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Ejaculate allocation by male sand martins, *Riparia riparia*

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Males of many species allocate sperm to ejaculates strategically in response to variation in the risk and intensity of sperm competition. The notable exception is passerine birds, in which evidence for strategic allocation is absent. Here we report the results of a study testing for strategic ejaculate allocation in a passerine bird, the sand martin (*Riparia riparia*). Natural ejaculates were collected from males copulating with a model female. Ejaculates transferred in the presence of a rival male contained significantly more sperm than ejaculates transferred in the absence of a rival male. There was no evidence that this difference was due to the confounding effects of the year of ejaculate collection, the identity of the model female, the colony, the stage of season or the period of the day in which ejaculates were collected. A more detailed examination of the ejaculate patterns of individual males, achieved by the DNA profiling of ejaculates, provided additional evidence for strategic allocation of sperm.

Keywords: copulation; ejaculate allocation; microsatellite DNA; sand martin; sperm competition

1. INTRODUCTION

Sperm competition occurs when the sperm from more than one male compete for the fertilization of a female's eggs (Parker 1970). Theoretical and empirical studies agree that, when sperm competition occurs, males transferring more sperm than rival males will, on average, fertilize a greater proportion of a female's eggs (Martin *et al*. 1974; Martin & Dzuik 1977; Smith 1979; Parker 1982, 1990*a*,*b*; Muller & Eggert 1989). One characteristic that is known to facilitate an increase in sperm number per ejaculate is testes mass (Parker *et al*. 1997; Birkhead & Møller 1998).

In addition to morphological adaptations to sperm competition, males might also have evolved the ability to allocate sperm strategically to multiple copulations through ecological time (reviewed by Parker 1998). The theory of strategic allocation of sperm to ejaculates relies on two main assumptions. First, that sperm competition occurs. In the absence of sperm competition, males would have no need to allocate sperm but should simply transfer the minimum number of sperm necessary for fertilization (Parker 1982). The second assumption is that sperm are a limited resource (Dewsbury 1982). If there were no limit on the availability of sperm, there would be no selection for males to exert prudent control over their use. Provided that these conditions are met, males will maximize their lifetime reproductive success by allocating sperm strategically in response to cues from their environment.

Parker (1990*a*,*b*) and Parker *et al*. (1996) envisaged two main models of sperm competition. In the 'risk' model, a copulating male is predicted to increase his ejaculate expenditure in proportion to the probability that sperm competition occurs with just one other male (Parker 1990*a*,*b*). In the 'intensity' model, a copulating male faces certain sperm competition and is predicted to decrease his ejaculate expenditure as the number of males that he is competing with increases above two (Parker *et al*. 1996). There is increasing empirical support that these models accurately describe allocation strategies in a range of taxa (reviewed by Parker *et al*. 1997; Birkhead & Møller 1998).

However, in passerine birds, where sperm competition is common (Westneat & Sherman 1997) and frequent copulation appears to leave males' sperm depleted (Birkhead 1991; Birkhead *et al*. 1995; Westneat *et al*. 1998; Nicholls 2000), there is no evidence so far that males strategically allocate sperm. This may be because few tests of strategic allocation have been possible because (i) there is great natural variation in the number of sperm per ejaculate (Amann 1981), and (ii) natural ejaculates are difficult to collect from birds (Møller 1988). However, Pellatt & Birkhead's (1994) technique of collecting ejaculates from males that will copulate with a model female has been successfully exploited in two species.

First, male zebra finches (*Taeniopygia guttata*) did not allocate their ejaculates differently when presented with two model females, either simultaneously or sequentially (Birkhead & Fletcher 1995). Rather, male zebra finches appear to operate under a physiological constraint and can only maximize the number of sperm ejaculated during extra-pair copulations (where they will face certain sperm competition with the pair male) by performing them outside their own copulation period when they are rested with large sperm reserves (Birkhead *et al*. 1995).

Second, Westneat *et al*. (1998) collected natural ejaculates from polygynous wild red-winged blackbirds (*Agelaius phoeniceus*) in order to test the predictions that (i) males would be less likely to copulate with a model female on their territory when their partners were fertile (i.e. when males would have reduced sperm reserves), and (ii) males would avoid copulating with a familiar female with whom they had recently copulated. However, they found no support for these predictions and, hence, no evidence that male red-winged blackbirds were allocating sperm strategically.

In addition to the above two studies, Hunter *et al*. (2000) found that non-passerine male Adélie penguins (*Pygoscelis adeliae*) preferentially allocated sperm to extra-pair females

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Figure 1. Two male sand martins competing to copulate with the model female. The male that transferred sperm (right) was defined as facing sperm competition risk from the competing male (left).

(i.e. under conditions of sperm competition). However, these male Adélie penguins were only able to do this by reducing the frequency of sperm transfer to their partners. Since Hunter *et al.* (2000) did not collect ejaculates using a model female, it is unknown whether these male Adélie penguins had control over the number of sperm contained in the ejaculates that they did transfer.

In the present study, we collected data on the sand martin (*Riparia riparia*), a small insectivorous passerine that breeds in dense colonies. The male sand martin is an ideal species in which to investigate ejaculate allocation strategies because its highly gregarious nature and keenness to copulate with a model female (Petersen 1955; Hoogland & Sherman 1976) mean that a large number of ejaculates can be collected. Furthermore, the two conditions under which strategic ejaculate allocation is predicted to occur are met by the sand martin: (i) sperm competition occurs in this species (Riley 1992; Alves & Bryant 1998), and (ii) after a bout of successive copulations, males show a reduction in sperm number per ejaculate (Nicholls 2000).

The aim of this study was to test the prediction that male sand martins allocate significantly more sperm to copulations in the presence of sperm competition risk than to copulations in the absence of sperm competition risk.

2. METHODS

(a) *Collection of ejaculates*

Fieldwork was conducted at three sand martin colonies on the river Tisza in Hungary (colony 1 in 1998 situated at 48°09.818′ N, 21°27.283′ E and colony 2 in 1998 and colony 3 in 1999 situated at 48°10.654′ N, 21°30.056′ E). Twelve female sand martins that had been killed but not damaged by a predator were preserved in a copulation solicitation position by injection of 5% formaldehyde and presented to males at the three colonies. The season was divided into three stages for each colony (the 'pre-fertile' stage, which was up to day −6, the 'fertile' stage, which was days −5 to +3 and the 'incubation' stage, which was days +4 to +18) relative to day 0, the modal laying date at each colony, which was calculated from frequent nest checks. Each day was divided into three periods ('morning', which was before 07.30, 'midday', which was between 11.00 and 13.00 and 'evening', which was after 16.30, all local times). A copulating male was defined as facing 'sperm competition risk' if, whilst making cloacal contact with the model, a second male made physical contact with him (figure 1). Such competition at the moment of sperm transfer is a common feature of natural copulation in this species (Nicholls 2000).

A model female fitted with a fresh false cloaca containing 5 μl of phosphate-buffered saline (PBS) was chosen at random for each presentation (Pellatt & Birkhead 1994). A wooden pole was used to suspend the model in the middle of the colony, at least 4 m from the observer. Following copulation, the model was retrieved and the ejaculate was removed with a Gilson pipette and diluted in a microfuge tube containing 95 μl of PBS and 100 μl of 5% formaldehyde–PBS to give a total of 200 μl of formaldehyde–PBS–sperm solution. Where ejaculates were located outside the cloaca, the 5 μl of PBS from the cloaca was used for hydrating the ejaculate and facilitating its transfer to the storage tube. Ejaculates collected from inside or outside the false cloaca were not significantly different in size ($t_{456} = 1.48$ and $p = 0.140$). Since the volume of the ejaculate was typically very small (< 5 μl), it was assumed to be negligible compared with the volume of PBS.

(b) *Determination of the number of sperm in ejaculates*

A standard curve was established between the spectrophotometric absorbance of a sperm sample and the concentration of sperm (Bakst & Cecil 1997). Three or four serial dilutions were made for each of ten different ejaculate samples, giving 34 separate 200-μl aliquots covering a range of sperm concentrations. The absorbance of 550-nm-wavelength light was measured for each aliquot using a spectrophotometer (Walden Precision Apparatus Ltd, Cambridge, UK) with a standard solution of 2.5% formaldehyde–PBS. Three absorbance readings were made for each aliquot and these showed high repeatability ($R = 0.994$, $F_{33,68} = 433$ and $p < 0.0001$). The mean absorbance of the same sample, as measured on two separate occasions, was also highly repeatable ($R = 0.976$, $F_{9,10} = 82.4$ and $p < 0.0001$). The sperm concentration of each aliquot was quantified using an Improved Neubauer Haemocytometer (Weber, UK). Six separate estimates were made and the outlier discarded in order to give five highly repeatable measures of sperm concentration for each aliquot ($R = 0.987$, $F_{33,136} = 375$ and $p < 0.0001$). The mean of the five measures (sperm μl^{-1}) was regressed on the mean absorbance of each aliquot ($r^2 = 0.938$, $F_{1,32} = 481$ and $p < 0.0001$) ($y = 1.80 \times 10^4 x + 0.067 \times 10^4$). This relationship was used for all subsequent samples in order to estimate the sperm concentration and, hence, the number of sperm per ejaculate from absorbance.

(c) *Identification of males using DNA profiling of ejaculates*

When a model female was presented at a colony, the number of males attempting to copulate and the speed with which they did so often made it difficult to be certain which male's ejaculate was in the false cloaca. This problem was overcome by using DNA profiling in order to identify males from the microsatellite signature of their ejaculates. Ejaculates were homogenized in 195 μl of PBS to give *ca.* 200 μl of sperm–PBS solution and 100 μl of this mixture was transferred to a microfuge tube containing 1 ml of 100% ethanol. The remaining 100 μl was

preserved in 100 µl of 5% formaldehyde and the number of sperm was quantified as above. The spectrophotometric absorbance of and, hence, the number of sperm contained in divided ejaculates was highly repeatable ($R = 0.996$, $F_{9,10} = 449$ and $p < 0.0001$), thereby supporting the assumption that the division of each ejaculate halved the number of sperm.

All DNA analyses were undertaken at the Natural Environment Research Council Molecular Genetics Facility at the University of Sheffield. A standard phenol–chloroform extraction protocol (Bruford *et al*. 1998) was used for extracting DNA from blood samples collected from 51 adults in 1997 and from 206 ejaculates collected in 1999 where only a single male had made cloacal contact with the model female. For DNA extraction from ejaculates, 1 µl of DTT was added to each sample during the proteinasing stage in order to induce decondensation of sperm nuclei (Gill *et al*. 1985). DNA microsatellite profiles (Ellegren 1992) were determined for each sample using PCR amplification, followed by silver staining at three microsatellite loci (*Hru*6 and *Hru*7 (Primmer *et al*. 1995) and *Pdo*µ5 (Griffith *et al*. 1999)) (see Nicholls (2000) for detailed methods).

The allele frequencies used in the calculation of expected genotype frequencies were obtained at the three loci of the 51 adults sampled in 1997. Samples showing a single allele at a locus were assumed to be homozygotes with two copies of the allele. Hardy–Weinberg and linkage equilibria were tested using GENEPOP v. 3.2 (Raymond & Rousset 1995). There was no significant deviation of the genotype frequencies from Hardy–Weinberg equilibrium. However, there was significant linkage disequilibrium ($p = 0.015$) between the loci amplified by *Pdo*µ5 and *Hru*7. Since *Hru*7 was not very polymorphic (heterozygosity = 0.638) and its inclusion only marginally increased the resolving power, it was not used in the analyses of ejaculate genotype frequency. There was no detectable linkage disequilibrium between the loci amplified by *Pdo*µ5 and *Hru*6 ($p = 1.00$) and the allele frequencies at these two loci were multiplied together in order to generate a two-locus genotype frequency q for each ejaculate. Where alleles in the ejaculates had not been observed previously, their frequency was estimated from genotyped ejaculates. The most common such allele had a frequency of 0.044. The highest value of q was 8.46×10^{-4} and the mean value of q (calculated from 137 ejaculates with different genotypes) was 8.23×10^{-5}. Therefore, the probability that two individuals in this data set would share the same genotype was small, leading to an overall expectation that a total of 1.74 pairwise comparisons would yield identical genotypes in this data set. As 28 different genotypes were observed between two and 16 times each, it is most likely that ejaculates with the same genotype came from the same individual. For these calculations, two assumptions were made: first, that the 51 adults sampled in 1997 were representative of the population as a whole and, second, that there had not been a significant shift in allele frequencies between 1997 and 1999.

(d) *Statistical analyses*

The effect of sperm competition risk on ejaculate expenditure was analysed using a fixed-factor multivariate ANOVA with colony, stage of the season, period of the day and sperm competition risk as factors. In this analysis, the assumptions of homogeneity of variance and normality of residuals were checked. Further to this analysis, a two-group randomization test (over 10 000 randomizations) was performed in order to estimate the probability that any difference in ejaculate expenditure between 'risk' and 'no-risk' males could have arisen by chance.

A second, more rigorous test of allocation, albeit with a small sample size, was performed using DNA profiles in order to identify donor males that had copulated with the model twice in 1 h. Males selected for this analysis had all copulated in the absence of sperm competition risk in their first copulation and the effect of sperm competition risk on their second ejaculate expenditure was analysed using a single factor ('risk' or 'no-risk') ANCOVA with first ejaculate expenditure as the covariate. In comparing the numbers of sperm transferred in successive copulations, two assumptions were made: first, that males did not copulate with another female between copulations with the model female and, second, that sperm replenishment between successive copulations was negligible. Since the interval between successive copulations was often a matter of minutes and never more than 1 h and the pattern of depletion from first to second ejaculate was so similar to that observed for the zebra finch (Birkhead *et al*. 1995), these assumptions seemed likely to be valid. All analyses were conducted with SPSS statistical software (Chicago, IL, USA).

3. RESULTS

(a) *Determination of the number of sperm in ejaculates*

We collected 500 ejaculates where a single male made cloacal contact with the model and where sperm was transferred. Evaporation from some of the stored samples ($n = 39$) meant that the sperm number per ejaculate was quantified for 461 samples. Since there was no significant difference between years in either sperm per ejaculate ($t_{459} = 0.85$ and $p = 0.40$) or in the frequency of copulating with sperm competition risk ($\chi^2_1 = 0.73$ and $p = 0.39$), the data from both years were combined. Since there was no evidence that the identity of the model female had any effect upon the sperm number per ejaculate ($F_{11,449} = 0.40$ and $p = 0.96$) or on the frequency of copulating with sperm competition risk ($\chi^2_{11} = 17.7$ and $p = 0.09$), the identity of the model female was ignored. The mean number of sperm per ejaculate was estimated at $2.82 \times 10^6 \pm 2.27 \times 10^6$ sperm (s.d.) ($n = 461$).

(b) *Ejaculate allocation*

As predicted, the absence or presence of sperm competition risk during copulation had a significant effect upon the number of sperm contained in ejaculates. Table 1 shows the output of the ANOVA for the main effects of the model. There was no evidence that either colony, stage of the season, period of the day or any of the higher order interactions of the model had any significant effect upon ejaculate expenditure. However, on average, males facing sperm competition risk allocated 1.16 times as many sperm to their ejaculates as males that copulated in the absence of sperm competition risk (figure 2) (no risk, $2.70 \times 10^6 \pm 2.23 \times 10^6$ sperm and risk, $3.14 \times 10^6 \pm 2.38 \times 10^6$ sperm) ($F_{1,405} = 6.57$ and $p = 0.011$) ($n = 453$ for this analysis due to eight copulations where sperm competition risk was not recorded). The two-group randomization test tended to support the validity of this result ($p = 0.057$).

There was a significant positive regression of second on first ejaculate expenditure for males that were identified from ejaculate DNA profiles to have copulated twice within the space of 1 h ($r^2 = 0.454$, $F_{1,12} = 9.99$ and

Table 1. *ANOVA table showing the effects of colony, stage of the season, period of the day and sperm competition risk on ejaculate expenditure*

(Higher-order interactions are not shown.)

effect	mean square	d.f.	F	p
colony	7.10	2	1.35	0.261
stage of the season	1.68	2	0.32	0.727
period of the day	0.55	2	0.11	0.901
sperm competition risk	34.63	1	6.57	0.011
error	5.27	405	—	—

$p = 0.008$) ($y = 0.343x + 0.339$). The slope of this regression was significantly less than 1 ($t_{12} = 6.03$ and $p < 0.001$), indicating a clear reduction in ejaculate expenditure from first to second copulation.

The ANCOVA revealed that, when controlling for the significant effect of first ejaculate expenditure on second ejaculate expenditure ($F_{1,11} = 7.56$ and $p = 0.019$), the sperm competition risk experienced during the second copulation had a significant effect on ejaculate expenditure ($F_{1,11} = 5.88$ and $p = 0.034$).

4. DISCUSSION

Male sand martins strategically allocated more sperm to copulations when there was sperm competition risk. Furthermore, in successive copulations, males subject to sperm competition risk allocated sperm to their copulations in a manner that was significantly different from males facing no sperm competition, which is consistent with the theory of strategic ejaculate allocation.

The assumption that each ejaculate was an independent measure of expenditure was implicit in the first analysis, i.e. the multivariate ANOVA of ejaculate expenditure. However, the DNA profiling of ejaculates for the second analysis revealed that some males had copulated with the model female on more than one occasion and, hence, the measures of ejaculate expenditure in the first analysis were not strictly independent. If the males in a state of sperm depletion were somehow able to avoid competition with rival males systematically, then it is theoretically possible that their depleted state could have accounted for the significantly lower ejaculate expenditure of males copulating in the absence of sperm competition risk. However, from the field observations it seemed unlikely that a copulating male had control over whether or not a rival male subjected him to sperm competition risk. Furthermore, the frequency of repeat copulations and, hence, the degree of pseudo-replication in this analysis was not high.

Indeed, it was the low level of repeat copulations that inhibited the sample size in the second analysis. Nevertheless, this provided further support for the suggestion that sand martins were strategically allocating sperm to their ejaculates. Together, these analyses yield, to the authors' knowledge, the first evidence that male birds can vary sperm number per ejaculate in response to the risk of sperm competition.

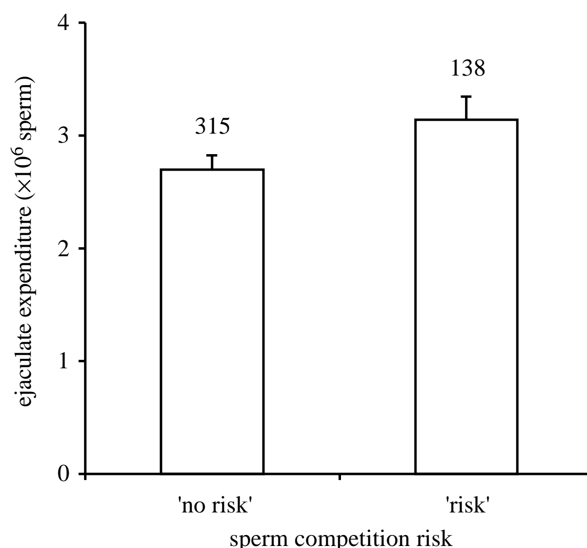

Figure 2. Mean ejaculate expenditure (±s.e.) in the absence and presence of sperm competition risk. The sample sizes are shown.

There are several possible explanations for the fact that ejaculate allocation has not been detected in the other passerines studied to date. First, the other species may not have met the conditions under which ejaculate allocation is predicted to occur. Sand martins have both a level of sperm competition that is typical of a passerine species and a finite sperm reserve that appears to become depleted following a bout of copulation. Since the sand martin's seminal glomera contain $8.14 \times 10^6 \pm 7.90 \times 10^6$ sperm ($n = 14$) (Nicholls 2000), the estimate of 2.82×10^6 sperm per ejaculate suggests that there are between two and three inseminations' worth of sperm stored in the combined seminal glomera of a sexually active male sand martin. Although the zebra finch has a similar number of inseminations' worth of sperm in its seminal glomera (Birkhead *et al.* 1995), its level of sperm competition is probably low since only an estimated 2.4% of offspring resulted from extra-pair fertilization (Birkhead *et al.* 1990). In contrast, the red-winged blackbird experiences a high level of sperm competition, with 24–35% of chicks on a male's territory being sired by an extra-pair male (Gibbs *et al.* 1990; Westneat 1993; Gray 1996). However, when rested, a male red-winged blackbird has enough sperm for six copulations and is able to replenish its sperm reserves rapidly following a bout of copulation (Westneat *et al.* 1998). This could mean that allocation of sperm to ejaculates was less important to these species and, hence, more difficult to detect than it was in the sand martin.

Alternatively, male zebra finches and red-winged blackbirds may not have been subjected to appropriate manipulations of sperm competition risk. For example, in a study of strategic ejaculate allocation in the Indian meal moth (*Plodia interpunctella*), it was found that male moths did not allocate sperm in response to the presence of a rival male (Cook & Gage 1995), even though this is a cue that influences male allocation strategies in several other species (e.g. Gage 1991; Gage & Baker 1991). However, the male moths did allocate sperm in response to the presence and size of a rival spermatophore in the female

reproductive tract (Cook & Gage 1995). This is probably because male Indian meal moths neither congregate around nor guard females prior to copulation, so female mating status rather than the presence of rival males is a more reliable measure of sperm competition risk in this species (Cook & Gage 1995).

Further research on ejaculate allocation is needed in the sand martin and other passerine species in order to determine the occurrence of this trait and understand the mechanism that underlies it. Candidate species should be those where males clearly have access to information on sperm competition risk in a natural situation. This is because, in the absence of information on sperm competition risk, males should treat all copulations equally and no allocation strategy would be expected, no matter how high the level of sperm competition (Parker *et al*. 1997; Ball & Parker 1998). In addition, care should be taken over how perceptions of sperm competition risk and intensity are manipulated. Finally, a large number of ejaculates may be needed in order to detect any allocation strategy above the noise caused by the extreme natural variation in the number of sperm contained in avian ejaculates.

In conclusion, the results presented in this study provide strong support for the hypothesis that some of the variation in sperm number per ejaculate in birds might be due to strategic allocation of sperm in response to variation in the risk of sperm competition.

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