Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy

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As inbreeding is costly, it has been suggested that polyandry may evolve as a means to reduce the negative fitness consequences of mating with genetically related males. While several studies provide support for this hypothesis, evidence of pure post-copulatory mechanisms capable of biasing paternity towards genetically unrelated males is still lacking; yet these are necessary to support inbreeding avoidance models of polyandry evolution. Here we showed, by artificially inseminating a group of female guppies with an equal number of sperm from related (full-sib) and unrelated males, that sperm competition success of the former was 10 per cent lower, on average, than that of the unrelated male. The paternity bias towards unrelated males was not due to different embryo survival, as the size of the brood produced by control females, which were artificially inseminated with the sperm of a single male, was not influenced by their relatedness with the male. Finally, we collected ovarian fluid (OF) from virgin females. Using computer-assisted sperm analysis, we found that sperm velocity, a predictor of sperm competition success in the guppy, was significantly lower when measured in a solution containing the OF from a sister as compared with that from an unrelated female. Our results suggest that sperm–OF interaction mediates sperm competition bias towards unrelated mates and highlight the role of post-copulatory mechanisms in reducing the cost of mating with relatives in polyandrous females.

Keywords: sperm competition; polyandry; inbreeding avoidance; ovarian fluid

1. INTRODUCTION

Mating with relatives can be costly, primarily as a consequence of the expression of deleterious recessive alleles [1,2]. It has been suggested that female multiple mating (polyandry) may have evolved as an inbreeding avoidance strategy mediated by post-copulatory mechanisms such as sperm–egg or sperm–female environment interactions [3–5], which could bias paternity towards unrelated or genetically compatible mates and hence reduce the costs of inbreeding. While growing evidence suggests that the genetic relatedness (or genetic similarity) between mating partners is associated with competitive fertilization success [6–14]—although not always in the predicted direction (i.e. fertilization success biased in favour of males that are more genetically similar to the female [15–18])—most of these studies cannot exclude the influence of pre-copulatory mate choice or behavioural control of post-copulatory processes, which could bias fertilization success towards unrelated mates. For example, in the fowl, the number of sperm transferred during a copulation is influenced by genetic relatedness/similarity between mates [19,20]. Clearly, the existence of purely post-copulatory processes is required to support the hypothesis that polyandry has evolved as a means to reduce the costs of mating with genetically related or incompatible males [21]. In contrast, behaviourally mediated post-copulatory mechanisms, although potentially capable of reducing the risk of inbreeding, do not explain per se why females mate multiply.

Despite the indirect evidence supporting post-copulatory biases towards unrelated males (see above), the few experiments specifically aimed at excluding processes mediated by pre-copulatory kin recognition, by using artificial insemination, failed to find evidence of purely post-copulatory mechanisms of inbreeding avoidance [16,22,23]. Despite these negative results, however, there is some evidence suggesting that sperm performance and sperm competition success are influenced by male–female interactions. For example, in the domestic fowl, artificial insemination of known numbers of sperm from two males revealed that sperm competition success of a particular male depends on the background against which his sperm compete for fertilization [24]. Similarly, a significant effect of male–female interaction on sperm competition success has been found in *Drosophila* [25]. In externally fertilizing fishes, it has been shown that the ovarian fluid (OF) released by the females with their eggs during spawning affects sperm swimming velocity [26,27], and this effect is influenced by the identity of the interacting male and female [28]. There is ample evidence that sperm swimming velocity and motility are important determinants of sperm competition success [29–31]. These results indirectly suggest that the effect of the OF on sperm velocity may act as a mechanism that biases fertilization success towards males with a particular genotype [28]. Thus, although indirect evidence suggests the existence of purely post-copulatory mechanisms favouring fertilizations by unrelated (or genetically dissimilar) males, direct experimental evidence is still lacking.

We investigated cryptic female preference for unrelated mates in the guppy (*Poecilia reticulata*), an internally fertilizing species in which the females mate multiply [32,33]. The guppy is an ideal model to study post-copulatory...
mechanisms of inbreeding avoidance [34]. First, there is ample evidence that inbreeding has deleterious fitness consequences in this species [35–39]. Second, while female guppies show a pre-copulatory preference for unfamiliar males or males with a rare colour phenotype [40–43], which indirectly reduces the risk of inbreeding, they are apparently unable to distinguish among related males when familiarity cues are controlled for [39,44,45]. Third, males can underuse female choice by means of gonopodial thrusting [46], a coercive mating tactic that allows males to forcibly inseminate females [47]. Paternity analysis in natural populations has revealed a paternity skew towards unrelated males [14], suggesting that mechanisms reducing fertilizations by genetically related mates do operate in this species. Whether this bias is determined at the gametic level, however, has not been demonstrated yet.

We used an established protocol of artificial insemination [48,49], controlling for behavioural effects on post-copulatory sexual selection [50,51], to evaluate the effect of a male’s genetic relatedness to his mate on sperm competition success. Male guppies vary in their sperm competition success, with some males being intrinsically superior to other males [52]. We therefore used a paired sperm competition test in which we artificially inseminated two unrelated females with an equal number of sperm taken from two males and analysed the difference in paternity success across females in relation to the difference in genetic relatedness among mates. We determined whether insemination from related males results in a reduced brood size, via differential fertilization success or embryo viability, by comparing the brood size of females that were artificially inseminated with the sperm from either a brother or an unrelated male. Finally, we went further by exploring the physiological mechanisms by which females mediate inbreeding avoidance. Specifically, we investigated the role of OF in mediating sperm competition success, by measuring in vitro sperm velocity in a solution containing OF from related and unrelated females.

2. MATERIAL AND METHODS

(a) Animals

Guppies used in this experiment were descendants of wild-caught fish from Lower Tacarigua, Trinidad, and were maintained in our laboratory (details on fish origin and maintenance are given in [49]). Full-sibs used in the experiments were obtained by randomly pairing males with unrelated, virgin females and raising offspring to maturity in single-sex groups.

(b) Paternity success trial

One brother and one virgin sister from each of two unrelated families (total number of families = 14) were randomly chosen and the two females were artificially inseminated with sperm from the two males. Thus, the same male (randomly chosen and labelled male B) was tested in a favourable condition (mated with an unrelated female) and an unfavourable condition (mated with a related female). This experimental design allows us to magnify the effect of genetic relatedness on sperm competition success and to control for intrinsic differences among male/ejaculate sperm competitiveness across males [52]. Furthermore, it avoids the stochastic effects owing to the random assignment of competitors that affect the variance in sperm competition success [53]. The following procedure was replicated for 14 fish quartets (in total, 28 males and 28 females). Sperm were collected from each male following an established procedure [48]. Briefly, each male was anaesthetized in a water bath containing MS-222 and placed on a slide under a stereomicroscope. A gentle abdominal pressure allowed the release of sperm in a drop of saline solution (NaCl 0.9%). Sperm in this species are packaged in discrete units called sperm bundles, which can be easily collected with a pipette. Sperm bundles were split into different aliquots, and the same number of sperm bundles (10) from each male were used to inseminate each female, according to an established procedure [48,49]. The paired mating design allowed us to control for any difference in the number of sperm per bundle, and more generally, in ejaculate quality, between males within each replicate [54,55].

Two broods (one for each female of the quartet) were obtained from 11 quartets, whereas in the other three quartets, one of the two females did not produce a brood and was excluded from paternity analysis. No post-partum mortality was recorded, and newborns were killed in an excess of anaesthetic (MS-222) within a few hours from birth. Tissues for DNA analyses (whole body of newborn offspring and fin clip from parents) were preserved in absolute ethanol until required.

DNA was extracted from offspring tissues using a Chelex protocol [56] and from adult fin clips using a standard salting-out protocol [57]. PCR products of six microsatellite loci (GenBank nos AF164205, BV097141, AF368429, DQ855573, DQ855605 and AF467907) were analysed on an ABI 3100 sequencer (Applied Biosystems). All offspring were unequivocally assigned to putative sires according to allele sharing. Sperm competition success of male B was determined from his paternity share (Pb). The observed difference in mean Pb in the related and unrelated condition (mean ΔPb-obs) was compared, after arc sine transformation, with the null expectation [23]. In particular, we calculated the expected difference in sperm competition success for each of the 11 pairs of males given the observed brood sizes and an equal probability of fertilizing the eggs using the function ‘dBinomialDev’ in Poptools [58]. We calculated the mean difference in sperm competition success from these 11 randomized values (mean ΔPb-rand) and resampled this statistic 10,000 times to obtain critical values using a Monte Carlo simulation.

(c) Effect of mate relatedness on brood size

In livebearing species, embryo survival can affect estimates of sperm competition success if paternity is measured at birth [59]. To test whether genetic relatedness affected embryo survival from fertilization to parturition, and hence paternity estimates in the guppy, we performed homospermic (sperm from single male) AI using 61 males and 131 females from 40 families. One ejaculate collected from each male was used to artificially inseminate at least one related (sister) and one unrelated virgin female (20 bundles each). After insemination, females were isolated and checked twice a day until they delivered their brood. Cases in which a female did not produce a brood within 60 days after insemination were considered unsuccessful. To control for unbalanced design owing to brooding failures, brood size was compared between experimental groups using a linear mixed model in which brood size was the dependent variable, male identity and
female family were entered as random factors, relatedness as a fixed factor and female body size (standard length, SL, measured from the snout to the base of the caudal fin) as a covariate.

(d) Effect of ovarian fluid on sperm swimming velocity
Sperm velocity was measured in three different solutions containing 40 per cent vol. of 150 mM KCl and 4 mg ml−1 BSA, and 60 per cent of (a) 0.9 per cent NaCl (control), (b) OF from a sister and (c) OF from an unrelated female, respectively. Intact sperm bundles from each male (*n* = 65, from 25 families) were placed on a multi-well slide containing solution a, b or c, and average path velocity (VAP) and curvilinear velocity (VCL, μm s−1) of sperm leaving the bundle were recorded using a CEROS sperm tracker (Hamilton-Thorne Research, Beverly, MA, USA) [54]. At least three bundles were used per male per analysis (mean number of motile sperm per analysis: 72.8 ± 29.1 s.d., *n* = 195). OF was collected from 65 virgin females (from the same 25 families as above) previously anaesthetized as described for males. Briefly, 3 μl of 0.9 per cent NaCl was gently injected into the female’s gonoduct and retrieved using a Drummond micropipette. This operation was repeated three times. Each female’s OF sample (approx. 9μl) was split into two aliquots and immediately used to measure sperm velocity from one related and one unrelated male, in random order. We first tested whether OFunrelated enhanced sperm velocity as compared with the control solution, using a paired *t*-test. Sperm velocity in OFunrelated and OFrelated solutions was then compared using a GLM in which male and female identities were entered as random factors. The use of a paired *t*-test and of GLM random factors allowed controlling for among-male differences in ejaculate quality and differences among OF samples owing to intrinsic differences among females or to sampling. For the sake of brevity, we present only results regarding VAP, but substantially identical results were obtained when VCL was considered.

3. RESULTS

(a) Paternity success trial
We obtained 196 offspring from 22 broods, one for each female in the 11 replicates of the sperm competition experiment (mean brood size = 8.91, s.d. = 2.93, *n* = 22). Our molecular analysis revealed that the focal male’s paternity share was lower, on average, when mated with the full-sib female than when mated with the unrelated female (mean difference in paternity, ΔF<sub>B.obs</sub> = −20.1%, *n* = 11; figure 1). Results of the Monte Carlo simulation revealed that the mean observed difference in paternity success between matings with related and unrelated females was larger than expected by chance (*p* < 0.002).

(b) Effect of mate relatedness on brood size
Brood size from artificially inseminated control females confirmed that this effect was not due to differential embryo mortality. Six of 131 artificially inseminated females died before delivering a brood (i.e. within one month from insemination), two from the related group and four from the unrelated group. Ten females, out of the 125 remaining females, did not produce a brood within two months from insemination, six in the related group (*n* = 62) and four in the unrelated group (*n* = 63, *p* = 0.53, Fisher’s exact test). Brood size did not vary between the two groups of females (linear mixed model: relatedness, *F*<sub>1,53</sub> = 0.77, *p* = 0.38; figure 2; female body size (SL, covariate), *F*<sub>1,72</sub> = 25.03, *p* < 0.0001). Male identity effect on brood size was not significant (*p* = 0.57), whereas female family significantly affected fecundity (*p* = 0.034, log-likelihood ratio test).

(c) Effect of ovarian fluid on sperm swimming velocity
Sperm swimming velocity (VAP) was higher in the KCl-OFunrelated solution as compared with the KCl-only (control) solution (paired *t*-test, *t*<sub>64</sub> = 3.502, *p* = 0.001; figure 3). Sperm showed a slower swimming velocity when tested with the KCl-OFrelated as compared with the KCl-OFunrelated (GLM, fixed factor: relatedness, *F*<sub>1,26</sub> = 8.13, *p* < 0.01).
that we documented here is unlikely to become evident unless a large sample size or a paired mating design is used. It is worth noting that our results are supported by a recent study on a wild Trinidadian population [14], which revealed that, in multiply sired broods, each sire’s paternity share is negatively correlated with his genetic similarity to the female (although the influence of pre-copulatory cues cannot be excluded in this type of studies).

We went further than previous studies and identified a post-copulatory mechanism that is likely to be responsible for the observed cryptic female preference for unrelated mates. Sperm velocity is an important determinant of sperm competition success in several external and internal fertilizers [30,31,60,61] and in our guppy population [62], and it is influenced by its interaction with female OF [26,63]. We first demonstrated that the guppy OF positively affects sperm velocity. Secondly, we showed that this effect was less pronounced when the OF was taken from a genetically related female (full-sib). Taken together, our results suggest that differences in sperm velocity, mediated by OF interaction, may be the underlying mechanism explaining the observed paternity bias towards unrelated males. Indeed, results from previous studies revealed a possible role of OF in cryptic female choice in two salmonid fishes, the Arctic charr (Salvelinus alpinus) [26] and the chinook salmon (Oncorhynchus tshawytscha) [28]. In these fishes, sperm swimming velocity differs significantly among males, and variation within males depends on the female’s OF in which the sperm is activated, leading to the suggestion that OF can mediate cryptic female choice [28]. However, whether or not genetic relatedness influences sperm velocity and sperm competition success has never been documented. Our results therefore demonstrate for the first time that the enhancing effect of OF on sperm motility is reduced when the male and female are genetically related. This is the first explicit evidence for a purely post-copulatory mechanism of inbreeding avoidance, a critical assumption for models of polyandry evolution based on costs of mating with relatives [3–5].

How the interaction between sperm and OF is influenced by mates’ genetic relatedness is presently unknown. Components of the OF, such as signalling peptides, may have chemokinetic effects on sperm. Rapidly evolving male and female reproductive proteins that mediate fertilization have been recognized in several taxa, from diatoms to humans [64]. While most of these peptides affect fertilization success when the sperm and the egg come into contact [65], some of them, which are found in both OF and sperm membranes, may potentially interact with the sperm cell surface before fertilization and influence sperm swimming performance (e.g. [66]). Obvious candidates are MHC peptides (e.g. [67]), and the role of similarity at a polymorphic MHC class II locus is presently under investigation in our laboratory. Understanding the mechanisms of sperm–OF interaction capable of influencing sperm performance differentially, according to male–female relatedness, is an exciting challenge for future research.

In conclusion, our experiments demonstrate that genetic relatedness influences the effect of OF on sperm performance by increasing the swimming velocity of sperm from unrelated males in the guppy. This result,
coupled with the evidence that unrelated males fertilized a larger proportion of the eggs of artificially inseminated females, strongly suggests that OF–sperm interaction works as a post-copulatory mechanism capable of reducing the probability of fertilizations from genetically related males. The incapacity of females to discriminate against related mates at the pre-copulatory level [39,43,44] and to avoid unwanted copulations [47,68], and the numerous fitness costs of inbreeding demonstrated in this species [14,35–38,69], has probably promoted the evolution of a purely post-copulatory mechanism of cryptic female choice favouring fertilizations by unrelated males in the guppy. Our results, coupled with previous evidence that reports fertilization biases in favour of unrelated or genetically dissimilar males in other species [6–13], underscore the importance of post-copulatory inbreeding avoidance as an evolutionary force promoting polyandry.

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