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Biases in sperm use in the mallard: no evidence for selection by females based on sperm genotype

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If we are to understand fully the factors influencing fertilization success, it is essential to untangle male and female effects on sperm use. In many species, differences in fertilizing ability have been found between males or male genotypes, but the impact of female effects is less clear and may vary between taxa. Here, we examine sperm use in the mallard (*Anas platyrhynchos*), a species of bird in which forced copulation forms a major component of the mating system, to investigate whether there is any evidence for post-insemination female choice or rejection of particular sperm genotypes. Current models of sperm use in birds suggest observed patterns of paternity are a result of passive sperm loss from the reproductive tract and the relative timing of inseminations. Although this type of model successfully predicted average values of last male precedence observed in this species, there was considerable variation between females in their pattern of sperm use, with a tendency for females to use sperm of a single genotype. However, females did not consistently prefer one genotype over another in repeated inseminations with identical sperm mixtures, suggesting that post-insemination female preference based on sperm genotype did not account for this variation.

Keywords: sperm competition; post-insemination choice; paternity; mallard

1. INTRODUCTION

When a female copulates with more than one male, not only may sperm from the different males compete inside the female's reproductive tract to fertilize her ova (Parker 1970) but females may also choose between their sperm to influence the paternity of their offspring (Eberhard 1996). This possibility has recently been the subject of considerable debate, but it has been difficult to demonstrate unequivocally due to the problem of untangling male and female influences on sperm use (Simmons *et al.* 1996; Birkhead 1998). However, in studies in which females are inseminated with sperm from more than one male, the fertilization success of sperm from different genotypes often differs; this can often be attributed to differences between males in their fertilizing ability (e.g. Simmons & Parker 1992; Dziuk 1996). However, recent studies have suggested that, in some species, female traits may also influence sperm use. Wilson *et al.* (1997) recently found evidence suggesting female genotype may influence the outcome of sperm competition. They conducted trials with the bruchid beetle (*Callosobruchus maculatus*) in which two males were both mated with three full sisters and three unrelated females; the proportion of offspring fathered by each male was highly repeatable between sisters but not between unrelated females. Furthermore, Olsson *et al.* (1996) found that, when female sand lizards (*Lacerta agilis*) copulate with more than one male, more offspring are fathered by the males that are most genetically dissimilar to the female. In contrast, a similar mammalian study found no evidence for any bias in sperm use by female common shrews (*Sorex araneus*) that had copulated with more than one male; relative fertilization success appeared to be explained by the sperm numbers inseminated (Stockley 1997).

It is of considerable importance to identify systems in which females can or cannot influence sperm use as these effects have wide implications for our understanding of the factors determining fertilization success. In birds, there is considerable evidence that females prefer particular males as their copulation partner, but despite considerable speculation, it remains unclear whether post-insemination female effects can influence sperm use. Patterns of paternity are, however, known to be influenced by sperm genotype and a strong last-male effect. Recent studies examining patterns of paternity following both natural copulations (Colegrave *et al.* 1995) and controlled artificial inseminations (Birkhead *et al.* 1995; Birkhead & Biggins 1998) have found that these patterns of last-male precedence were best explained by passive sperm loss (PSL) models (Lessells & Birkhead 1990). These models assume that, in a situation where two equally sized inseminations are made some time apart, sperm from the first insemination are released at a constant rate from a female's sperm storage sites over the

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period of egg laying (passively lost) and, when a second insemination occurs some time later, these sperm are also lost at the same rate. However, because sperm numbers from the first male have already started to decline before the second insemination, at any given time there will be more sperm from the second male available to fertilize an egg. If a higher representation of sperm means a higher probability of fertilizing an egg (Parker's (1990) fair raffle principle), the second insemination would fertilize more eggs as more sperm would be present from this second insemination at any given time. Although current models take into account differences in the fertilization success of sperm from different genotypes, no attempt has yet been made to untangle whether these stem from inherent differences in the fertilizing ability of sperm from different males or the biased use of sperm of a given genotype by individual females.

Mechanisms of sperm selection or rejection might be expected to be most apparent in species where behavioural control of paternity is not possible. Therefore, in this paper we examine sperm use in the mallard (*Anas platyrhynchos*), a species in which forced copulation is a major component of the mating system (Barash 1977). These copulations do not appear to be initiated by females in order to incite copulations from preferred males and, furthermore, when males are ranked directly from observations of female preference, male rank does not influence the outcome of forced copulation attempts (Cunningham 1997). We first examine whether PSL models can explain patterns of paternity in the mallard following different patterns of insemination and, second, whether any bias in sperm use would appear to stem from male or female effects.

2. CAN MALE TRAITS AND CURRENT MODELS OF SPERM USE ACCOUNT FOR PATTERNS OF PATERNITY IN THE MALLARD?

Mallards show relatively high levels of last-male precedence compared to other species (Cheng *et al.* 1983) so we first examined whether current models of sperm use can explain the observed patterns of paternity in this species.

Birkhead *et al.* (1995) expressed the PSL model mathematically as

$$\log_e(P/1-P) = d + \mu T - \log_e I,$$

where P is the proportion of offspring fathered by the second male, d is any difference in fertilizing capacity between the sperm of the two inseminations, μ is the instantaneous rate of loss of sperm from the reproductive tract, T is the time interval between inseminations and I is the size difference between the two inseminations.

We calculated values for these parameters from a combination of new, experimentally derived data, and a series of sperm competition experiments using the recessive white plumage gene of the mallard as a genetic marker (Cheng *et al.* 1983).

(a) *Methods and material*

(i) *Calculating μ—the rate of passive sperm loss*

Mallards lay one egg each day and all sperm that are present at the site of fertilization become trapped between the perivitelline layers that surround the yolk as the egg is formed shortly after fertilization takes place. Hence, the number of sperm in successive eggs in a clutch provides a measure of the rate at which sperm are lost from sperm storage tubules, providing no copulations occur once egg laying has commenced (Wishart 1987; Brillard & Bakst 1990).

Twenty mallards were housed as pairs in individual pens (8 m × 2 m) with a nesting cover and a 2 m stretch of free-flowing water, and fed *ad libitum* on commercial duck mix. Pairs were allowed to copulate freely until the first day of egg laying when males were removed to prevent any further inseminations. Eggs were collected daily and replaced with dummy eggs until each female had laid a complete clutch. After collection, sperm counts were made according to the techniques of Wishart (1987). The rate of sperm loss from the sperm storage tubules was estimated from the point in the clutch where sperm uptake was complete by calculating the regression for each female of the $\log_e$ number of sperm in successive eggs in a clutch in relation to time. The slope of this relationship is the instantaneous *per capita* loss of sperm per day (Wishart 1987; Lessells & Birkhead 1990).

(ii) *Calculating P—the proportion of offspring fathered by a given insemination*

Seven groups of 16 female mallards were inseminated with equal amounts of sperm from eight males of two genotypes, DW (recessive white plumage) and GF (wild-type plumage) (see Lancaster 1963; Cheng *et al.* 1983); one group of DW females received the two sperm types mixed together and two groups of DW females were each inseminated with the two sperm types, in reciprocal order, 1, 3 or 6 h apart (for further details see Cheng *et al.* (1983)). All inseminations occurred outwith 'the insemination window', a period lasting *ca.* 1 h following egg laying when the contractions of the oviduct associated with egg laying may influence the uptake of sperm (Birkhead *et al.* 1996). When a female is inseminated by two males, the pattern of sperm use can be expressed numerically as the proportion of offspring fathered by a particular insemination; in our experiments, this was the proportion of offspring from DW inseminations. These proportions can be combined for each female to give a value for P, the probability that a chick will be fathered by DW sperm when DW and GF inseminations are made a certain time apart.

(iii) *Calculating d—differences in fertilizing capacity between males and between sperm types*

Birkhead *et al.* (1995) suggested that d can be estimated by

$$d = 1/2(\log(P_1/1-P_1) + \log(P_2/1-P_2),$$

where P_1 and P_2 are the probabilities of being a DW chick in two experiments that differ only in the order of inseminations. This measure of differential fertilizing capacity may reflect a bias in sperm use by females in favour of sperm of a particular genotype or inherent differences between males in their fertilizing ability. Differential fertilizing capacity was first investigated by examining fertilization success following single inseminations with each genotype. The differential fertilizing capacity d was then calculated from experiments where sperm were mixed and inseminated together. Data from the experimental inseminations were not used to calculate d; this avoided estimating one of the variables being used to generate the model's predictions from the same data that are being used to test these predictions.

Table 1. *Ratios of progeny following inseminations at different time intervals with sperm from domestic white mallard (DW) and wild-type game farm mallard (GF)*

(Variation around the predicted values of sperm precedence given by the passive sperm loss model and results from Kolmogorov–Smirnov one-sample tests were used to examine whether the variation is significantly greater than would be predicted by binomial variation alone.)

order of inseminations	time interval between inseminations	mean proportion of offspring sired by the second insemination	coefficient of variation (%)	probability that variation is greater than expected binomial variation
mixed GF–DW	none	0.51	56	0.005
GF–DW	1 h	0.23	155	0.002
DW–GF	1 h	0.50	82	<0.001
GF–DW	3 h	0.56	77	<0.001
DW–GF	3 h	0.28	49	<0.001
GF–DW	6 h	0.86	29	<0.001
DW–GF	6 h	0.42	72	<0.001

(iv) *Comparison of predicted and observed results*

Predicted patterns of paternity were calculated from the PSL model using the parameters detailed above. They were then compared with the observed levels of paternity in the sperm competition trials described.

(b) *Results*

(i) *Rate of sperm loss*

Seven complete clutches were obtained with a mean clutch size of ten eggs (range 6–14 eggs). The mean *per capita* rate of loss over the whole clutch was $0.0490\,h^{-1}$ (s.e. $=0.0052$ and $n=7$ females).

(ii) *Proportion of offspring sired by each male*

The mean proportions of offspring sired by each insemination in the series of experiments are displayed in table 1. The variation in the patterns of paternity displayed by individual females around average *P*-values was high, as shown by the coefficients of variation (table 1), and was higher than that expected from binomial variation alone.

(iii) *Differential fertilizing capacity*

Cheng *et al.* (1983) controlled for individual differences between males within one sperm type by pooling semen from eight different males. However, they found a difference in the duration that fertile eggs were produced by females inseminated by the two genotypes (Cheng *et al.* 1983). Following single inseminations, the pattern of fertilization success differed from day 6 onwards, suggesting that differences in fertilizing capacity may stem from differences in longevity between the two sperm genotypes (figure 1). Inseminations with DW sperm continued to produce more fertile eggs over the latter part of the clutch than GF sperm, suggesting a difference in survivorship between the two sperm types ($F=15.44$, d.f. $=1,11$ and $p=0.003$). Between days 1 and 5 $d=0.096$ (s.e. $=0.3047$) and between days 6 and 14 $d=-0.2492$ (s.e. $=0.3941$).

Although the two genotypes displayed a difference in their fertilizing ability there was no difference in the hatching success of eggs fertilized by the two sperm types (Mann–Whitney, $W=161$, $n=12$ and $p=0.54$) once these differences in fertility were taken into account.

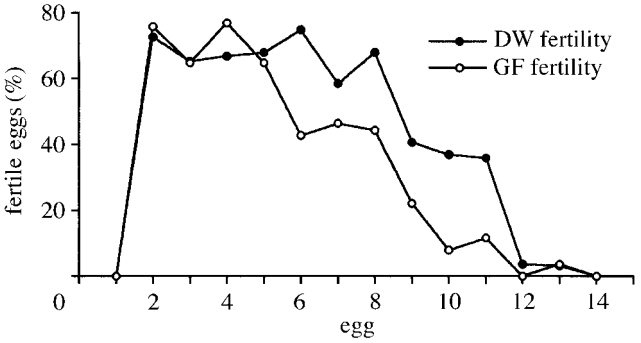

Figure 1. Percentage of fertile eggs produced over the laying sequence following single inseminations of sperm from domestic white mallard (DW) and wild-type game farm mallard (GF).

(iv) *Comparison of predicted and observed results*

Data from days 1–5 and 6–14 should be examined separately to account for sperm from the different males behaving differently after day 6 (see below). To be statistically correct, the results for each period should then be combined and each clutch used as a single data point. However, this would increase the associated standard error and, hence, bias the likelihood of finding the model to be consistent with the data. However, as the clutch sizes were large, data were examined separately for the two periods as this is more biologically realistic. The results are summarized in tables 2 and 3. The observed patterns of paternity following multiple inseminations 1, 3 and 6 h apart did not differ significantly from those predicted by the PSL model in the first half of the clutch (days 1–5) (table 2). In the latter part of the clutch, four out of six multiple inseminations resulted in patterns of paternity that did not differ significantly from those predicted by the model (table 3). However, there was a significant difference in two of the experiments; experiment DW–GF3 where sperm were inseminated 3 h apart and experiment GF–DW6 where sperm were inseminated 6 h apart. However, their reciprocal experiments did not follow the same pattern. These exceptions departed from the expected pattern in different directions, one being a higher level of last-male precedence than expected and the other being lower than expected. The standard errors were large, as

Table 2. *Comparison of observed patterns of paternity following double inseminations with patterns predicted from the passive sperm loss model between days 1 and 5*

(p is significant at 0.025 after Bonferroni corrections as each data set is tested twice, once for the first half of the clutch and once for the second.)

		observed		expected		
experiment	number of ducks	$\log(p/1-p)$	s.e.	$\log(p/1-p)$	s.e.	probability value
GF–DW1	16	−1.1427	0.6998	0.145	0.3047	0.091
DW–GF1	16	−0.0720	0.4966	0.047	0.3047	0.841
GF–DW3	16	−0.0881	0.4649	0.243	0.3051	0.779
DW–GF3	16	0.3847	0.4938	−0.051	0.3051	0.453
GF–DW6	16	1.4631	0.6747	0.390	0.3062	0.147
DW–GF6	16	0.2696	0.4073	−0.198	0.3062	0.357

Table 3. *Comparison of observed patterns of paternity following double inseminations with patterns predicted from the passive sperm loss model between days 6 and 14*

(p is significant at 0.025 after Bonferroni corrections as each data set is tested twice, once for the first half of the clutch and once for the second.)

		observed		expected		
experiment	number of ducks	$\log(p/1-p)$	s.e.	$\log(p/1-p)$	s.e.	probability value
GF–DW1	16	−1.1472	0.6998	−0.2000	0.3941	0.242
DW–GF1	16	0.2696	0.4888	0.2980	0.3941	0.368
GF–DW3	16	0.8712	0.6635	−0.1020	0.3944	0.207
DW–GF3	16	2.7339	0.5587	−0.3960	0.3944	0.0002[a]
GF–DW6	16	1.8070	0.5036	0.0450	0.3958	0.006[a]
DW–GF6	16	0.3722	0.4803	−0.5430	0.3958	0.126

[a] Significantly different from passive sperm loss.

expected from the distribution of the values of P, which showed considerable variance around the predicted patterns of paternity.

3. DO FEMALES CONSISTENTLY PREFER PARTICULAR SPERM TYPES?

The two genotypes of male described in the above experiments differ in their fertilizing success. This difference persists in non-competitive situations suggesting that this difference may arise from an inherent difference in the fertilizing ability of DW and GF sperm. However, female effects cannot be excluded. Therefore, to examine this possibility further, we examined whether females show a consistent preference for sperm of one particular genotype.

If it is assumed that preference for particular males remains constant over a breeding season, the possibility that females may select sperm on the basis of sperm type can be examined by investigating whether females repeatedly use the same sperm type under standardized conditions. As birds store sperm for relatively short periods of time and can produce several clutches in a season, repeated measurements of sperm precedence can be obtained from the same female in one breeding season. Male effects arising from differences in sperm numbers and rate of sperm transfer can be controlled for using artificial insemination techniques.

(a) *Materials and methods*

Sperm were collected and pooled from eight different males of each genotype (DW and GF). Five DW females were artificially inseminated with equal volumes of DW and GF sperm mixed together on three separate occasions, allowing time for sperm from previous inseminations to be lost from the sperm storage tubules between trials. A further five DW females were inseminated with equal mixtures of DW and GF sperm on two separate occasions. Following insemination, eggs were collected daily and incubated artificially. Paternity was again assigned on the basis of plumage. Whether females consistently used the sperm of one genotype in a series of identical inseminations was examined by calculating the repeatability of the proportions of offspring fertilized by the two sperm genotypes for a series of clutches from each female (Lessells & Boag 1987). All proportional data were arcsin transformed.

(c) *Results*

If paternity was simply a result of the relative numbers of sperm present in the reproductive tract, the proportion of offspring fathered by each genotype would be predicted to be 0.5 when equal quantities of sperm from males of the two genotypes were mixed and inseminated together. On average, the proportion of offspring sired by each genotype did not differ from 0.5 ($t=0.2$, $n=28$ and $p=0.84$), but again a wide variation in sperm precedence patterns occurred (coefficient of variation = 56.11%). Furthermore, examination of the kurtosis of the data

suggested that the patterns of paternity tended towards a bimodal distribution ($g^2 = -1.44$, -1.38 and -1.13 in each of the three trials), suggesting that females are more likely to use sperm from one insemination than the other. However, there was no preference for one particular sperm type; repeatability analyses showed that individual females were not consistent in the sperm genotype they used between clutches ($r = -0.02$, $F_{2,22} = 0.78$ and $p = 0.42$).

4. DISCUSSION

We first examined whether current models of sperm use can explain patterns of paternity in the mallard. In general, the observed patterns of paternity did not differ significantly from the model. However, in two cases, paternity in the latter part of the clutch deviated significantly from that predicted by the model but were not consistent in the direction in which they deviated. However, in the mallard, egg fertility starts to decline nine days after insemination (Elder & Weller 1954), so patterns of sperm use occurring after this period could be influenced by any sudden release of any remaining sperm left in the sperm storage tubules as they degenerate at the end of egg laying (Briskie 1996).

Despite the model correctly predicting mean values of paternity in 10 out of 12 experiments, there was a high degree of variance around the predicted values of paternity throughout the entire clutch. This has been a feature of similar studies in other taxa (Lewis & Austad 1990) and studies that have separated male and female effects have shown that part of this variation can be attributed to differences between males, for example in their ejaculate size and copula duration (Simmons & Parker 1992; Dziuk 1996). However, the artificial insemination techniques employed in our experiments should standardize many of these variables, yet considerable variation around predicted patterns of paternity still remains, with a tendency for females to use more of one sperm type than expected. Several factors may account for this.

One possibility is that females may be influencing sperm use following insemination. Females have the potential to manipulate sperm use at two levels: (i) differential storage or use on the basis of male phenotype, assessed before or during copulation, or (ii) by detecting differences in sperm types on the basis of sperm phenotype after insemination. We tested this second possibility in our second experiment. If sperm selection or rejection occurred at the level of sperm phenotype, it would be predicted that females would consistently use the same sperm genotype when repeatedly inseminated with the same sperm combination. However, female mallards were not found to be consistent in the genotype of sperm they used. Furthermore, despite the apparent differences between male genotypes in sperm longevity observed following single inseminations with each sperm type (figure 1), there was also no consistent male effect as might be expected. This suggests that sperm use may be influenced by other factors. The observation that the distribution of paternity shows a tendency towards a bimodal distribution, with females likely to produce offspring mainly from a single insemination, may occur because sperm do not mix evenly and remain grouped together in the sperm storage sites. Current models of sperm competition assume that sperm from different inseminations mix completely and evenly. Whether sperm may remain grouped together after insemination, which could be reflected in subsequent sperm use, now requires further investigation.

However, variation in patterns of paternity may also stem from several other sources. Some clutches also showed an increase in sperm numbers on the second and third eggs of the clutch despite no copulations occurring after the first egg was laid. This suggests that complete sperm uptake may take longer than 24 h, as the model assumed. Some of the variation may also arise from the experimental procedure. In a separate series of experiments of single inseminations, 11.4% of all inseminations produced a whole clutch of infertile eggs, suggesting that some inseminations were not effective. Furthermore, while inseminations were standardized for semen volume, they were not controlled for semen concentration, and the same volume of semen may vary in the number of sperm; but this variation was minimized by pooling semen from different males.

In summary, while observed patterns of paternity in the mallard are consistent with the patterns of paternity generated by the PSL model, mean values mask the considerable variation in sperm use patterns shown by individual females. The sources of this variation now need to be examined in more detail, but the findings of this study suggest that post-insemination sperm selection on the basis of sperm phenotype does not account for biases in sperm use in this species.

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