Male Grijalva mosquitofish (Heterophallus milleri Radda, 1987) increase individual mating preferences in front of an audience

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Abstract
Socially influenced mate choice behavior is currently a growing field in the study of sexual selection and evolution. Here, we provide the first description of male Grijalva mosquitofish (Heterophallus milleri) courtship behavior, and further report on an unparalleled "audience effect" in that species. Lab-reared male Grijalva mosquitofish significantly increased their preference for an initially preferred female in a full contact design in the presence of another male. This is somewhat unexpected as previous studies found males of other members of the family Poeciliidae to interact more evenly with stimulus females when faced with an audience, and thus decreasing their preference for the initially preferred female. As those "audience effects" are assumed to represent male tactics to prevent sperm competition risk arising through male mate choice copying, we argue that male mate choice copying might not play a major role in the reproductive biology of H. milleri.

Zusammenfassung

Résumé
Le comportement de choix du partenaire sous influence sociale est actuellement un champ d’investigation croissant dans l’étude de la sélection et de l’évolution sexuelles. Nous proposons ici la première description du comportement de la parade de reproduction du mâle Heterophallus milleri. Des mâles de cette espèce élevés en laboratoire augmentaient leur préférence pour une femelle déjà choisie par un comportement de plein contact en présence d’un autre mâle. C’est chose plutôt inattendue dans la mesure où d’autres études ont montré que les mâles d’autres membres des Poeciliidae interagissent de même davantage avec des femelles stimulés en présence d’un groupe et donc qu’ils diminuent leur intérêt pour la femelle initialement préférée. Comme ces « effets de groupe » sont sensés
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Sommario

Il comportamento socialmente influenzato della scelta del compagno è attualmente un settore in crescita nello studio della selezione sessuale e dell'evoluzione. Qui forniamo la prima descrizione del comportamento di corteggamento del maschio di gambusia Grijalva (Heterophallus milleri) e un resoconto su un “effetto spettatore” senza precedenti in questa specie. I maschi di gambusia Grijalva allevati in laboratorio aumentano significativamente la loro preferenza per una femmina inizialmente prescelta in un disegno sperimentale a pieno contatto in presenza di un altro maschio. Questo è un risultato inaspettato, poiché studi precedenti hanno evidenziato che maschi di altri membri della famiglia Poeciliidae interagiscono di fronte a un pubblico in modo più uniforme con le femmine stimolo, diminuendo l’interesse per la femmina inizialmente preferita. Poiché si presume che tale “effetto pubblico” rappresenti una tattica del sesso maschile per prevenire il rischio di competizione da spermatozoi derivante dal copiare la scelta della compagna da parte di un maschio, si sostiene che questo fenomeno di imitazione non svolgerebbe un ruolo importante nella biologia riproduttiva di H. milleri.

INTRODUCTION

Gregarious animals often choose their mating partners within social aggregations, i.e. in front of conspecifics, so mate choice decisions and any other communicatory interactions between choosing individuals, their potential mates, and by-standing individuals are usually part of extensive communication networks (Matos & Schlupp 2005; McGregor & Peake 2000; Peake 2005; Earley & Dugatkin 2005). By-standing individuals may extract and subsequently use information from observed (sexual) interactions to modify their own mating decisions (‘social eavesdropping’; Naguib et al. 2004; Dabelsteen 2005; Earley 2010); for instance, male fiddler crabs (Uca mjoebergi Rathbun, 1924) use rivals’ courtship behavior to detect receptive females (Milner et al. 2010). Another form of social eavesdropping is mate choice copying, during which a female’s propensity to mate with a given male increases after it had observed that male interact sexually with another female (Pruett-Jones 1992, Galef 2008; Vakirtzis 2011). M mate choice copying appears to be widespread throughout the Animal Kingdom (teleost fishes: Dugatkin 1992; Schlupp et al. 1994; Witte 2006; birds: Freed-Brown and White 2009; mammals: Galef et al. 2008, including humans: Waynforth 2007, Place et al. 2010). Most studies reported on female mate choice copying, but males, too, were found to copy other males’ mating decisions (poeciliid fishes: Schlupp & Ryan 1997; Bierbach et al. 2011a; pipefish: Widemo 2006; humans: Waynforth 2007; Place et al. 2010).

However, at least in internally fertilizing species, copulating with a previously mated female clearly elevates sperm competition risk for both, the copier and the copied male (Plath & Schlupp 2008; Plath & Bierbach 2011). Males that are at risk of being copied by another male are predicted to respond by trying to prevent the rival from copying. Indeed, males may alter their mating behavior strategically when observed by a sexually active rival (i.e., by a potential copier; Plath et al. 2008a,b,c; Ziege et al. 2009; Dosen & Montgomery 2004; Bierbach et al. 2011b, 2012; Wronski et al. 2012). Such audience effects have been examined most thoroughly in male Atlantic mollies (Poecilia mexicana Steindacher 1863, family Poeciliidae), which decrease sexual activity and cease expressing mating preferences when a rival male is nearby (Plath et al. 2008a,b,c; 2010; Ziege et al. 2009; Bierbach et al. 2011b, 2012). Moreover, males interacted first with the previously non-preferred female when an audience male was presented, thereby potentially exploiting male mate choice copying behavior so as to lead the rival away from the preferred mate (‘deceptive behavior’, Plath et al. 2008c; 2010; Bierbach et al. 2012).

These behavioral responses to an audience male were confirmed in a comparative study by Bierbach et al. (2012) for 9 different species of poeciliid fishes (P. mexicana, P. orri Fowler, 1943, P. latipinnia (LeSueur, 1821), P. latipunctata Meeck, 1904, P. reticulata Peters, 1859, P. picta (Regan, 1913), Limia tridens (Hilgendorf, 1889), L. sulphurophilia Rivas, 1980, Gambusa sexradiata Hubbs, 1936) and also found in male sand gazelles (Gazella marica, Wronski et al. 2012). The only exception from this general pattern appears to be the Grijalva mosquitofish (Heterophallus milleri Radda, 1987), a close relative to the genus Gambusa (Hrbek et al. 2007 lists H. milleri in the genus Gambusa) that is endemic to the Rio Grijalva/Usumacinta drainage in southern Mexico (Miller et al. 2005). Interestingly, males in this species appear to increase their mating preferences when confronted with a rival (Ziege et al. 2008).

However, in contrast to the comparative study by
Bierbach et al. (2012) as well as the study first describing male deceptive mating behavior (Plath et al. 2008c), which both established male mating preferences through measuring direct sexual interactions, the study by Ziege et al. (2008) used association times instead. Furthermore, wild-caught fish were used by Ziege et al. (2008) instead of lab-reared individuals that were used to being held in glass tanks and had previously been exposed to handling stress. Thus comparability of the seemingly contradictory results from Ziege et al. (2008) with the results of other studies is difficult.

We, thus, hypothesize that laboratory-reared H. milleri males would show similar behavioral responses to the presence of an audience male - reduced sexual activity and cessation in the expression of mating preferences - as reported for other lab-reared poeciliids. Our present study, therefore, made use of laboratory stocks of H. milleri that were established during the past three years to better control for effects related to handling stress while we further measured direct sexual interactions to establish male mating preferences, which increases comparability with the data from other poeciliid species. Thus, the current study (a) describes the behavioral repertoire of male H. milleri in terms of courtship and mating behavior, and (b) tests for audience effects in the controlled environment of a lab-based experimental setup.

MATERIAL AND METHODS

Origin and maintenance of the study organism: All fish used in this study were lab-reared descendants of wild-caught fish collected in 2006 in a small creek, Arroyo Bonita, a tributary to the Río O xo lotán near the village of Tapijulapa (for details see Riesch et al. 2011). Test fish were maintained as randomly outbred mixed-sex stocks in 1000-L tanks in a greenhouse of the Aquatic Research Facility of the University of Oklahoma in Norman, U.S.A. Experiments were conducted in November 2010. Test fish were kept at a temperature of 25°C, under a natural light: dark regime and fed daily ad libitum with commercially available flake food (TetraMin™).

We isolated focal (N = 30) males in 38-l tanks for four days prior to the tests to ensure that they were motivated to mate (Schlupp & Plath 2005). Each focal male was tested only once; however, due to the limited number of males available from our stocks, some males were also used as audience males after they were used as a focal male, but never on the same day. All fish used in a trial were taken from different stock tanks to avoid confounding effects of familiarity (Bierbach et al. 2011b)

Experimental design: We used a full contact design in which focal males’ mating behavior towards two types of females was videotaped by a ‘Canon 3CCDF Digital Video Camcorder’ and videos were used for a qualitative description of courtship displays (a sample tape is provided in the online supplement). To analyze males’ change in mating behavior when presented with a by-standing rival, sexual behaviors towards the two stimulus females were scored as a measure of mating preferences. Each focal male was tested for its “baseline” mating preference and was then retested either without an audience (control) or with an audience male present (audience treatment). This allowed us to examine the change in focal males’ behavior from the 1st to the 2nd part of the tests while we could differentiate between effects induced by an audience and changes that would occur over the course of the experiment even without an audience present (Plath et al. 2008c,c).

The test tank (50 cm length x 30 cm width x 30 cm height) was filled to 20 cm with aged tap water of 27-28°C. Black plastic covered all sides except the front. Prior to the tests, we selected two different-sized stimulus females [mean (± SE) SL; large: 33.3 ± 0.5 mm; small: 25.0 ± 0.7 mm] from the stock tanks and introduced them into the test tank. We exchanged stimulus females after each trial and retransferred them to the stock tanks, so that some females may have been used more than once (but never on the same day). Poeciliid males typically prefer to mate with larger females (e.g., Bisazza et al. 1989; Herdman et al. 2004) as fecundity usually correlates positively with body size (e.g., Pires et al. 2011) - a pattern that was also confirmed for H. milleri (Riesch et al. 2011). Afterwards, we introduced the focal male (22.2 ± 0.5 mm, N = 30) into a transparent Plexiglas cylinder (10 cm diameter) in the center of the tank and left the fish undisturbed for 5 minutes. After this habituation period, we gently lifted the cylinder. During a 10-min observation period, male sexual behaviors (nipping, gonopodial thrusts, and courtship displays) were scored, and we noted with which female the male interacted first. We decided a priori to terminate a trial if the male did not show any sexual behavior during the 1st part of the test; N = 6 trials (out of 30) were discarded based on this criterion so that data
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Fig. 1. Videotaped courtship sequence of H. milleri. For a description see main text.
Fig. 2. Influence of a by-standing male on male mate choice behavior in Grijalva mosquitofish. During the 1st Part, the focal male could interact with two stimulus females (large (black bars) or small (gray bars)) without an audience present. During the second part of the trials, half of the males were visually presented with an audience male (2nd Part with Audience), while another half of trials were repeated without an audience (2nd Part Control). Depicted are means (± SE) for (A) nipping and (B) thrusting, along with results from paired t-tests. Also given are (C) fractions of males that interacted with either the large or small female first when released from their acclimatization cylinder (Binomial tests).
but in one half of the trials, an audience male was presented, while the other half was repeated without an audience (control). To initiate this 2nd part of a trial, we reintroduced the focal male into the acclimatization cylinder. An audience male (21.5 ± 0.5 mm) was placed in another transparent cylinder in the central back of the tank, while for the control an empty cylinder was presented. The audience male was confined in his cylinder throughout the experiment. After another 5 minutes for habituation (during which all four fish could interact visually), measurement of male preferences was carried out as described above. Interactions between males were not quantified; qualitatively, we often observed synchronized swimming up and down of the two males. After a trial was completed, all fish were measured for standard length (SL) to the closest millimeter.

Statistical analysis: All data were tested for normal distribution using Shapiro-Wilk tests and, throughout, all relative data were arcsine (square root)-transformed prior to the statistical analyses. However, as courtship displays were performed by only 16% (4 out of 24) of males in both parts of the test, 40%, (10 out of 24) showed courtship in at least one part, we did not quantitatively analyze this behavior.

Poeciliid males tend to reduce sexual activity (determined herein as the sum of nipping and thrusting with both stimulus females) upon exposure to a rival (Bierbach et al. 2012). In our first analysis, we therefore compared the change of male sexual activity from the 1st to 2nd part of the tests. We used the relative sexual activity during the second part of the tests (as a fraction of the sexual activity during both parts) as dependent variable in a univariate GLM (general linear model) including ‘treatment’ (with or without audience) as factor. As covariates we included focal males’ SL, as well as the size difference between the large and small stimulus female (SL large – SL small female), but removed both as they had no significant effects (‘focal male

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**Fig. 3.** (A) Change in male sexual activity during the 2nd part of the tests (as fraction of the sum of sexual behaviors during both parts). During the 2nd part males were randomly assigned to either a control treatment (control, black bars) or a treatment during which their mate choice was observed by a rival male (audience, gray bars). Values above 0.5 indicate increased sexual activity during the 2nd part. (B) Changes in individual focal males’ mating preferences induced by the presence of an audience male. Depicted are preference scores (see main text), whereby negative values indicate that male preferences decreased in strength. Data in (A) and (B) are means ± SE.
body size': $F_{1,22} = 0.09, P = 0.76$; ‘female body size difference': $F_{1,21} = 0.01, P = 0.94$).

To explore the direction of male preferences, we compared numbers of sexual behaviors with the large and the small stimulus female during either of the two test parts using paired $t$-tests; nipping and gonopodial thrusting were analyzed separately in this analysis. Moreover, males were predicted to alter their individual mating preferences under the influence of an audience (Plath & Schlupp 2008; Plath & Bierbach 2011). To explore this effect, a preference score, based on focal males' individual preferences, was calculated as: \((\text{fraction of nipping and thrusting directed toward the initially preferred female during the 2nd part of a trial}) - (\text{fraction of nipping and thrusting with the same female during the 1st part})\). Hence, the score expressed the change between the two parts of the tests, whereby negative values would indicate that preferences decreased in strength while positive values would indicate an increase in strength of preference (Plath et al. 2008; Ziege et al. 2009; Bierbach et al. 2011). Data were compared in a GLM using 'treatment' (with or without audience) as a factor. In the initial model, we included 'focal male body size' ($F_{1,22} = 1.93, P = 0.18$) and 'female body size difference' ($F_{1,21} = 0.18, P = 0.68$) but removed both covariates from the final model as they had no significant effect.

Finally, males of other poeciliids, including the closely related G. sex radiata, initially approach the previously non-preferred female upon presentation of a rival (Bierbach et al. 2012). We asked whether a similar pattern would be uncovered in H. milleri and first compared the numbers of males that directed their first sexual behavior towards the large or small female using Binomial tests. Data from the 1st and 2nd part of the tests were analyzed separately. Second, we compared the proportion of males that altered their individual preferences from the 1st to 2nd part between the control group and the audience treatment using a chi$^2$-test. All statistical analyses were run using SPSS 16.0.

**RESULTS**

**Description of male courtship displays:** Heterophallus milleri males showed conspicuous courtship displays during our mate choice tests. During courtship, males usually start to swim calmly behind the female's anal fin and follow the female in exactly this position for up to one minute. From this position males move to nip at the female's gonopore similar to other poeciliids. Males then suddenly start to circle around the female, which typically remains stationary throughout (Fig. 1 A-F). During these circles males rest for a short period in front of the female's head and bend their bodies to an S-shape (Fig. 1 G-I). In this position males raise their tails and vibrate them while folding all unpaired fins. Additionally, males heavily swing their gonopodia at this stage (Fig. 1 G-H). After this episode, they either quickly swim behind the female and repeat the circling performance or they slowly swim backwards with all unpaired fins folded (Fig. 1 J-L) until both the male's and the female's heads are at the same level (Fig. 1 L). They then quickly turn around and try to thrust the female, which now typically swims away (Fig. 1 M-O).

We were also able to observe those courtship circles from above in natural habitats of H. milleri, as this species often swims directly below the water surface (see Riesch et al. 2011).

**Changes in sexual activity induced by an audience male:** Even though visual inspection of our data suggests that males slightly increased sexual activity when observed by an audience (Fig. 2a), no significant difference was detected in the GLM (mean square = 0.04, $F_{1,23} = 0.94, P = 0.34$).

**Audience-induced changes in the overall direction of male mating preferences:** Males tended to direct more nipping and thrusting towards the larger female during the 1st part of the tests, however, this effect was not significant (see Fig. 3a, b for results from paired $t$-tests), and males in both treatments also did not show a significant preference during the 2nd part (Fig. 3a-b).

**Audience-induced changes in individual male mating preferences:** When comparing the strength of change in individual male mating preferences from the 1st to 2nd part of the tests using GLM (i.e., preference scores) - irrespective of whether the larger or smaller female was preferred - a significant difference between treatments was uncovered (mean square = 0.49, $F_{1,23} = 5.49, P = 0.028$), and males increased their initial preference in response to the presence of an audience (Fig. 2b).

**First sexual interaction:** When released from the acclimatization cylinder most focal males first interacted with the larger stimulus female during the 1st part of the tests (see Fig. 2c for $P$-values from Binomial tests). This pattern remained virtually unchanged during the 2nd part of the tests in both treatments (Fig 2c), and even though the effect was not significant, qualitatively, most males...
still approached the larger stimulus female first during the second test part. Comparing the fraction of males that switched to another female during the 2nd test part, we did not find a significant difference between the control and audience treatments (chi²-test; \( \chi^2 = 1.15, df = 1, P = 0.28 \)).

**DISCUSSION**

Surprisingly, some males showed vigorous courtship behavior, e.g., circled around a female and performed a swinging movement of their gonopodium directly in front of a female. This was somewhat unexpected since courtship is reduced and rare in the closely related genus Gambusa (e.g. Rosen & Tucker 1961; Hughes 1985; Pilastro et al. 1997). However, if put into a larger phylogenetic context, the tribe Gambusini also comprises genera and species (e.g. the genus Xiphophorus or the pike killifish, Belonesox belizanus Kner 1860; Hrbek et al. 2007) that are known to often vigorously court their females (Farr 1989). Furthermore, females of at least some members of the genus Gambusa were found to exhibit a mating preference for males with large gonopodia (see Langerhans et al. 2005 for discussion, but see Gasparini et al. 2011 for absence of such a preference in P. reticulata), so we suspect courtship displays of male *H. milleri* to be an ‘advertising strategy’ that might exploit such a female preference.

Our mate choice tests confirmed previous results from Ziege et al. (2008) in which the authors established male mating preferences and the influence of an audience by measuring association times in a dichotomous design. Males increased their preferences for the initially preferred female when observed by an audience. Nevertheless, as wild-caught individuals were used by Ziege et al. (2008) we initially hypothesized that those effects might have been an artifact of testing wild caught fish rather than representing the species’ normal behavioral repertoire; wild *H. milleri* were clearly not accustomed to the handling stress. While being stressed, wild-caught *H. milleri* males may not have perceived a fourth fish (the audience male) as a rival, but “safety-in-numbers” due to the addition of another shoal mate may have had a calming effect (Queiroz & Magurran 2005), resulting in an increased motivation to mate. However, after confirming these results in the present study, we now ask why male *H. milleri* behave differently compared to other poeciliids – which typically decrease their sexual activity and interact more with the initially non-preferred female when presented with an audience male – especially compared to members of the closely related genus Gambusa (see Bierbach et al. 2012).

One hypothesis that has been put forward to explain the unusual behavior of *H. milleri* males was that males could try to monopolize preferred females and defend them from other males (Ziege et al. 2008). Preliminary data from a comparison of male aggressive behavior in several poeciliids, however, identify *H. milleri* as being little aggressive (D. Bierbach et al., unpublished data) while high aggression would be predicted if overt competition with rivals played a major role.

Generally, it was argued that audience effects (i.e., reduced sexual behavior, reduced mating preferences, and approach of the usually non-preferred female first) can be interpreted as tactics to reduce sperm competition risk arising from male mate choice copying (see Schlupp & Ryan 1997; Plath & Schlupp 2008; Plath & Bierbach 2011). However, little is known about male mate choice copying in the genus *Heterophallus* as well as in the related genus Gambusa. Possibly, male mate choice copying does not play a major role in the reproductive biology of *H. milleri* (for a similar argument see Ziege et al. 2008). In this context, it will be important to not only establish whether or not males copy other males’ choices under laboratory conditions, but especially knowledge about the probability for male mate choice copying to occur under natural conditions is vital to our understanding of the evolutionary forces shaping male behavioral responses to rivals (Plath & Bierbach 2011). In summary, we are as yet lacking a compelling answer as to the question of why *H. milleri* males differ in their response to an audience from any other poeciliid investigated so far. Our study, therefore, identifies *H. milleri* as being of central importance for future studies looking into adaptive explanations of audience-induced behavioral modification in male poeciliid fishes, especially when trying to answer the question why closely related species differ in their behavioral repertoire.

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