

Female choice and the relatedness of mates in the guppy (*Poecilia reticulata*)

Mate choice and inbreeding depression

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Abstract Several studies suggest that females may offset the costs of genetic incompatibility by exercising pre-copulatory or post-copulatory mate choice to bias paternity toward more compatible males. One source of genetic incompatibility is the degree of relatedness among mates; unrelated males are expected to be genetically more compatible with a female than her relatives. To address this idea, we investigated the potential for inbreeding depression and paternity biasing mechanisms (pre- and post-copulatory) of inbreeding avoidance in the guppy, *Poecilia reticulata*. Inbreeding resulted in a reduction in offspring number and quality. Females mated to siblings gave birth to significantly fewer offspring compared to females mated to non-siblings and inbred male offspring took longer to reach sexual maturity. There was no evidence of inbreeding avoidance in pre-copulatory behaviors of females or males. Sexual responsiveness of females to courting males and the number of sexual behaviors males directed at females did not decrease as a function of the relatedness of the two individuals. We also tested whether female guppies can use post-copulatory mechanisms to bias sperm usage toward unrelated males by comparing the number of offspring produced by females mated to two of their siblings (SS), two males unrelated to the female (NN), or to one unrelated male and a sibling male (NS). We found that NS females produced a number of offspring not significantly different than what would be expected if fertilization success were

halfway between completely outbreeding (NN) and completely inbreeding (SS) females. This suggests that there is no significant improvement in the number of offspring produced by females mating to both related and unrelated males, relative to that which would be expected if sperm from both males were used equally. Our results suggest that female guppies do not discriminate against closely related males or their sperm.

Keywords Genetic compatibility · Inbreeding depression · Mate choice · Cryptic female choice · Sperm competition

Introduction

An important, incompletely resolved question in the field of sexual selection is: Why are females choosy about their mates in species where males provide only sperm and there are no obvious direct benefits? Mating is often costly and these costs can include increased levels of predation, loss of energy, and decreased foraging time (Pomiankowski 1987; Rowe 1994; Kokko et al. 2002). In light of these costs, any adaptive explanation for the persistence of female choice in non-resource based mating systems requires a counterbalancing benefit to the female. This suggests that females may benefit from mate choice through genetic benefits to their offspring. Potential genetic benefits include the acquisition of good genes or compatible genes (reviewed in Neff and Pitcher 2005).

The good genes hypothesis proposes that females should bias paternity towards males with the most elaborate ornaments because these traits are genetically correlated with traits conferring high fitness (e.g. Zahavi 1975; Andersson 1982, 1986; Pomiankowski 1988; Iwasa and

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Pomiankowski 1991). The genetic compatibility hypothesis proposes that females should bias paternity towards males with the most compatible genomes because interactions between male and female genomes can be important in determining offspring fitness (e.g. Trivers 1972; Zeh and Zeh 1996, 1997, 2003). Although genetic incompatibility may operate through a range of mechanisms, including endosymbionts, selfish genetic elements, and coadapted gene complexes, the genetic similarity or relatedness of parents is undoubtedly a major source of incompatibility (reviewed in Tregenza and Wedell 2000).

The relatedness of parents can have important implications for offspring fitness because the risk of inbreeding depression increases with genetic similarity. Deleterious effects of inbreeding, including reduced offspring numbers and survival in domesticated animals and in natural populations, are well documented (reviewed in Keller and Waller 2002). To avoid these costs, individuals could recognize and avoid mating with kin (i.e. pre-copulatory processes), and/or females could discriminate against the sperm of kin (i.e. post-copulatory processes). Pre-copulatory avoidance of kin has been demonstrated in some vertebrates and invertebrates (e.g. Simmons 1991; Stow and Sunnucks 2004) but not others (e.g. Keller and Arcese 1998; Keane et al. 1996). If multiply mating females are unable to recognize or avoid mating with related individuals, the ability to discriminate against the sperm of related males at the post-copulatory level would enable them to reduce costs of inbreeding (see Tregenza and Wedell 2000; Simmons 2005). The strongest evidence to date for post-copulatory female mate choice for unrelated males comes from field crickets (*Gryllus bimaculatus*), in which females multiply mate and apparently bias paternity in favor of more distantly related males (e.g. Tregenza and Wedell 2002; Bretman et al. 2004; but see Jennions et al. 2004 for another species of cricket). If this ability of females to bias sperm use away from kin is shared by other species, where the costs and risk of inbreeding are high, this form of genetic incompatibility avoidance may be an important factor promoting the evolution of multiple mating in species with non-resource based mating systems.

Guppies are live-bearing fish with internal fertilization and a promiscuous, non-resource based mating system in which both female pre- and post-copulatory mate choice play a role in determining paternity (e.g. Houde 1988; Evans et al. 2003a; Pitcher et al. 2003). Genetic and behavioral studies indicate that multiple mating is common in female guppies (e.g. Evans and Magurran 2000; Kelly et al. 1999; Neff et al. 2007). There is already some evidence for inbreeding depression in wild and domestic populations of this species (e.g. Farr and Peters 1984; Sheridan and Pomiankowski 1997; Shikano and Taniguchi 2002; Nakadate et al. 2003; van Oosterhout et al. 2003; Mariette et al. 2006). The possibility that guppies could

suffer inbreeding in natural populations is likely for several reasons: small groups of guppies can become isolated for several months in small pools of water during the dry season (Griffiths and Magurran 1997), and relatedness is apparently high within isolated sections of stream (based on Y-linked color patterns (Rodd and Pitcher pers. obs.) and microsatellite data (Hain and Neff 2007)). Hain and Neff (2007) found that 16% of randomly chosen pairs of individuals in a wild population of guppies were more related than half-siblings. Therefore, one might expect that female guppies would have evolved the ability to avoid copulating with and using the sperm of kin.

In this study, we asked whether guppies suffer deleterious effects of inbreeding with respect to key life history variables and, if so, whether females or males discriminate against related individuals via pre- or post-copulatory paternity biasing mechanisms. To test these questions, pairs of unrelated females were allowed to interact freely with related and/or unrelated males. We observed the mating trials for evidence of pre-copulatory behavioral avoidance of kin, and we then isolated the females and reared their offspring to maturity to look for evidence of inbreeding depression. We also compared offspring number among female mating treatments to see whether there was any evidence for post-copulatory inbreeding avoidance (see Tregenza and Wedell 2002).

Materials and methods

Experimental fish

Fish used in this study were descendents of wild caught fish (held in the lab for two generations) from the Oropuche River in the Northern Range, Trinidad (10°39.570' N 061°07.868' W). This river is a high-predation locale where guppies co-exist with several species of predator including the cichlid, *Crenicichla alta*. Guppies used in this experiment were obtained by isolating females inseminated by a single male (a different male for each female) in individual 20 l aquaria. Once females gave birth, the offspring were reared in family groups in 20 l aquaria; female and male offspring were separated as they approached sexual maturity and before males developed color patterns. Females were raised in visual isolation from adult males because exposure to male patterns can influence subsequent mate choice decisions (Breden et al. 1995; Rosenqvist and Houde 1997; Hughes et al. 1999). Males were housed with unrelated females once they reached sexual maturity to ensure that they developed normal sperm stores and sexual behavior (Bozynski and Liley 2003; Field and Waite 2004). All fish were sexually mature and 4–6 months old when used in mate choice trials.

Behavioral observations and mating treatments

Trials ($n = 60$) were conducted in 60 l aquaria containing an air stone and beige colored gravel, with tan paper attached to the back and sides to provide a uniform visual environment. Tanks were maintained at 25°C and illuminated on a 12:12 light:dark cycle (starting at 07:00 EST) with a fluorescent bulb (Vitalite Ltd.) placed 30 cm above the water surface. Observations were conducted in an otherwise darkened room during the morning hours between 0800 EST and 1200 EST.

There were two treatments, both consisting of two females swimming and interacting freely with male dyads differing in their degree of relatedness to the females (see Fig. 1). The “no choice” treatment consisted of two females, unrelated to each other, and two males that were brothers of one female and unrelated to the other female ($N = 40$, see Fig. 1a). The *no choice* designation refers to the fact that the females had no choice in terms of whether they would have to inbreed or outbreed. The “choice” treatment consisted of two females, unrelated to each other, and two males, each was a brother of one female and unrelated to the other female ($N = 20$, see Fig. 1b). The *choice* designation refers to the fact that females in this treatment have a choice of a related and unrelated male.

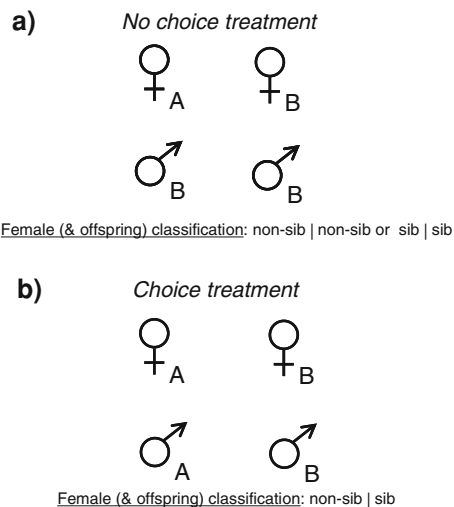


Fig. 1 Schematic representation of the two treatments (see section ‘Materials and methods’ for details) and the designations of females and their offspring. The *no choice* designation indicates that the females had no choice in terms of whether they must inbreed or outbreed; the *choice* treatment indicates that females had a choice between a related and unrelated male. The letters “A” and “B” refer to the individual’s family. Females (and their offspring) from the *no choice* treatment were classified as non-sib/non-sib (NN) for the female that was unrelated to both males and sibsib (SS) for the female related to both males. Females (and their offspring) from the *choice* treatment were all classified as non-sibsib (NS) because both females had access to both a related and unrelated male

An even sex ratio was used in both treatments in an attempt to minimize any potentially confounding effects of male-male competition. The identity of individual males was established using differences in color patterns (spot colors and locations) and individual females were identified with small fin clips of the caudal fin (top or bottom corner of the fin).

Females and their offspring were classified based on the males present in the mating trials (see Fig. 1). For the *no choice* treatment, females and their offspring that were unrelated to either male are called non-sib/non-sib (NN); females and their offspring related to both males are called sibsib (SS) (see Fig. 1a). Females and their offspring from the *choice* treatment are called non-sibsib (NS) because females had access to a related and unrelated male (see Fig. 1b). NN (outbred) and SS (inbred) offspring were compared for effects of inbreeding depression (see below).

Because female guppies from some populations prefer larger males (e.g. Reynolds and Gross 1992; Endler and Houde 1995) and males with relatively more black and orange coloration (Endler and Houde 1995), each male dyad was chosen by eye to be of similar body size and coloration. In our study population, females do not appear to show any preference for any particular spot color but do show an aversion to males with black and blue/violet coloration (Endler and Houde 1995). Also, to avoid biases among males due to their preference for larger females (e.g. Dosen and Montgomerie 2004), females in each dyad were chosen by eye to be of similar body size.

For each trial, the experiment began at 08:00 a.m. (EST) when both females were placed in an aquarium. So that females could see, but not mate with, the males, each male was placed in a separate clear rectangular Plexiglas holding pen (15 cm × 15 cm) that reached above the surface of the water and was situated on either the far left or far right side of the experimental tank. These pens possessed small flow-through holes on all four sides in order to allow water and any olfactory cues to pass in and out of the pen. The fish were left for 24 h to acclimate to the tank and each other. At 8:00 a.m. the following morning, the male pens were removed at which point the males and females could freely interact (i.e. free-swim) for 2 h. The foursome was observed continuously for the entire 2 h for the *choice* treatment and for the first hour for the *no choice* treatment. The difference in the amount of observation time in each treatment was due to logistical constraints of collecting behavioral data for each grouping (i.e. SS, NN, SN) in 1 day. More prolonged behavioral data was collected for SN pairings to ensure that complete mating information was collected for females that could have potentially mated with both the unrelated and related males, in case there was evidence of post-copulatory biasing of sperm usage in this group.

A male's most intense courtship display, the sigmoid, is characterized by the male orientating himself in front of a female and vibrating his body in an S-shaped posture (Houde 1997). A measure of female sexual responsiveness to a particular male can be estimated by the percentage of sigmoids to which a female responds to in a positive fashion (see Houde 1988, 1997; Pitcher et al. 2003). During the free swim observation period, the sexual responsiveness of each of the females to each of the males was calculated as the total (i.e., summed across the entire observation period) proportion of sigmoids to which a female responded to positively. Following Houde (1988), we deemed a response positive when the female, at a minimum, oriented towards the male and glided towards him (indicating her willingness to copulate). This proportional measure of female responsiveness controls for variation in display rate among males and is a good predictor of mating success (e.g. Houde 1988, 1997; Pitcher et al. 2003). Copulations were recorded when males made gonopodial contact with the female following courtship. When females are less receptive, males attempt sneak copulations (i.e. gonopodial thrusts) which may result in sperm transfer (e.g. Pilastro and Bisazza 1999; Evans et al. 2003b). A gonopodial thrust was recorded when a male did not court the female before swinging his gonopodium forward and attempting to insert it into the genital pore of the female.

At the completion of the 2 h free swim period, the males and females were removed from the tank. Males were anaesthetized in a water bath containing buffered MS-222 and then photographed using a digital camera (Nikon CoolPix 950, see Pitcher and Evans 2001 for details). Area of colors and standard length were quantified using NIH Image analysis software (ImageJ available at <http://rsb.info.nih.gov/ij/>) to examine whether there were any differences in these variables between males in the dyads for each treatment. The total surface area of each male was measured in order to calculate the relative area covered by orange and black. Females were isolated in 20 l aquaria and held until they had given birth to two broods, at which time they were measured using calipers to examine whether there were any differences in body size within the dyads of females in each treatment.

Assessing the effects of inbreeding

To assess potential costs of inbreeding, all females from the *no choice* mating trials (NN and SS females) were isolated in 20 l aquaria and allowed to produce up to two broods, of which the second brood was reared to sexual maturity. Life history traits of individuals from first broods were not considered because these broods tended to be

relatively small in size, which would result in poor statistical power.

Offspring life history traits

In order to minimize effects of density, we isolated eight haphazardly chosen offspring from each of the second broods of NN and SS females. As the NN (outbred) and SS (inbred) male offspring in these groups approached maturity, they were examined every 2 or 3 days for sexual maturation. When males were deemed mature (i.e. when the hood grew even with or beyond the tip of the gonopodium, see Houde 1997), the age and size of each male were recorded. Because sperm related parameters (e.g. number and abnormalities) have often been associated with inbreeding depression (e.g. Wildt et al. 1987; Roldan et al. 1998; Margulis and Walsh 2002; Gage et al. 2006), we also examined sperm number in the inbred and outbred male offspring.

To estimate sperm number (see Pitcher et al. 2007), 3 days after a male reached sexual maturity, he was anaesthetized and placed on a Petri dish under a dissecting microscope. The gonopodium was swung forward and slight pressure was applied to the side of the abdomen, just anterior to the gonopodium (where the testes are located) which releases the spermatozeugmata (i.e. sperm bundles). This procedure was performed several times until no more sperm were ejected. The sperm bundles were then drawn up a pipette and added to a fixed amount (e.g. 100 μ l) of saline solution. To distribute sperm evenly on the counting grid, samples were repeatedly drawn up and expelled from the pipette. Sperm counts were calculated by counting sperm cells in an "improved Neubauer chamber" haemocytometer under 400 \times magnification. The numbers of sperm in each of five larger squares on the haemocytometer were counted. There are 25 of these large squares on the haemocytometer and each of these large squares has 16 smaller squares within it. Sperm are counted in the four large corner squares and the large center one (80 smaller grids). The mean number of sperm per large square count (i.e. mean of the 5 counts) was multiplied by 25 (to obtain the mean per 5 \times 5 large-square grid) and again by 10 (the depth of the chamber in μ m). This number was then multiplied by the initial volume of the sample to estimate the sperm number.

Dam life history traits

Both the length of the brood cycle and brood size were measured because both contribute to lifetime reproductive success. Interbrood interval is the number of days between

a female's first and second broods. We present analyses for first and second broods separately, because the number of offspring in the first brood is usually small and can be variable.

Statistical analyses

To control for 'trial' effects in our analysis of female pre-copulatory behavior, relative female responsiveness was calculated. In the *no choice* treatment, relative female responsiveness to the unrelated male was calculated as the responsiveness of one female to both of the unrelated males (unrelated response: calculated as the mean of the proportional responses to each male) minus the responsiveness of the other female to both of the related males (related response: calculated as the mean of the proportional responses to each male) divided by the overall female responsiveness for the trial. For the *choice* treatment, relative female responsiveness to the unrelated male was calculated for each female separately as her responsiveness to the unrelated male (unrelated response: calculated as the mean of the proportional responses to each male) minus her responsiveness to the related male (related response: calculated as the mean of the proportional responses to each male) divided by the overall female responsiveness for the trial. For both of these measures, each pair of females was the unit of replication because the dependent variable was either the difference in proportional responsiveness between the two females within a tank ($n = 40$ pairs for the *no choice* treatment) or was the mean difference in proportional responsiveness across the two females within a tank to the unrelated and related males ($n = 20$ pairs for the *choice* treatment). We used one sample *t*-tests to ask whether relative female responsiveness to the unrelated male (responsiveness to unrelated male minus responsiveness to related male) differed significantly from zero. A significant positive difference from zero in these one sample *t*-tests would indicate that females are showing a pre-copulatory preference for unrelated males.

All males were observed for the numbers of sigmoids, copulations, and thrusts they directed at each female. We used one sample *t*-tests to ask whether differences in the relative male sexual behavior (i.e. sigmoids, and thrusts) directed to unrelated females (e.g. sigmoid number directed to the unrelated female minus sigmoid number directed to the related female) differed significantly from zero, using each pair of males in a tank as a replicate for both the *no choice* and *choice* treatments. A significant positive difference from zero in these one sample *t*-tests would indicate that males are showing a preference for unrelated females.

Based on the prediction that inbreeding is deleterious, we used directional statistical tests to determine whether sons' life history traits (age and size at maturity, and sperm number controlling for male body size at maturity) or dam life history traits (interbrood interval and offspring number controlling for female body size) were negatively affected where females were allowed to interact with related versus unrelated males. We used nested ANOVAs, with female identity nested in treatment and as a random effect, using Proc Mixed in SAS (SAS 1989), to test whether or not relatedness of mates explained some of the variation in offspring age of maturity, size at maturity and sperm number (with the natural logs of male body size as a covariate). We used *t*-tests to assess whether or not relatedness of mates explained some of the variation in the time to first brood and the interbrood interval.

An ANCOVA was used to assess whether or not relatedness to the males (NN, SS, or NS) explained some of the variation in offspring number in first and second broods, independent of female body size. The slopes for the female body length-offspring number relationships were homogeneous ($P > 0.4$), so the interaction terms were removed from the ANCOVA models and the adjusted means were calculated using the common within group slope (Huitema 1980).

In light of evidence of inbreeding depression (i.e. offspring numbers were reduced in females mated to siblings (SS) compared to females mated with non-siblings (NN) (see below)), we asked whether there was any evidence of paternity biasing in favor of unrelated males relative to related males in the *choice* treatment. To determine whether females are able to preferentially fertilize their eggs with sperm from unrelated males, we compared offspring number in first and second broods of females from the different treatments using one-sample *t*-tests. Specifically, we compared the adjusted mean number of offspring (from the ANCOVA) produced by NS females versus the adjusted mean number of offspring produced by NN and SS females (from the ANCOVA). A significant positive difference from the combined NN and SS offspring number mean would demonstrate that there is a significant improvement in offspring number in polyandrous females mating to both unrelated and related males, suggesting a bias in sperm usage towards unrelated males (see Tregenza and Wedell 2002).

A more refined test of the paternity biasing prediction would account for the amount of sperm contributed by the related and unrelated males in the NS trials. We used the comprehensive behavior data collected for the NS males and females to estimate the amount of sperm each male contributed to the each female. Although we could not directly estimate actual ejaculate size in our study, we assumed that each gonopodial thrust (sneak copulation)

that made contact with the female genital opening allowed a male to contribute on average 1.13×10^4 sperm and each cooperative copulation allowed a male to contribute 6.13×10^5 sperm (estimates are based on a study of the efficacy of sperm delivery via copulations and thrusts using the same population, Cheng 2004). These sperm number estimates were then used to construct an expected distribution of paternity for each male based on a “fair raffle” model (Parker et al. 1990; see also Cook et al. 1997; Evans and Magurran 2001). Under this null model, individual sperm from each male have an equal chance of fertilizing the eggs. Therefore, the proportion of ova fertilized by a male is equal to the proportion of total sperm that is his. In this case, $P_i = S_i / (S_i + S_j)$, where P_i is the paternity of the i th male, and S_i and S_j represent the sperm load of the i th and j th male in a dyad. For example, i and j respectively represents the unrelated and related male in a trial. We then compared the adjusted mean number of offspring (from the ANCOVA and corrected for via the fair raffle model calculations) produced by NS females versus the adjusted mean number of offspring produced by NN and SS females (from the ANCOVA). A significant positive difference from the combined NN and SS offspring number mean would demonstrate that there is a significant improvement in offspring number in polyandrous females mating to both unrelated and related males, suggesting a bias in sperm usage towards unrelated males.

All means are reported plus or minus one standard error, except where noted otherwise. All female responsiveness and color data were arcsine square root transformed, offspring number was log transformed, and offspring sperm number, age at maturity and size at maturity were transformed using natural logarithms to render them normally distributed (Zar 1999). All statistical tests were performed using SPSS (v. 12), except where noted otherwise.

Results

Consequences of inbreeding

For inbred sons (SS), compared to outbred sons (NN), there was delayed sexual maturity (days) (85.4 ± 2.7 vs. 79.1 ± 2.2 ; $F_{1,33} = 3.42$, $P = 0.035$). There was not a significant difference in male size at maturity (mm) between inbred and outbred offspring (15.47 ± 1.3 vs. 15.47 ± 1.2 ; $F_{1,33} = 0.01$, $P = 0.48$). Sperm number was apparently reduced in inbred sons ($F_{1,31.5} = 4.14$, $P = 0.025$) but the difference was not significant when two outliers (>3 studentized residuals) were removed from the analysis (least squares means (\pm s.e.): outbred: 1.17×10^6 (0.09×10^6), inbred 1.00×10^6 (0.09×10^6); $F_{1,31.9} = 1.81$, $P = 0.095$). When we compared SS and NN females,

there was a significant difference in the number of days it took females to give birth to first (38.7 ± 1.5 vs. 35.4 ± 1.3 , $t_{44} = 1.82$, $P = 0.045$), but not second broods (interbrood interval: 31.4 ± 1.3 vs. 29.8 ± 0.7 ; $t_{41} = 1.51$, $P = 0.23$).

An analysis of covariance revealed that there was a significant effect of the relatedness of sires (NN, SS, NS) on the number of offspring born in second broods (ANCOVA $F_{2,60} = 4.47$ and $P = 0.015$; Figs. 2, 3), but not first broods (ANCOVA $F_{2,67} = 0.55$ and $P = 0.58$). Post hoc analyses of offspring number revealed that the significant effect in second broods was due to smaller brood sizes born to females with access to two brothers relative to females with access to at least one unrelated male (Tukey tests: SS vs. NN, $P = 0.001$; SS vs. NS, $P = 0.038$; Fig. 3).

Behavioral data

Our experimental design called for dyads of males that were similar in terms of body size and coloration. As planned, the dyads of males did not differ in terms of body length in the *choice* (paired difference: $0.12 \text{ mm} \pm 0.35$; $t_{19} = 0.33$, $P = 0.74$) or *no choice* treatments (paired difference: $0.3 \text{ mm} \pm 0.2$; $t_{39} = 0.33$, $P = 0.74$). There was also no difference among the male dyads in terms of the amount of relative orange coloration in the *choice* (paired difference: $0.16 \pm 0.3\%$; $t_{19} = 0.62$, $P = 0.54$) or *no choice* treatments (paired difference: $0.21 \pm 0.27\%$; $t_{39} = 0.86$, $P = 0.40$). Finally, there was no difference in the amount of relative black coloration in male dyads in the *choice* (paired difference: $3.2 \pm 2.5\%$; $t_{19} = 1.25$, $P = 0.23$) or *no choice* treatments (paired difference:

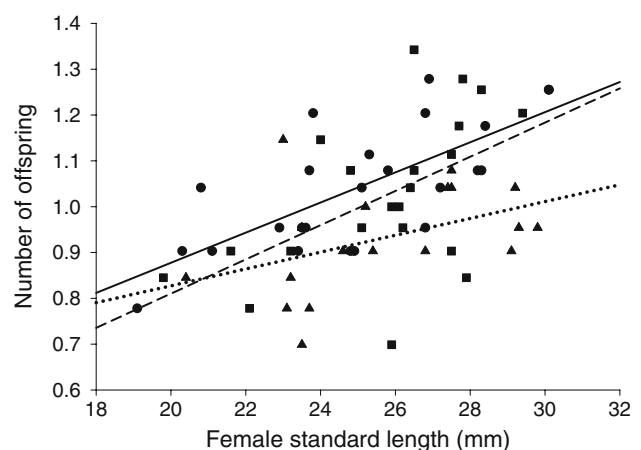


Fig. 2 Relationship between female body size and the number of offspring (log transformed) produced from second broods for each of the treatments. The lines and symbols represent the three treatments: non-sibnon-sib (NN) (solid line and ●); sibsib (SS) (dashed line and ▲); and non-sibsib (NS) (dotted line and ■)

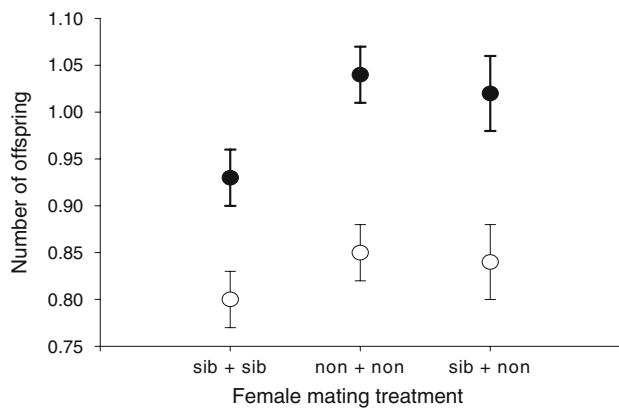


Fig. 3 The number of offspring (log transformed) from (a) first (open circles) and (b) second (closed circles) broods controlling for female body size (adjusted means from the ANCOVA) across mating treatments (means and standard errors). Females were either mated to two siblings (sib + sib), two non-siblings (non + non), or a sibling and non-sibling (sib + non)

$0.14 \pm 1.6\%$; $t_{39} = 0.04$, $P = 0.97$). Our experimental design also called for dyads of females that were similar in terms of body size. As planned, there was no significant differences in the length of females used in each of the dyads for the *choice* (paired difference: $0.22 \text{ mm} \pm 0.67$; $t_{19} = 0.31$, $P = 0.74$) or *no choice* (paired difference: $0.31 \text{ mm} \pm 0.58$; $t_{39} = 0.28$, $P = 0.78$) treatments.

Neither females nor males behaved differently towards related versus unrelated individuals. Relative female responsiveness to the unrelated males (female responsiveness to unrelated male minus responsiveness to related male) did not differ significantly from zero in either the *no choice* treatment (unrelated vs. related: $8.3 \pm 0.8\%$ vs. $7.5 \pm 0.6\%$, $t_{39} = 0.33$, $P = 0.74$) or the *choice* treatment (unrelated vs. related: $7.7 \pm 0.8\%$ vs. $6.2 \pm 0.7\%$, $t_{19} = 0.75$, $P = 0.46$) indicating that females did not show a pre-copulatory preference for unrelated males. Female cooperation is needed in order for males to make sustained gonopodial contact and there was no difference in the number of cooperative copulations achieved by unrelated and related males in either the *no choice* treatment (0.99 ± 0.08 vs. 0.73 ± 0.53 ; $t_{39} = 0.63$, $P = 0.52$) or the *choice* treatment (1.4 ± 0.17 vs. 1.0 ± 0.14 ; $t_{19} = 1.1$, $P = 0.30$).

We found no evidence that males showed a preference for unrelated females. The number of sigmoids (per min) directed towards the unrelated female(s) minus the number of sigmoids directed towards the related female(s) did not differ significantly from zero in either the *no choice* treatment (unrelated vs. related: 0.19 ± 0.1 vs. 0.20 ± 0.1 ; $t_{39} = 0.24$, $P = 0.82$) or the *choice* treatment (unrelated vs. related: 0.17 ± 0.11 vs. 0.16 ± 0.1 ; $t_{19} = 0.08$, $P = 0.94$). Likewise, there was also no difference in the number of thrusts (sneak copulation attempts) directed

towards unrelated female(s) versus thrusts directed towards related female(s) in the *no choice* (unrelated vs. related: 0.094 ± 0.01 vs. 0.1 ± 0.01 ; $t_{39} = 0.07$, $P = 0.92$) or *choice* treatment (unrelated vs. related: 0.097 ± 0.01 vs. 0.1 ± 0.01 ; $t_{19} = 0.12$, $P = 0.90$).

Effect of mating treatment on offspring number

Given the reduction in brood size for inbred matings (see above), we could ask whether females were able to bias sperm usage towards unrelated males. To ask whether females were using disproportionately more of the sperm from unrelated males, we compared the offspring number of females in the *choice* trials (i.e. sibling and non-sibling males were available) to what would be expected if fertilization success (and the subsequent offspring number) were halfway between completely outbreeding (NN) and completely inbreeding (SS) females (i.e. random sperm usage) (see Tregenza and Wedell 2002). One-group *t*-tests for offspring number in first and second broods revealed that females in mating trials with a sibling and a non-sibling produced a number of offspring not significantly different than what would be expected if fertilization success were halfway between completely outbreeding and completely inbreeding females (i.e. there was no evidence of a bias in sperm usage towards unrelated males) (first brood: $t_{24} = 0.66$, $P = 0.52$; second brood: $t_{20} = 2.26$, $P = 0.07$; see Fig. 3). This suggests that there is no significant improvement in offspring number in polyandrous females mating to both related and unrelated males, relative to that which would be expected if sperm from both males were used equally. However, because there was a statistical trend in second broods suggesting females may be able to bias paternity towards unrelated males we re-analyzed the data to control for sperm inputs from related and unrelated males. A more refined test of the paternity biasing ability of females might predict that the paternity bias in favor of the unrelated male's sperm should increase as he contributes more sperm relative to the related male; in contrast, the paternity bias of the unrelated male's sperm should decrease as the related male contributes more sperm than the unrelated male (see Parker et al. 1990). After controlling for sperm contribution, one-group *t*-tests for offspring number in first and second broods revealed that females in mating trials with a sibling and a non-sibling produced a number of offspring not significantly different than what would be expected if fertilization success were halfway between completely outbreeding and completely inbreeding females (i.e. there was no evidence of a bias in sperm usage towards unrelated males) (first brood: $t_{24} = 0.83$, $P = 0.42$; second brood: $t_{20} = 1.59$, $P = 0.13$).

Discussion

In this study we found costs of inbreeding, which should favor discrimination against mating kin. Females that mated with kin (SS), relative to those that mated with non-kin (NN), produced smaller broods and their male offspring had delayed sexual maturity. Despite these costs of mating with kin, we found no evidence for pre-copulatory discrimination against kin by either males or females. Likewise, we found no evidence for post-copulatory discrimination against the sperm of kin by females. These data suggest that, despite real costs to inbreeding in guppies, adult females apparently do not discriminate against kin at either the pre- or post-copulatory stage.

Evidence for the effects of inbreeding in guppies

Females mated to siblings produced offspring that exhibited evidence of inbreeding depression. Male offspring from sib–sib matings took longer (6.3 days) to reach sexual maturity. The developmental delay represents a $\sim 10\%$ increase in age at maturity, which could mean a significant decrease in fitness given the relatively short lifespan of guppies in the wild (Reznick et al. 1996). The existence of inbreeding depression demonstrated in our experiment is consistent with previous studies suggesting other negative consequences of consanguineous matings in wild guppies (reduced male ornamentation and courtship behavior, Sheridan and Pomiankowski 1997; van Oosterhout et al. 2003; Mariette et al. 2006).

We also found that female guppies mated to siblings took longer to produce first broods and produced fewer offspring than those mated to non-siblings (Figs. 2 and 3). Differences in the amount of time females took to give birth to first broods may represent differences in development rate among inbred and outbred offspring due to inbreeding depression. Alternatively, females might have delayed fertilization of the embryos in order to obtain more compatible sperm at a later time. The reduced brood size of females mated to siblings could be the result of; (i) inbreeding depression through reduced survivorship of fertilized ova, (ii) female guppies selectively aborting offspring based on their genetic compatibility, or (iii) females providing yolk to fewer ova when fertilized by a related male.

Pre-copulatory behavior

Our results, in combination with previous studies demonstrating inbreeding depression in guppies, suggest that there should be selection against mating with kin in this species, perhaps in both sexes. Yet, we found no evidence of pre-copulatory discrimination by either sex. Females did

not discriminate against related males in pre-copulatory mate choice (see Viken et al. 2006 for similar results in a different population). One possibility is that adult females simply cannot recognize kin unless reared with them (i.e. using familiarity as a cue for relatedness), as has been observed in guppies from other populations (e.g. Griffiths and Magurran 1999; but see Kelley et al. 1999). Our data cannot address this hypothesis because we intentionally reared guppies separately because previous studies have shown that exposure to mature males can influence female mate choice decisions (Breden et al. 1995; Rosenqvist and Houde 1997; Hughes et al. 1999) and because we wanted to separate out the effects of familiarity from relatedness. Our data do suggest that guppies in our study population cannot innately identify kin. In the wild, females may instead use other cues to avoid mating with related individuals; for example, previous studies have shown that female guppies prefer males with rare or unfamiliar phenotypes (Farr 1977; Hughes et al. 1999).

There was no evidence that males discriminated against related females in pre-copulatory mate choice. Males directed not significantly different amounts of sexual behaviors (i.e. sigmoids and sneak copulation attempts) towards kin (SS) and non-kin (NN) females. If sperm are relatively cheap to produce, then the costs to males of mating with related females may be relatively small. If indeed males are under weaker selection than females to avoid or minimize the costs of mating with relatives, this will set the scene for sexual conflict over the willingness to mate with related individuals (see Waser et al. 1986; Manson and Perry 1993; Pizzari et al. 2004).

Post-copulatory processes

We found no evidence that females use post-copulatory mechanisms to bias fertilization toward non-kin and the power of our tests was moderate to strong (at $\sim 70\%$). Female guppies that had an opportunity to mate with both a non-sibling and sibling (NS) produced an intermediate number of offspring compared to numbers produced by SS and NN females, as would be expected if there were no sperm use biasing. Our data do not suggest that inbreeding avoidance favors multiple mating in guppies as has been suggested in other taxa. There are several correlative studies which suggest that females may benefit from multiple mating by biasing paternity towards unrelated males (e.g. Madsen et al. 1992; Olsson et al. 1996; Denk et al. 2005), while others find no evidence of any such mechanism (e.g. Stockley 1997; Simmons 2001). To date, there have been only a few studies that manipulated the relatedness of potential mates to examine evidence for post-copulatory paternity biasing (reviewed in Simmons 2005). For example, Tregenza and Wedell (2002) found that

female crickets mated to a sibling and non-sibling had the same reproductive success (i.e. hatching success) as females mated to two non-siblings. A subsequent genetic analysis revealed that unrelated males were much more successful in gaining paternity than were sibs (Bretman et al. 2004). This would be most likely to arise if sperm from non-siblings were disproportionately successful at fertilization (but see Zeh and Zeh 2006; Garcia-Gonzalez and Simmons 2007). In contrast, Jennions et al. (2004) repeated Tregenza and Wedell's (2002) experiment using another species of cricket and found no evidence that females used post-copulatory mechanisms to bias fertilization towards unrelated males. It remains to be seen whether post-copulatory mechanisms to avoid inbreeding are widespread and how important they are in the evolution and maintenance of multiple mating.

Although our study suggests that female guppies do not bias sperm use to avoid the costs of inbreeding depression, paternity biasing mechanisms may be favored to avoid other types of genetic incompatibilities (reviewed in Tregenza and Wedell 2000). Further study is needed in a broad range of taxa to assess how common pre- or post-copulatory paternity biasing mechanisms for genetic incompatibility avoidance are in nature.

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