers exact test: $p = 0.74$).

11-Ketotestosterone

Males that attempted to mate with female sailfin mollies had significantly higher KT responsiveness relative to those that had not attempted to mate with sailfin mollies (Unpaired *t*-test: $df = 17$;

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

Individual	*n*	Premating hormone	Day 1 ± SE (pg/sample)	Day 2 ± SE (pg/sample)
Male sailfin molly	19	KT	152.05 ± 54.14	133.67 ± 23.63
	19	E	882.56 ± 243.27	948.93 ± 356.61
	14	T	1045.42 ± 163.67	1253.30 ± 211.66
Female sailfin molly	18	KT	36.16 ± 13.21	
	18	E	480.59 ± 84.02	
	14	T	1198.14 ± 237.13	
Amazon molly	19	KT	35.39 ± 9.36	
	19	E	955.70 ± 329.16	
	16	T	1300.62 ± 191.66	

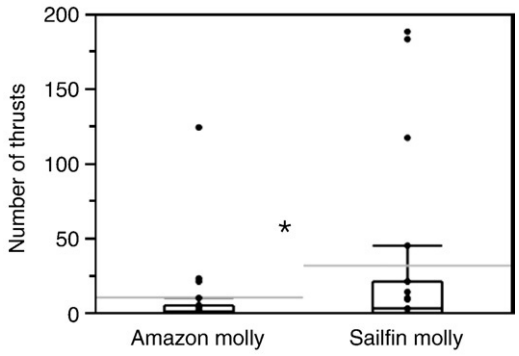

Fig. 1. Box plots representing the number of thrusts (mating attempts) by male sailfin mollies ($n = 19$) directed at Amazon mollies and sailfin mollies. The upper and lower horizontal lines of the boxes represent the first and third quartiles, the middle horizontal lines represent the medians, and the bars indicate the range. The grey horizontal lines indicate the mean, * indicates $p = 0.01$.

$t = 2.07$, $p = 0.05$; Fig. 2A). Moreover, the KT responsiveness of female sailfin mollies were significantly greater after males mated with them than when males had not mated with them (Unpaired t-test: $df = 15$; $t = 2.98$, $p = 0.01$; Fig. 2B). In contrast, males did not significantly differ in their KT responsiveness as a result of just being paired with female sailfin vs. Amazon mollies (Paired t-test: $n = 19$, $t = -0.49$, $p = 0.63$; Fig. 2C), and males that attempted to mate with Amazon mollies did not have significantly different KT responsiveness to those that had not attempted to mate (Unpaired t-test: $df = 17$; $t = -0.11$, $p = 0.91$; Fig. 2A). Additionally, there was no significant difference in the KT responsiveness of Amazon mollies whether males attempted to mate with them or not (Unpaired t-test: $df = 16$; $t = 0.42$, $p = 0.68$; Fig. 2B).

There was no relationship between male KT responsiveness and the time to first thrust with Amazon mollies (Kendall's τ: $n = 19$; $\tau = 0.00$, $p = 1.0$; Fig. 3A). The shorter the time to first thrust with female sailfin mollies, however, the greater the male KT responsiveness (Kendall's τ: $n = 19$; $\tau = -0.40$, $p = 0.02$; Fig. 3A). There was no correlation between male KT responsiveness and number of thrusts directed at Amazon mollies (Kendall's τ: $n = 19$; $\tau = 0.026$, $p = 0.88$; Fig. 3B), but male KT responsiveness was positively correlated with the number of thrusts directed at female sailfin mollies (Kendall's τ: $n = 19$; $\tau = 0.451$, $p = 0.01$; Fig. 3B). There was no correlation between Amazon or female sailfin molly KT responsiveness and the time to first thrust (Kendall's τ: Amazon mollies, $n = 18$, $\tau = -0.049$, $p = 0.786$; female sailfin mollies, $n = 17$, $\tau = -0.312$, $p = 0.095$). There was also no correlation between Amazon molly KT responsiveness and the number of thrusts directed at them by males (Kendall's τ: $n = 18$, $\tau = 0.234$, $p = 0.198$: Fig. 3C). In contrast, there was a positive correlation between female sailfin molly KT responsiveness and the number of thrusts directed at them (Kendall's τ: $n = 17$, $\tau = 0.418$, $p = 0.026$; Fig. 3C).

Estradiol

Males that attempted to mate with Amazon and sailfin mollies did not have significantly different E responsiveness to those that had not attempted to mate (Unpaired t-test: Amazon mollies, $df = 16$; $t = 0.50$, $p = 0.62$; sailfin mollies $df = 16$; $t = -2.05$, $p = 0.07$; Fig. 2D). There was no significant difference in the E responsiveness of Amazon mollies or female sailfin mollies and whether males attempted to mate with them or not (Unpaired t-test: Amazon mollies, $df = 16$; $t = 0.19$, $p = 0.85$; sailfin mollies, $df = 15$; $t = -1.69$, $p = 0.13$; Fig. 2E). Males

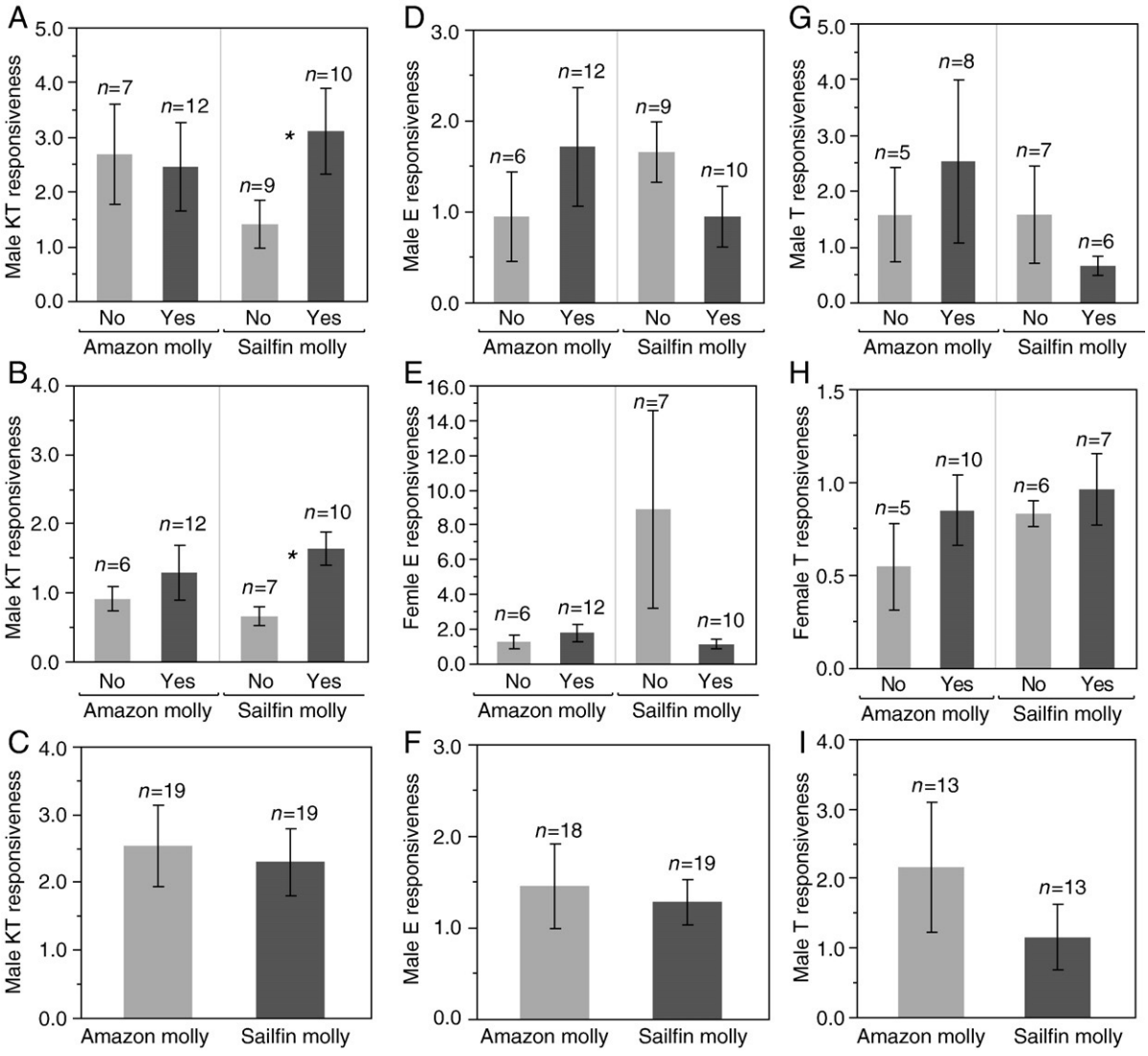

Fig. 2. Mean ± SE responsiveness (postmating/premating samples) of: 11-ketotestosterone (KT), estradiol (E), and testosterone (T) for male sailfin mollies that attempted to mate (Yes) or not (No) when paired with Amazon mollies or female sailfin mollies (panels A, D, G); KT, E and T for Amazon mollies and female sailfin mollies when males attempted to mate or not (panels B, E, H); and KT, E and T for male sailfin mollies when tested with Amazon mollies or female sailfin mollies, irrespective of whether males attempted to mate or not (panels C, F, I). * indicates $p < 0.05$.

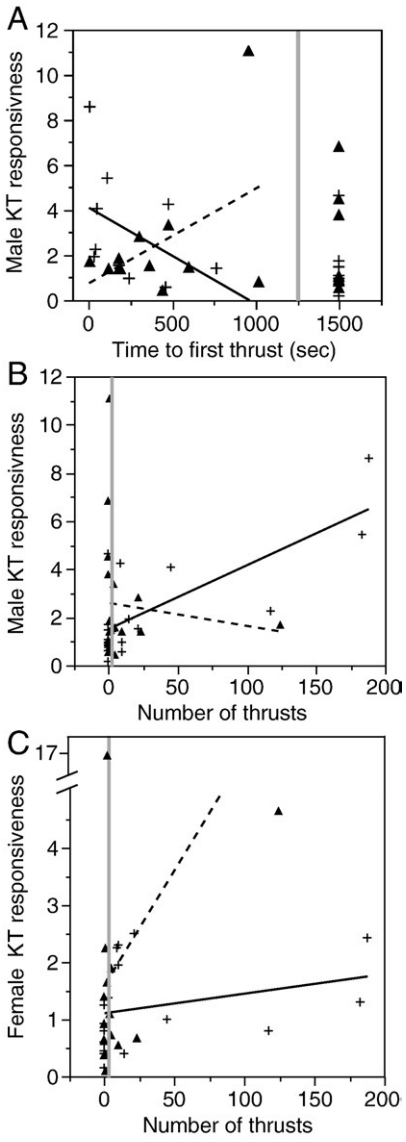

Fig. 3. The correlation between 11-ketotestosterone (KT) responsiveness (postmating/premating samples) and: (A) the time to first thrust (in seconds) for male sailfin mollies when tested with female sailfin mollies (black line and plus symbol) and Amazon mollies (dashed line and triangle symbol; 1500 s indicates no thrusts), (B) the number of thrusts (mating attempts) by male sailfin mollies when they were paired with female sailfin mollies or Amazon mollies, and (C) the number of thrusts by male sailfin mollies for female sailfin and Amazon mollies. The data to the right (A) and left (B, C) of the gray line indicates data for males that did not thrust.

did not significantly differ in their E responsiveness when they were paired with female sailfin or Amazon mollies (Paired t-test: $n=17$, $t=0.57$, $p=0.57$; Fig. 2F).

There was no relationship between male E responsiveness and the time to first thrust with Amazon mollies or female sailfin mollies (Kendall's τ: Amazon molly, $n=18$; $\tau=0.042$, $p=0.82$; sailfin molly $n=17$; $\tau=0.082$, $p=0.65$). There was no correlation between male E responsiveness and number of thrusts directed at Amazon mollies or female sailfin mollies (Kendall's τ: Amazon mollies, $n=18$; $\tau=0.099$, $p=0.59$; sailfin mollies, $n=17$; $\tau=-0.0901$, $p=0.63$). There was no correlation between Amazon or female sailfin molly E responsiveness and the time to first thrust (Kendall's τ: Amazon molly, $n=18$, $\tau=0.076$, $p=0.67$; sailfin molly, $n=17$, $\tau=0.344$, $p=0.066$). There was also no correlation between Amazon and female sailfin molly E responsiveness and the number of thrusts directed at them by males (Kendall's τ: Amazon mollies $n=18$, $\tau=-0.092$, $p=0.61$; sailfin mollies, $n=17$, $\tau=-0.257$, $p=0.17$).

Testosterone

Males that attempted to mate with Amazon and female sailfin mollies did not have significantly different T responsiveness to those that had not attempted to mate (Unpaired t-test: Amazon mollies, $df=11$; $t=-0.05$, $p=0.96$; sailfin mollies $df=11$; $t=-0.85$, $p=0.41$; Fig. 2G). There was no significant difference in the T responsiveness of Amazon or female sailfin mollies and whether males attempted to mate with them or not (Unpaired t-test: Amazon molly, $df=11$; $t=1.14$, $p=0.29$; sailfin molly, $df=11$; $t=0.31$, $p=0.77$; Fig. 2H). Males did not significantly differ in their T responsiveness when they were paired with female sailfin or Amazon mollies (Paired t-test: $n=12$, $t=-1.54$, $p=0.15$; Fig. 2I).

There was no relationship between male T responsiveness and the time to first thrust with Amazon or female sailfin mollies (Kendall's τ: Amazon molly, $n=13$; $\tau=-0.097$, $p=0.66$; sailfin molly $n=13$; $\tau=0.11$, $p=0.64$). There was no correlation between male T responsiveness and number of thrusts directed at Amazon mollies or female sailfin mollies (Kendall's τ: Amazon molly, $n=13$; $\tau=0.17$, $p=0.45$; sailfin mollies, $n=13$; $\tau=-0.075$, $p=0.74$). There was no correlation between Amazon or female sailfin molly T responsiveness and the time to first thrust (Kendall's τ: Amazon molly, $n=15$, $\tau=-0.07$, $p=0.72$; sailfin molly, $n=13$, $\tau=-0.202$, $p=0.36$). There was also no correlation between Amazon molly and female sailfin molly T responsiveness and the number of thrusts directed at them by males (Kendall's τ: Amazon mollies $n=15$, $\tau=1.54$, $p=0.45$; sailfin mollies, $n=13$, $\tau=0.23$, $p=0.30$).

Discussion

Consistent with previous studies, we found that male sailfin mollies preferred to mate with conspecific females relative to Amazons mollies (Ryan et al., 1996; Gabor and Ryan, 2001; Heubel and Schlupp, 2008; Robinson et al., 2008). This species difference in male mating effort was associated with several endocrine events. While males, as expected, had higher premating KT levels than did females, 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. Male and female premating KT, T and E levels did not differ whether males attempted to mate or not with female sailfin or Amazon mollies. However, we found that both male and female sailfin mollies showed higher KT production (responsiveness) when males attempted to mate with female sailfin mollies as compared to when males did not attempt to mate with female sailfin mollies. We found no such relationship when males were paired with Amazon mollies. Note, however, that males mated less with Amazon mollies and this may have affected their KT production. Male sailfin mollies also showed a positive relationship between KT production and time to first thrust and mating attempts only when paired with female sailfin mollies. Additionally, female sailfin molly KT production increased with increasing male mating attempts but there was no relationship between Amazon molly KT production and male mating attempts. In contrast, E and T production did not significantly change under these conditions. Taken together these results indicate that the degree to which female sailfin mollies respond to male mating attempts with increased KT affects both KT production and mating attempts in males. This was not the case with Amazon mollies. Thus, KT production during courtship in both males and females appears to be related to male species discrimination in this complex unisexual–bisexual mating system but E and T production do not.

Mate choice and species recognition are a continuum in animal communication such that the same forces that make the signaler a more attractive mate might also influence species recognition (Ptacek, 2005; Phelps et al., 2006). Here, we found that both male and female sailfin mollies showed an increase in KT production as a result of mating, whereas Amazon mollies did not. It is possible that this KT response by sailfin mollies is an ancestral component of the mating

system and the outcome is that the KT response also provides a mechanism by which males can differentiate conspecifics from heterospecifics. Comparisons of our results to those from animals from allopatric populations would be required to test this hypothesis.

Both sexes of the sailfin molly show an increase in KT production in response to mating, while the Amazon mollies do not, which indicates the loss or lack of hormone response in the unisexual species, rather than any specific change in male preference/discrimination (Sorensen and Scott, 1994). The apparent loss of the female response in Amazon mollies appears to be driving male species-specific androgenic and behavioral responses to mating. One alternative hypothesis for the lack of endocrine responsiveness in Amazon mollies is that their other parental species, *P. mexicana* also lack this endocrine response and Amazon mollies inherited it from *P. mexicana*. We are currently examining this hypothesis.

We found a positive correlation between premating E and male mating attempts, which is consistent with a large literature on E and male mating behavior in tetrapods (reviewed in Nelson, 2005). However, it is not clear that this relationship holds for teleost fishes, as Bayley et al. (1999) found that increased E caused decreased mating behavior and sperm production in guppies, while increasing the number of sperm cells in ejaculates (Toft and Baatrup, 2001). We do not think this increase in mating attempts with increase in E is providing the mechanism of species recognition because once males were paired with females their E production did not significantly differ whether they were paired with female sailfin or Amazon mollies.

It is not clear from the present data whether KT is acting within animals on the regulation of their behavior, or as a water-borne signal that is directly synchronizing behavior between males and females. The idea that sex hormones are important chemical cues that are not species specific in fishes (Sorensen and Scott, 1994; Stacey, 2003), is consistent with both Aspbury et al. (2010), who found that male sailfin mollies could not discriminate between female sailfin and Amazon mollies based on chemical cues alone, and our current finding that female sailfin and Amazon mollies do not differ in their premating KT, E and T levels. Our results suggest that, with regard to KT, males need to begin interacting and perhaps mating with females, before female hormone levels lead to the ability for males to discriminate between species. The finding that males increase KT only when mating with sailfin mollies also suggests a mechanism to explain how males rapidly produce sperm (within an hour) while they are mating with female sailfin mollies, but not when mating with Amazon mollies (Robinson et al., 2008). KT is well recognized for its role in stimulating spermatogenesis in fish and specifically poeciliids (Schreibman et al., 1986). The observed increases in KT that result from interacting with females (this study) should be sufficient to drive the observed increase in sperm production observed by Robinson et al. (2008).

Our results suggest a bidirectional interaction between KT and social behavior (see above), and this appears to be the first demonstration of a single hormone being involved in bidirectional interactions between males and females during courtship. Thus far, most studies of the effects of social interactions on hormones have only examined unidirectional effects (review by Hirschenhauser and Oliveira, 2006; birds: Wingfield et al., 1990; review by Goymann et al., 2007; fish: Hirschenhauser et al., 2004; Remage-Healey and Bass, 2004; Earley et al., 2006; frogs: Burmeister and Wilczynski, 2000; lizards: Greenberg and Crews, 1990; Yang and Wilczynski, 2002; rodents: Graham and Desjardins, 1980; Bronson and Desjardins, 1982). In fact, Goymann et al. (2007) indicated that for birds, most studies of the "Challenge Hypothesis" did not examine hormone levels as a direct outcome of male–male or male–female social interactions but upon further studies, found that this was an important variable. In fish, Hirschenhauser et al. (2004) found that water-borne KT, but not T increased in males upon exposure to ovulating females and to males in some species. Here we examined both male and female KT production and found that both male and female sailfin mollies showed an increase in KT production when mating together, but males did not show such a response when mating with Amazon mollies, nor did the Amazon mollies show a clear response in KT production to male mating attempts. Because we could only examine the change in KT levels after an interaction, we do not know if female sailfin molly KT production increased before male KT production or if it occurred simultaneously.

One way to explore the potential timing of KT production during the mating interaction is to look at the time to first thrust and number of thrusts in relation to the relative increase in KT production (Figs. 3A–C). The correlation, in sailfin mollies, between the relative increase in male KT production and the time to first thrust and the number of thrusts suggests that some males recognize conspecific females sooner than Amazon mollies and then once they are mating with conspecifics, male KT levels increase. Whereas when males are paired with Amazon mollies, male production of KT does not increase once they first thrust nor while thrusting. On the other hand, we found a positive correlation between female sailfin molly KT production and the number of male mating attempts. Thus, our data are more consistent with the hypothesis that the observed elevation in male KT during courtship is a by-product of his increased mating attempts towards females, and an increase in male androgen levels following interactions with females. Such a result has been shown in a variety of vertebrate species (birds: Sorenson et al., 1997; Pinxten et al., 2003; Goymann et al., 2007; fish: Kobayashi et al., 1986; Hirschenhauser et al., 2004; rodents: Graham and Desjardins, 1980; Bronson and Desjardins, 1982).

The persistence of unisexual lineages is an evolutionary paradox. Amazon mollies share disadvantages of both sexual reproduction (the costs of finding a mate and costs of mating) and unisexual reproduction (accumulation of deleterious mutations that cannot be purged by recombination (Muller, 1964)). Yet Amazon mollies continue to persist. Prior studies have found that male sailfin mollies from populations sympatric with Amazon mollies show stronger preference to mate with conspecific females (Gabor and Ryan, 2001) and produce more sperm when with conspecific females over Amazon mollies (Aspbury and Gabor, 2004; Robinson et al., 2008). However, males clearly also make mating mistakes, such as when Amazon mollies are larger than conspecific females (Gumm and Gabor, 2005). Here we have found another mechanism by which Amazon mollies persist, male sailfin mollies did not significantly differ in their overall KT, E and T production whether they were tested with sailfin or Amazon mollies (Figs. 2C, F, I). However, when males mated with female sailfin mollies both sexes showed KT response that was not found for Amazon mollies (Figs. 3A–C). These results suggest that males need to mate with a female before initiating the female endocrine response that mediates mate choice and subsequent species recognition in this unisexual–bisexual complex. This may also explain how males still make mating mistakes. Occasional mating with Amazon mollies could occur because males require input (a lack of KT production by Amazon mollies) following their first mating attempt before they decrease mating with Amazon mollies. In the absence of this experience (for example in naïve fish), sailfin males may be more likely to make mating mistakes.

In conclusion, we have found that female KT production, but not E and T, can provide a mechanism for species recognition by male sailfin mollies. Our data suggest that male sailfin mollies prefer to mate with conspecifics because male KT production and subsequent mating attempts are responsive to female KT production, but only conspecific sailfin mollies increase KT production in response to male mating attempts. Instead of high levels of androgens driving males to mismate, an endocrine interaction by which males recognize conspecific females drives divergence in the frequency of mating, and thus may serve as a mechanism for premating reproductive

isolation. This bidirectional neuroendocrine response may be another important factor to consider in future studies of the mechanisms of reproductive isolation for other closely related species in sympatry.

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