Behavioural Processes xxx (xxxx) xxx-xxx

Contents lists available at ScienceDirect

Behavioural Processes

journal homepage: http://ees.elsevier.com

Smaller rival males do not affect male mate choice or cortisol but do affect 11-ketotestosterone in a unisexual-bisexual mating complex of fish

Diana Kim[a], Andrea S. Aspbury[a], J. Jaime Zúñiga-Vega[b], Caitlin R. Gabor[a,*]

[a] Department of Biology, Texas State University, 601 University Drive, San Marcos, TX 78666, USA
[b] Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México, Cuidad Universitaria 04510, Distrito Federal, Mexico

ARTICLE INFO

Keywords
Androgens
Glucocorticoid
Gynogenetic
Mate choice
Poeciliidae

ABSTRACT

Male mate discrimination may be affected by the social environment (presence or absence of rival males or mates), which can also affect stress and sex hormones (e.g., cortisol and 11-ketotestosterone (11-KT)). The Amazon molly, *Poecilia formosa*, is an all-female fish species dependent on sperm from mating with male *P. latipinna*. We investigated male mate choice in *P. latipinna* between conspecific females and *P. formosa* with a rival male present and no rival male present. We measured cortisol and 11-KT release rates from all fish. The presence of a rival male had no effect on male mate choice for conspecific females nor overall mating effort. Male 11-KT decreased on the second day after exposure to a rival male on the first day. Focal male 11-KT is positively correlated with the size of the rival male. Both conspecific and heterospecific females released more 11-KT when in the rival male treatment than when not. Neither male nor female cortisol was affected by the presence or absence of the rival male. We did not find an effect of rival males on male mate choice in contrast to our prediction. Instead, our findings may indicate a hormonal response to social competition.

1. Introduction

The social environment can strongly influence individual mating decisions and preferences even when the mating choice seems maladaptive (West-Eberhard, 1983). For example, when mate-choice copying occurs, individuals may increase preference for conspecific mates that are preferred by other individuals, including heterospecifics (Auld and Godin, 2015; Schlupp et al., 1994). In addition, audience effects occur with the presence of a mating rival and can change mating preferences for conspecific or heterospecific partners (Auld and Godin, 2015; Mautz and Jennions, 2011; Plath et al., 2008a,b). For example, in *Poecilia mexicana*, males reduce overall mating activity, decrease preference for conspecific females, and initiate mating with heterospecific females, when in the presence of rival males (Plath et al., 2008a,b). Audience effects are mediated by various physiological processes (Aspbury, 2007; Cummings et al., 2008; Desjardins et al., 2015), but little is known about the hormonal basis of changes in mating preferences. Understanding the hormonal mechanisms that mediate these mating and social behaviors can help us elucidate how the social environment affects mating behaviors. Social environments of animals often include competitive interactions which can mediate changes in concentrations of androgenic and glucocorticoid hormones (reviewed by Briffa and Sneddon, 2007; Oliveira, 2004; Schreck, 2010; Teles and Oliveira, 2016).

In teleosts, one of the primary androgens, 11-ketotestosterone (11-KT), regulates male mating behavior (Borg, 1994), male response to social challenges (Clement et al., 2005; Hirschenhauser et al., 2004), and may mediate species recognition in male mate choice (Gabor and Grober, 2010). Social dominance, male ornamentation or coloration may also correlate with higher 11-KT levels in fish (Butts et al., 2012; Cardwell and Liley, 1991; Oliveira et al., 2008). Sex steroid hormone receptors are found in key brain regions known to modulate social behaviors in teleost fish and across vertebrates (Munchrath and Hofmann, 2010) indicating a potentially strong role of androgens in the effects of social competition on mate choice. Glucocorticoids, such as cortisol, are involved in the stress response and have more complex effects on reproduction (Milla et al., 2009). Increases in cortisol decreases selectivity in mate choice, reduce sexual receptivity, and suppress sexual behavior of subordinates (Davis and Leary, 2015; Vitousek and Romero, 2013). However, small increases in cortisol may also allow individuals to mobilize energy stores for metabolically demanding aspects of reproductive behaviors, such as courtship displays or challenges by other males (Clement et al., 2005; Teles and Oliveira, 2016).

* Corresponding author.
Email address: gabor@txstate.edu (C.R. Gabor)

https://doi.org/10.1016/j.beproc.2019.103916
Received 27 Febraury 2019; Received in revised form 18 July 2019; Accepted 22 July 2019
Available online xxx
0376-6357/

A suitable system for investigating hormonal modulation of social interactions and species recognition is a unisexual-bisexual complex of fish, where females of a unisexual species rely on matings with closely related males of a bisexual species. The Amazon molly, *Poecilia formosa*, is a gynogenetic livebearing species of fish that most likely arose from a hybrid crossing between male *P. latipinna* and female *P. mexicana* (Alberici da Barbiano et al., 2013; Avise, 2008; Hubbs and Hubbs, 1932; Warren et al., 2018). Gynogens are all-female lineages that require sperm from males of closely related species to initiate embryogenesis, but inheritance is strictly maternal. Evolutionary persistence of gynogens requires matings by males of the bisexual species. Both male *P. latipinna* and *P. mexicana* prefer to mate with conspecific females over female *P. formosa*, but this preference is stronger in male *P. latipinna* than in male *P. mexicana* (Gabor et al., 2012; Gabor and Ryan, 2001; Ryan et al., 1996). Mating systems differ between these two closely-related species: male *P. latipinna* exhibit alternative mating tactics, whereas male *P. mexicana* show a dominance hierarchy (Farr et al., 1989; Ptacek, 1998). The alternative mating tactics employed by male *P. latipinna* include courting and coercive males. However, male *P. latipinna* show extreme continuous variation in a suite of morphological and behavioral traits (Snelson, 1985). At one end of the variation, large males typically exhibit striking coloration, an exaggerated sail-like dorsal fin, and perform courtship behavior (Ptacek and Travis, 1996; Travis and Woodward, 1989). There is a genetic basis to these size-dependent tactics and male size does not change once males reach sexual maturity. Female *P. latipinna* prefer to mate with large males (MacLaren et al., 2004; Ptacek, 1998). Conversely, smaller males do not have exaggerated secondary sexual characteristics and are more likely to secure matings via coercive (e.g., forced copulation) behavior. However, intermediate-sized males exhibit a greater degree of plasticity in behavior depending on the relative size of males within the social environment (Fraser et al., 2014; Travis and Woodward, 1989).

The presence of a rival male influences male reproductive behavior in *P. mexicana* and both reproductive behavior and physiology in *P. latipinna*. In *P. mexicana*, presence of a rival conspecific male significantly decreases a male's initial mate preference, but males retain their initial choice when there is no rival male present (Plath et al., 2008a,b). Male *P. latipinna* prime more sperm prior to mating and expend more sperm when mating with conspecific females in the presence of male competitors, suggesting that males respond physiologically to sperm competition risk (Aspbury, 2007). Furthermore, Gabor and Grober (2010) measured male and female *P. latipinna* 11-KT-response (post-mating/pre-mating hormone release rates) and found that both sexes show an increase in 11-KT-response when they mate with each other but this response is absent when male *P. latipinna* mate with the unisexual *P. formosa*. Populations of *P. latipinna* form loose social aggregations called shoals, which provide ample opportunities for audience effects, mate-choice copying, and other social behaviors (Schlupp and Ryan, 1996).

Sperm competition risk theory and empirical findings (e.g., Aspbury, 2007), as well as audience effects (Plath et al., 2008a,b), suggest that the presence of a rival male can affect male mating behavior and physiology. Here we test the hypotheses that the presence of a rival male affects: (1) male mating effort and male conspecific mate choice, and (2) androgen (11-KT) and glucocorticoid (cortisol) responses of male and female *P. latipinna*. We predict that, in the presence of a rival male, male *P. latipinna* will show a higher overall mating effort and increase mating attempts with heterospecific females. Additionally, we predict that the presence of a rival will increase cortisol production of male *P. latipinna*, but not females, as a function of the social challenge. Finally, we predict that, in the presence of a rival male, male *P. latipinna* will have more 11-KT than males not in the presence of a rival male. Any increases in 11-KT of males with rivals may also lead to increases in 11-KT production of conspecific females that are paired with males in the presence of rivals as was shown in a previous study (Gabor and Grober, 2010).

2. Materials and methods

2.1. Animal collection and maintenance

We collected *P. latipinna* and *P. formosa* from a sympatric population in Northern Tamaulipas, Mexico (25.11 °N, 97.56 °W) in September 2012 and brought them back to laboratory facilities at Texas State University, San Marcos, TX. We quarantined fish for 90 days and maintained fish in 37.8 L aquaria (54 × 29 x 33 cm) at a constant temperature (25 °C) on a 14:10 h light-dark cycle with UV fluorescent lighting. We fed fish twice daily with fish food (Purina AquaMax 200) and supplemented with live brine shrimp nauplii. Prior to testing, we isolated females of both species from males for a minimum of 30 days to standardize levels of receptivity in females. We isolated males for 7 days prior to testing. We performed behavioral experiments from 0700 to 1500 h, June - August 2013. We only used mature females (≥ 32 mm in standard length; SL) across all trials (Robinson et al., 2011). All research with animals was conducted with approval from Texas State University Institutional Animal Care and Use Committee (IACUC) under protocol #0815_0319_19.

2.2. Experimental design

We tested male mate choice for conspecific or heterospecific females in two treatments using a repeated measures design: with a smaller rival and without a rival male. We tested males in both treatments across two days of testing and randomized the order that focal males received each treatment. We divided a 37.8 L test tank into three separate, unequal-sized compartments (Fig. 1), using clear, perforated dividers. The clear divider was perforated to allow for both visual and chemical cues to be transferred between all fish. We placed individual focal males (n = 25) with a filter into one compartment of the test tank, and size-matched conspecific and heterospecific females in another compartment for at least a 17 h (up to 21 h) acclimation period. Water filters were used to oxygenate the water and to remove waste from test tanks between trials. Conspecific and heterospecific females did not differ in SL (mean ± S.E. mm = *P. formosa*: 35.63 ± 0.46; *P, latipinna*: 35.75 ± 0.45; Wilcoxon Signed Rank Test: V = 171.5, p = 0.315). We placed a rival male or no male, depending on the treatment, at the back third compartment of the tank. The focal males and rival males did differ in SL (mean ± S.E. mm = Focal males: 36.52 ± 0.72;

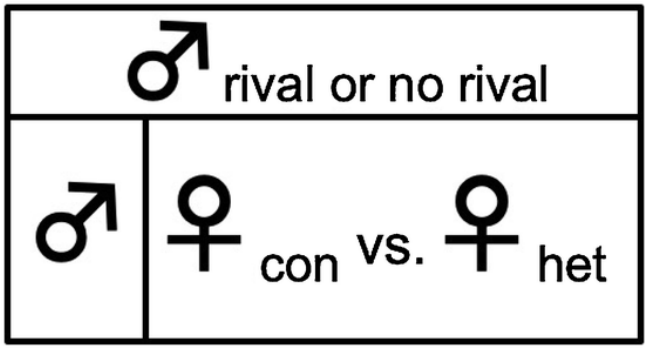

Fig. 1. Male mate choice experimental tank set-up in the treatment with the presence of a rival male prior to mating trial.

Rival males: 27.75 ± 0.45; Wilcoxon Signed Rank Test: V = 300, $p < 0.001$).

The following day, we collected water-borne hormones by placing the focal male and both conspecific and heterospecific females in individual 250 mL sterile beakers with 100 mL of de-chlorinated water for 1 h to measure hormone release rates (following methods of Gabor and Grober, 2010). Water-borne hormone collection is a non-invasive method to obtain hormone release rates using repeated measures without compromising health and behaviour. We then immediately returned the focal male and both females to the testing tank, removed the filter, and removed the divider to allow these fish to freely interact for the mating trial. We removed the filter during mating trials so that it would not obstruct behavioural interactions between fish. During rival treatment mating trials, the rival males were left in their separate compartments. We recorded focal male mating attempts (gonopodial thrusts) toward conspecific and heterospecific females for 25 min. After the mating trial, we returned the filter to the tank and restored the divider to separate the focal male from a new pair of conspecific and heterospecific females. After at least another 17 h (up to 21 h) acclimation period, we repeated the hormone collection and mating trial as described above with the other treatment (rival male present or rival male absent, Fig. 2). Thus, each male (n = 25) was tested twice in random order. We did not use focal males and rival males that were previously housed together in the same trial to avoid any effects of familiarity between males. We stored all water-borne hormone samples at −20 °C until hormones could be assayed (Ellis et al., 2004).

2.3. Hormone extraction and assay

We extracted hormones using a solid-phase extraction (SPE) protocol and assayed using enzyme immunoassay (EIA) methods (modified from Gabor and Grober, 2010). The correlation between water-borne hormone release rates and plasma steroid levels were previously validated for both cortisol and KT in *P. latipinna* (Gabor and Contreras, 2012; Gabor and Grober, 2010). Briefly, we extracted hormones from water samples using Sep-Pak C18 columns (Waters Corp., Milford, MA) placed on a vacuum manifold. We activated columns with 4 mL washes of methanol, followed by 4 mL washes of distilled water. We then ran our water-borne hormone sample through the C18 column to collect hormones and eluted hormones using 4 mL of methanol from C18 columns into borosilicate test tubes. We evaporated the eluent using nitrogen gas and resuspended the hormone residue with 5% ethanol and vortexed then added 95% EIA buffer. We assayed hormones using EIA kits (Cayman Chemical, Ann Arbor, MI) for cortisol and 11-KT. We adhered to protocols provided by the manufacturer for duplicate samples on 96-well plates, which we read on a spectrophotometer at 412 nm (Powerwave XS, Bio Tek Instruments, Inc., Winooski, VT). We ran 11 plates which included a control sample (a pooled mix of hormone suspension from many *P. latipinna*) across all plates and determined 12.5% inter-assay variation with a range of 0.5% to 15.8% for intra-assay variation for 11-KT. The inter-assay variation for 9 cortisol EIA plates was 14.6%, and the intra-assay variation ranged from 3.6% to 18.5%. Plate sensitivity for minimum 11-KT was 1.3 pg/mL and 35 pg/mL for cortisol.

2.4. Statistical analyses

We standardized hormone release rates to SL (standard length) for each fish by multiplying the hormone release rates (pg/mL/h) by the reconstitution volume of the hormone residue (1 mL), dividing by SL (mm), and then ln-transformed the data to better fit the assumptions of parametric analyses (see Table 1 for non-corrected hormone values). We conducted all analyses in R version 3.2.3 (R Core Development Team, 2015). We first tested whether there was male mate preference for conspecific females, regardless of rival treatment, using a paired Wilcoxon Signed Rank test with gonopodial thrusts as the response variable and female species as the predictor variable.

To determine the effects of a smaller rival on male mate choice, we used a generalized linear mixed model with the glmmPQL function from the MASS package (Venables and Ripley, 2002) with the number of mating attempts (gonopodial thrusts) directed at females as the response variable. We used a quasi-Poisson distribution because our initial analysis with a Poisson distribution for count data (number of gonopodial thrusts) revealed that the data were overdispersed. We included the following fixed effects: species of female, rival treatment, treatment order, and all interactions. Male identity was included as a random factor.

We also tested the hypothesis that treatment (rival male presence or absence) affects male hormone release rates. We used two linear mixed

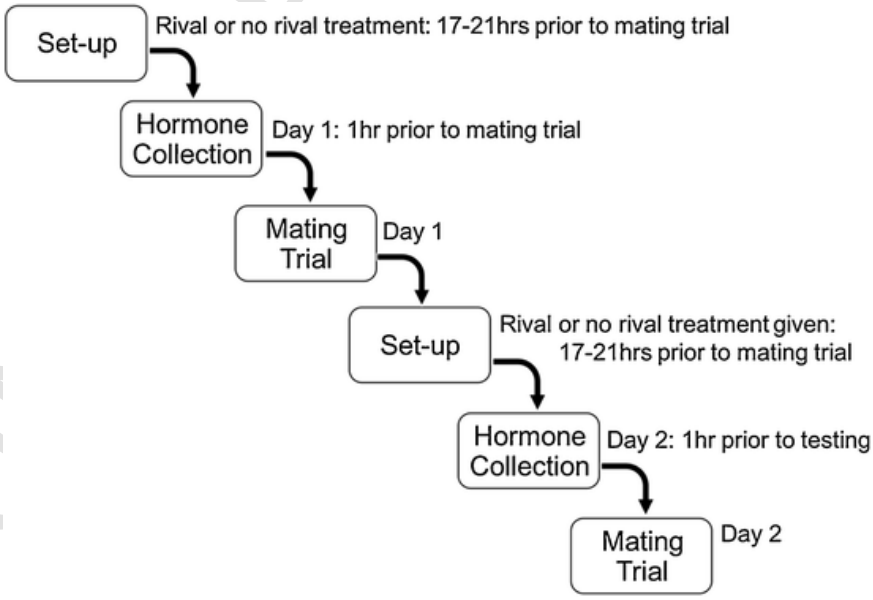

Fig. 2. Summary of the experimental procedure for our repeated measures design.

Table 1
Mean ± S.E. untransformed values for number of gonopodial thrusts and hormone release rates (pg/mm SL/h) of males and female fish by rival and no rival treatments.

Treatment	Rival		No rival	
	Mean	S.E.	Mean	S.E.
Gonopodial thrusts	5.96	1.87	9.46	3.31
Male 11-KT	307.94	134.50	319.81	98.34
Male cortisol	14764.38	1827.47	18498.34	2228.12
Female *P. latipinna* 11-KT	2.12	0.56	2.36	0.42
Female *P. formosa* 11-KT	2.36	0.29	1.64	0.16
Female *P. latipinna* cortisol	9244.99	1847.62	9027.92	2106.79
Female *P. formosa* cortisol	11028.21	2161.72	11593.23	2853.96

effect models with the lme function from the nlme package (Pinheiro et al., 2018) with male hormone release rates (11-KT and cortisol) as the response variables. We used rival treatment, treatment order, and their interaction as fixed effects, and male identity as a random factor. Female species was not included as a factor, as we only had a measure of male hormones when the male was in the presence of both species of female simultaneously. We used a simple regression to determine the relationship between male 11-KT release rates and SL of the rival male.

Similar to above, we also tested the hypothesis that treatment (rival male presence or absence) can affect female hormone release rates. We used two linear mixed effect models with female hormone release rates (11-KT and cortisol) as the response variables. We used species of female, rival treatment, treatment order, and all interactions as fixed effects, and male identity as a random factor to account for both non-independent observations of female species, and for repeated measures between treatments.

3. Results

As has been found for many sympatric populations (Gabor and Grober, 2010; Gabor and Ryan, 2001), male *P. latipinna* mated more often with conspecific than with heterospecific females (mean thrusts ± S.E. = conspecific: 14.54 ± 3.55; heterospecific: 0.88 ± 0.25, V = 139, $p =$ 0.003). When testing the effects of rival treatment on male mating attempts to female *P. latipinna* or *P. formosa*, there were no significant model effects or interactions (Table 2). The presence of a smaller rival male did not affect overall mating effort of the focal male to either of the females (main effect of Rival Treatment in GLMM, Table 2).

Males that did not encounter a rival on the first day had significantly higher 11-KT than males without a rival on the second day (treatment x order effect: Table 3, Fig. 3). There was also an overall main effect of rival treatment on the focal male 11-KT release rates (Table 1, Fig. 3). Post-hoc comparisons showed significant decreases

Table 2
Fixed effects from a quasi-Poisson GLMM examining social effects on male *P. latipinna* mating attempts (gonopodial thrusts) with male identity as a random factor.

x	Estimate ± S.E.	t	*p*
Female Species	−3.404 ± 1.92	−1.769	0.081
Rival Treatment	−0.080 ± 1.65	−0.049	0.961
Treatment Order	−0.812 ± 0.61	−1.335	0.186
Female Species x Rival Treatment	2.009 ± 2.59	0.775	0.441
Female Species x Treatment Order	0.475 ± 1.27	0.375	0.709
Rival Treatment x Treatment Order	0.381 ± 1.11	0.344	0.732
Female Species x Rival Treatment x Treatment Order	−1.635 ± 1.91	−0.858	0.394

Table 3
Fixed effects from a linear mixed effects model examining social effects on male *P. latipinna* hormone release rates (pg/mm SL/h) with male identity as a random factor. Significant *p*-values are in bold.

Male	Estimate ± S.E.	t	*p*
11-KT			
Rival Treatment	2.672 ± 1.26	2.128	**0.037**
Treatment Order	0.282 ± 0.43	0.652	0.516
Rival Treatment x Treatment Order	−1.855 ± 0.83	−2.226	**0.029**
Cortisol			
Rival Treatment	0.313 ± 1.00	0.313	0.755
Treatment Order	−0.497 ± 0.36	−1.382	0.171
Rival Treatment x Treatment Order	−0.022 ± 0.66	−0.033	0.974

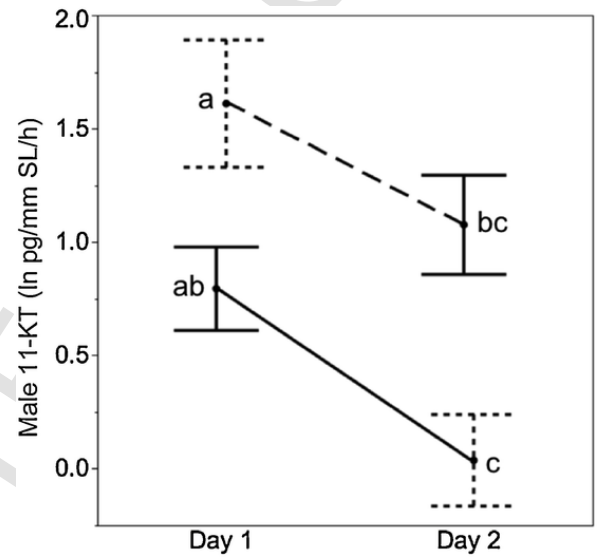

Fig. 3. Mean ± S.E. of male Ln 11-KT release rates (pg/mm SL/h) by rival treatment (no rival: dashed error bars; rival: solid error bar) and by treatment order (no rival presented on first day: dashed line; rival presented on first day: solid line). Ln-transformed data are shown. Post-hoc comparisons show grouping by lowercase letters. Male 11-KT release rates of either treatment order decreased by the second day. However, male 11-KT release rates in the absence of a rival were lower on the second day after exposure to a rival on the first day.

in male 11-KT release rates on the second day regardless of treatment order (Fig. 3). There was also a significant positive relationship between the size of the rival male and 11-KT release rates by the focal male ($R^2 = 0.34$, $p =$ 0.002, Fig. 4). The presence of a rival, treatment order, or their interaction did not affect male cortisol release rates (Table 3), and there was also no significant relationship between rival male size and cortisol release rates ($R^2 = 0.03$, $p =$ 0.420).

Female 11-KT release rates were higher in the presence of a rival male (and did not differ between the two species), but were not affected by any of the other model predictors or interactions (Table 4, Fig. 5). Female cortisol release rates were not affected by the presence of a rival, treatment order, species of female, or any of the interactions (Table 4).

4. Discussion

Understanding the proximate basis of audience effects will further elucidate how the social environment affects mating behaviors. Similar to other studies (Gabor et al., 2013; Gabor and Ryan, 2001), we show significant male preference for conspecific females based on male mating attempts. Male *P. latipinna* mating preference for conspecific fe-

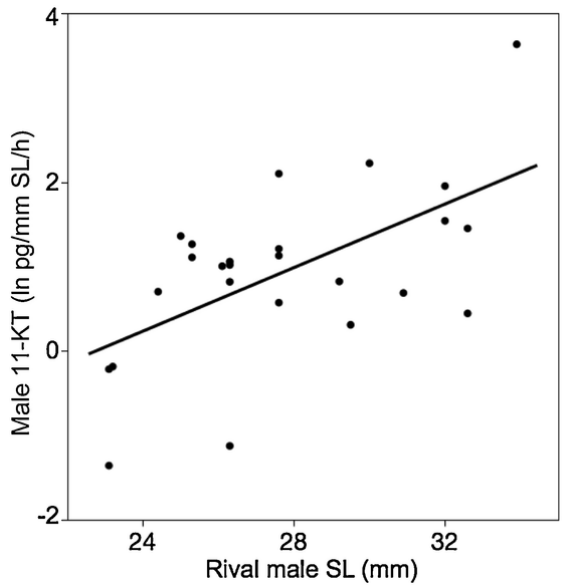

Fig. 4. Correlation between male Ln 11-KT release rates (pg/mm SL/h) and the SL of the rival male. Ln-transformed data are shown.

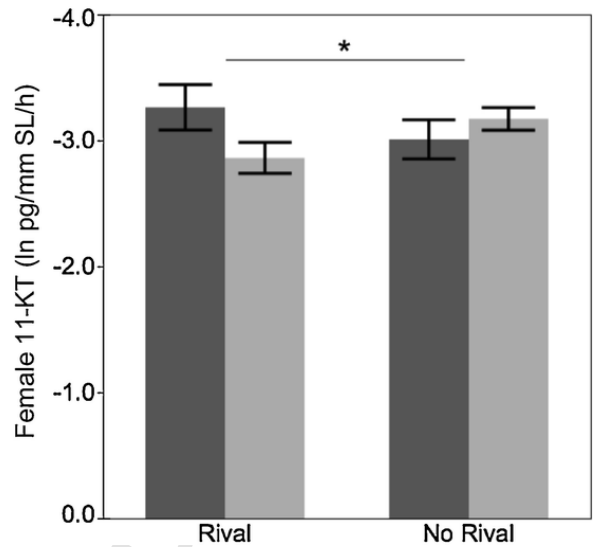

Fig. 5. Mean ± S.E. of female Ln 11-KT release rates (pg/mm SL/h) by rival treatment and by female species (conspecific: dark gray bars; heterospecific: light gray bars). Ln-transformed data are shown. * indicates significant difference ($p < 0.05$) between rival treatments. There was no significant difference between species and no species by treatment interaction.

Table 4
Fixed effects from linear mixed models examining social effects on female hormone release rates (pg/mm SL/h) with male identity as a random factor. Significant *p*-values are in bold.

Female	Estimate ± S.E.	t	*p*
11-KT			
Female Species	0.825 ± 0.55	1.492	0.141
Rival Treatment	1.392 ± 0.68	2.037	**0.046**
Treatment Order	0.188 ± 0.28	0.674	0.503
Female Species x Rival Treatment	−1.299 ± 0.76	−1.702	0.094
Female Species x Treatment Order	−0.274 ± 0.34	−0.803	0.425
Rival Treatment x Treatment Order	−0.769 ± 0.44	−1.743	0.086
Female Species x Rival Treatment x Treatment Order	0.487 ± 0.48	1.010	0.316
Cortisol			
Female Species	0.299 ± 0.83	0.362	0.719
Rival Treatment	1.138 ± 1.00	1.141	0.259
Treatment Order	0.168 ± 0.41	0.409	0.684
Female Species x Rival Treatment	0.043 ± 1.15	0.037	0.971
Female Species x Treatment Order	−0.082 ± 0.51	−0.160	0.874
Rival Treatment x Treatment Order	−0.803 ± 0.64	−1.250	0.217
Female Species x Rival Treatment x Treatment Order	0.042 ± 0.72	0.059	0.954

males seems to be ubiquitous across *P. latipinna* populations. However, we did not find support for our hypothesis that the presence of a smaller rival male would affect male mating preference for conspecific over heterospecific females. Although mate-choice copying among females exists in this species (Schlupp et al., 1994), males do not mislead their potential competitors as seen in *P. mexicana*, where males show reduced preference for conspecific females in the presence of a rival male (Plath et al., 2008a,b). Male *P. latipinna* may not have a reduced conspecific mate preference with a smaller rival male because they have a stronger overall conspecific mating preference than *P. mexicana* (Ryan et al., 1996). If male *P. latipinna* have a strong conspecific mate preference, then a slight decrease of this initial preference may not be detectable (i.e., a decrease in a strong preference results in a weaker preference, but still results in an overall preference for conspecific females). However, a decrease in a weak conspecific preference would possibly lead to the expression of either no preference, or a switch to a heterospecific preference as found with *P. mexicana*. In addition, the presence of a smaller rival male did not affect the overall mating effort of males. Focal males in our study were exposed to a rival male for 17–21 hours prior to the mating trials, which may have been enough time for them to behaviourally habituate to the presence of rival males thus, unintentionally, diminishing their response to rivals. Alternatively, smaller males may not be considered significant rivals. However, we do not consider this a likely explanation for the lack of a behavioural mating effort effect because we did observe an effect of the smaller male rival on the focal male hormones. In addition, small males use an alternative mating tactic to obtain coercive copulations so that fitness may be equal to that of larger courting males. Thus, the hypothesis that smaller males may not be considered significant rivals is not very likely.

We also predicted that the presence of a rival male would elicit an increase in 11-KT release rates. Indeed, we found that the presence of a smaller rival male affected 11-KT release rates, but the relationship between a rival male's presence and 11-KT was time-dependent. Males with no rival on day one had greater 11-KT release rates than males with no rival on day two. Prior studies have shown that isolated males have lower or no difference in androgens levels than males faced with a rival (Dijkstra et al., 2011; Galhardo and Oliveira, 2014), which is counter to our results of the higher 11-KT release rates in the no rival male treatment on the first day as compared to the second day. In our study, male 11-KT decreased on day two of the experiment regardless of treatment possibly due to down-regulation of 11-KT. However, there was greater down-regulation in the no-rival male treatment than the rival treatment, suggesting a relationship between the presence of a competitor and 11-KT.

After 34–42 hours (day two of the experiment), we observed a reduction in overall male *P. latipinna* 11-KT release rates. One hypothesis is that male 11-KT is down-regulated after initial increases from exposure to a new social environment. Data on the timing of 11-KT changes in response to social challenges or to the presence of mates in fish species are not universally consistent. Males of several cichlid species

show increases in 11-KT after one hour of exposure to a simulated territorial intruder (Hirschenhauser et al., 2004), and shoaling male zebrafish have increased 11-KT release rates 30 min after males engage with rival males (Teles and Oliveira, 2016). However, there is no difference in 11-KT of nest-holding male Siamese fighting fish, *Betta splendens*, 20 min after treatment with or without a male audience, but 11-KT is significantly lower in the presence of a female audience (Dzieweczynski et al., 2006). These studies suggest that changes in 11-KT can occur at relatively shorter time scales in response to the presence of social rivals or mates, but our study suggests that overall release rates of 11-KT decrease after longer time periods, which can mask any effects of social rivals on male release rates of androgens.

Focal male 11-KT release rates are positively correlated with the size of the rival male. Male *P. latipinna* have alternative mating phenotypes, they vary greatly in body size and they also engage in aggressive interactions that include chasing, nipping, and aggressive displays. Larger males are preferred by females (Ptacek and Travis, 1997) and may pose a greater threat in mating competition which could explain increases in 11-KT of focal males in the presence of larger rival males. Audience effects on male mate choice are greater when males are confronted with large rivals (Auld et al., 2017; Bierbach et al., 2011). In the shell-brooding cichlid, *Lamprologus callipterus*, large nest-holding males increased 11-KT when confronted with other large nest holders or intermediately sized sneakers, but not when confronted with the much smaller dwarf male (von Kuerthy et al., 2016). The relative size or competitive ability of rival males may have an important role in the androgen response of males, which could be explored in future studies.

Female *P. latipinna* and *P. formosa*, in our study, show an increase in 11-KT in the presence of a smaller rival male. This increase in female 11-KT is relatively smaller than the changes seen in the focal male 11-KT. Androgens are predominantly associated with male physiology and behavior but increases in female 11-KT release rates may be a physiological byproduct in response to mating interactions (Stacey, 2003, 2015) or may allow males to discriminate between species (Gabor and Grober, 2010). Although we found small increases in female 11-KT release rates, we did not find any differences in 11-KT release rates between the two species of female. We interpret this result with caution because our result does not match the results of (Gabor and Grober, 2010), who found increases in 11-KT of conspecific females when mated with male *P. latipinna,* but no such increase in *P. formosa* that mated with male *P. latipinna*. In the prior study, Gabor and Grober (2010) tested males with one species of female at a time (i.e., sequential mate choice trials) which may explain differences between our results. The presence of both species of females in our study (i.e. simultaneous mate choice trials) may further affect female hormones and suggests that males would have greater difficulty in using 11-KT release rates of females as a cue for species identification in natural populations.

We found no support for the hypothesis that male and female (both conspecific and heterospecific) cortisol release rates are affected by the presence of a smaller rival male. Cortisol plays a role in short-term mobilization of energy stores for energetically demanding mating behaviours, such as courtship and male-male aggression (Wingfield and Sapolsky, 2003). One possible reason for a lack of differences between the rival present and the rival absent treatments in cortisol release rates in our focal fishes is because the rival male was never in direct physical contact with them. Male zebrafish, *Danio rerio*, do not have higher cortisol release rates when faced with mirrors and male chemical cues, but do have higher cortisol release rates when they are allowed to directly compete with rival males and win in social competitions (Teles and Oliveira, 2016). Another hypothesis for the lack of variation in cortisol across the rival male treatments is because of high male 11-KT release rates, especially on the first day of testing. In trout, 11ß- hydroxysteroid dehydrogenase (11ß-HSD) catalyzes 11-KT production but may also play a role in protecting the gonadal tissue from circulating cortisol (Fernandino et al., 2013).

Although the social environment is an important component of male mating behavior in other species, we found no evidence to support the hypothesis that the presence of a smaller rival male affects species recognition in mate choice of male *P. latipinna.* However, we did find that the social environment has an effect on male physiology. The presence of a single rival male is enough to elicit a change in male androgen release rates, which may translate into changes in behaviour in subsequent encounters with other rival males or females. In addition, males may not be able to discriminate between species when in a complex social environment such as the set-up in this study where both species of females are presented together, possibly due to both females releasing similar amounts of 11-KT. This result could partially account for the maintenance of the unisexual species in this system.

Author contributions

C.G. and A.A. designed the study. J.J.Z.V. obtained permits for the study. D.K. conducted the experiment and analyzed the data. All authors contributed to the writing of the manuscript.

Uncited reference

.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by the National Science Foundation [IOS-1021873]; and Sigma Xi Grants-in-Aid of Research. We thank the Government of Mexico for granting permission to collect fish in Tamaulipas, Mexico (HOODGIPA-D8-OT-033). We also thank the GASP Lab for past and present assistance with fish handling and maintenance.

References

Alberici da Barbiano, L., Gompert, Z., Aspbury, A.S., Gabor, C.R., Nice, C.C., 2013. Population genomics reveals a possible history of backcrossing and recombination in the gynogenetic fish *Poecilia formosa*. Proc. Natl. Acad. Sci. U. S. A. 110, 13797–13802. doi:10.1073/pnas.1303730110.

Aspbury, A.S., 2007. Sperm competition effects on sperm production and expenditure in sailfin mollies, Poecilia latipinna. Behav. Ecol. 18, 776–780. doi:10.1093/beheco/arm044.

Auld, H.L., Godin, J.G.J., 2015. Sexual voyeurs and copiers: social copying and the audience effect on male mate choice in the guppy. Behav. Ecol. Sociobiol. 69, 1795–1807. doi:10.1007/s00265-015-1992-z.

Auld, H.L., Ramnarine, I.W., Godin, J.G.J., 2017. Male mate choice in the Trinidadian guppy is influenced by the phenotype of audience sexual rivals. Behav. Ecol. 28, 362–372. doi:10.1093/beheco/arw170.

Avise, J.C., 2008. Clonality: the Genetics, Ecology, and Evolution of Sexual Abstinence in Vertebrate Animals, Oxford University Press, New York, NY.

Bierbach, D., Girndt, A., Hamfler, S., Klein, M., Mucksch, F., Penshorn, M., Schwinn, M., Zimmer, C., Schlupp, I., Streit, B., Plath, M., 2011. Male fish use prior knowledge about rivals to adjust their mate choice. Biol. Lett. 7, 349–351. doi:10.1098/rsbl.2010.0982.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 109, 219–245. doi:10.1016/0742-8413(94)00063-g.

Briffa, M., Sneddon, L.U., 2007. Physiological constraints on contest behaviour. Funct. Ecol. 21, 627–637. doi:10.1111/j.1365-2435.2006.01188.x.

Butts, I.A.E., Love, O.P., Farwell, M., Pitcher, T.E., 2012. Primary and secondary sexual characters in alternative reproductive tactics of Chinook salmon: associations with androgens and the maturation-inducing steroid. Gen. Comp. Endocrinol. 175, 449–456. doi:10.1016/j.ygcen.2011.11.041.

Cardwell, J.R., Liley, N.R., 1991. Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. Gen. Comp. Endocrinol. 81, 7–20. doi:10.1016/0016-6480(91)90120-u.

Clement, T.S., Parikh, V., Schrumpf, M., Fernald, R.D., 2005. Behavioral coping strategies in a cichlid fish: the role of social status and acute stress response in direct and displaced aggression. Horm. Behav. 47, 336–342. doi:10.1016/j.yhbeh.2004.11.014.

Cummings, M.E., Larkins-Ford, J., Reilly, C.R.L., Wong, R.Y., Ramsey, M., Hofmann, H.A., 2008. Sexual and social stimuli elicit rapid and contrasting genomic responses. Proc. R. Soc. B Biol. Sci. 275, 393–402. doi:10.1098/rspb.2007.1454.

Davis, A.G., Leary, C.J., 2015. Elevated stress hormone diminishes the strength of female preferences for acoustic signals in the green treefrog. Horm. Behav. 69, 119–122. doi:10.1016/j.yhbeh.2015.01.005.

Desjardins, J.K., Becker, L., Fernald, R.D., 2015. The effect of observers on behavior and the brain during aggressive encounters. Behav. Brain Res. 292, 174–183. doi:10.1016/j.bbr.2015.06.019.

Dijkstra, P.D., Wiegertjes, G.F., Forlenza, M., van der Sluijs, I., Hofmann, H.A., Metcalfe, N.B., Groothuis, T.G.G., 2011. The role of physiology in the divergence of two incipient cichlid species. J. Evol. Biol. 24, 2639–2652. doi:10.1111/j.1420-9101.2011.02389.x.

Dzieweczynski, T.L., Eklund, A.C., Rowland, W.J., 2006. Male 11-ketotestosterone levels change as a result of being watched in Siamese fighting fish, Betta splendens. Gen. Comp. Endocrinol. 147, 184–189. doi:10.1016/j.ygcen.2005.12.023.

Ellis, T., James, J.D., Stewart, C., Scott, A.P., 2004. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. J. Fish Biol. 65, 1233–1252. doi:10.1111/j.1095-8649.2004.00499.x.

Farr, J.A., Meffe, G.K., Snelson, F.F., Jr., 1989. Sexual selection and secondary sexual differentiation in poeciliids: determinants of male mating succcess and the evolution of female choice. In: Meffe, G.K., Snelson, F.F., Jr. (Eds.), Ecology and Evolution of Livebearing Fishes,, Prentice Hall, Englewood Cliffs, NJ, pp. 91–123.

Fernandino, J.I., Hattori, R.S., Moreno Acosta, O.D., Strüssmann, C.A., Somoza, G.M., 2013. Environmental stress-induced testis differentiation: androgen as a by-product of cortisol inactivation. Gen. Comp. Endocrinol. 192, 36–44. doi:10.1016/j.ygcen.2013.05.024.

Fraser, B.A., Janowitz, I., Thairu, M., Travis, J., Hughes, K.A., 2014. Phenotypic and genomic plasticity of alternative male reproductive tactics in sailfin mollies. Proc. R. Soc. B Biol. Sci. 281. doi:10.1098/rspb.2013.2310.

Gabor, C.R., Aspbury, A.S., Ma, J., Nice, C.C., 2012. The role of androgens in species recognition and sperm production in Atlantic mollies (*Poecilia mexicana*). Physiol. Behav. 105, 885–892. doi:10.1016/j.physbeh.2011.10.024.

Gabor, C.R., Contreras, A., 2012. Measuring water-borne cortisol in *Poecilia latipinna*: is the process stressful, can stress be minimized and is cortisol correlated with sex steroid release rates? J. Fish Biol. 81, 1327–1339. doi:10.1111/j.1095-8649.2012.03411.x.

Gabor, C.R., da Barbiano, L.A., Aspbury, A.S., 2013. Geographic variation in male mate choice in a gynogenetic species complex: Evaluating long-term data across mating contexts. Evol. Ecol. Res. 15, 653–666.

Gabor, C.R., Grober, M.S., 2010. A potential role of male and female androgen in species recognition in a unisexual-bisexual mating complex. Horm. Behav. 57, 427–433. doi:10.1016/j.yhbeh.2010.01.012.

Gabor, C.R., Ryan, M.J., 2001. Geographical variation in reproductive character displacement in mate choice by male sailfin mollies. Proc. R. Soc. B Biol. Sci. 268, 1063–1070. doi:10.1098/rspb.2001.1626.

Galhardo, L., Oliveira, R.F., 2014. The effects of social isolation on steroid hormone levels are modulated by previous social status and context in a cichlid fish. Horm. Behav. 65, 1–5. doi:10.1016/j.yhbeh.2013.10.010.

Hirschenhauser, K., Taborsky, M., Oliveira, T., Canàrio, A.V.M., Oliveira, R.F., 2004. A test of the `challenge hypothesis' in cichlid fish: simulated partner and territory intruder experiments. Anim. Behav. 68, 741–750. doi:10.1016/j.anbehav.2003.12.015.

Hubbs, C.L., Hubbs, L.C., 1932. Apparent parthenogenesis in nature, in a form of fish of hybrid origin. Science 76, 628–630. doi:10.1126/science.76.1983.628.

MacLaren, R.D., Rowland, W.J., Morgan, N., 2004. Female preferences for sailfin and body size in the sailfin molly, Poecilia latipinna. Ethology 110, 363–379. doi:10.1111/j.1439-0310.2004.00974.x.

Mautz, B.S., Jennions, M.D., 2011. The effect of competitor presence and relative competitive ability on male mate choice. Behav. Ecol. 22, 769–775. doi:10.1093/beheco/arr048.

Milla, S., Wang, N., Mandiki, S.N.M., Kestemont, P., 2009. Corticosteroids: friends or foes of teleost fish reproduction? Comp. Biochem. Physiol. A Mol. Integr. Physiol. 153, 242–251. doi:10.1016/j.cbpa.2009.02.027.

Munchrath, L.A., Hofmann, H.A., 2010. Distribution of sex steroid hormone receptors in the brain of an African cichlid fish, Astatotilapia burtoni. J. Comp. Neurol. 518, 3302–3326. doi:10.1002/cne.22401.

Oliveira, R.F., 2004. Social modulation of androgens in vertebrates: mechanisms and function. Adv. Study Behav. 34, 165–239. doi:10.1016/s0065-3454(04)34005-2.

Oliveira, R.F., Canario, A.V.M., Ros, A.F.H., 2008. Hormones and alternative reproductive tactics in vertebrates. In: Oliveira, R.F., Taborsky, M., Brockmann, H.J. (Eds.), Alternative Reproductive Tactics: An Integrative Approach, Cambridge University Press, New York, pp. 132–173.

Pfennig, K.S., 2007. Facultative mate choice drives adaptive hybridization. Science 318, 965–967. doi:10.1126/science.1146035.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R-Core-Team, 2018. nlme: Linear and nonlinear mixed effects models. R Package Version 3, 1–137.

Plath, M., Blum, D., Schlupp, I., Tiedemann, R., 2008. Audience effect alters mating preferences in a livebearing fish, the Atlantic molly, Poecilia mexicana. Anim. Behav. 75, 21–29. doi:10.1016/j.anbehav.2007.05.013.

Plath, M., Richter, S., Tiedemann, R., Schlupp, I., 2008. Male fish deceive competitors about mating preferences. Curr. Biol. 18, 1138–1141. doi:10.1016/j.cub.2008.06.067.

Ptacek, M.B., 1998. Interspecific mate choice in sailfin and shortfin species of mollies. Anim. Behav. 56, 1145–1154. doi:10.1006/anbe.1998.0909.

Ptacek, M.B., Travis, J., 1996. Inter-population variation in male mating behaviours in the sailfin mollie, Poecilia latipinna. Anim. Behav. 52, 59–71. doi:10.1006/anbe.1996.0152.

Ptacek, M.B., Travis, J., 1997. Mate choice in the sailfin molly, Poecilia latipinna. Evolution 51, 1217–1231. doi:10.2307/2411051.

Robinson, D.M., Konkin-Garcia, T., Espinedo, C.M., Gabor, C.R., Aspbury, A.S., 2011. Seasonal effects on female fecundity and male sperm availability in a thermally stable temperate population of sailfin mollies (*Poecilia latipinna*). Am. Midl. Nat. 166, 394–403. doi:10.1674/0003-0031-166.2.394.

Ryan, M.J., Dries, L.A., Batra, P., Hillis, D.M., 1996. Male mate preferences in a gynogenetic species complex of Amazon mollies. Anim. Behav. 52, 1225–1236. doi:10.1006/anbe.1996.0270.

Schlupp, I., Marler, C., Ryan, M.J., 1994. Benefit to male sailfin mollies of mating with heterospecific females. Science 263, 373–374. doi:10.1126/science.8278809.

Schlupp, I., Ryan, M.J., 1996. Mixed-species shoals and the maintenance of a sexual-asexual mating system in mollies. Anim. Behav. 52, 885–890. doi:10.1006/anbe.1996.0236.

Schreck, C.B., 2010. Stress and fish reproduction: The roles of allostasis and hormesis. Gen. Comp. Endocrinol. 165, 549–556. doi:10.1016/j.ygcen.2009.07.004.

Snelson, F.F., 1985. Size and morphological variation in males of the sailfin molly, Poecilia latipinna. Environ. Biol. Fishes 13, 35–47. doi:10.1007/bf00004854.

Stacey, N., 2003. Hormones, pheromones and reproductive behavior. Fish Physiol. Biochem. 28, 229–235. doi:10.1023/B:FISH.0000030540.99732.2c.

Stacey, N., 2015. Hormonally derived pheromones in teleost fishes. In: Sorensen, P.W., Wisenden, B.D. (Eds.), Fish Pheromones and Related Cues, John Wiley & Sons, Chichester, pp. 33–88.

Teles, M.C., Oliveira, R.F., 2016. Androgen response to social competition in a shoaling fish. Horm. Behav. 78, 8–12. doi:10.1016/j.yhbeh.2015.10.009.

Travis, J., Woodward, B.D., 1989. Social context and courtship flexibility in male sailfin mollies, *Poecilia latipinna*(Pisces: Poeciliidae). Anim. Behav. 38, 1001–1011. doi:10.1016/s0003-3472(89)80139-3.

Venables, W.N., Ripley, B.D., 2002. Modern Applied Statistics With S, Springer, New York.

Vitousek, M.N., Romero, L.M., 2013. Stress responsiveness predicts individual variation in mate selectivity. Gen. Comp. Endocrinol. 187, 32–38. doi:10.1016/j.ygcen.2013.03.009.

von Kuerthy, C., Ros, A.F.H., Taborsky, M., 2016. Androgen responses to reproductive competition of males pursuing either fixed or plastic alternative reproductive tactics. J. Exp. Biol. 219, 3544–3553. doi:10.1242/jeb.143974.

Warren, W.C., García-Pérez, R., Xu, S., Lampert, K.P., Chalopin, D., Stöck, M., Loewe, L., Lu, Y., Kuderna, L., Minx, P., Montague, M.J., Tomlinson, C., Hillier, L.W., Murphy, D.N., Wang, J., Wang, Z., Garcia, C.M., Thomas, G.C.W., Volff, J.-N., Farias, F., Aken, B., Walter, R.B., Pruitt, K.D., Marques-Bonet, T., Hahn, M.W., Kneitz, S., Lynch, M.,

Schartl, M., 2018. Clonal polymorphism and high heterozygosity in the celibate genome of the Amazon molly. Nat. Ecol. Evol. 2, 669–679. doi:10.1038/s41559-018-0473-y.
West-Eberhard, M.J., 1983. Sexual selection, social competition, and speciation. Q. Rev. Biol. 58, 155–183.
Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: when and how. J. Neuroendocrinol. 15, 711–724. doi:10.1046/j.1365-2826.2003.01033.x.