

Innate responses to male sexual harassment in female mosquitofish

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Abstract Male mosquitofish are very persistent in their sexual activity and harass any female they encounter. Gravid females pay a large tribute to this intense male sexual activity in terms of reduced foraging efficiency. Previous observations have demonstrated that gravid females, when chased by a male, dilute male harassment by moving closer to other females to form shoals. They also approach other males to promote male competition, and when males differ in size, they preferentially target large males, whose harassment is less intense. In this study, we tested whether the modulation of females' social preferences in response to male harassment is innate or learned. We tested social preference in three groups of females that differed in experience of sexual harassment and in the factors affecting it. Females of the first group were reared without any sexual experience, and pregnancy was induced through artificial insemination. The second group was composed of naive females kept singly with a male; these females experienced sexual harassment but were prevented from experiencing the effects of male–male competition and shoaling on the amount of male sexual harassment. In the third group (controls), females were reared in multi-male, multi-female groups and could experience the modulating

effects of social interactions on sexual harassment. When exposed to a harassing male, females of the three groups immediately reduced their distance from another female, approached a group of males or moved toward the larger of two available males. Moreover, the results for these three groups of females were similar to those obtained in wild-caught females that were tested in the same three tests in a previous study (Dadda et al. *An. Behav.*, 70:463–471, 2005). This suggests that the strategies adopted by females in response to male sexual harassment do not need to be learned through specific experience of the social contexts.

Keywords Sexual harassment · Shoaling · Mosquitofish · Poeciliid fish

Introduction

In recent years, the classical paradigm of sexual selection as an adaptive process (i.e. a benefits-based process) has been challenged by the recognition of the role of sexual conflict in the co-evolution of male and female reproductive strategies (Parker 1979; Rice 1996; Holland and Rice 1998; Hosken et al. 2001; Chapman et al. 2003; Arnqvist and Rowe 2005). The classical view of reproduction as a predominantly cooperative process among mates has therefore been progressively challenged by evidence that the two sexes diverge in their evolutionary interests, for example, regarding the optimal mating rate (Bateman 1948; Parker 1979). Sexual conflict may thus give rise to an antagonistic co-evolutionary process in which adaptation by one sex promotes the evolution of counter-adaptations in the other sex (Parker 1979; Rice 1996; Arnqvist and Rowe 2002). Models of sexually antagonistic co-evolution predict that male and female traits should evolve in response to

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antagonistic selection forces and be genetically determined (Parker 1979; Holland and Rice 1998; Rowe et al. 2005; Hardling and Smith 2005). Evidence that sexual conflict determines the evolution of antagonistic traits is growing (Chapman et al. 1995; Rice 1996; Holland and Rice 1999; Hosken et al. 2001; Pitnick and Garcia 2002; Arnqvist and Rowe 2002; Wigby and Chapman 2004; Linder and Rice 2005; Friberg 2005). To the best of our knowledge, however, the genetic basis of sexually antagonistic traits has thus far been studied only for morphological and physiological traits, and evidence that complex behavioural counter-strategies have evolved in response to sexual antagonism is still lacking.

In this study, we provide evidence that a complex, innate social strategy has evolved in female eastern mosquitofish (*Gambusia holbrooki*), a poeciliid fish in which male sexual harassment is intense (Bisazza et al. 1996; Pilastro et al. 2003). Poeciliids, a group of freshwater fish with internal fertilization, represent a paradigmatic case of sexual conflict in mating frequency. Male poeciliids are among the most persistent in the animal kingdom, their sexual activity reaching one sexual act per minute (Magurran and Seghers 1994a; Bisazza et al. 1996). Conversely, females can store sperm for months, and only a few copulations ensure the fertilization of all their eggs for the whole breeding season (Dulzetto 1928; Constantz 1984). This intense male sexual activity is likely to be costly to the female. In guppies, male courtship attracts predators that usually prefer to capture the female (Pocklington and Dill 1995). Males may even exploit female anti-predator vigilance and try to sneak-copulate with a female reacting to a predator (Magurran and Nowak 1991; Evans et al. 2003a). In several species, male sexual behaviour has also been shown to interfere with females' foraging activity (Magurran and Seghers 1994a; Griffiths 1996; Schlupp et al. 2001; Pilastro et al. 2003). In the eastern mosquitofish, *G. holbrooki*, a poeciliid species in which males rely mainly on sneak-mating attempts for fertilization, it has been found that male presence can halve a female's foraging efficiency (Pilastro et al. 2003). In this species, the amount of sexual harassment and consequent reduction of foraging efficiency vary according to shoal size and sex ratio. For example, females in shoals receive fewer mating attempts than isolated females, the dilution effect increasing with shoal size (Pilastro et al. 2003). Small males harass more intensely than large males and single males more than grouped males, since males compete for access to the female and have less opportunity to attempt copulations while engaged in a fight (Bisazza and Marin 1995; Pilastro et al. 2003). Consistently with these observations, it has been found that, in the presence of a sexually active male, single gravid females change their social preferences and (1) move closer to other females to form shoals, (2) move toward groups of males, and/or (3)

move toward a larger male (Dadda et al. 2005; Agrillo et al. 2006).

Here, we investigated whether the strategies used by females to reduce harassment are innate or whether females learn them through social experience. In principle, females could assess the consequences of their social decisions by measuring the intensity of male harassment in different situations. Each female may therefore learn how to reduce the cost of male sexual harassment through the association between their social decisions and the experienced level of male harassment (and/or foraging efficiency). Since, however, the direct costs of sexual harassment are high (Pocklington and Dill 1995; Pilastro et al. 2003) and the reproductive life of a female mosquitofish is rather short (Hughes 1985; Reznick et al. 2006), we expect natural selection to favour behavioural responses that are innate, so that the costs of sexual conflict can be minimised from the beginning of reproductive life.

Reaction to male sexual harassment was studied in three groups of females raised in the laboratory; the first group was prevented from experiencing the influence of male size, male–male competition, and shoaling on the levels of male sexual harassment; the second group was totally deprived of experience with male sexual harassment; a third group (controls) was raised and maintained in a mixed-sex group and learnt how to reduce sexual harassment. Furthermore, we compared the data of these three groups of females with those of wild-caught that were tested in the same three tests in a previous study (Dadda et al. 2005).

Materials and methods

Fish maintenance and experimental conditions

Individuals were collected from Valle Averte, the Venetian lagoon, in Italy. The fish were carried to the laboratory and maintained in groups (20–25 individuals) in several stock tanks (150 l, provided with gravel and an air-filter). Young sexually undifferentiated fish (TL < 15 mm) were separated from the others and placed in aquaria. Aquaria were provided with live plants (*Ceratophyllum*), illuminated by one 15-W fluorescent light, maintained at a constant temperature ($25 \pm 1^\circ\text{C}$) and photoperiod (0600–2000 hours). Fish were fed twice a day with commercial food flakes and live *Artemia* nauplii.

In order to obtain virgin females, we inspected the fish daily for sexual development. In this species, individuals that develop into males can easily be recognised several weeks before sexual maturation, since the anal fin slowly develops into a copulatory organ, the gonopodium. Development starts with the thickening, and then elongation of

the third, fourth and fifth rays of the anal fin (Dulzetto 1928). Developing males were recognised before they started sexual activity and immediately removed from the tank. After sexual maturation, females were assigned to one of three different treatments.

In treatment A (control group), females were offered a conventional life experience in a multi-male, multi-female group. In this treatment, groups of four females were kept for a period of eight days in a large tank ($160 \times 50 \times 40$ cm $w \times d \times h$) with four adult males of different size (largest male, 27.31 mm; smallest male, 20.87 mm; XSD, 23.24 ± 1.53 mm) (sex ratio 1:1). The tank was divided into four identical sectors ($40 \times 60 \times 36$ cm $w \times d \times h$) by means of three rectangular plastic panels provided with a circular opening (\varnothing 5 cm) that allowed the fish to move from one sector to another.

In treatment B, females could experience sexual activity but they were prevented from experiencing the effects that male–male competition, size-associated variations in male sexual behaviour and shoaling have on sexual harassment (Pilastro et al. 2003). In this treatment, each female was kept singly with one male for 8 days in tanks of the same size ($40 \times 60 \times 36$ cm $w \times d \times h$) as the sectors in treatment A. Four replicate tanks were used and males within each tank were matched for size (XSD, 21.02 ± 1.23 mm). Females were switched daily between tanks so that they were paired with all four males.

In treatment C, females had no experience of males, and we induced pregnancy through artificial insemination. The procedure for artificial insemination was similar to that described by Evans et al. (2003b). Sperm were manually stripped from males following Matthews et al. (1997). In *G. holbrooki* males, as in other poeciliid fish, sperm are packaged in bundles (spermatozeugmata), each containing on average about 4,000 individual sperm cells (Constantz 1989). For each insemination, a virgin female was anaesthetised in a water bath containing a mild dose of MS222 and placed in a polystyrene ‘cradle’ with her genital pore exposed. A micropipette was used to inseminate twelve sperm bundles from each stripped ejaculate (suspended in 10 l of 0.9% NaCl) into the female gonoduct (penetration depth approximately 2 mm). After insemination, females were maintained alone for 2 weeks in order to verify the success of the insemination. Insemination was successful in 83% of the cases.

Forty-four females were used in this study: 16 assigned to treatment A, 16 to treatment B and 12 to treatment C. All females were gravid at the time of the test as judged by rounded belly and by the presence of a black ‘gravid spot’. Three different experiments were performed to estimate the responses to sexual harassment. Experiment 1 measured social distance among females according to whether an adult male was visible to the females or not; experiment 2

tested the tendency of a single female to approach a group of males prior to and after the introduction of a harassing male into the tank; experiment 3 was aimed at investigating the tendency of a single female to approach large or small males prior to and after the introduction of the harassing male into the tank. All females were tested first in experiment 1. Half of the females used in experiment 1 were also used in experiment 2, while the remaining half were used in experiment 3. It is worth noting that, in experiment 1, females could not experience sexual harassment and had only visual (not direct) contact with males.

All subjects were fed to satiation before the beginning of the trials. Sexually mature males were used for the experiments. No mortality was observed during the experiments, and all fish were released back into the capture locality at the end of the experiments. The standard length (XSD) of the fish was measured from the digital recordings of the experiments.

The females used in this study, including the control group, had limited experience with adults of their species and with other features of their natural environment. To verify whether females that reached sexual maturity in a complex natural environment showed any differences in social behaviour compared with females matured in the laboratory, we compared the data of the three groups of females of this study with those of wild-caught females (Dadda et al. 2005).

Experiment 1: female aggregation in the presence of a male

The apparatus used was the same as that described by Dadda et al. (2005). Briefly, the experimental tank was a circular arena (diameter, 65 cm) filled with 15 cm of water and illuminated with four 8-W fluorescent lamps. A hollow and transparent plexiglas cylinder (diameter, 19.5 cm; height, 16.5 cm) was placed in the centre of the arena to enclose the stimulus. A second, opaque cylinder (diameter, 19 cm; height, 16.5 cm) was inserted into the first and suspended on a monofilament line attached to a pulley system allowing us to move it up and down. Two females from the same treatment, matched in size (standard length difference, ≤ 2 mm), were introduced into the arena and allowed to settle for 1 h. After this period, one male mosquitofish was put into the central cylinder. The male could be hidden or shown to the females by movement of the opaque plastic cylinder up or down.

We tested eight female pairs from treatment A, eight female pairs from treatment B and six female pairs from treatment C. Each trial consisted of eight observation periods, including four observations with the male visible and four with the male hidden. Each observation period lasted 30 min and was separated by a 10-min interval. To randomise the order of presentation (male present, male

absent), half of the trials of each treatment started with a period with the male visible in the central cylinder and half with the male kept hidden from the shoaling females. A video camera, positioned about 2 m above the centre of the apparatus, was used to record the trials. Video recordings were subsequently digitalised. We examined three frames per minute and, by means of a computer program (originally developed in our lab, written in Delphi5 Borland), we took two measures of the distance between the females: (1) the linear distance between the two females and (2) the angle obtained by virtually connecting the head of the two females with the centre of the apparatus. In total, we obtained 720 distance measurements for each trial (360 with the stimulus fish visible to the two experimental females and 360 with the stimulus fish hidden). From these measures, we calculated the mean distance and the mean angle between the two experimental females for each 30-min period.

Experiment 2: movement towards male groups by harassed females

The experimental apparatus was the same as that used in a previous study (Dadda et al. 2005). Briefly, the apparatus (Fig. 1) consists of two tanks. The first one (subject chamber; $40 \times 60 \times 36$ cm $w \times d \times h$) was placed in front of the second tank (stimulus tank; $30 \times 60 \times 36$ cm $w \times d \times h$). The subject chamber was divided into two communicating compartments by a 2×2 mm net in a 25×36 cm plastic

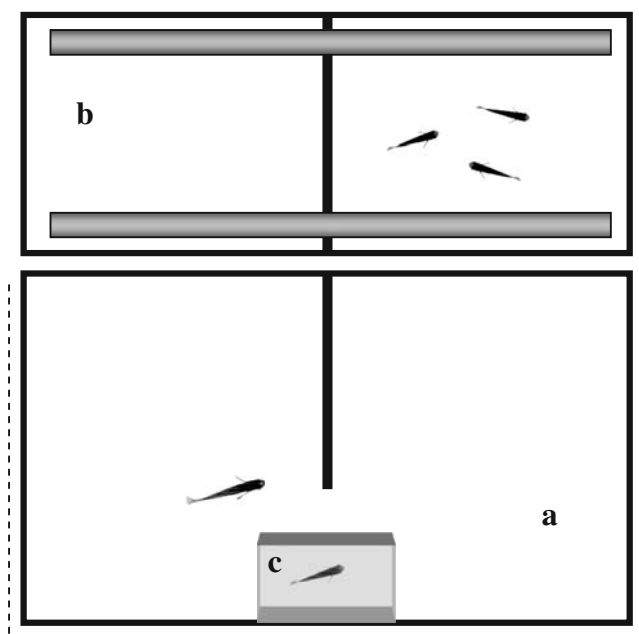


Fig. 1 Schematic representation of the apparatus used in the second and third experiments. Subject was inserted in the subject tank (a) placed in front of the stimulus tank (b). Harassing male was placed in the start-box (c) and was provided with a trapdoor leading into the subject chamber

frame attached to the back of the chamber, allowing the test female to swim from one side to the other. A small plastic ‘start-box’ ($10 \times 7.5 \times 20$ cm $w \times d \times h$) was attached to the front of the subject chamber. The start-box contained a male (mean standard length, 20.7 ± 1.08 mm, $n=22$) and was provided with a trapdoor leading into the subject chamber. By raising the door, the male could enter the subject chamber. The stimulus tank was divided in half by a plastic partition. Two 15-W fluorescent lamps were placed on the two sides of the stimulus tank. A video camera was mounted about 1 m above the subject chamber and was used to record the position of the female during the trials. The evening before the test, a female was taken from the treatment tank and put into the subject chamber immediately preceding the introduction of three sexually mature males (21.7 ± 2.63 mm, $n=48$) into one of the two side compartments of the stimulus tank (right and left positions alternated between trials). The following morning, a sexually mature male was put in the start-box and allowed to settle for 10 min. The position of the female was then recorded for a period of 30 min (preliminary period). At the end of this first observation period, the male was released into the subject chamber, and the female’s position was recorded for a further 30 min (test period).

We tested eight females with treatment A, eight females with treatment B and six females with treatment C. As in a previous study (Dadda et al. 2005), the preference of the female was determined using a scan-sampling method (Altmann 1974). The position of the female was determined every 15 s from the video recordings. Three lines superimposed on the video allowed us to virtually divide the subject chamber into three equal-sized longitudinal sectors, and time spent in the compartments was inferred from a total of 120 observations without the male and 120 observations with the harassing male present. The preference determined with this method is a close approximation of preference computed directly from the time spent in each compartment (Agrillo et al. 2006), where the females were considered to be associating with a stimulus when they were in a sector of 13.3 cm (corresponding to one third of the tank size) from the glass facing the stimulus tank.

Experiment 3: vicinity preference for large males by harassed females

The apparatus and procedure used for this experiment were the same as that described for experiment 2, except that in the stimulus tank, we put one large male (28.5 ± 1.44 mm, $n=22$) into one side compartment and one small male (18.8 ± 1.32 mm, $n=22$) into the other side compartment. We tested eight females with treatment A, eight females with treatment B and six females with treatment C; 22 harassing males were used in this experiment (21.2 ± 1.96 mm).

Statistical methods

Data were tested for normality and homogeneity of variance. Proportions were arcsin (square root)-transformed. All tests were two-tailed. SPSS 11.5.1 was used for all statistics. Means are given \pm SD.

Results

Experiment 1: female aggregation in the presence of a male

Data were analysed by use of a repeated measures analysis of variance (ANOVA) in which the presence of the male and succession of the observation periods were the within-group factors, while the order of presentation (whether the experiment started with the male visible or not visible to the females) was the between-group factor.

Control group females (with experience of a multi-male, multi-female group) swam significantly closer together (linear distance $F_{1,6}=36.869$, $p<0.001$) when the male was visible than when he was not visible. While the distances between females were not influenced by presentation order ($F_{1,6}=1.399$, $p=0.282$), they decreased significantly with time ($F_{3,18}=3.274$, $p=0.045$). None of the interactions were significant.

Females from treatment B (a single female kept with a male) exhibited the same behaviour, swimming significantly closer (linear distance $F_{1,6}=20.487$, $p=0.004$) when the male was visible. While the distances between subjects were not influenced by presentation order ($F_{1,6}=0.008$, $p=0.931$), they decreased significantly with time ($F_{3,18}=3.192$, $p=0.049$). None of the interactions were significant.

Females from treatment C (females artificially inseminated) swam significantly closer (linear distance $F_{1,4}=54.430$, $p=0.002$) when the male was visible. Shoaling distance was not influenced by presentation order ($F_{1,4}=0.193$, $p=0.683$)

and did not decrease significantly with time ($F_{3,12}=1.739$, $p=0.212$). None of the interactions were significant.

Similar results were found for each group when the angle between females was considered. As shown in Table 1, repeated measures ANOVA revealed that, when the male was visible, females swam significantly closer than when he was not visible in all the three treatments. No other factors nor interactions were significant.

An analysis of the pooled data from the three treatments was performed with a repeated measures ANOVA with treatment (control group, B or C) as between-group factor. Females swam significantly closer together (linear distance $F_{1,16}=74.343$, $p<0.001$; Fig. 2) when the male was visible (mean distance $11.18\text{ cm}\pm 2.29$) than when he was not visible ($7.19\text{ cm}\pm 1.17$). The distance between females was not influenced by presentation order ($F_{1,16}=0.338$, $p=0.574$) while it decreased significantly with time ($F_{3,48}=3.593$, $p=0.007$). No difference was found between the three treatments ($F_{2,16}=2.178$, $p=0.148$, power=0.375, effect size partial eta-squared $\eta_p^2 = 0.225$). None of the interactions were significant. Similar results were found when the angle between females was considered.

Comparing the shoaling behaviour of the three different treatments (control group, treatments B and C) with the results of a previous study (Dadda et al. 2005), we verified that the behaviour of these females did not differ from that of wild-caught females (linear distance: $F_{3,25}=1.765$, $p=0.18$, power=0.403, $\eta_p^2 = 0.275$; angle between females: $F_{3,25}=0.724$, $p=0.547$, power=0.181, $\eta_p^2 = 0.109$).

Experiment 2: movement towards male groups by harassed females

During the preliminary period (before we released the male into the subject chamber), females of both experimental treatments tended to avoid the sector near the stimulus tank on both sides of the subject chamber, as did the control

Table 1 Female aggregation in the presence of a male

Treatments	Mean angle between females		Succession of observation periods	Order of presentation
	Male visible (mean \pm SD)	Male hidden (mean \pm SD)		
Controls (treatment A)	25.42 \pm 3.42° $F_{(1, 6)}=9.826$, $p=0.020$	35.69 \pm 10.32	$F_{(3, 18)}=2.069$, $p=0.137$	$F_{(1, 6)}=0.464$, $p=0.521$
Treatment B	23.11 \pm 2.91 $F_{(1, 6)}=23.170$, $p=0.003$	37.35 \pm 6.80	$F_{(3, 18)}=1.423$, $p=0.269$	$F_{(1, 6)}=2.107$, $p=0.197$
Treatment C	24.77 \pm 3.55 $F_{(1, 6)}=32.675$, $p=0.005$	31.59 \pm 3.46	$F_{(3, 12)}=2.007$, $p=0.167$	$F_{(1, 6)}=1.125$, $p=0.349$

The table represents the results of the repeated measures ANOVA in which the presence of the male and succession of the observation periods were the within-group factors, while the order of presentation (whether the experiment started with the male visible or not visible to the females) was the between-group factor. When the male was visible, females swam significantly closer than when he was hidden in all the three treatments. Mean angle \pm SD in degrees is given.

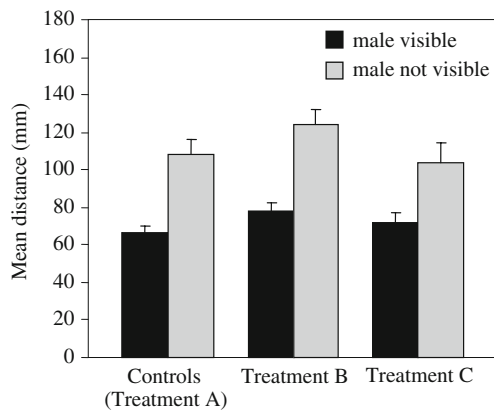


Fig. 2 Female aggregation in the presence of a male. Mean distance \pm SE in millimetres between two experimental females with a male visible to or hidden from the females. Each bar represents the mean of four observation periods each lasting 30 min. Control females (treatment A) were maintained in a multi-male–multi-female group. In treatment B, each single female was maintained with a single male, and in treatment C, females were artificially inseminated and had no sexual experience

group (treatment A; Fig. 3). The number of observations in the sectors near the stimulus tank was significantly below the chance level for all three treatments (one sample t test, $p < 0.05$ in all tests). Females spent a similar proportion of time in the two halves of the subject chamber, and there were no significant differences between the three treatments (control group 0.51 ± 0.04 , $t_7 = 0.914$, $p = 0.391$; treatment B 0.50 ± 0.02 , $t_7 = 0.991$, $p = 0.981$; treatment C 0.51 ± 0.03 , $t_5 = 0.725$, $p = 0.501$).

Once the harassing male was released into the subject chamber, the female increased occupancy of the sector that

was in front of the three males. The number of observations in this sector was significantly above chance in all three treatments (one sample t test, $p < 0.001$ in all tests). During the test phase, females from all three treatments preferentially occupied half of the tank in front of the three males (control group 0.63 ± 0.06 , $t_7 = 6.469$, $p < 0.001$; treatment B 0.66 ± 0.03 , $t_7 = 13.894$, $p < 0.001$; treatment C 0.67 ± 0.02 , $t_5 = 15.558$, $p < 0.001$).

An analysis of all three treatments together was performed with repeated measures ANOVA in which the preliminary and test periods were the within-group factors while the treatment was the between-subjects factor. In contrast with the preliminary period, after the release of the male, females preferentially occupied the side of the subject chamber that was in front of the males ($F_{1,19} = 152.46$, $p < 0.001$). There was no significant effect owing to the treatment ($F_{2,19} = 0.576$, $p = 0.572$, power = 0.131, $\eta_p^2 = 0.056$) nor to the interaction between factors ($F_{2,19} = 1.097$, $p = 0.354$, power = 0.211, $\eta_p^2 = 0.203$). The behaviour of females in all three treatments of this study did not differ from that exhibited in the same test by females maturing in the wild (Dadda et al. 2005; treatment effect, $F_{3,26} = 0.312$, $p = 0.817$, power = 0.102, $\eta_p^2 = 0.036$; preliminary and test periods, $F_{1,26} = 117.06$, $p < 0.001$; interaction, $F_{3,26} = 1.497$, $p = 0.239$).

Experiment 3: vicinity preference for large males by harassed females

During the preliminary period, females in all treatments tended to avoid the sectors near the large and the small males (Fig. 4). The number of observations in the sectors

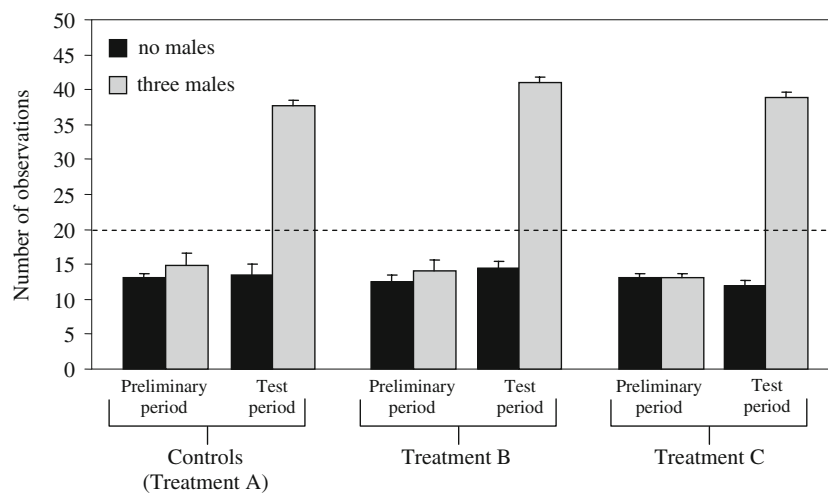


Fig. 3 Vicinity preference for male groups by harassed females in control females (maintained in a multi-male–multi-female group), treatment B (female maintained with a single male) and in treatment C (females artificially inseminated). Frequency (number of observations and standard error) of occupancy by the test female of the sectors near

an empty compartment or near a compartment containing a group of three males before (preliminary period) and after (test period) the introduction of a harassing male. The dotted line indicates the occupancy expected by chance

near the stimulus tank was significantly lower the chance level for all three treatments (one sample t test, $p < 0.01$ in all tests). Females spent a similar proportion of time in the two halves of the subject chamber and there was no significant difference between the treatments (control group 0.49 ± 0.02 , $t_7 = 0.271$, $p = 0.794$; treatment B 0.50 ± 0.02 , $t_7 = 0.505$, $p = 0.629$; treatment C 0.49 ± 0.05 , $t_5 = 0.235$, $p = 0.853$).

Once the harassing male was released, the female increased occupancy of the sector in front of the larger male. The number of observations in this sector was significantly above chance for the three treatments (t test, $p < 0.001$ in all tests). During the test phase, females from all three treatments preferentially occupied half of the tank in front of the larger male (control group 0.61 ± 0.02 , $t_7 = 13.896$, $p < 0.001$, treatment B 0.62 ± 0.03 , $t_7 = 11.066$, $p < 0.001$, treatment C 0.66 ± 0.05 , $t_5 = 7.906$, $p < 0.001$).

An analysis of all three treatments together was performed with a repeated measures ANOVA. Compared with the preliminary period, after the release of the harassing male, females preferentially occupied the side of the subject chamber that was in front of the larger male ($F_{1,19} = 197.58$, $p < 0.001$). There was no significant effect owing to the treatment ($F_{2,19} = 1.205$, $p = 0.322$, power = 0.231, $\eta_p^2 = 0.107$) nor to the interaction between factors ($F_{2,19} = 3.016$, $p = 0.073$, power = 0.516, $\eta_p^2 = 0.235$). The behaviour of females in all three treatments did not differ from that exhibited in the same test by females maturing in the wild and observed by Dadda et al. (2005; treatment effect, $F_{3,28} = 1.878$, $p = 0.189$, power = 0.431, $\eta_p^2 = 0.214$; preliminary and test periods, $F_{1,28} = 252.87$, $p < 0.001$; interaction, $F_{3,28} = 1.872$, $p = 0.157$).

Discussion

The results of these three experiments confirm and extend earlier observations of female strategies to reduce sexual harassment (Dadda et al. 2005; Agrillo et al. 2006). Like females maturing in their natural environment, gravid females maturing in the laboratory with experience of a mixed-sex group in a complex environment exhibited a set of strategies to reduce the costs imposed by intense male sexual activity. At the appearance of a harassing male, they changed their social preferences: They significantly reduced their distance to another female, approached groups of males or preferred to stay close to the larger of two available males. We have shown previously that these changes in the social behaviour are specific to male presence and are not elicited by the sight of another female or by a sham liberation of a harassing male (Dadda et al. 2005). All three strategies have previously been shown to reduce the intensity of sexual harassment with an immediate beneficial effect on the foraging efficiency of the female (Pilastro et al. 2003). The same three strategies were adopted by treatment B females that, prior to the experiment, only had experience of single, same-size males, and therefore could not learn that shoaling can dilute sexual harassment and that male size and male competitive interactions can affect the intensity of harassment. Even females from treatment C that were artificially inseminated and had no experience of sexual harassment prior to the experiment showed the same change in shoaling strategies the first time they were exposed to a sexually active adult male. Collectively, these results indicate that, in *G. holbrooki*, the ability of females to adopt context-dependent

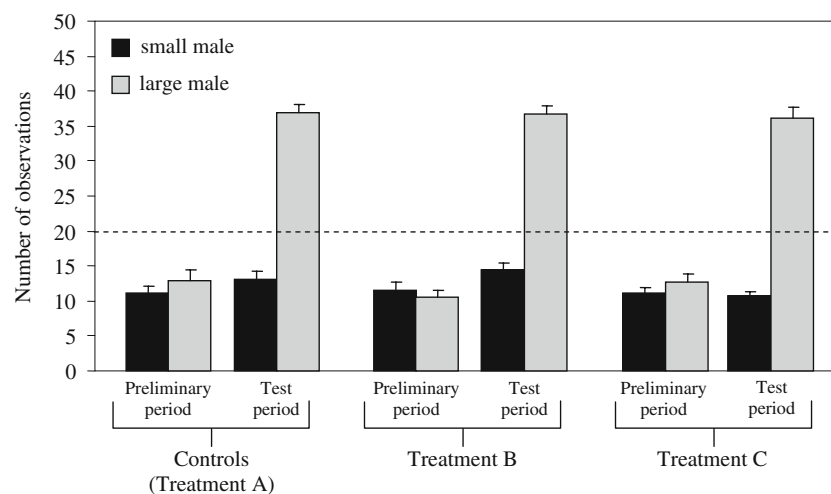


Fig. 4 Vicinity preference for large males by harassed females in the three treatments. Frequency (number of observations and standard error) of occupancy by the test female of the sectors near a large or a

small male, before (preliminary period) and after (test period) the introduction of a harassing male. The dotted line indicates the occupancy expected by chance

shoaling strategies, which minimise the costs of male sexual harassment, has no bearing on their previous experience. Obviously, this does not exclude the possibility that females can make subtle and adaptive adjustments to their anti-harassment repertoire, and that learning from experience plays an important role in reducing the costs of sexual conflict.

The ability to recognise a biologically relevant situation and respond with the appropriate behaviour on the first encounter is often crucial. Many animal species, for example, show an innate ability to recognise their predators, and they exhibit the appropriate anti-predator response without prior experience (van der Ven et al. 2000), as a single failure to escape a predator causes death. In species that lack prolonged parental care, individuals may need to recognise their food innately (Irvine and Northcote 1983; Schuler and Hesse 1985), as the failure to select an appropriate diet may lower their survival in the period just after birth. Sometimes, however, the type of predator or the type of food may vary spatially or temporally in unpredictable ways, and thus, natural selection may favour behavioural plasticity and finely tuned responses that can only be acquired through individual experience (Kelley and Magurran 2003).

The opportunity to learn an appropriate response critically depends on the availability of information during an individual's life course. Mate choice criteria, for example, are frequently innate (Houde and Endler 1990), as individuals cannot assess the fitness consequences of their decisions. Life length and the presence of parental care may thus be crucial variables. In species with a short life and without parental care, selection should favour the ability to recognise predators and to choose the appropriate foods and mates without the need for a long training process. On the other hand, species that have parental care, live longer and reproduce several times in their lives may have a greater opportunity to learn from experience and may rely more often on plastic responses to environmental demands.

In nature, female mosquitofish swim in shallow waters searching for food either alone or in small shoals that change their composition frequently. Males are very sexually active (approximately one mating attempt per minute), and therefore each female spends several hours each day followed by one or more harassing males (McPeck 1992; Bisazza and Marin 1995). Females have many opportunities to measure sexual harassment in different circumstances and to learn the consequences of their shoaling decisions.

There are several possible reasons why this route has not been followed in the course of mosquitofish evolution. One possibility is that the learning process is constrained under natural conditions. Many different factors have been shown to influence the intensity of sexual harassment in poeciliids,

including the distance from other females, the body size of shoal mates, the size of the shoal, the number of males in the shoal, the body size of the harassing male, the reproductive condition of the female and the perceived predation risk (Bisazza et al. 1989; Magurran and Nowak 1991; Pilastro et al. 2003; Dadda et al. 2005; Agrillo et al. 2006). Other factors, such as the physical structure of the environment, vegetation density, the temperature and the water turbidity, are likely to affect the intensity of the sexual harassment. An association between a shoaling decision and its effects on harassment intensity might therefore be masked by the concurrent action of numerous other factors, making it difficult for the female to integrate all of this information.

A second circumstance is when the cost of an inappropriate response is very high. Male sexual harassment has been shown to hamper female foraging in several poeciliids (Magurran and Seghers 1994a; Schlupp et al. 2001), and in laboratory experiments with eastern mosquitofish, the rate of food intake has been shown to halve in a female harassed by a single male (Pilastro et al. 2003). Few mosquitofish survive the winter to have a second reproductive season, and thus most females are expected to produce two or three broods in their whole life (Reznick et al. 2006; Hughes 1985). During this period, females also invest in somatic growth that will ultimately affect the number and the size of sons produced (Bisazza et al. 1989). Even a reduction of food intake far less than the 50% observed in the laboratory and only for a few days at the beginning of their reproductive life could be extremely detrimental to their future reproduction. Females may be undermined in their mate choice and thus also suffer indirect costs (Pilastro et al. 1997; Pilastro and Bisazza 1999; Matthews and Magurran 2000; Bisazza and Pilastro 2000; Bisazza et al. 2001; Evans et al. 2003a). The sum of all these costs could be very high.

Both classical models of the evolution of male sexual secondary traits through female choice and recent models of sexually antagonistic co-evolution require that male and female characteristics are genetically determined (e.g. Fisher 1930; Lande 1980; Kirkpatrick and Ryan 1991; Holland and Rice 1998). To date, research on the genetic basis of female traits has mainly focused on female mate preferences (Houde and Endler 1990; Bakker 1993; Morris et al. 1996; Wilkinson et al. 1998; Bisazza and Pilastro 2000; Brooks and Endler 2001; Iyengar et al. 2002; Muhlhauser and Blanckenhorn 2004; Ritchie et al. 2005; Haesler and Seehausen 2005) and on physiological aspects of female resistance (e.g. Linder and Rice 2005; Friberg 2005). Male poeciliids have developed the most extraordinary morphological and physiological adaptations to circumvent female control of paternity and mating rates. These traits include the smallest size and the longest copulatory organ that can be observed in a vertebrate

(Henn 1912; Chambers 1986): The presence of hooks at the tip of the gonopodium is observed only in some invertebrates (Rosen and Gordon 1953; Constantz 1984; Magurran and Seghers 1994b), and so is the ability to engage in one mating attempt per minute for the whole breeding season (Magurran and Seghers 1994b; Godin 1995; Bisazza and Marin 1995). Females did not obviously evolve comparable morphological counter-adaptations. By varying their shoaling propensity, by choosing between shoalmates on the basis of their phenotype and between shoals on the basis of their size and by inciting male–male competition, female mosquitofish are likely, however, to obtain a substantial reduction of the costs generated by sexual conflict (Pilastro et al. 2003).

One major concern in our results is the sample size. In order to clarify the results, we calculated power and effect size (partial eta-squared). The effect size obtained in our study is often close to zero, and indicates the reliability of the null hypothesis. As reported (Cohen 1988), unlike the significance test, the effect size is largely independent of sample size and represents a measure of the strength of the relationship between variables. According to this, the absence of significant differences between the three treatments reported in this study appears to be consistent despite the relatively small sample size.

The present study represents a partially successful attempt to investigate the origin of female counterstrategies to reduce the costs of sexual harassment. Our findings suggest that female mosquitofish are equipped with a set of complex shoaling strategies that presumably, although flexible and context-dependent, do not require learning. Further research with greater sample size, however, is needed.

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