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Variation in the Number of Spermatozoa in Blue Tit and Great Tit Eggs

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In birds, fertilization takes place in the infundibulum at the top of the female reproductive tract. To reach this site, spermatozoa must travel from the hostile environment of the vagina, or from sperm-storage tubules located at the utero-vaginal junction, and on through the oviduct (Birkhead et al. 1993a). Only a minute fraction of the inseminated spermatozoa reach the site where eggs are fertilized, but the actual number varies considerably across species (Birkhead et al. 1994). By counting the number of spermatozoa in or penetrating the perivitelline layers of fresh eggs, it is possible to obtain an index of the number of spermatozoa present in the infundibulum at the time of fertilization (Birkhead and Fletcher 1994).

When released from the ovary, the ovum is covered by the inner perivitelline layer. Fertilization takes place 15 to 30 min after ovulation with one of several spermatozoa that penetrate the inner perivitelline layer (Bakst and Howarth 1977). Shortly after fertilization, the outer perivitelline layer is laid

down on the ovum, trapping other spermatozoa present in the infundibulum (Wishart 1987). The number of holes in the inner perivitelline layer is correlated with the number of spermatozoa trapped on the outer layer (Birkhead and Fletcher 1994, Birkhead et al. 1994).

In this paper, we report on variation in the number of spermatozoa in eggs of two passerine species, the Blue Tit (*Parus caeruleus*) and the Great Tit (*P. major*), with a special focus on variation within and between clutches of individual females. The results are discussed in light of the mating system of these species.

Study areas and methods.—Eggs were collected in early May 1997 from two populations of Blue Tits and Great Tits that nested in boxes in southeastern Norway. One study site was located on the island of Jomfruland (58°52'N, 9°36'E) off the coast of Telemark County, where tit populations have been studied for several years (Krokene et al. 1998). On Jomfruland, we collected a maximum of three eggs from each clutch, which did not interrupt the normal nesting events. At the other study site, in Maridalen, Oslo (59°58'N, 10°47'E), we collected entire clutches of both species to study variation in total number of spermatozoa over the laying sequence. For the Blue Tit, the overall sample for analysis consisted of 107

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eggs from 21 females (35 eggs from 13 clutches on Jomfruland, and 72 eggs from 8 clutches in Maridalen). For the Great Tit, we analyzed a total of 94 eggs from 26 females (52 eggs from 21 clutches on Jomfruland, and 42 eggs from 5 clutches in Maridalen). We attempted to randomize the eggs collected on Jomfruland with respect to laying order, but because the population was not monitored continuously and eggs often had to be taken before clutches were complete, the eggs collected on Jomfruland were biased toward the earlier part of the laying sequence. For the Blue Tit, eggs analyzed from Jomfruland clutches were on average 1.3 eggs earlier in the laying sequence than those from Maridalen clutches ($t = 2.66$, $df = 19$, $P = 0.016$). For the Great Tit, Jomfruland eggs were on average 1.9 eggs earlier in the laying sequence than those from Maridalen ($t = 3.97$, $df = 24$, $P < 0.001$).

Eggs were collected during the laying period, which ensured that they had not been incubated. They were stored at 4°C for one to six days until shipped by express courier to TRB's lab in Sheffield for immediate examination. In the lab, eggs were opened with scissors and the perivitelline layers were removed, placed on a microscope slide, and stretched out flat. Next, a drop of the fluorescent Hoechst dye 33342 was added to stain the sperm nuclei, and the layers were examined under a microscope at 20× and 40× power. The number of spermatozoa on the inner and outer perivitelline layer of each egg and the number of holes in the inner perivitelline layer were recorded (see Birkhead et al. 1994). Unfortunately, several eggs were damaged during shipment. Except for two Blue Tit eggs and six Great Tit eggs, however, the perivitelline tissue or a part of it could be rescued and examined. The number of trapped spermatozoa was then extrapolated from counts on the intact part. In a total of 27 Blue Tit eggs and 35 Great Tit eggs, the inner perivitelline layer with the blastodisc could not be inspected. In these cases, we estimated the number of holes from the number of spermatozoa trapped on the outer perivitelline layer, using a regression for intact eggs.

Results.—In both species, there was a positive correlation between the number of holes made by spermatozoa penetrating the inner perivitelline layer and the number of spermatozoa trapped on the outer layer (Blue Tit, $r = 0.59$, $n = 20$, $P = 0.006$; Great Tit, $r = 0.64$, $n = 24$, $P = 0.0007$). A similar relationship has been documented in other species (Birkhead et al. 1993b, 1994; Birkhead and Fletcher 1994). Holes in the inner perivitelline layer were concentrated on the blastodisc. The proportion of holes located on the blastodisc in eggs with intact membranes amounted to 77% ($n = 68$) in the Blue Tit and 71% ($n = 61$) in the Great Tit.

The total number of spermatozoa (i.e. holes plus trapped spermatozoa) averaged $168 \pm SE$ of 16 ($n =$

21) in Blue Tit clutches and 299 ± 35 ($n = 26$) in Great Tit clutches. Clutch means ranged between 49 and 275 spermatozoa per egg for the Blue Tit and 0 and 741 for the Great Tit. The within-clutch variance was high compared with the between-clutch variance, especially for Great Tits. Consequently, the repeatability (Lessells and Boag 1987) of the total number of spermatozoa calculated across eggs within the same clutch was low in both species (0.27 for Blue Tit and 0.08 for Great Tit) and was statistically significant only for the Blue Tit ($F = 2.84$, $df = 20$ and 86, $P = 0.0004$; Great Tit, $F = 1.31$, $df = 25$ and 68, $P = 0.19$).

One Great Tit clutch from Maridalen was exceptional in that all seven eggs seemed to lack spermatozoa entirely; no spermatozoa were trapped in the eggs, and two eggs each had a single hole in the inner perivitelline layer (one on the blastodisc) that might have been caused by spermatozoa. Hence, the entire clutch seemed to be infertile. No other Great Tit eggs had fewer than 53 total spermatozoa. In Blue Tit eggs, the minimum total number of spermatozoa was 34.

The total number of spermatozoa in eggs of complete clutches at Maridalen declined with laying order in the Blue Tit but not in the Great Tit (Fig. 1). All eight clutches of Blue Tits showed a significant negative slope in the number of total spermatozoa plotted against laying order. The mean correlation coefficient ($r = -0.39$) was significantly different from zero ($t = -4.43$, $df = 7$, $P = 0.003$). For Great Tits, the mean correlation coefficient was not significantly different from zero ($r = -0.20$, $t = -1.07$, $df = 3$, $P = 0.36$), but with the small sample of only four clutches (excluding the infertile clutch), we cannot rule out the possibility that a negative relationship also existed in this species.

The average total number of spermatozoa per egg did not differ between Great Tit clutches from Jomfruland ($\bar{x} = 293 \pm 35$, $n = 21$) and Maridalen ($\bar{x} = 407 \pm 107$, $n = 4$, excluding infertile clutch; $t = -1.23$, $df = 23$, $P = 0.23$) nor between Blue Tit clutches from the two sites (Jomfruland, $\bar{x} = 185 \pm 21$, $n = 13$; Maridalen, $\bar{x} = 128 \pm 15$, $n = 8$; $t = 1.70$, $df = 19$, $P = 0.11$). We adjusted values from Jomfruland to represent the middle egg of the clutch, using the average slope of the regressions of total number of spermatozoa and laying order shown in Figure 1.

Discussion.—We found that the total number of spermatozoa in Great Tit eggs was about twice as high as in Blue Tit eggs, despite the ovum being only 30% larger. Interestingly, Kempnaers et al. (1994) reported that the total sperm load in sperm-storage tubules of female Great Tits was nearly double that found in female Blue Tits. These strikingly similar findings provide additional support for the claim (Wishart 1987, Birkhead et al. 1994) that the total number of spermatozoa in eggs not only reflects the abundance of spermatozoa in the infundibulum, but also that within the entire oviduct. Previous studies

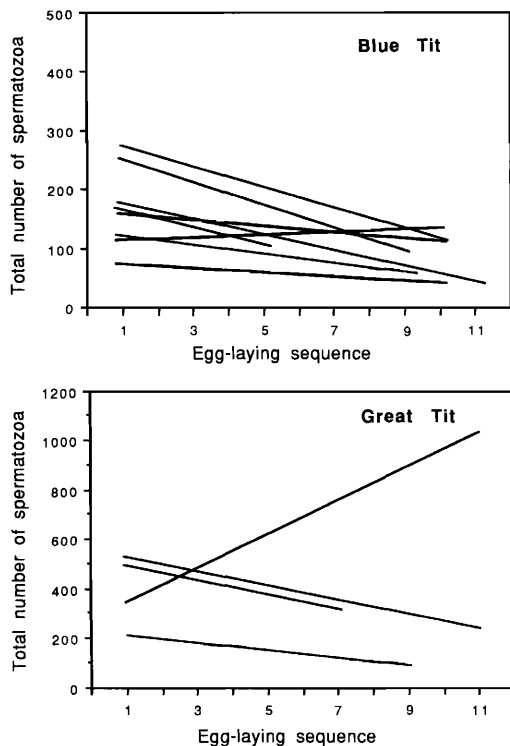


FIG. 1. Linear regression lines for the change in total number of spermatozoa with laying sequence for individual complete clutches of Blue Tits ($n = 8$) and Great Tits ($n = 4$) from Maridalen. The regression line for one Great Tit clutch that appeared to be infertile is not shown.

have also found that the number of spermatozoa counted in eggs is positively correlated with the number stored in sperm-storage tubules (Brillard and Bakst 1990) and with the number that were artificially inseminated (Bramwell and Howarth 1992).

The total number of spermatozoa declined with laying order in Blue Tit clutches. This pattern is consistent with the observation that copulation rates in this species decline over the laying period after a peak on the day before laying the first egg (B. Kempenaers pers. comm.). Nevertheless, in both species the number of spermatozoa in eggs varied widely within clutches, which might be due to variation in copulation rate during the laying period.

Great Tits have a lower copulation rate than Blue Tits (Kempenaers et al. 1994). Thus, it is puzzling that Great Tits had about twice as much oviductal sperm. Similarly, it is striking that Dunnocks (*Prunella modularis*) have an extraordinarily high copulation rate (Davies 1992), yet had the lowest level of total spermatozoa in their eggs in a comparative study of 24 bird species (Birkhead et al. 1994). This suggests a general negative relationship between

copulation rate and number of oviductal spermatozoa across species, which could be associated with the intensity of female-driven sperm competition. Perhaps it is easier for females to ensure paternity through extrapair copulations when the abundance of oviductal spermatozoa from her social mate is low. This could be achieved through a high rate of passive sperm loss from the oviduct and optimal timing of extrapair copulations. A good example of a species with high rates of copulation and sperm loss is the Bearded Tit (*Panurus biarmicus*; Birkhead and Hoi 1994, Sax et al. 1998).

One complete clutch of Great Tits, out of 26 sampled, evidently was infertile because no spermatozoa were detected in any of the eggs. Although no general pattern can be inferred from a single case, it is interesting that such a case occurred at all. In our study of paternity in the Jomfruland population of both tit species (Krokene et al. 1998), it was proposed that low (but biologically significant) proportions of males were infertile. The speculation arose from the fact that all young in three broods (1 of 47 [2.1%] Blue Tit broods and 2 of 55 [3.6%] Great Tit broods) were sired by an extrapair male. In all other broods, the frequency of extrapair offspring was low (5% in both species) and was randomly distributed among broods. Similar cases of a few single broods with complete extrapair paternity have been documented in other studies of these tit species (Kempenaers et al. 1997, Verboven and Mateman 1997). In the present case of clutch infertility, the female may have been paired with an infertile male but did not successfully engage in any copulation with a fertile extrapair male. Although the success of copulations seems to be under female control (Birkhead and Møller 1992), there may be severe constraints on the female's ability to pursue extrapair copulations (Birkhead 1996, Slagsvold and Lifjeld 1997) because she must successfully escape mate guarding and find a suitable male that is available for copulation. The males of both tit species exhibit mate guarding (Björklund and Westman 1986, Kempenaers et al. 1995). Moreover, female Zebra Finches (*Taeniopygia guttata*) paired to vasectomized males showed no greater tendency to seek extrapair copulations than females paired to intact males (Birkhead and Fletcher 1995).

Wetton and Parkin (1991) documented a positive association between the occurrence of extrapair offspring and unhatched eggs in the House Sparrow (*Passer domesticus*), which they interpreted as evidence for the hypothesis that females seek extrapair copulations to minimize the risk of male infertility. However, other interpretations are also possible (Lifjeld 1994). Birkhead et al. (1995) found that more than 80% of unhatched House Sparrow eggs contained spermatozoa and therefore had been fertilized, and only 1.2% (6 of 503) of all eggs appeared to be infertile. Hence, the evidence for male infertility was rather weak and indirect in this species. Very lit-

tle is known about the occurrence of infertility in wild birds, but anecdotal evidence of complete sterility exists (e.g. Birkhead and Møller 1992:199). An interesting question that begs further empirical and theoretical work is: How high must the frequency of infertile males be for females to enjoy a net reproductive benefit by performing insurance copulations with extrapair males?

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