Ejaculate features and sperm utilization in peafowl *Pavo cristatus*

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SUMMARY

We estimated the number and quality of sperm that male peafowl (*Pavo cristatus*) transferred during copulation to examine differences in these features between males. Ejaculate size was estimated in two ways: directly from sexually rested males copulating with a stuffed female (mean number of sperm per ejaculate $124 \times 10^6 \pm 39.73 \times 10^6$ s.d.); and indirectly as the number of sperm on the outer perivitelline layers of laid eggs (1386 ± 116 s.d.). Neither method revealed any significant difference between males. The mean percentage of live sperm in ejaculates was $84\% \pm 12$ s.d. ($n = 6$ males) and the number of live sperm per ejaculate was significantly and positively correlated with the number of sperm found on the outer perivitelline layers of eggs laid by females inseminated by those males. There was no significant difference between males in the proportion of live sperm per ejaculate. The instantaneous rate at which sperm were lost from the female tract was 0.0067 sperm $h^{-1} \pm 0.0008$ s.e. and the mean duration of sperm storage was 26 days ± 8.6 s.e. ($n = 9$ females), both these values are intermediate between those found in other galliformes. Beacause neither of the two methods for estimating of the numbers of sperm per ejaculate size differed significantly between males, it seems unlikely that female peafowl could obtain reliable direct fertility benefits from choosing to copulate with particular males.

1. INTRODUCTION

The patterns of copulation in birds are better known than in any other animal taxon (Birkhead *et al.* 1987; Birkhead & Møller 1992*a*), but the numbers of sperm that males transfer during copulation and the way that females utilize sperm are known for very few species (e.g. domestic fowl *Gallus domesticus*, Brillard & Antoine 1990; zebra finch *Taeniopygia guttata*, Birkhead *et al.* 1993). The females of most birds copulate several times for each clutch, yet it has been demonstrated in several species that a single insemination can provide sufficient sperm to fertilize an entire clutch even though successive ova of a clutch may be fertilized over a period of one or more weeks (Howarth 1971; Birkhead & Møller 1993). Among lekking birds, females usually copulate only once or a few times during a breeding cycle. In these species sperm competition appears to be relatively rare, female choice is relatively unconstrained and in the majority of species most females on a lek copulate with the same male or subset of males (Alatalo *et al.* 1991; Wiley 1991; Birkhead & Møller 1993). As females of lekking species obtain nothing other than semen from the males they copulate with, most studies have assumed that they gain only indirect (genetic) benefits from their choice of copulation partner (Bradbury & Gibson 1983; Alatalo *et al.* 1991). However, as Reynolds & Gross (1990) and Kirkpatrick & Ryan (1991) have pointed out (see also Avery 1984; Sheldon 1994), females may also obtain direct benefits by copulating with a particular male and the most likely of these is the quantity and quality of semen they receive. In some lekking species a female's choice of copulation partner appears to be based on the male's display rate (Gibson & Bradbury 1985) and Mjelstad (1991) found in a study of capercaillie *Tetrao urogallus*, that males with the highest display rate also had the highest quality ejaculates.

The peafowl *Pavo cristatus* is a lekking species whose breeding biology is known in some detail: females copulate preferentially with a small subset of males at a lek and only once or a few times for each clutch of eggs several days before egg laying starts (Petrie *et al.* 1991, 1992; Petrie & Halliday 1994). There is good evidence that female peafowl obtain indirect benefits from the males they copulate with because the females that copulate with the most attractive males produce faster growing offspring which subsequently have a greater chance of survival (Petrie 1994). This result does not preclude the possibility that female peafowl also gain direct fertility benefits in the form of ejaculate quality from copulating with a particular male. This study reports some preliminary estimates of the size and quality of peacock ejaculates and describes how female peafowl utilize inseminated sperm.

2. METHODS

The study was conducted on peafowl (10 males and 20 females) breeding in captivity. Estimates of ejaculate size and quality were obtained in two ways. First, we collected entire ejaculates from males copulating with a stuffed peahen. Our

original intention was to use a false cloaca to collect semen (see Pellatt & Birkhead 1994), but the posture adopted by male peafowl during copulation was more extreme than our mounted female would permit and so ejaculates were usually deposited on the wooden base supporting the stuffed hen. Entire ejaculates were placed in 1 ml of phosphate buffered saline (PBS). A subsample was taken and we recorded the proportion of live and dead sperm by staining with eosin and nigrosin (Cooper & Rowell 1958). This method is simple to apply under field conditions and provides results similar to those use more complex, laboratory-based procedures (e.g. Chaudhuri *et al.* 1988). The rest of the ejaculate was diluted and fixed in 10% formalin, and stored for later counting using an Improved Nuebauer Chamber. Successive samples were separated by 48 h or more to minimize any depletion effects (see Bakst & Cecil 1981). Only six of the ten males copulated with the stuffed female: for these we obtained between one and three ejaculate (1 in one case, 2 in two cases and 3 in three cases). Second, the same ten males were allowed to copulate with two different live females each and we then recorded the total numbers of sperm attached to the outer perivitelline layer and penetrating the inner perivitelline layer of laid eggs (see Wishart 1987; Birkhead *et al.* 1994). The sum of the number of holes in the inner perivitelline layer and the number of sperm on the outer perivitelline layer provides an estimate of the total number of sperm in the female's infundibulum at the time of fertilization, and in poultry this is closely correlated with the numbers of sperm in the sperm storage tubules and with the original number inseminated (Wishart 1987; Brillard & Antoine 1990; Brillard & Bakst 1990). Twenty different female peafowl were allowed to copulate once with particular males and the sperm penetrating and attached to the perivitelline layers of all eggs laid subsequently by these females was recorded as follows: eggs were collected on the day of laying and kept cool for between 1 and 6 days before being examined. Eggs were opened and the perivitelline layers from around the yolk removed, washed in PBS and mounted on a microscope slide. Holes in the inner perivitelline layer were observed and counted with a $\times 10$ objective using dark field optics. On the same preparation sperm on the outer perivitelline layer were counted by staining their nuclei with the Hoechst dye 33342, and observed and counted using a $\times 25$ objective. Counting methods were the same as those described by Birkhead *et al.* (1994), which comprised counting the numbers of sperm nuclei and holes in known areas of perivitelline membrane at and away from the germinal disc, measuring the diameter of the germinal disc region and the entire ovum (to the nearest mm), and calculating the total numbers of sperm (the sum of holes plus nuclei) on the entire ovum.

To determine whether any correlations existed between the ejaculate features and male plumage characteristics we measured tail, length, eye spot width and the number of tail feathers with eye spots for the six males from which we obtained sperm samples. These tail features have previously been shown as important in female peafowl mating preferences (Petrie *et al.* 1991; Petrie & Halliday 1994). However, there were no significant correlations between any male ejaculate feature and any aspect of tail morphology (all correlations, $p > 0.05$). This could arise if the tail morphology of our subsample of males was particularly uniform, and there was some evidence that this was the case. We compared the coefficient of variation for three tail features in our sample of six males with the eight males in Petrie & Williams' (1993) study: repectively these values were, tail length: 7.3% and 6.8%, eye spot width: 4.9% and 6.2%, and the number of feathers with eye spots: 4.5% and 8.5%. As it is difficult to draw any conclusions from these data they are not discussed further.

The duration of sperm storage in female birds is the number of days over which they lay fertile eggs after the last copulation (Lake 1975). Because we were unable to classify unincubated eggs as fertile or infertile on the basis of the appearance of the germinal disc (c.f. Kosin 1944) here, and because we destroyed eggs to remove the perivitelline layers before any incubation, we defined the end of the fertile period as the day on which the first egg with no detectable sperm on the outer perivitelline layer was laid.

In female birds, sperm are released from the sperm storage tubules at a constant rate and are then transported to the infundibulum, where fertilization takes place (Bakst *et al.* 1994). The way female peafowl utilized stored sperm was examined by determining the relation between the total numbers sperm on the perivitelline layers of successive eggs and time. In fact, we estimated the instantaneous per capita rate of loss of sperm from the reproductive tract from the slopes of the relation $\log_e$ (total sperm on the perivitelline layers) with time for each female (see Lessells & Birkhead 1990). Because female peafowl sometimes laid at irregular intervals, it was impossible to compare the numbers of sperm on eggs on specific days between females. We therefore used the intercept of the relations $\log_e$ (total sperm on the perivitelline layers) and time as an index of the initial number of sperm in the female reproductive tract to make comparisons between males.

3. RESULTS

(*a*) *Males*

The overall mean ejaculate size was 124.08×10^6 sperm $\pm 39.72 \times 10^6$ s.e. (range of means for males: 92×10^6 to 191.5×10^6; range of all values: 4.2×10^6 to 248×10^6) and there were no significant differences between males in ejaculate size (ANOVA, $F_{4,12} = 0.774$, $p > 0.5$). The lack of difference between males was not due to inaccuracies in the methods used to count sperm as the repeatability (Lessells & Boag 1987) of counts within samples was high ($r = 87.2\%$, $F_{16,84} = 35.0$, $p < 0.0001$ using data for all samples and $r = 88.2\%$, $F_{5,29} = 38.44$, $p < 0.0001$ using a single randomly chosen sample for each male). The overall mean percentage live sperm was $83.66\% \pm 12.13$ s.e. (range of means for males: 98.7 to 63.5; range of all values 98.7 to 41.7). Again, there were no significant differences between males in the percentage (arcsin transformed) live sperm (ANOVA, $F_{4,10} = 1.194$, $p > 0.4$). The overall mean number of live sperm per ejaculate was $103.07 \times 10^6 \pm 34.10 \times 10^6$ s.e. (range of means for males 73.23×10^6 to 161.05×10^6, range of all values 1.75×10^6 to 207.6×10^6), with no significant differences between males ($F_{4,10} = 0.637$, $p > 0.6$). Across males there was no correlation between mean ejaculate size and the mean proportion of live sperm ($r_s = 0.314$, 4 d.f., $p > 0.05$).

There was a tendency for the mean number of sperm in an ejaculate to be positively related to the intercept of the relation between $\log_e$ (total sperm numbers on perivitelline layers) and time for each male although this was not significant ($r = 0.601$, 4 d.f., $p < 0.2$; $r^2 = 0.361$). However, the relation between the total number of live sperm and the intercept of the relation

between $\log_e$ (total sperm on the perivitelline layers) and time for each male, was significant ($r = 0.838$, 4 d.f., $p < 0.05$; $r^2 = 0.702$). In other words, the number of live sperm in an ejaculate was positively correlated with the estimated number of sperm reaching the site of fertilization. Although the sample sizes are small and hence the power of these tests, low, the difference in the r^2 values for these relations is as predicted, since only live sperm are taken up by the female's sperm storage tubules following insemination (Allen & Grigg 1957). Brillard & Antoine (1990) also found a significant correlation between numbers of sperm inseminated and numbers on perivitelline layers in domestic fowl with a much larger sample of females.

There was no difference between males in the intercepts of the relations between $\log_e$ (total sperm on the perivitelline layers) and time (ANOVA, $F_{8,15} = 0.40$, $p > 0.8$). We estimated the mean overall value by back transformation across males to be 1386 sperm $\pm$ 116 s.d.. Although there was considerable variation in the intercepts between females, the lack of any significant difference between males is consistent with our direct estimates of ejaculate size (above). Neither method for estimating ejaculate size therefore provided evidence for any consistency of ejaculate features within males.

Not all behaviourally successful copulations resulted in insemination: only 0.715 $\pm$ 0.30 s.d. of copulations resulted in sperm on perivitelline tissue. Samples sizes were too small (range 2–6 per male) however, to determine whether individual males differed significantly in this respect. Some females had started to lay eggs when copulations occurred, In poultry it is known that the uptake of sperm following artificial insemination is reduced among laying females compared with those that have yet started to lay (Brillard & Bakst 1990). To determine whether a female's reproductive state could account for differences in sperm transfer we compared the success of copulations in relation to whether egg laying had started and to the interval between copulation and egg laying. Overall, 18 copulations occurred before egg laying started, nine (50%) of which were successful. The mean interval between copulation and egg laying did not differ significantly between successful (11.00 days $\pm$ 5.74 s.d.) and unsuccessful copulations (12.33 $\pm$ 7.66 s.d.; $t =$ 0.418, 16 d.f., n.s). Thirteen copulations occurred after egg laying had started and 10 (77%) of these were successful (binomial test, 2-tailed, $p > 0.05$). Again, the mean interval between copulation and egg laying did not differ between the two groups (successful: 1.00 days $\pm$ 1.05 s.d.; unsuccessful 1.67 $\pm$ 1.53 s.d., Mann-Whitney U test, $p > 0.05$). This suggests that neither the timing of copulation in relation to whether egg laying had started nor the time of egg laying affected the likelihood of successful sperm transfer. The fact that not all behaviourally successful copulations result in the transfer of sperm may explain why, unusually for lekking birds, some peahens copulate several times with the same male during a single breeding cycle (Petrie *et al.* 1992). Another possibility is that females ejected the ejaculate of some males, as in known to occur in other species (see Howarth 1971; T. R. Birkhead, unpublished data), and then remated.

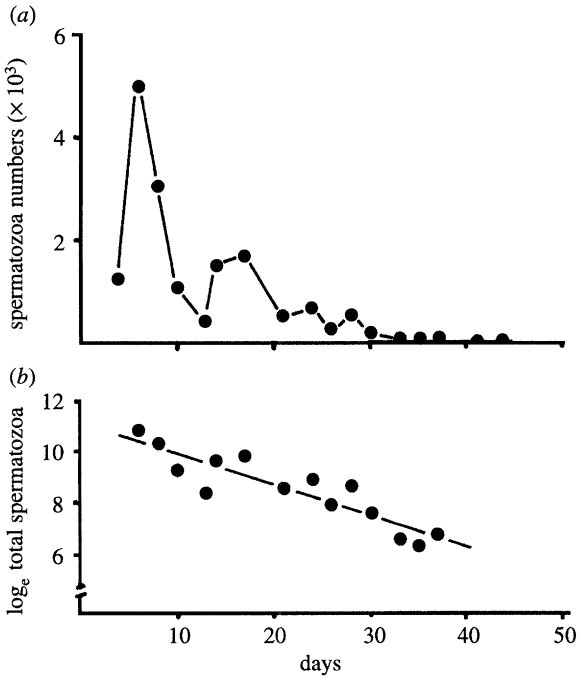

Figure 1. An example of the total numbers of sperm on successive eggs (from left to right) laid by one peahen in relation to the number of days after a single copulation: (*a*) absolute numbers (*b*) $\log_e$ numbers of sperm, (excluding the first initial low point, prior to sperm numbers reaching a maximum (see text) and two zero points).

(*b*) *Females*

The numbers of sperm on successive eggs increased initially over the first five days after insemination and then showed an exponential decline in numbers similar to that recorded in other species (Wishart 1987; Birkhead *et al.* 1993; Birkhead & Fletcher 1994). An example is shown in figure 1. The initial increase in sperm numbers has not previously been reported in birds, and may be due to a lag between insemination and sperm reaching the infundibulum. The numbers of sperm and their instantaneous rate of loss from the female reproductive tract were characterized by the intercept and slope, respectively, of the relation between $\log_e$ (sperm numbers on the perivitelline layers) with time since copulation (Lessells & Birkhead 1990; Birkhead & Fletcher 1994). Excluding the data from the first five days after copulation when sperm numbers increased, the mean instantaneous rate of loss (calculated from the slope for each female) was 0.0067 sperm $h^{-1} \pm 0.0006$ s.e. ($n = 12$ females). The duration of sperm storage varied from 9 to 35 days between females, with a mean maximum of 26.22 days $\pm$ 8.628 s.d. ($n = 9$). The duration of sperm storage across females was positively correlated, although not significantly, with our index of initial sperm numbers, that is, the intercept ($r = 0.527$, 7 d.f., $p < 0.2$) but not with the rate of loss, that is, the slope ($r = -0.288$, 7 d.f., $p = p > 0.2$). In these latter two analyses we treated females as independent because although several of them were inseminated by the same male (but no more than two females per male), there were no consistent male effects (see above). Not all females provided data that we could use in this analysis, either because they

produced no fertile eggs, or in one case because the peahen destroyed the eggs before we could collect them.

We used the relation between sperm numbers on the perivitelline layers and time since copulation to estimate the total numbers of sperm interacting with the ovum. We assumed: (i) a constant rate of sperm loss from the female tract, regardless of whether an egg was laid on a particular day, as Wishart (1987) found in the domestic fowl; and (ii) that sperm numbers interacting with the ovum did not reach a maximum until 5 days after insemination (see figure 1*a*). We calculated the area under the curve for the eight females that laid no later than the fifth day after copulation: the estimated mean number of inseminated sperm interacting with the ova was 127057 ± 110641 s.d. (range: 500–360833). With a mean ejaculate size of 124×10^6 sperm, the mean proportion of sperm interacting with the ova was 0.102%. Comparable figures for the turkey and the domestic fowl are: 0.054% and 0.105%, respectively (Birkhead *et al.* 1993). The proportion of inseminated sperm reaching the site of fertilization in the peafowl, and in other birds, is substantially higher than for mammals (Suarez *et al.* 1990; Birkhead *et al.* 1993). This is probably because in birds the ovum is relatively large and hence requires more sperm to ensure fertilization (Birkhead *et al.* 1993).

4. DISCUSSION

The mean size of ejaculate in peafowl, measured either directly, or indirectly as the number of sperm on the outer perivitelline layers of laid eggs, showed no consistency within males. Some of the variation in sperm numbers estimated from ejaculates obtained directly may have been a consequence of the way in which samples were acquired, but this is not likely to be the case for the indirect method as this relied on natural inseminations. In addition, neither the percentage of live sperm nor the absolute number of live sperm differed consistently between males. In studies of poultry in which semen samples have been obtained manually, ejaculate features from different males are also highly variable, although with large samples consistent differences between males have also been recorded (Wishart & Palmer 1986). The variability in ejaculate features in domestic mammals and humans is also high (Amann 1981; Handelsman *et al.* 1984). The idea that females choose between males on the basis of differences in their ability to fertilize eggs (e.g. Sheldon 1994) requires there to be consistent differences between males in ejaculate features. The lack of consistency in ejaculate features we found between males suggests that peahens would be unlikely to obtain any reliable fertility benefits from choosing to copulate with a particular male. Moreover in this study the time between ejaculates for a particular male was controlled, but in nature variation between males in the time since last ejaculation would further reduce the likelihood of reliable differences between males in their ability to fertilize eggs.

The mass of both testes combined (9.43 g, 0.2% male body mass (4766 g: Petrie *et al.* 1995)) is 23% lower than that predicted (12.3 g) by body mass from Møller's (1991) equation based on a comparative study of 247 bird species. However, other lekking species also have relatively small testes (Birkhead & Møller 1992*a*). Similarly, the estimated ejaculate size recorded in the present study (124×10^6 sperm) is 27% lower than that predicted (170×10^6) from the mass of the two testes combined using Møller's (1988) equation based on a comparative study of 33 bird species. This indicates that ejaculate size in the peacock is relatively small, a feature which may be explained by the relatively low incidence of sperm competition in lekking birds (Birkhead & Møller 1992*a*). There are no data on ejaculate size from other lekking birds for comparison.

The mean maximum duration of sperm storage in the peafowl (26 days) is relatively long compared with most other birds (Birkhead & Møller 1992*b*), but intermediate between that of the turkey (45 days; Lorenz 1950; McCartney 1951; Hale 1955) and the domestic fowl (12 days; Polge 1951) and Japanese quail (6 days; Sittman & Abplanalp 1965; Birkhead & Fletcher 1994). Interestingly, the instantaneous rate of sperm loss in the female peafowl (0.0067 sperm h^{-1}) also falls between that recorded in the turkey (0.003 sperm h^{-1}; Wishart 1988) and the domestic fowl and Japanese Quail (*Coturnix japonica*) (0.013 and 0.015 sperm h^{-1}, respectively; Wishart 1987; Birkhead & Fletcher 1994). Across a range of bird species the duration of sperm storage is positively correlated with the spread of egg laying (i.e. clutch size × the interval between successive eggs) (Birkhead & Møller 1992*b*). In the wild peafowl lay an average clutch of five or six eggs laid at two day intervals, a sperm storage duration of 26 days may at first sight seem excessive. However, female peafowl typically copulate about 12 days before laying their first egg (M. Petrie, cited in Birkhead & Møller 1993). Given that sperm numbers on perivitelline layers tended to increase over the first five days after copulation, presumably because of a lag between insemination and an accumulation of sperm in the infundibulum, this early timing of copulation relative to the start of egg laying may be a consequence of females allowing sufficient time for sperm to reach the sperm storage tubules and the infundibulum. It is possible that the relatively long period of time to reach the infundibulum in peafowl may in part be a consequence of the relatively large size and length of the female peafowl reproductive tract. In the domestic fowl and turkey, it took three and two days after insemination for sperm numbers in the sperm storage tubules to reach their maximum (Brillard & Antoine 1990; Brillard & Bakst 1990). In a study of artificial insemination in Houbara bustards *Chlamydotis undulata* Saint Jalme *et al.* (1994) found that insemination had to take place at least three days before egg laying to produce fertile eggs.

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