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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