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Sperm precedence in the domestic fowl

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SUMMARY

The aim of this study was to examine last-male sperm precedence in the domestic fowl. We used sperm from two different genotypes to assign paternity, and in seven experiments females were artificially inseminated with either equal or unequal numbers of sperm at intervals of 4 or 24 h. We were unable to replicate the results of a previous study by Compton *et al.* (1978) in which a strong last-male precedence effect had been recorded when two equal sized inseminations were made 4 h apart. We observed no marked last-male sperm precedence and our results did not differ significantly from that predicted by the passive sperm loss model, in which a last-male effect is determined by the rate at which sperm are lost from the female tract and the interval between successive inseminations. The most likely explanation for the disparity between our result and Compton *et al.*'s is a difference in the timing of inseminations. The implications of this for studies of sperm competition in birds is discussed.

1. INTRODUCTION

When a female copulates with two or more males during a single reproductive episode, the outcome is sperm competition (Parker 1970, 1984). Most birds are socially monogamous, but extra-pair copulation, sperm competition and extra-pair paternity are widespread (Birkhead & Møller 1992). The factors that determine the outcome of sperm competition – that is, which copulations result in paternity – are poorly known. However, in birds and insects it appears that the sperm from the last male to copulate have an advantage over that from previous inseminations, an effect referred to as last-male sperm precedence (Birkhead & Hunter 1990). For both birds and insects the use of mathematical models has allowed researchers to focus their empirical studies on the most plausible mechanisms for last-male sperm precedence (Lessells & Birkhead 1990; Parker 1990*a, b*; Parker & Simmons 1990; Parker *et al.* 1990; Eady 1994).

References to a last-male mating advantage in the domestic fowl *Gallus domesticus* date back to Aristotle (cited in Payne & Kahrs 1961), and several other studies subsequently reported a similar effect, using either natural matings (Crew 1926; Warren & Kilpatrick 1929; Krallinger 1930) or artificial insemination (Bonnier & Trulsson 1939; Warren & Gish 1943; Compton *et al.* 1978; DeMerritt 1979; Haije 1990, and summarized in Birkhead & Møller 1992). Last-male sperm precedence has also been reported in other species: turkey *Meleagris pavo* (Payne & Kahrs 1961), mallard *Anas platyrhynchos* (Cheng *et al.* 1983), ring dove *Streptopelia roseogrisea* (Sims *et al.* 1987) and zebra finch *Taeniopygia guttata* (Birkhead *et al.* 1988). One of the most detailed, and influential, studies of poultry is that of Compton *et al.* (1978), who used

genetic markers to assign paternity and artificial insemination to control for sperm numbers. When they inseminated equal numbers of sperm sequentially, with a 4 h interval between inseminations, a greater proportion (77%) of offspring was fathered by the second insemination, regardless of the order in which semen of each genotype was used. Their results indicated that the proportion of offspring fathered by the second insemination was significantly different from one half, and Compton *et al.* (1978) proposed that this occurred because, with an interval of four or more hours, the sperm from successive inseminations remain stratified within the female's sperm storage tubules, and that a 'last-in-first-out' system operated, with the last male's sperm having first access to the ova.

Martin *et al.* (1974), showed that when sperm from two genotypes were mixed in different proportions and artificially inseminated simultaneously, paternity was proportional to the relative number of sperm from each genotype. These results, combined with those of Compton *et al.* (1978), indicated that patterns of paternity in birds depend on the interval between successive inseminations; it has been proposed that when inseminations occur close together in time the sperm from different males mix before entering the sperm storage tubules, but when the interval is four or more hours the sperm from successive inseminations remain stratified within the sperm storage tubules (Cheng *et al.* 1983; McKinney *et al.* 1984).

Although stratification seems a plausible explanation, it has proved difficult to perform an empirical test of this idea. Van Krey *et al.* (1981) used autoradiography in an attempt to establish whether stratification of ejaculates occurred. They artificially inseminated domestic fowl twice, 4 h apart, one insemination contained sperm labelled with [³H]thymi-

dine and the other with unlabelled sperm. However, the results were inconclusive. Subsequently the same experiment was performed, again with domestic fowl, using unlabelled sperm and sperm labelled with the vital fluorescent dye Hoescht 33342 (which works well with mammalian sperm: Cummins *et al.* 1986), but this also failed to establish whether stratification of sperm occurs (Bakst 1994; M. R. Bakst, personal communication; G. J. Wishart & T. R. Birkhead, unpublished data). This technique was unsuccessful because once inside the sperm storage tubules the sperm dyed with Hoescht 33342 also labelled the unlabelled sperm, as well as the sperm storage tubules themselves, making it impossible to distinguish the sperm from different inseminations.

Using a series of mathematical models Lessells & Birkhead (1990) attempted to identify the most likely mechanisms responsible for the level of last-male sperm precedence in the domestic fowl reported by Compton *et al.* (1978). Lessells & Birkhead (1990) found that the stratification of sperm within sperm storage tubules could not account for the observed levels of sperm precedence either. The model predicted that if stratification of sperm from successive ejaculates occurred in the sperm storage tubules, last-male sperm precedence should decline over time, as sperm from the initial insemination were 'uncovered'; but Compton *et al.* (1978) did not record any such effect, and in fact found that the proportion of offspring fathered by the two inseminations remained constant over time. Lessells & Birkhead (1990) considered also the passive sperm loss model, which assumes that after their acceptance by the sperm storage tubules, sperm are lost from the female reproductive tract at a constant rate, and that when a female is inseminated twice with equal numbers of sperm the second insemination is likely to have precedence simply because, on average, more sperm are present from the more recent insemination when each egg is fertilized. The magnitude of this effect depends upon the rate of loss of sperm and the interval between successive inseminations (Lessells & Birkhead 1990). Empirical studies of domestic fowl have shown that sperm are indeed lost from the female tract at a constant rate (Wishart 1987; see also Brillard & Antoine 1990). However Lessells & Birkhead (1990) showed that the passive sperm loss model could not account for Compton *et al.*'s (1978) results and concluded that the most likely mechanism for their results was the displacement of the first male's sperm by those of the second.

We intended to test the sperm displacement hypothesis empirically and commenced by repeating Compton *et al.*'s (1978) experiment. However, we were unable to replicate their results. The main aims of this paper are therefore to describe our results, and compare them with Compton *et al.*'s (1978) and with predictions based on the passive sperm loss model.

2. METHODS

We used pure-bred Rhode Island Red (RIR) and Light Sussex (LS) domestic fowl (AFRC Institute of Animal Health, Houghton) to assign paternity on the basis of

Table 1. Summary the details of sperm competition experiments conducted on domestic fowl

experiment ^a	insemination 1st:2nd	time interval	no. sperm
		between inseminations (h)	1st:2nd ($\times 10^6$)
2	LS:RIR	4	120:120
3	RIR:LS	4	120:120
4	RIR:LS	24	120:120
5	RIR:LS	4	120:120
6	RIR:LS	4	20:20
7	RIR:LS	4	120:20
8	RIR:LS	4	240:240

^a Experiment 1 comprised a mixture of equal numbers of sperm (120×10^6) from each genotype (see text).

plumage markers. LS birds are homozygous for the dominant silver (S) allele, whereas RIR are homozygous for the recessive gold(s) (Smyth 1990). Paternity could be determined in the offspring of RIR hens by either 'silver' (LS father) or brown-patched (RIR father) down on hatched chicks or late embryos. Semen from six males of each genotype was pooled, diluted in Lake's pH 7.1 medium (Lake & Ravie 1979) to an appropriate concentration, mixed and artificially inseminated into RIR females. We obtained two pooled semen samples from each of the genotypes immediately before each insemination. Eggs were collected daily for 30 days after the last insemination and hatched in a commercial incubator. Eggs were laid at intervals of approximately 24 h and fertilization of the next ovum occurs within one hour of laying (Lake 1975). To ensure, therefore, that sperm from each insemination could potentially fertilize eggs, we recorded the paternity of eggs laid at least 44 h after the second insemination. The birds had not been inseminated before experiment 1, and thereafter the same individual adult birds were used in all experiments. To ensure that sperm stored in the female reproductive tract from an earlier trial could not affect subsequent trials, all experiments were separated by at least 30 days, which is the longest known duration of sperm storage in the domestic fowl (Lake 1975). The mean storage duration is 12 days (Lake 1975) but because not all females laid each day or laid eggs which were fertile or hatched, sample sizes are usually less than 12 per female within experiments. We used a total of 30 females, but as not all females laid in each experiment, their numbers vary between experiments. We conducted a total of eight experiments. All, except experiment 1, comprised two successive inseminations of either equal or unequal numbers of sperm separated by either 4 or 24 h. Where inseminations were separated by 4 h, the first was performed at 19h00 and the second at 23h00. In experiment 4 where inseminations were 24 h apart, both inseminations took place at 19h00. We timed inseminations to occur as long as possible after egg laying (which occurred in the morning) hence avoiding the time when inseminations are least likely to be successful (Brillard *et al.* 1987).

For experiment 1 we inseminated all females once with a mixture of equal numbers (120×10^6) of sperm from each of the two genotypes, to determine whether any differential fertilizing capacity (Lanier *et al.* 1979) occurred between the two strains of males. We used 120×10^6 sperm in most inseminations since this is similar to the number thought to be transferred during natural copulations (Van Krey 1990).

For experiments 2–8 the details are recorded in table 1. Note that experiments 2 and 3 differ only in the insemination order, that experiment 5 is a repeat of experiment 3, that all

Table 2. Results of sperm competition experiments showing for each experiment: the number of females inseminated; the number of chicks; the proportion, p , of LS offspring (s.e.); the corresponding logit value, $\log [p/(1-p)]$; and s.e.

experiment	no. hens	no. chicks	p	s.e.	$\log (p/1-p)$	s.e.
2 ^a	12	83	0.56	0.09	0.24	0.38
3	19	110	0.67	0.08	0.72	0.34
4	7	40	0.73	0.09	0.97	0.46
5	25	125	0.45	0.07	-0.20	0.30
6	16	48	0.55	0.10	0.19	0.39
7	18	62	0.51	0.10	0.06	0.40
8	10	28	0.33	0.13	-0.70	0.58

^a Note: only in experiment 2 were LS sperm inseminated first, so the proportion of offspring fathered by the second insemination in this experiment was 0.44 (s.e. = 0.09).

except experiment 4 had a 4 h interval between inseminations, and that only experiment 7 did not use equal numbers of the two sperm types.

We compared observed results with those of Compton *et al.*'s (1978) and those predicted by Lessells & Birkhead's (1990) passive sperm loss model.

(a) Statistical analyses

Each experiment provides an estimate of the probability (and an associated standard error) of an egg being fertilized by the sperm of one of the two genotypes; we work with the probability that a chick was fathered by LS sperm, p . In any experiment each clutch provides a proportion of LS chicks. How these proportions should be combined to give an estimate for p based on that experiment depends on whether these proportions of LS chicks differ significantly over clutches. When they are homogeneous the only source of variation is binomial, otherwise the data are more dispersed than would be expected under the hypothesis of homogeneity. In the next few sections we describe how we tested for homogeneity, and then describe how we estimated p .

(b) Testing homogeneity

As the numbers of offspring produced by particular females were sometimes small (see table 2), the expected values are also small and the χ^2 test for homogeneity will not be valid. We therefore followed the procedure suggested in McCullagh & Nelder (1989, §4.2.5) and made comparisons using z values, based on formulae for mean and variances of the χ^2 statistic given in Haldane (1939) and Plackett (1974: 124). The statistics for each individual experiment can also be added over separate experiments to provide an overall test, which provides clear evidence that there is indeed over-dispersion in experiments 2–8 (see Appendix A). The likely cause of this over-dispersion is described in the §4 (below).

(c) Estimating p

When the clutches in an experiment are homogeneous, p and its standard error are estimated on the assumption that the individual eggs provide independent observations; thus p is simply the overall proportion of LS chicks. When the clutches in an experiment are not homogeneous, as in experiments 2–8, the proportions for all hens are averaged and the standard error of this mean computed. For experiment 1 there is no reason to doubt homogeneity so the first procedure is appropriate, but in fact the other method yields a very similar value.

It is usual, and has substantial technical advantages, to

Table 3. Comparison with Compton *et al.*'s results

experiment	Δ^a	s.e.	cf Compton	
			z	p -value
3	0.48	0.51	-2.55	0.011
4	0.73	0.59	-2.08	0.038
5	-0.44	0.48	-3.80	< 0.001
6	-0.04	0.54	-3.13	0.002
7	-0.18	0.55	not applicable	
8	-0.93	0.69	-3.73	< 0.001

^a Δ in Compton *et al.*'s experiment was 2.49 (s.e. = 0.6), see text.

compare probabilities on the 'logit' scale (see for example Cox 1970). This means that, rather than comparing values of p , values of $\log [p/(1-p)]$, the log odds ratio, are compared (natural logarithms are used throughout). When \hat{p} is an estimate of p with standard error s then the standard error of $\log [\hat{p}/(1-\hat{p})]$ is $s/[\hat{p}(1-\hat{p})]$ (see Kendall & Stuart 1977, §10.6). When there is only binomial variation the estimation of the log odds ratio can be refined slightly (see Plackett 1974, §4.4, p. 40), and this was done for experiment 1 and for Compton *et al.*'s data.

(d) Measuring last-male precedence

If p_1 and p_2 are the probabilities of a chick being LS in two experiments then the advantage (in terms of LS chicks produced) in the second experiment over the first can be measured by:

$$\Delta = \log [p_2/(1-p_2)] - \log [p_1/(1-p_1)]. \quad (1)$$

Then, for a reciprocal pair of experiments, in which the only difference is the insemination order, Δ measures the magnitude of the last-male effect (with $\Delta = 0$ corresponding to no effect). Furthermore, and this is one of the major benefits of this measure, Δ is independent of any differential fertilizing capacity between the types (see Cox 1970, §2.4; McCullagh & Nelder 1989, §4.3). The estimated variance of Δ is the sum of the variances of the two parts, and from this its standard error is obtained.

Comparisons with Compton *et al.*'s results were made by calculating Δ (above) from their data (Compton *et al.* 1978; table 3) and comparing it with the results of our experiments using z -values.

(e) Comparing our results with the passive sperm loss model

Let μ be the instantaneous rate of sperm loss for domestic fowl (determined empirically to be 0.0128 h^{-1} by Wishart

(1987), a personal communication cited in Lessells & Birkhead 1990), T be the time interval between inseminations (either 4 or 24 h) and I the number of times bigger the first insemination is than the second (either 1 or 6). Thus at the time of the second insemination the ratio between the numbers of sperm present from first and second inseminations is $I e^{-\mu T}$: 1. Let d be the differential fertilizing capacity of the LS genotype (d is the log odds that a chick is LS when an equal mixture of the sperm from the two genotypes is present). Then, when LS is first, the passive loss model predicts:

$$\log [p/(1-p)] = d - \mu T + \log I, \quad (2)$$

whereas when it is second the prediction is:

$$\log [p/(1-p)] = d + \mu T - \log I. \quad (3)$$

In each case the standard error will be $\sqrt{[s.e.(d)^2 + T^2 s.e.(\mu)^2]}$.

The differential fertilizing capacity can be estimated from experiment 1, see §3, and this can be used to calculate (using the formulae above) the value for $\log [p/(1-p)]$ expected on the basis of the model in each experiment, and its standard error. This prediction can be compared with the value actually obtained using z -values.

If p_1 and p_2 are the probabilities of a chick being LS in two experiments that differ only in the order of the inseminations then d , the differential fertilizing capacity, can be estimated by:

$$d = \frac{1}{2} \{ \log [p_1/(1-p_1)] + \log [p_2/(1-p_2)] \}. \quad (4)$$

3. RESULTS

In experiment 1 we obtained 101 offspring from 21 females of which 0.605 (s.e. = 0.049) were fertilized by LS sperm. This was significantly greater than the expected 50:50 ratio ($p = 0.03$). There was thus a differential fertilizing capacity effect with LS sperm being 1.5 times as likely to fertilize eggs as RIR sperm. This experiment gives a value of $d = 0.42$ (s.e. = 0.20). In comparing our results with those of Compton *et al.* the method used (above) means that any differential fertilizing capacity is accounted for. However, the differential fertilizing capacity effect ($d = 0.42$) obtained in this experiment is important in calculating the predicted values in the passive sperm loss model (see below).

In experiments 2–8, the proportion of offspring fathered by second inseminations varied between 0.33 and 0.73 in the different experiments (see table 2). In

the next two sections we compare these results with those of Compton *et al.* (1978) and with the passive sperm loss model.

We also estimated d from experiments 2 and 3 ($d = 0.48$, s.e. = 0.26) and from experiments 2 and 5 ($d = 0.02$, s.e. = 0.24), and compared these experiments with that from experiment 1; neither differs significantly from the value obtained in experiment 1.

(a) Comparison with Compton *et al.*'s result

We compared our results with Compton *et al.*'s (1978) using Δ , as described earlier. Δ was calculated by comparing the results from experiments 3–8 with those of experiment 2 (which was the only one in which LS semen was inseminated first). From the data in Compton *et al.* (1978, see table 3) $\Delta = 2.49$ (s.e. = 0.314). The s.e. is based on the assumption that the only source of variation in Compton *et al.*'s experiment is binomial sampling. However, as the results our experiments show (Appendix 1), this may not be the case and the true s.e. in Compton *et al.*'s experiment is likely to be larger. In the absence of the data from individual females in Compton *et al.*'s experiment, which would have allowed us to calculate the s.e. directly, we have assumed the s.e. to be the same magnitude as in our experiments, using a value of 0.6. Note that making this assumption reduces the chances of obtaining a statistically significant difference between our results and those of Compton *et al.* (1978).

As table 3 shows, all the values of Δ we obtained were lower than that of Compton *et al.* (1978) and, even assuming a s.e. of 0.6, all the comparisons are statistically significant. The most meaningful comparisons are between Compton *et al.*'s results and our experiments 3 ($p = 0.011$) and 5 ($p < 0.001$), although even in experiments 6 and 8, where the numbers of sperm from each genotype differ markedly from those used by Compton *et al.*, the differences are highly significant ($p < 0.002$). Finally, it is reasonable to expect that experiment 4 should produce an effect of at least the magnitude of Compton *et al.*'s, since the longer interval between inseminations (24 h) should increase rather than decrease the degree of last-male precedence. However, the comparison with experiment 4 also produces a significant result ($p = 0.038$). Overall these results provide convincing evidence that in this study we did not find a last-male effect of the magnitude recorded by Compton *et al.* (1978).

Table 4. Comparison with predictions from the passive sperm loss model

experiment	hens	observed		predicted		p -value
		$\log (p/1-p)$	s.e.	$\log (p/1-p)$	s.e.	
2	12	0.24	0.38	0.37	0.20	ns
3	19	0.72	0.34	0.47	0.20	ns
4	7	0.97	0.46	0.72	0.21	ns
5	25	-0.20	0.30	0.47	0.20	ns
6	16	0.19	0.39	0.47	0.20	ns
7	18	0.06	0.40	-1.32	0.20	0.002
8	10	-0.70	0.58	0.47	0.20	ns

(b) Comparison with the passive sperm loss model

The proportions of offspring fathered by LS sperm predicted by the passive sperm loss model are compared with the observed values for each of our experiments, in table 4. In all cases except one, the two values did not differ significantly. Only in experiment 7 was there a significant difference. This was the only experiment in which we used different numbers of sperm in the successive inseminations, with six times as many RIR sperm (120×10^6) as LS sperm (20×10^6). We might therefore have predicted a relatively small proportion of offspring to be fertilized by LS sperm, but as table 2 shows, this was not the case, and 51% of offspring were of the LS phenotype.

4. DISCUSSION

We found no evidence for last male precedence of the magnitude reported by Compton *et al.* (1978) when two inseminations were separated by 4 or 24 h. Our results were, however, consistent with Lessells & Birkhead's (1990) passive sperm loss model (with the exception of experiment 7). Here we consider differences in protocol between Compton *et al.*'s experiments and ours that could account for the difference in our results, and heterogeneity in paternity between clutches; then we attempt to draw some general conclusions from our results.

(a) Protocol differences from Compton *et al.*

Our experimental design was similar to that of Compton *et al.* (1978) although, because we assumed their last-male effect to be robust, we did not attempt to replicate their experiment in every detail. As a result, our experiments differ in three ways: sperm numbers; differential fertilizing capacity; and the timing of inseminations.

(i) Sperm numbers

Compton *et al.* inseminated females with 0.023 ml of semen, equivalent to about 115×10^6 sperm (Van Krey 1990), which is slightly less than the 120×10^6 we used in most of our experiments. This difference is unlikely to account for the difference in our results.

(ii) Differential fertilizing capacity

Compton *et al.* reported no differential fertilizing capacity between the genotypes they used, whereas in our study there was (see above). Compton *et al.* used two genotypes, normal (DwDw) and dwarf (dwdw), which were chosen because 'of a reported lack of competitive fertilization between the normal and dwarf gametes (Hutt 1959)'. Furthermore, they inseminated females with a single dose of heterozygous (Dwdw) semen and, as they found no significant deviation from a 1:1 ratio of normal to dwarf offspring, they concluded that the 'competitive fertilization between the gametes was not a matter of concern'. However, no competitive

matings between genotypes are reported by Hutt (1959). Instead he simply demonstrates that the mode of inheritance is consistent with Mendelian expectations for a sex-linked recessive; Compton *et al.* assumed this to be equivalent to a lack of competitive fertilization between sperm of the two genotypes. Similarly, in their own study (rather than testing for a differential fertilizing capacity between the sperm from different bird genotypes) Compton *et al.* compared the sperm from heterozygous males whose sperm carried either the normal (Dw) or the dwarf allele (dw). In other words they erroneously assumed that differential fertilizing capacity is a property of the sperm themselves, rather than of the bird that produced them. Moreover, despite their assertion for no differential fertilizing effect, it is clear from their data that when sperm of the dwarf genotype were inseminated first they fertilized a greater proportion of eggs than the normal genotype did when it was first. We estimated the differential fertilizing capacity in favour of the dwarf genotype to be $d = 0.36$ (s.e. = 0.157), which is not dissimilar to $d = 0.42$ obtained in experiment 1 of this study. As already noted in the §3, standard errors based on Compton *et al.*'s data must be viewed with caution as they probably exaggerate precision. However, because of the method of analysis used, a difference in the fertilizing capacity between the genotypes in Compton *et al.*'s (1978) study would not account for the difference between our results.

(iii) Timing of inseminations

Compton *et al.*'s inseminations took place during the day (M. M. Compton, personal communication), whereas ours took place in the evening. It is well established that the likelihood of an insemination resulting in fertilization depends upon its timing relative to oviposition: inseminations close to oviposition are significantly less likely to result in fertilization (e.g. Brillard *et al.* 1987). Compton *et al.* state that their birds 'generally had empty oviducts' at the time of insemination demonstrating (see Gilbert 1971) that their inseminations took place much closer to oviposition than did ours. Compton *et al.* excluded females that 'laid within 90 min after insemination', indicating that they were aware of the period of reduced fertility immediately before oviposition. However, if the period of reduced fertility starts more than 90 minutes before oviposition (and this might be the case: see Parker 1945; Brillard *et al.* 1987) some of Compton *et al.*'s first inseminations will have had relatively low fertilization success. Much more importantly however, several workers (e.g. Brillard *et al.* 1987) have shown that the period of reduced fertility also lasts for at least 1 h after oviposition; a period not avoided by Compton *et al.* Overall therefore it seems likely that many of Compton *et al.*'s first inseminations took place when the likelihood of success was relatively low, hence explaining the marked last (i.e. second) male precedence they observed. In our study females laid in the morning and, specifically to avoid the period of low fertility, inseminations were made at least 7 h later when all females had a hard-shelled egg in the

oviduct: the optimum time for insemination (Lake & Stewart 1978). Thus in contrast to Compton *et al.*'s study, the timing of our first and second inseminations could have resulted in approximately equal likelihood of fertilization, leading to a much less marked last male effect. The difference in the timing of inseminations therefore appears to be the most plausible explanation for the discrepancy between our results and Compton *et al.*'s.

(b) *Variation in clutches*

We found high variability the proportion of LS chicks between clutches, significantly more than can be attributed to binomial variation alone (see Appendix). In some instances, even with as many as nine chicks, only one of the two genotypes fertilized any of a particular female's eggs. Without access to Compton *et al.*'s raw data we cannot assess whether the level of variability in their experiments was similar to ours. Much of the variation we detected is likely to be due to the fact that female domestic fowl (and other birds) eject a large (> 90%) proportion of the sperm from the cloaca soon after insemination (Allen & Grigg 1957). It seems plausible that this results in variability in the numbers of sperm that remain in the female tract and hence in the number that eventually interact with the ova at the site of fertilization (Wishart *et al.* 1992). For example, in 17 females each inseminated with 100×10^6 sperm the number of sperm per 5.5 mm^2 of outer perivitelline tissue of the first fertile egg laid after insemination varied by a factor of 13 (from 11 to 143; mean 65.1, coefficient of variation 61.9) (Wishart *et al.* 1992). Random variation in the number of sperm ejected after each artificial insemination would account for the variability in our results.

Although sperm ejection is known to occur following natural matings in birds (T. R. Birkhead, unpublished data), little is known about the proportion of sperm ejected in either the domestic fowl or other birds. We cannot exclude the possibility that diluting semen (to adjust the control sperm numbers in sperm competition experiments) facilitates ejection by reducing semen viscosity. Caution is needed therefore in extrapolating from studies of domesticated birds involving artificial insemination and diluted semen, to wild birds.

5. CONCLUSION

If our interpretation for the difference between the results presented here and those of Compton *et al.* (1978) is correct, the timing of inseminations relative to egg laying is an additional factor determining the outcome of sperm competition. The fact that the uptake of sperm by females is reduced around the time of egg laying (Brillard *et al.* 1987) appears at first sight to be at odds with Cheng *et al.*'s (1983) concept of an 'insemination window'. This proposes that during the hour or so following laying when there is no hard-shelled egg in the oviduct, sperm can travel unimpeded

up the oviduct to fertilize the next ovum to be ovulated (i.e. the next egg to be laid). An 'insemination window' implies a favourable time for insemination, but Cheng *et al.*'s (1983) results using artificial insemination with ducks confirm that this is not the case: only 25% of the next day's eggs were fertilized by inseminations at this time, compared with 80% (excluding the next egg to be laid) for inseminations made at other times. In wild birds little is known about the efficacy of inseminations soon after egg laying, but because the majority of species copulate only infrequently once egg laying has started and hence tend not to utilize the insemination window (see Birkhead & Møller 1993), the timing of inseminations relative to egg laying might not be an especially important factor in determining the outcome of sperm competition in most wild birds.

Overall, our results suggest that a 4 h interval between two successive inseminations containing equal numbers of sperm away from the timing of egg laying, does not result in marked last-male sperm precedence, and that Compton *et al.*'s result was an artifact of the timing of their inseminations. Instead, our results are more consistent with the passive sperm loss model. Colegrave *et al.* (1995) also found that the passive sperm loss model adequately accounted for the observed pattern of sperm precedence reported by Birkhead *et al.* (1988) in the zebra finch. It therefore seems possible that rather than there being two separate sperm competition mechanisms depending upon the interval between successive inseminations, as suggested by Cheng *et al.* (1983) and McKinney *et al.* (1984), only a single mechanism exists determined by the passive loss of sperm from the female tract. If this is true then the outcome of sperm competition and any last-male precedence effect will be determined partly by the relative numbers of sperm from each male getting into the female's sperm storage tubules and partly by the interval between successive inseminations from different males (i.e. the time available for passive loss to have occurred and hence affect the relative numbers of sperm still present in the female tract). At least two factors will affect the number of sperm getting to the sperm storage tubules: (i) the relative number of sperm inseminated by each male; and (ii) the proportion of sperm retained by the female, which in turn will be determined in part by the timing of insemination relative to egg laying. Empirical tests with a range of species are now needed to test these ideas.

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APPENDIX

Homogeneity

The table gives the results of the test for homogeneity within each experiment.

Table A1. *Tests for homogeneity*

experiment	hens	chicks	<i>z</i>	<i>p</i> -value
1	21	101	−0.4	> 0.1
2	12	83	4.2	< 0.001
3	19	110	4.6	< 0.001
4	7	40	0.1	> 0.1
5	25	125	5.5	< 0.001
6	16	48	1.0	> 0.1
7	18	62	3.3	< 0.001
8	10	28	2.1	< 0.5

Experiment 1 (which used mixed semen) is consistent with binomial variation. Of the experiments that involved two inseminations, experiments 2, 3, 5 and 7 provide strong evidence against homogeneity. The remainder, experiments 4, 6 and 8, give no reason to doubt homogeneity; but these are experiments that produced the fewest chicks, so their power to detect a lack of homogeneity will be lowest. A portmanteau test for inhomogeneity, by combining results from experiments 2 to 8 provides overwhelming evidence against homogeneity (z -score = 7.69, $p < 0.0001$).