Repeated inseminations required for natural fertility in a wild bird population

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In most bird species, pairs copulate many times before egg laying. The exact function of repeated inseminations (i.e. successful copulations) is unknown, but several suggestions have been made. We tested the hypothesis that repeated inseminations are required to ensure fertilization of eggs, by using an experimental method where free-ranging male collared flycatchers (*Ficedula albicollis*) were prevented from inseminating their mates. We show that egg fertility was lower when females had not copulated during the studied part of their fertile period. By counting sperm on the inner perivitelline layer of eggs, we estimated that a minimum of 86 sperm must reach the site of fertilization to ensure average fertility. Using the timing of inseminations and the numbers of sperm on successive eggs, we show that repeated copulations are necessary to achieve an average rate of fertilization of a single clutch. Our results thus provide evidence that repeated inseminations function to ensure fertilization success. We discuss possible constraints on sperm production and utilization that may have contributed to this pattern.

Keywords: egg fertility; insemination pattern; sperm store; wild bird population

1. INTRODUCTION

In most birds the female copulates more than once during her fertile period (Birkhead *et al.* 1987). The female may copulate several times with the same partner (repeated copulation), or copulate with several partners (multiple copulation). It is difficult to explain repeated copulation from an evolutionary aspect because a single insemination can sometimes be sufficient for the fertilization of an entire clutch (Lake & Rave 1975; Birkhead 1988), and copulation may well carry costs for both sexes (Daly 1978; Birkhead & Møller 1992b). It seems expedient to seek adaptive explanations for this copulatory behaviour from the females' perspective, as there is increasing empirical evidence that, in most bird species, females can determine the successfulness of copulation by cooperation to achieve cloacal contact and thus control copulations (Lifjeld & Robertson 1992; Kempenaers *et al.* 1995; Sheldon *et al.* 1999; Michl *et al.* 2002).

According to Hunter *et al.* (1993), females may engage in repeated copulation with a male to:

- (i) devalue the sperm obtained from an inferior male (McKinney *et al.* 1983);
- (ii) assure a male of his paternity and, as a consequence, make him invest more effort into paternal care (Davies *et al.* 1992);
- (iii) reduce the likelihood that her partner can copulate successfully with other females (Petrie 1992; Eens & Pinxten 1995);
- (iv) facilitate the formation of a pair (Birkhead & Lessells 1988); and
- (v) assess the male's quality (Lens *et al.* 1997).

In their review, Hunter *et al.* (1993) also discussed the fertilization assurance hypothesis (Birkhead *et al.* 1987), according to which the female might seek repeated or multiple copulation if one copulation is not enough to fertilize all the eggs that she will lay. Hunter *et al.* (1993) concluded that there was no evidence to support this hypothesis.

Birds' eggs are fertilized sequentially, shortly after ovulation when each egg enters the infundibulum. Eggs are ovulated at intervals of *ca.* 24–48 h and fertilized by sperm that were previously accumulated in the sperm storage tubules at the utero-vaginal junction (Mero & Ogasawara 1970; Birkhead & Møller 1992b). As sperm are released from the sperm stores at a constant rate, in a single early insemination, fewer and fewer sperm reach the infundibulum as egg laying proceeds (Wishart 1987). Recent data have shown that, in several species, the rate of sperm loss from the female's reproductive tract can be very high, indicating the necessity of repeated or multiple inseminations for the sake of adequate fertilization success (Birkhead *et al.* 1996; Sax *et al.* 1998). The depletion of stores may result in the potential cost of infertile eggs appearing in a relatively large clutch that may enhance evolution of multiple or repeated copulation.

The hatchability of eggs in wild songbird populations is usually high (Koenig 1982). As dead embryos can be found in some of the unhatched eggs (Wetton & Parkin 1991), and embryo mortality may occur very early in development due to genetic incompatibilities between mates (Bensch *et al.* 1994; Kempenaers *et al.* 1996) it is probable that the fertility rate of eggs is higher than their hatchability rate. It is well known that in some domesticated or captive species the number of sperm trapped on the inner perivitelline layer (PVL) is positively correlated with the probability of fertilization and the amount of

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sperm stored in the female's genital tract (Wishart 1987; Birkhead & Fletcher 1998). To maintain the amount of stored sperm at a relatively high level during the fertile period and thus fertility, females may require several inseminations even though multiple or repeated copulations may be costly.

The aim of this study was to determine how frequency and timing of inseminations and amount of transferred sperm influenced fertility of eggs in a free-ranging population of the collared flycatcher (*Ficedula albicollis*). We used a method that enabled us to prevent a male from inseminating his mate (Michl *et al.* 2002) and counted the number of sperm trapped on the PVL of the egg yolk to assess the quantity of stored sperm in female genitalia.

In accordance with the fertilization assurance hypothesis we predicted that

- (i) females with higher insemination frequency achieved higher egg fertility;
- (ii) females should suffer from sperm shortage before a focal insemination (Hunter *et al.* 1993).

2. MATERIAL AND METHODS

We conducted this study from late April to mid-May 1999 on an experimental plot in a continuous oak forest near Budapest, Hungary (47°43' N, 19°01' E). The collared flycatcher is a small, migratory hole-nesting passerine. Males arrive in Hungary in mid-April, a few days before females. Sexes are dichromatic, with males having a conspicuous white forehead patch that is known to be a sexually selected trait. In pair formation the females prefer males with large forehead patches (Gustafsson *et al.* 1995). Extra-pair copulation and insemination is on average 22% in the studied population (Michl *et al.* 2002; Garamszegi *et al.* 2002). Females lay six or seven eggs and incubate alone, but both parents feed young. A detailed description of the study site, the breeding population and the established ringing protocol is given elsewhere (Török & Tóth 1986; Michl *et al.* 2000).

We collected the clutches of 20 pairs (15 experimental and five control pairs) under licence from the Nature Protection Office of Hungary. We removed any newly laid eggs every day and prevented nest abandonment by replacing them with dummy eggs. The whole clutch (six or seven eggs) was collected from each brood. Eggs were stored in a refrigerator (5 °C) until examination.

One prediction of the fertilization assurance hypothesis is that insemination (successful copulation) frequency correlates with the proportion of fertilized eggs (Hunter *et al.* 1993). To test this prediction, we had to manipulate the frequency of inseminations. In an experiment, we used a recently developed method on 15 experimental pairs 3–4 days before onset of egg laying (Michl *et al.* 2002). Randomly chosen males were prevented from transferring sperm during copulation with their mates from at least 2 days before the start of laying eggs by fitting males with a rubber ring around their cloaca. We checked the males with a telescope regularly and found that the position of the rings never changed and that they remained attached to the male throughout. Pair bonds were maintained during egg laying and no unusual behaviour could be observed in the meantime. The rubber ring did not obstruct defecation. Presumably, the rubber ring inhibited its bearer's sperm transfer, because it was twice as long as the males' and females' combined cloaca protuber-

ance length and, according to our observations, the dimension of the ring made it impossible to reach the natural position of copulation. The rubber ring was removed after clutch completion. Elsewhere (Michl *et al.* 2002) we report further evidence that this method is effective in preventing the male from inseminating the female, derived from studies of paternity in collared flycatcher families, and from sperm counts on eggs produced by female zebra finches (*Taeniopygia guttata*) isolated with single males. Furthermore, five randomly chosen pairs as control pairs were involved in the study where the male could copulate freely with his mate.

Sperm counts on egg membranes provide a unique opportunity to follow, through a non-invasive method, the changes in the number of sperm within the female's genital tract during the fertile period. The number of countable sperm trapped on the PVL of an egg correlates closely with the number of sperm stored in the sperm storage tubules (Wishart 1987) *ca.* 48 h earlier (Colegrave *et al.* 1995). To determine the number of sperm on the PVL, we used the technique introduced by Wishart (1987). Sperm counts were made by J.B. blindly for the predictions under test. An insemination event could be detected as a dramatic peak in the number of sperm on the inner PVL and could be dated to *ca.* 2 days earlier than the egg had been laid (Michl *et al.* 2002). The height of a peak is proportional to the number of sperm transferred during an insemination (Wishart 1987; Brillard & Bakst 1990) and therefore provides a means of estimating the relative number of transferred sperm. We calculated the height of a peak by subtracting the number of sperm trapped on the PVL before the current insemination from the number of sperm counted after insemination. In a given egg the number of sperm that would be present before insemination was estimated based on the number of sperm counted on the previous egg by taking the rate of passive sperm loss into account. The rate in collared flycatchers is $0.019 \pm 0.008 \ln \text{ sperm h}^{-1}$ (Michl *et al.* 2002).

We counted the sperm on the eggs of each clutch and inferred the timing of insemination events from the distribution pattern of peaks. The sperm peaks indicated that the insemination events were generally sharp, with an increase in the number of sperm being limited to a single day (Michl *et al.* 2002). As we could not distinguish between one and several copulations on the same day both were 'an insemination event' as scale of analysis was per day. In those rare events when the peaks lasted for 2–3 days, we concluded that an insemination event had taken place on every consecutive day. As insemination events are rare in collared flycatchers (Michl *et al.* 2002), our method reliably showed the timing pattern of inseminations in a clutch. Taking into account the height of sperm peaks, the rate of sperm loss and the relation between the number of sperm counted on PVL and the probability of the fertilization of an egg, we calculated the fertilization capacity of each insemination. The fertilization capacity shows how many consecutive eggs could be fertilized at a level of equivalent to the average rate of fertility in the population. To investigate the relation between the number of sperm counted on the PVL and the probability of an egg being fertile, we determined the fertility status of each egg by macroscopic observation ($n = 109$). Embryonic development starts when fertilization occurs in the infundibulum of a bird's oviduct. Consequently, when the egg is laid, the embryo is generally composed of 40 000–80 000 morphologically undifferentiated blastodermal cells designated as stage X by Eyal-Giladi & Kochav (1976). Fertility can be assessed by eye in fresh, unincubated eggs by distinguishing the regular spherical appearance of the

fertile blastoderm (area opaca and area pellucida) from the amorphous condensed knot of opaque material and surrounding crater-like lacunae, which represent the degenerating germinal disc (blastodisc) of the unfertilized egg (Kosin 1945). Eggs showing these structural changes were classified as fertile eggs, whereas those without a spherical blastoderm were categorized as infertile.

During data analyses we applied log transformation for sperm numbers and arcsine transformation for fertility rates to achieve normality. We used STATISTICA for Windows (v. 4.5) in all statistical analyses.

3. RESULTS

We detected no sperm peaks on eggs of 60% of the experimental pairs ($n=9$), which were categorized as group INS(-). Group INS(+) included experimental pairs with insemination ($n=6$) and five control pairs without rubber ring. In group INS(+) each female engaged in insemination(s) one to three times during the studied fertile period (between days -1 and +3 where the day 0 is the date of the first egg laid). As we detected sperm on the first egg of each pair of the two groups (INS(-) group $x=60$, s.d. = 64, $n=9$; INS(+) group $x=104$, s.d. = 115, $n=11$) we presumed that each female had already engaged in at least one successful copulation, before the studied fertile period. The mean sperm number on the first eggs did not differ between the two groups (Student's t -test, $t=1.032$ n.s.), which indicated that the early insemination pattern of the groups was similar. In the INS(+) group we examined 59 eggs of 11 clutches, among which four eggs proved to be infertile and the fertility rate of the clutches was 94% (95% c.i.: 88.3–99.6). Hereafter, we use this value as the average rate of fertility in the studied collared flycatcher population. In INS(-) pairs we found that out of 50 eggs 12 were infertile in nine clutches. Within this group the fertility rate of clutches turned out to be 76%. The fertility rate of the clutches in the INS(+) group was significantly higher than in INS(-) pairs (Student's t -test $t=2.10$, $p<0.05$), showing that an increase in the frequency of inseminations resulted in a higher rate of fertilization in collared flycatchers.

The sperm numbers per egg varied between 0 and 440, and the number of sperm was a strong predictor of the probability that an egg would be fertilized (figure 1 logistic regression, $\chi^2=12\,035$, $p=0.000\,52$, $n=109$). From the fitted relation, the number of sperm required for fertilization success to be equal with the population average, 0.94 can be estimated as 86.

We dated insemination events ($n=15$) and, by counting the number of sperm on the PVL, estimated the amount of sperm that had been stored on the day before a focal insemination. Using the calculated rate of loss of sperm from the female's sperm stores, we estimated the number of sperm present on the day of insemination. In only three of 15 cases was the estimated number of sperm present greater than 86, the number calculated as being required to ensure fertilization at the population mean rate.

The size of the peak in sperm numbers, which indicated the amount of transferred sperm, varied between 61 and 398 (mean = 219 ± 28.4 s.e.m.). Only four of 15 insemination events (27%) could have fertilized an entire six-egg clutch (figure 2).

Figure 1. The probability of an egg being fertile in relation to the number of sperm counted on its PVL obtained from data of 109 eggs. The dashed lines indicate that 86 sperm on PVL are needed to reach the mean egg fertility rate of the population. The curve is derived from the logistic regression model with equation $y = \exp(0.793 + (0.021) * x) / (1 + \exp(0.793 + (0.021) * x))$.

Figure 2. The fertilization capacity of inseminations in the collared flycatcher. The columns show the proportion of insemination events that provide sufficient sperms to fertilize the indicated number of consecutive eggs (horizontal axis) at the average rate of fertility in the population.

In INS(+) pairs, a total of 15 insemination events occurred during the females' fertile period. The insemination events were timed, and the highest frequency took place on day 0, when the first egg was laid (figure 3, χ^2 -test $h=14.80$, $p<0.0006$, to achieve the minimal cell sample size we pooled 0 and +1; +2 and +3 days' data, respectively). To minimize the cost of copulations females should time their single insemination to day -2 or even earlier.

4. DISCUSSION

One prediction of the fertilization assurance hypothesis is that pairs with a higher insemination frequency achieve higher egg fertility (Hunter *et al.* 1993). Our results showed that successful copulation during the fertility period increased the rate of fertility of eggs, and that multiple or repeated copulation may be necessary to ensure fertilization of the entire clutch. As every female was inseminated

Figure 3. The distribution of inseminations during the studied part of the fertile period of collared flycatcher females (day 0 is when the first egg was laid) showing a non-random distribution peaking on day 0. As the insemination events before -2 day cannot be monitored we could not determine the inseminations for -2 day (for dating inseminations see § 2).

at least once before the studied part of the fertile period, we have shown that female collared flycatchers must copulate successfully at least twice to assure high fertilization success of the clutch. In addition, the very early inseminations are unable to contribute to the fertilization success of clutches, as sperm storage tubules are still undeveloped and their sperm storage capacity is limited (Birkhead *et al.* 1997).

According to another prediction of the fertilization assurance hypothesis, the sperm storage tubules of females should contain a relatively small amount of sperm before the focal insemination, as one function of the copulation is to refill these stores. Our results supported this prediction, because the number of sperm remaining from previous inseminations was lower than the value predicted to ensure fertility at the rate equal to the average in the population. Hence, it appears that female collared flycatchers were short of sperm before inseminations, and by copulating again increased the likelihood of eggs being fertilized. Our findings thus support the fertilization assurance hypothesis.

A second finding of our study provides further support for the fertilization assurance hypothesis. In the population as a whole, only 27% of inseminations could potentially have fertilized an entire clutch. Taking account of the number of transferred sperm, the clutch size, the rate of sperm loss and the relation between sperm numbers and fertility, in 11 out of 15 inseminations the amount of sperm transferred was not enough to ensure fertility of the clutch corresponding to the average rate in the population. In § 4a–f we discuss possible constraints on copulation and fertilization in the collared flycatcher.

(a) Clutch size

In the collared flycatcher, clutch size varies between four and eight eggs, with most clutches being of six or seven eggs. Fertilization success (in terms of the proportion of eggs fertilized) of an early insemination is negatively correlated with clutch size, because the probability of fertilization of eggs that are laid later decreases owing to passive sperm loss (Birkhead & Møller 1992b). Our results

indicate that a relatively large clutch size might be partly responsible for multiple or repeated inseminations. Only a quarter of the inseminations were sufficient for the fertilization of a six-egg clutch at a level equivalent to the average rate in the population; however, no successful insemination could be found that would have fertilized a seven-egg clutch at this rate (figure 2).

(b) Timing of inseminations

To reduce the costs of multiple or repeated copulation a female should time her single insemination just before the onset of egg laying. Consequently, the number of sperm counted on eggs should be highest during the early stages of the fertile period. However, we found that this variable reached its minimum during this period (Michl *et al.* 2002). The frequency of insemination showed a peak at day 0 and day +1; however, we found low insemination rates at early stages of the fertile period. This insemination pattern indicated that females timed their insemination not to minimize the number of copulations but perhaps to achieve other aims like securing good genes or more paternal care (Sheldon *et al.* 1997, 1999; Sheldon & Ellegren 1999; Michl *et al.* 2002).

(c) Number of transferred sperm

In passerine birds the frequency of copulations is positively correlated with testis size, and the latter determines the rate of sperm production (Møller 1991; Birkhead *et al.* 1993a,b). As the insemination and replenishment rate in collared flycatchers is low (Michl *et al.* 2002), it may be that sperm production capacity of this species is also low. This suggestion is supported because the sibling species, pied flycatcher (*Ficedula hypoleuca*), in which the copulation rate is also low, has a high body mass : testis size ratio (Pitnick & Karr 1996). In species where the rate of sperm production is low, the size of sperm reserves drops dramatically after even a single insemination; replenishment of sperm stores may take several days (Birkhead & Fletcher 1995). This may explain why the amount of transferred sperm (judged by the height of peaks in sperm numbers) varied considerably, and why many insemination events resulted in relatively few sperm being transferred to the females. An alternative explanation is that the testis size of males varies, as in the house sparrow (*Passer domesticus*) and the greenfinch (*Carduelis chloris*) which could result in a different rate of sperm production (Birkhead *et al.* 1994a; Merilä & Sheldon 1999). In collared flycatchers copulation behaviour is probably under female control (Sheldon *et al.* 1999; Michl *et al.* 2002), which excludes the possibility of insufficient sperm transfer caused by incorrect cloaca contact.

(d) Rate of passive sperm loss

The collared flycatcher is a socially monogamous bird with sperm competition (Sheldon & Ellegren 1999). Among the bird species that have already been studied and that follow a similar mating system, the female's rate of passive sperm loss in the collared flycatcher is one of the lowest (Birkhead *et al.* 1994a; Sax *et al.* 1998). These data would indicate that the rate of sperm release *per se* is not likely to be the constraint of multiple or repeated insemination. However, the evolution of multiple copulations can modify some physiological traits of female

birds (e.g. an increase in the rate of passive sperm loss), which may lead to the relative shortage of sperm in the genital tract (see § 4f).

(e) *Sperm-egg interactions*

Only a portion of the sperm that reaches the inner PVL is able to penetrate the layer itself. The ratio of trapped to penetrating spermatozoa varies between species, which may be due to species-specific sperm penetrating capacity or the thickness of the PVL (Birkhead *et al.* 1994b). The large numbers of spermatozoa penetrating the avian ova (physiological polyspermy) may be explained by the difficulty of sperm in finding the germinal disc (pronucleus). The germinal disc in birds constitutes a relatively small target for spermatozoa on the surface of the large yolky ovum (Bakst & Howarth 1977; Hosken & Stockley 1998). It is possible that in different species different numbers of sperm are needed to find the germinal disc. Consequently, at least two kinds of mechanism can make it possible for two species to differ in sperm-egg interactions.

Comparing the zebra finch (Birkhead & Fletcher 1998) and the collared flycatcher we found differences in sperm-egg interactions. The average number of sperm counted on the PVL per egg was 30–40 in the former and 80–90 in the latter considering similar egg fertility. Based on the rate of sperm loss in the collared flycatcher, *ca.* 2 days are needed to reduce the number of sperm counted on the PVL from 90 to 40. This indicates that if the sperm of the collared flycatcher fertilized the ova with an efficiency similar to that of the zebra finch then a single insemination could fertilize two more eggs at a level equivalent to the average rate of fertility in the population. However, the rates of sperm competition also differ in these two species. On average, *ca.* 15% of offspring in *ca.* 30% of broods are fathered by extra-pair copulations in the collared flycatcher (Sheldon & Ellegren 1999; Veen *et al.* 2001). In the zebra finch the corresponding figures are 2.4% and 8%, respectively (Birkhead *et al.* 1993b). Hence, male collared flycatchers may be selected to inseminate more sperm into females than male zebra finches, a fact that may have led to differences in sperm utilization efficiency between females of the two species.

(f) *Evolution of multiple copulations and risk of infertile eggs*

Recent studies found no positive correlation between the males' mate-guarding intensity and within-brood paternity (Gowaty & Bridges 1991; Kempenaers *et al.* 1995; Currie *et al.* 1998). It was also revealed that females seek extra-pair copulations actively (Otter *et al.* 1994; Smiseth & Amundsen 1995) and choose males as extra-pair partners that are of better quality than their social mate, thus gaining genetic benefits (Hasselquist *et al.* 1996; Sheldon & Ellegren 1999; see for review Birkhead 1998). All these findings provided convincing evidence that the female is predominantly able to control copulations and thus, in most of the studied species, sperm competition appears female driven (Birkhead 1998). Analyses of copulatory behaviour demonstrated that female birds stop copulating with their social partner early, still a few days before laying the first egg, which provides an opportunity for them to manipulate paternity (Birkhead & Møller 1993). Based on the last-male sperm

precedence rule, if the female waits for 1 or 2 days and then engages in extra-pair copulation(s) with the preferred male, the chance of the latter to fertilize the ova will grow, because in the meantime the sperm of the pair-male mostly empties from the female's genital tract (Birkhead & Møller 1993). In the process of the evolution of multiple copulations, those females whose individual rate of sperm loss was higher than average had better ability to manipulate paternity, gaining reproductive benefits. Furthermore, to facilitate the success of last-male fertilization, some physiological traits influencing egg-sperm interaction may have changed, as a result of which the fertilization became more difficult. During the evolution of female-driven sperm competition, the rate of sperm loss could increase and more sperm could be necessary for fertilization so the sperm demand might outweigh the sperm production ability of the testis, which increased the chance that the female would lay some infertile eggs. By way of compensation, repeated or multiple copulations were needed to maintain the natural fertility of the clutch. The supposed evolutionary route is in line with the result of a comparative study that found an inverse relation between the frequency of copulations and the duration of sperm storage (Birkhead & Møller 1992a).

On the level of inseminations, our results demonstrated that the successful copulations of females are temporarily separated, as they are usually not inseminated on days -2 and -1 (Michl *et al.* 2002) and as this study showed, this results in a shortage of sperm during the egg-laying period. In the collared flycatcher, if the fertilizations were as 'easy' as in zebra finches (Birkhead & Fletcher 1998) and the rate of sperm loss was as low as in lekking birds with weak sperm competition (Sax *et al.* 1998), then the 2-day sperm loss would not jeopardize the fertility of eggs. To avoid the risk of laying infertile eggs, flycatcher females filled their sperm storage tubules by well-timed insemination(s). For cuckolding females, it implied extra-pair insemination(s), which resulted in an extra-pair-male biased sperm ratio (5 : 1), indicating that the female is indeed able to manipulate paternity significantly (Michl *et al.* 2002). As occurrence of multiple copulations depended on pair-male quality, repeated copulations were needed for females that did not cuckold as well.

We suggest that female-driven multiple copulations as a mating tactic creates a trade-off between the high genetic quality offspring and risk of laying infertile eggs. The evolution of multiple copulations may alter some physiological traits (duration of sperm storage, sperm-egg interaction) and the copulatory behaviour of females, which may increase the risk of laying infertile eggs.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.