Multiple mating and reproductive skew in Trinidadian guppies

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Male offspring production in promiscuously mating species is typically more skewed than female offspring production. It is therefore advantageous for males to seek as many mating partners as possible. However, given the documented benefits of polyandry we expect females, as well as males, to mate multiply. We tested these ideas using Trinidadian guppies, *Poecilia reticulata*. Fishes were collected from the wild, housed in groups of 10 males and 10 females and allowed to reproduce freely over a period of three months. We used hypervariable microsatellite loci to identify the parents of 840 offspring and to quantify the variance in mating success. As anticipated, and in line with the Bateman gradient, there was greater skew in the number of progeny produced by males. By contrast, we found no sex difference in mating partner number over the duration of the experiment. A median of two males fathered each brood and there was marked turnover in the identities of the sires of successive broods. Female partner turnover was, however, less than expected under random mating. We suggest that partner switching over time, as well as polyandry within broods, could contribute to the maintenance of genetic diversity in guppy populations.

Keywords: mating success; microsatellites; paternity; reproductive skew; sexual selection

1. INTRODUCTION

Sexual reproduction means that each offspring has precisely one father and one mother. The total number of progeny produced by females will thus equal the total produced by males (Arnold 1983). However, in many animal species females are limited in the number of young they can produce whereas males have the potential to sire numerous progeny. It follows that if some males are highly prolific, others will have few offspring, or none at all. In promiscuous mating systems therefore, particularly where male–male competition and/or female choice reinforce inequality in male mating opportunities, offspring production by males is likely to be more skewed than that by females. An extreme example is the Northern elephant seal *Mirounga angustirostris*. Male seals can father up to 100 pups in a lifetime, whereas the maximum number produced by females is only eight (Le Boeuf & Reiter 1988).

The benefit to males of mating with as many different females as possible was elegantly demonstrated in a classic investigation of *Drosophila* by Bateman (1948). Bateman found a linear increase in the number of offspring sired by males with the number of copulation partners. By contrast, under normal rearing conditions, a single male provided sufficient sperm to fertilize all of a female's eggs. However, recent investigations have demonstrated that females profit from mating multiply (polyandry) (Zeh & Zeh 2001). Tregenza & Wedell (2002) for instance discovered that female crickets, *Gryllus bimaculatus*, can avoid the negative effects of genetic incompatibility by mating with several males, while Newcomer *et al.* (1999) showed that polyandry increases the reproductive success of female pseudoscorpions, *Cordylocherbes scorpioides*.

Furthermore, as Birkhead (2000) points out, food was limited in some of the experiments of Bateman (1948), and because of an attendant decline in sperm production, these female *Drosophila* needed to mate repeatedly to replenish their sperm stores. The traditional perception of coy females and ardent males (Darwin 1871; Bateman 1948; Trivers 1972) is thus being replaced by the recognition that females actively seek out a variety of mating partners (Birkhead 2000; Knight 2002; Judson 2003).

The Trinidadian guppy is an excellent species in which to examine male and female patterns of reproductive skew and multiple mating. During brief receptive periods (Liley 1966) female guppies solicit matings from males that have been chosen on the basis of colour pattern, body size or courtship behaviour (Houde 1997); males also compete for mating opportunities (Kelly & Godin 2001). Recent work has revealed that there is variation among and between female guppies in mate choice (Brooks & Endler 2001; Kodric-Brown & Nicoletto 2001) and that they improve both the quality and quantity of their brood by mating multiply (Evans & Magurran 2000). In addition, female guppies, particularly those in high-predation populations, are constantly the target of sneaky mating attempts (Magurran & Seghers 1994). Because female reproduction is constrained by body size (Reznick & Miles 1989), we predict, in line with Bateman's study, greater skew in the number of progeny produced by males. By contrast, for the reasons articulated above, we expect both females and males to mate with multiple partners. Our specific goal here is to quantify the extent of polyandry when multiple mating within a brood cycle is combined with partner switching between brood cycles. However, given the importance of pre-copulatory (Houde 1997) and post-copulatory (Evans *et al.* 2003b) sexual selection in the guppy mating system, we do not anticipate that the turnover in sire identity between successive broods will be random. We test these ideas by assigning parentage to the offspring of wild-caught guppies over a period of three

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months—an ecologically realistic timeframe given guppy mortality risk in the wild (Rodd & Reznick 1997). This analysis also provides an opportunity to examine the relationship between male phenotype and mating success.

2. MATERIAL AND METHODS

(a) *Fishes*

Adult guppies were imported from Trinidad in January 2001. Our guppies came from Trinidad's Lower Aripo River where they are exposed to a range of predators, notably the pike cichlid, *Crenicichla alta*. Wild fishes were collected by seine to ensure that the size distribution in our investigation reflected the natural one. After a short period of quarantine we allocated 10 male and 10 female guppies to each tank (60 cm × 30 cm × 30 cm); there were no differences in female ($F_{9,69} = 1.26$; $p = 0.27$) or male ($F_{9,76} = 1.37$; $p = 0.22$) size among the 10 replicate tanks. A year-long survey of guppies in the Lower Aripo River showed that the population sex ratio differed from 50 : 50 in only four out of the 12 months (Pettersson *et al.* 2004). The average temperature was 24.4 °C. Tanks were furnished with gravel and filters. Dense weeds provided cover for newborn fishes. Tanks were checked daily, or every other day, for offspring. All offspring that were born during the first month were discarded. This ensured that females had an opportunity to mate with males in their tank and that stored sperm did not dominate subsequent broods (see below). The investigation then ran for a further three months (from early March until early June 2001). All newborn fishes ($n = 840$) that were collected during this period were humanely killed by overdose of anaesthetic (benzocaine) and preserved for subsequent genotyping (see below). Total adult mortality was 16% for males and 18% for females. Any adults that died were also preserved where possible for genotyping, yielding genotypes for 98% of adult males and 100% of adult females. However, only fishes that survived until the end of the investigation could be used for behavioural analysis and sperm counting.

(b) *Behavioural and morphological measurements*

At the end of three months, each group of fishes was taken from its home tank and placed in an observation tank. Each male's ($n = 84$) courtship behaviour was recorded (as frequency of sigmoid displays and sneaky mating attempts per 10 min, following 10 min of equilibration (Evans & Magurran 1999)). Males were then humanely killed by anaesthetic overdose and immediately photographed using a digital camera. Measures of standard length (SL), body area, gonopodium length and area of red and black coloration were obtained from these photographs by using NIH-image software (<http://rsb.info.nih.gov/nih-image/>). Repeated-measures analysis of variance revealed no significant differences between repeated morphological measures on the same subject ($F_{1,14} = 0.05$, $p > 0.75$), indicating high consistency in our measurements. Finally, a sperm count was made for each male using the methods outlined in Matthews *et al.* (1997). To do this the gonopodium was swung forward and gentle pressure was applied to the side of the abdomen, just anterior to the base of the gonopodium. This action released sperm in the form of a number of spermatozeugmata (sperm bundles). The procedure was repeated to ensure that all sperm bundles were removed. Following removal, sperm bundles were drawn up a Gilson pipette and added to 100 µl of water. Sperm number was determined by multiplying the mean sperm count (from five counts using an 'improved Neubauer

chamber' haemocytometer under magnification, ×400) by the sample's dilution factor and initial volume. Females ($n = 82$) were also photographed and measured.

(c) *Genotyping*

A panel of eight highly variable guppy-specific microsatellite loci was cloned and characterized *de novo* (Becher *et al.* 2002). A further highly polymorphic locus, Pr67, was developed from preserved library clones at a later stage. Characteristics for Pr67 (GenBank accession number AF533589) are as follows: Forward primer (5' to 3'): CAG GTT GGC TTT TGT TTG TC, reverse primer (5' to 3'): ATG ATT TGA TGA TGA TTT GAA GC, annealing temperature $T_a = 53$ °C. The Pr67 primers amplify a (TG)₁₃A(GT)₃₈GCC(GT)₃ repeat motif with an original clone length of 238 bp.

Four polymorphic loci (table 1), Pr40, Pr67, G49 and TTA4, were selected for automated fluorescent genotyping on a Beckman-Coulter CEQ 2000 XL eight-channel capillary sequencer. These loci were specifically chosen for their absence of overlap in PCR product range, and for their high degree of polymorphism in samples from 48 Lower Aripo individuals initially tested. Distinct PCR product size ranges permitted labelling of Pr67 and Pr40 with D3pa (green), and of G49 and TAA with D4pa (blue) Beckman dyes (ResGen, Invitrogen). While PCR reactions themselves were not multiplexed, this strategy allowed us to score four loci simultaneously on the CEQ 2000, thus yielding a maximum of 384 genotypes per run.

DNA for PCR amplification was extracted from whole body tissue of day-old guppies and from tail and peduncle tissue of adult guppies, using either the standard phenol-chloroform method (Sambrook *et al.* 1989), or alternatively by salting out (MasterPure kit, Epicentre Technologies).

PCR amplification conditions for automated genotyping were as follows: 20 ng of genomic DNA were PCR amplified in 15 µl reactions containing 0.2 mM each dNTP, ×1 buffer (16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, 0.01% Tween-20), 2 mM MgCl₂, 3 pmol of each primer, 1 U of Taq (Bioline) with the following cycling parameters: 90 °C for 3 min, 30 cycles of 90 °C for 30 s, primer-specific annealing temperature for 20 s, 72 °C for 20 s, and final elongation at 72 °C for 5 min.

Offspring were collected daily or every other day, killed humanely with an overdose of anaesthetic, and preserved in tubes containing 96% ethanol until DNA extraction. Separate tubes were used for each tank and collection date. Parentage assignment benefited from the knowledge that guppy brood cycles last approximately four weeks (Houde 1997, p. 31). This meant that females that had given birth within a window of 7–21 days prior to the day a juvenile was collected could be excluded as candidate mothers. Following DNA extraction, the mother of each juvenile was identified by comparing its genotype with that of the candidate adult females present in the juvenile's tank. Once the mother had been unambiguously identified, the remaining unallocated allele of each of her offspring was compared with those of candidate fathers in the juvenile's tank at each of the four loci. Parentage was assigned when three out of the four loci indicated the same candidate individuals. The fourth locus was used in cases where microsatellite locus stuttering or possible mutation events resulted in one of the alleles not exactly matching the parental genotype. Individuals for whom no matching paternal genotype could be identified were assumed to have been sired by stored sperm.

Table 1. PCR product size ranges, GenBank accession numbers and references for the microsatellite loci used for parentage analysis.

(Although PCR product size ranges show an area of overlap between the Dpa4-labelled G49 and TAA loci, in practice only eight out of the total of 1024 individuals analysed showed allele size overlap at these two loci. These genotypes were resolved by re-analysis without multiplexing.)

primer	PCR product range	dye label	accession number	reference
Pr40	233–425	D3pa	AF467904	Becher <i>et al.</i> (2002)
Pr67	134–196	D3pa	AF533589	this paper
G49	124–259	D4pa	AF026459	Parker <i>et al.</i> (1998)
TTA4	113–158	D4pa	AF164205	Taylor <i>et al.</i> (1999)

Figure 1. Total offspring production by male (filled bars) and female (open bars) guppies over the three months of the investigation.

3. RESULTS

Up to four loci were screened for each adult and juvenile in the investigation. We were able to assign parentage to more than 95% ($n = 799$) of offspring. Out of these, 75% ($n = 596$) were fathered by males in our aquaria; the remainder were attributed to sperm stored from matings in the wild prior to capture. In the case of broods resulting from stored sperm, we determined the minimum number of males responsible by counting the number of male-derived alleles per locus. For example, more than two male-derived alleles in a group of offspring with unassigned paternity meant that at least two fathers were involved. In total, females produced 242 broods (tank mean $\pm$ s.d. = 24.2 ± 3.12) and had an average of 2.65 (s.d. ± 0.885) broods over the course of the investigation. There was no significant difference among tanks in the number of offspring produced by male ($F_{9,92} = 0.662$, $p = 0.74$) and female ($F_{9,89} = 1.18$, $p = 0.32$) guppies. However, as expected, the pattern of offspring production differed markedly between the sexes (figure 1). We used the program SKEW 1.1 (Krieger & Keller 1997) to calculate separate skew indices for males and females in each tank and then examined the difference (treating tanks as replicates) using a one-sample t -test. This revealed that reproductive skew was greater for males than for females ($t_9 = 2.66$, $p = 0.026$; mean male skew = 0.148; mean female skew = 0.062). In both sexes, however, the observed skew was significantly different (95% level) from the values expected under the assumption of random mating (10 out of 10 cases for males and 9 out of 10 cases for

Figure 2. Mating partner number over three months. Male (filled bars) partner number represents the number of females that a male successfully inseminated and produced offspring by. Female (open bars) partner number is the number of males in the home tank that sired a female's offspring.

Figure 3. Relationship between sire number and brood size for the $n = 242$ broods produced during the three-month investigation.

females). A Kolmogorov–Smirnov-two sample test (Sokal & Rohlf 1995) confirmed that the overall distribution of offspring (all tanks summed together) differed significantly between males and females ($D = 0.263$, $n_1, n_2 = 86, 100$, $p < 0.01$). The median number of offspring per individual was five (males) and eight (females).

By contrast, for this population under these conditions, the median number of partners did not differ between the sexes ($t_9 = 0.274$, $p = 0.79$; figure 2). A Kolmogorov–Smirnov two-sample test revealed that there was no significant difference in the overall distributions of partner number ($D = 0.054$, $n_1, n_2 = 86, 100$, $p > 0.05$).

Figure 4. Observed (filled bars) and expected (open bars) levels of partner turnover for the $n = 52$ females producing three broods over the course of the investigation. Observed turnover expresses the actual number of fathers as a proportion of the total possible number of fathers. For example, the score for a female that had three broods, each fathered by two males, and where five different males were involved, would be $5/6 = 0.83$. A value of 1 means that different males fathered each brood. Expected turnover shows the distribution of this turnover index based on 1000 randomizations assuming random mating (from a pool of $n = 10$ males) over three broods with a median of two fathers per brood. The observed and expected distributions of turnover differ significantly (Kolmogorov–Smirnov goodness-of-fit test, $D_{0.5} = 0.30$, $p < 0.01$).

The median number of fathers per brood was two (figure 3). This is consistent with previous estimates based on inherited colour patterns (Houde 1997) and DNA markers (Evans & Magurran 2000). Larger females had more partners ($r_s = 0.23$, $z = 2.06$, $p = 0.04$). There was also a positive association between sire number and the size of individual broods ($r_s = 0.51$, $z = 7.97$, $p < 0.001$; figure 3). The minimum number of sires per brood is of course determined by brood size. We detected a marked turnover in the sires of successive broods (figure 4). This turnover was, however, lower than expected under a model of random mating (see figure 4 for details).

The number of offspring was, as anticipated, related to female standard length ($F_{1,77} = 6.76$, $p = 0.01$). We uncovered a highly significant correlation between the number of partners that males had and the number of offspring they produced ($r = 0.80$, $n = 100$, $p < 0.001$). A stepwise multiple regression involving seven morphological and behavioural variables, and with tank as a factor, revealed that, overall, the only significant predictor of partner number was SL ($F_{1,83} = 6.37$, $p = 0.01$, table 2); smaller males successfully inseminated more females and thus sired more offspring. When the effect of body size was removed by calculating the residual number of partners against standard length, the proportion of body area covered by red colour spots was found to predict male mating success ($F_{1,83} = 3.73$, $p = 0.05$). There was no correlation between male SL and the proportion of red ($r = -0.13$, $n = 86$, $p = 0.24$), or between male SL and the size of female partners ($r = 0.08$, $n = 69$, $p = 0.50$).

4. DISCUSSION

Reproductive skew describes the extent to which reproductively active individuals partition mating activity (Bourke 2001; Sumner *et al.* 2002). As expected (Bateman 1948; Arnold 1983), some guppy males were much more successful than others at fathering offspring (figure 1). Intriguingly, small males sired a disproportionate number of progeny. One possibility is that small males are preferred by females. The literature offers no consensus on this form of choice. Reynolds & Gross (1992) discovered that larger males had an advantage in no-choice mating trials. However, an extensive investigation of female preferences in 11 guppy populations uncovered only one case in which small males were preferred (Endler & Houde 1995). In 9 out of the 11 populations, male size had no influence on female choice and in one case, large males were preferred. Alternatively, male mating tactics may drive the relationship. Houde (1997) notes that males engage in ‘more sneaky’ mating (gonopodial thrusting) when they are small relative to their competitors. Our study confirmed that the frequency with which this tactic is employed is inversely related to male size ($r_s = -0.225$; $z = -2.08$, $p = 0.04$). Despite this, sneaky mating rate could not account for variation in partner number (table 2). Finally, it could be events that take place after mating, rather than male or female courtship activity *per se*, that explain the success of small males. Evans *et al.* (2003b) used artificial insemination to disentangle aspects of male phenotype from potentially confounding variables such as mating order and ejaculate size. The smaller male in a pair sired a greater proportion of the brood than would be expected by chance. Males with more orange coloration were also relatively more successful. Since sperm numbers were identical, Evans *et al.* (2003b) speculate that more successful males produce competitively superior ejaculates. The replication of their finding in our unrestricted and long-term mating experiment reinforces the conclusion that post-copulatory mechanisms may play an important role in determining male reproductive success in guppies.

Female fecundity, by contrast, tracked the distribution of body size. However, females, like males, mated with several different partners (figure 2). It is already known that females mate multiply, and that two or three fathers per brood is not uncommon (Houde 1997; Evans & Magurran 2000; figure 3). In addition, female guppies vary not only in their level of choosiness but also in their preferences for particular male ornaments (Brooks & Endler 2001). A novel finding of our study (figure 4) is the marked turnover in the identities of these partners between successive broods. For this population, under these conditions, this meant that the distribution of partner number in females converged on the male distribution. Nevertheless, females did not switch partners at random; there was greater consistency in sire identity between successive broods than would be expected by chance.

There are at least two explanations for partner switching between broods. First, females may be inconsistent over time in their choice of partners. We are not aware of any tests of repeatability of female choice between brood cycles. However, there is evidence that preferences can change due to copying behaviour (Dugatkin & Godin

Table 2. Predictors of partner number in male guppies.

variables included in final model ^a	coefficient	s.e.	<i>t</i>	<i>p</i>
constant	8.317	3.437	2.420	0.018
male SL (mm)	-0.412	0.163	-2.534	0.013
variables excluded from final model				
proportion of red ^b	1.926	1.605	1.200	0.234
number of sneaky mating attempts ^c	-0.508	0.483	-1.050	0.297
proportion of black ^b	1.460	1.609	0.908	0.367
gonopodium length (mm)	0.206	0.559	0.369	0.713
number of courtship displays ^c	-0.115	0.322	-0.358	0.722
sperm store ($\times 10^6$) ^c	-0.084	0.351	-0.238	0.813
tank	0.008	0.033	0.241	0.810

^a Stepwise multiple regression. *F* to enter, 4; *F* to remove, 3.996; dependent variable: number of females by which males fathered offspring.

^b Following arcsine transformation.

^c Following $\log(x + 1)$ transformation.

1992), while virgins tested at six and 12 months of age show different preferences for ornamentation (Kodric-Brown & Nicoletto 2001). A related explanation, that females prefer unfamiliar males (Hughes *et al.* 1999), is unlikely to hold here as fishes shared the same tank for four months although females may be avoiding previously mated males. Fertility insurance (Birkhead 2000), inbreeding avoidance (Tregenza & Wedell 2002) and genetic complementarity (Reusch *et al.* 2001) may also explain why receptive females mate with more than one male and switch partners from one brood to the next.

Second, post-copulatory mechanisms may select among ejaculates. Females mate multiply during a brood cycle (Houde 1997; Evans & Magurran 2000) and receive additional sperm from sneaky matings (Matthews & Magurran 2000; Evans *et al.* 2003a). Previous studies have demonstrated that the number of sires is fewer than the number of males known to transfer sperm (Evans & Magurran 2000, 2001). Eighty-eight per cent of broods had one or two fathers (figure 3)—an observation consistent with post-copulatory sperm selection. How the winners of sperm competition are chosen remains unclear (but see Evans *et al.* 2003b) although a recent investigation (Pilastro *et al.* 2002) indicates that females may control the number of sperm inseminated during cooperative matings. Irrespective of the mechanisms involved, a sampling effect, whereby a different subset of available inseminates is used on each occasion, could account for the observed sire turnover. These explanations are not mutually exclusive and further research is needed to differentiate between them.

This study has shown that partner skew in females can match that of males. Kelly *et al.* (1999) argue that levels of multiple paternity vary geographically in Trinidad and that sire number is higher in females from high-predation localities, such as the Lower Aripo (see Neff & Pitcher 2002). It would thus be instructive to contrast male and female partner distribution in populations experiencing different levels of predation risk or where sex ratio (Rodd & Reznick 1997) or level of sexual conflict (Magurran 2001) varies, as well as in conditions that more closely resemble the natural stream environment. This

work could help answer a number of topical questions including the mechanisms responsible for the maintenance of high levels of male colour polymorphism in genetically depauperate populations. It is already clear that females do not always share the same mating preferences (Brooks & Endler 2001); turnover in sire identity in successive broods will also limit the extent of directional sexual selection within a population.

The implications of these results extend beyond the guppy system. It would, for example, be interesting to examine patterns of female partner turnover in other species and to ask how this is related to the demography or the ecology of the population concerned. Our work also underlines the importance of obtaining long-term data on individual rates of polygyny and polyandry.

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