Females of the grasshopper *Chorthippus parallelus* (Zett.) do not remate for fresh sperm

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The evolution of female multiple mating is still a largely debated field. Among the benefits that have been proposed to explain this risky behaviour is the replenishment of sperm reserves. Apart from an increase in total sperm number, it can be an expression of post-copulatory mate choice or can be directed towards the uptake of fresh sperm. Using fresh sperm for fertilization instead of sperm aged by storage in the female genital tract may avoid a lowered fertilization capacity, an increase in deleterious effects or a skewed offspring sex ratio. We investigated the influence of sperm age on female fitness in the grasshopper *Chorthippus parallelus*, a species where females mate multiply. After copulation, females store sperm over the course of weeks until fertilization. An average ejaculate of 250 000 spermatozoa exponentially declined with time within the female's spermatheca. The number of days since copulation better explained the variation in actual sperm number than the number of pods or eggs laid. We investigated differences in female fitness parameters in two treatments. In the first, females were mated only once, while in the second, females always had freshly ejaculated sperm available. Although in our experiment, multiply mated females had heavier offspring than singly mated females, egg number per pod, hatching and fertilization success, their composite effects and offspring sex ratio did not vary with respect to season or sperm age. We therefore reject the hypothesis that the reason for remating in females of this species is the uptake of fresh sperm.

Keywords: multiple mating; sex ratio; sexual selection; sperm age; sperm use

1. INTRODUCTION

Females of many species mate more frequently than necessary to fertilize their lifetime's egg production (e.g. Thornhill & Alcock 1983; Ridley 1988; Birkhead & Møller 1992). This is an intriguing and long-debated subject because there are direct and indirect costs of mating (Magnhagen 1991; Chapman *et al.* 1995; Clutton-Brock & Langley 1997). Direct and indirect fitness benefits that have been proposed to explain this risky behaviour of females include fertility insurance, decreased sexual harassment, lower probability of infanticide, the attraction of parental care by more than one male, the delivery of material gifts at various stages of the mating act as well as increased viability or attractiveness of offspring (summarized by Møller 1998). Remating not only avoids sperm depletion (Andersson 1994; Sax *et al.* 1998) it can also serve to increase the number of sperm of a preferred male (Brown 1997) and is suggested to provide fresh sperm (Andersson 1994). The availability of fresh sperm can be crucial in avoiding the effects of lowered fertilization capacity or an increase in deleterious effects of aged sperm. A decrease in sperm quality with sperm age, here defined as the duration of storage within the female's storage organs, seems to be supported by empirical arguments. The trade-off between longevity and swimming speed (Bolton & Havenhand 1996) makes aged sperm potentially less motile (but see Gee & Zimmer-Faust 1997) and leads to a lower likelihood of fertilization. A sharp decrease in fertilization capacity, an

obvious character of sperm quality, has been demonstrated in many external (Lillie 1919; Levitan *et al.* 1991; André & Lindegarth 1995) and internal fertilizers (Lanmann 1968; Lodge *et al.* 1971; Smith & Lodge 1987). Another consequence of sperm ageing could be a differential survival according to the sex chromosomes as suggested by Hewitt *et al.* (1989) and Bella *et al.* (1992). Thus, sperm ageing in the female can lead to a biased offspring sex ratio (Sapp & Martin-DeLeon 1992; Corkery & Martin-DeLeon 1996). Birkhead & Møller (1998) summarized these arguments into the general assumption that the storage and hence ageing of sperm can lead to a decrease in the quality of sperm and, thus, offspring.

We tested the hypothesis that sperm aged by storage in the female genital tract does lead to a decrease in offspring quality and hence, female fitness. Insects provide a good example for studying the influence of sperm age on fertilizing capacity. After copulation, female insects store sperm in special organs for several days to several years (Tschinkel 1987) until fertilization at the moment of oviposition (Wigglesworth 1972; Okelo 1979). However, when investigating the effect of sperm age on fitness, two effects often overlap that cannot be easily separated: a variation in fertilization success due to a decrease in sperm quality or due to female factors. The latter can include a seasonal variation in fertilization success (López-León *et al.* 1994) or senescence effects in fertilization ability as suggested by K. Reinhardt and J. Meister (unpublished data). We investigated the effects

that the use of stored and thus aged sperm has on female fitness in the grasshopper *Chorthippus parallelus* (Zett.), a species in which females do not gain material benefits from multiple mating but fertility insurance could be important (Reinhardt & Köhler 1999). Female fitness in this species is mediated by maternal investment, as Monk (1985) reports that hatchling dry weight can be adjusted according to site quality. We carried out experiments that allowed us to separate the effects of sperm age and laying order by comparing a treatment of females with free access to males with one where females were mated only once. Since last-male sperm precedence is commonly found in grasshoppers (Simmons & Siva-Jothy 1998) and also in our study species (Bella *et al.* 1992; K. Reinhardt, unpublished data) it is reasonable to assume that multiply mated females always have a certain level of fresh sperm and that the most recently received sperm is used for fertilization. We designed an experiment where the interaction term of laying order and mating frequency, corrected for female age effects, can be interpreted as sperm age effects.

2. METHODS

(a) General rearing conditions and female treatments

Last-instar nymphs captured in the field at a site near Jena, Germany, were reared in cages (measuring 15 cm × 30 cm × 50 cm) in a greenhouse under natural light conditions. After the imaginal moult the sexes were separated in order to guarantee virginity. At the onset of the experiments, females were housed individually in cages measuring 15 cm × 15 cm × 25 cm. Males were kept in groups of 20 to 30 individuals in cages measuring 15 cm × 30 cm × 50 cm. Bunches of wheat leaves in 100 ml water-filled bottles were provided as food and changed at approximately three-day intervals. Sand-filled plastic cups served as oviposition sites. They were checked daily.

Sixty-nine females were randomly devoted to one of two treatments. In treatment 1, which was run between 25 July and 1 September 1997, 54 females were assigned to the once-mated group and subdivided into six groups with nine individuals each. Females of one of these six groups were sacrificed immediately after copulation (no oviposition) whereas the others were to be sacrificed after one, two, three, five and eight oviposition events, respectively. When a female was found freshly dead it was investigated and devoted to the group with the respective number of ovipositions. Some females were not found immediately after their death and did not allow investigation. Thus, 41 females were left for analysis and the groups did not have equal sample sizes. Fifteen females were assigned to treatment 2 representing the multiple-mated females and to be investigated after the eighth oviposition. This treatment ran between 17 July and 22 August 1997. One female that died due to fly parasitism was excluded.

Daily, between 08.30 and 12.00 Central European Summer Time, treatment 2 females were provided with a male randomly taken from a pool of 15 for 2 h. Every ten days all males were replaced with 15 virgin ones. Mating durations were recorded for all matings in both treatments. We kept the two treatments in separate but similar rooms in the greenhouse. The positions of the cages within treatments were changed daily.

Oviposition in this species cannot be inhibited by lacking mating opportunities (Kriegbaum & Von Helversen 1992;

K. Reinhardt & G. Köhler, unpublished data). Females of this and related species mate at a maximum of one time per oviposition (Renner 1952; Haskell 1958; Hartmann & Loher 1996; K. Reinhardt and G. Köhler, unpublished data; but see Bella *et al.* 1992) as they are not receptive between copulation and oviposition (Renner 1952; Hartmann & Loher 1996). They can reject males for more than one oviposition (Haskell 1958; Kriegbaum & Von Helversen 1992; K. Reinhardt and G. Köhler, unpublished data).

(b) Number of sperm stored

After females were anaesthetized in ester, the spermatheca was removed and put into 0.5 ml locust saline. The spermatheca was slightly ruptured, homogenized by drawing the solution 20 times through a syringe and counted in an Improved Neubauer Chamber. Morisita's index of dispersion (Krebs 1989) did not indicate a significant departure from a random distribution for any of the samples. Therefore, we calculated the mean of 25 inner quadrats of the Neubauer chamber. The number of sperm counted in females that were killed within 2 h of copulation is herein defined as ejaculate size. In order to guarantee uniformity, all sperm counts were done by the same observer (K.R.) with a repeatability (intraclass correlation coefficient; Lessels & Boag 1987) of $r = 0.97$ (one-way ANOVA $F_{9,20} = 60.65$, $p < 0.001$).

(c) Female fitness parameters

We used five female fitness characters, namely egg number per pod, hatching success, fertilization success, offspring sex ratio and mean hatchling dry weight. To account for a possible synergistic effect of three of the female fitness parameters we calculated a very simple factor, 'composite effects' $W = \text{egg number per pod} \times \text{hatching success} \times \text{mean offspring dry weight}$.

All egg pods (152 from treatment 1 and 112 from treatment 2) were stored separately and kept permanently wet in sand-filled film tubes. Approximately four weeks after the last pod had been laid, the egg pods were transferred to the refrigerator and stored for five months at about 5 °C. After that time, which is sufficient to complete diapause in this species (Köhler 1991), egg pods were stored at 25 °C, expecting hatching from about day 11 (Köhler 1983). Because of the large amount of egg pods, these hatching procedures were staggered over about two weeks. During daily surveys newly hatched nymphs were collected and killed by freezing for 2 h at -30 °C. They were sexed under a ×30 binocular microscope by their difference in the genital plate (Sokolow 1926). Seven nymphs with shrunken abdomens remained unsexed and were removed from the analysis of sex ratio but not of hatching success. After sex identification, nymphs were dried at 60–70 °C for 96 h and individual dry masses weighed to the nearest 0.1 mg.

After 35 days, the hatching period was terminated. We collected the egg pods, dissected them and counted the number of non-hatched eggs under a binocular microscope. We defined hatching success as the proportion of hatchlings per pod. Non-hatched eggs were further subdivided into infertile eggs, dried eggs and those where embryos were visible but hatching failed. We defined fertilization success as the number of hatchlings plus the number of non-hatched embryos, divided by the total number of eggs. A total of 51 egg pods (19.3%) were excluded from the analysis of fertilization status as they partly contained dried eggs. As the hatching success may be one indicator of the accumulation of deleterious effects in the sperm translated to the offspring (Thibault 1970), we decided to include hatching success

Figure 1. Number of spermatozoa in the storage organ in relation to the number of days after a single copulation in females of the grasshopper *Chorthippus parallelus*, where y is sperm number at time i and D_i is the number of days since copulation. Each filled circle represents an individual female.

as a separate fitness measurement, despite the fact that it was highly dependent on fertilization success:

$$\text{hatching success} = 0.948 + 0.823 \times \text{fertilization success} \\ (r^2 = 0.949).$$

Minimum and maximum final sample sizes for the traits were: 25–40 (pod 1), 17–29 (pod 2), 12–23 (pod 3), 3–15 (pod 4), 1–8 (pod 5), 4–8 (pod 6), 1–5 (pod 7) and 2–5 (pod 8). The statistical analysis of female fitness parameters was done using a linear mixed-model approach (Laird & Ware 1982; Bates & Pinheiro 1997) in the statistical package S-Plus 4 (1998), with mating as a treatment and laying order as a random effect. Female age at oviposition was included as an additional covariate. Sex ratios were calculated when five or more nymphs hatched from one pod. Proportional data were normalized by arcsine square-root transformation (Sokal & Rohlf 1995). A mean hatchling dry weight per pod was calculated when four or more nymphs hatched from a pod. Nymph dry masses do not differ between sexes (Reinhardt & Köhler 1999).

3. RESULTS

(a) Variation in sperm numbers in singly and multiply mated females

Ejaculate size of ten males was $249\,000 \pm 42\,000$ sperm (± 1 s.e.) with a range of 66 000–414 000. A slight tendency of an increase in ejaculate size with copulation duration (Spearman's rank correlation, $r_s = 0.557$, $n = 10$, $p = 0.095$) was possibly not important in this study as neither the duration of the first copulation (one-way ANOVA $F_{1,64} = 0.912$, $p = 0.764$) nor that of all copulations combined ($F_{1,152} = 1.459$, $p = 0.229$) differed between the treatments.

In females that mated only once, the sperm number successively declined within the female's storage organ (figure 1). The most important factor explaining the variation in sperm content was time since copulation (table 1). This decline was best explained by an exponential model (adj. $r^2 = 0.50$) rather than either linear (adj.

Table 1. Statistics of a linear regression model (forward selection) for the relationship between the $\ln$ number of sperm stored and independent variables in female grasshoppers, *C. parallelus*

(Females were mated once and dissected after discrete time intervals. β is the partial regression coefficient.)

variable	B	β	t	P
constant	12.29		15.808	0.000
days since copulation	−0.173	−0.452	−3.205	0.027
not introduced into the model:				
egg number		−0.20	0.50	n.s.
number of hatchlings		−0.07	−0.33	n.s.
number hatchlings + embryos		−0.04	−0.19	n.s.
number of egg pods		−0.10	−0.21	n.s.

$r^2 = 0.45$) or hyperbolic (adj. $r^2 = 0.36$). There was no significant increase in the explanatory power when other variables were stepwise introduced to the model. Ln-transformed data revealed a half-life of 4.0 days of an average ejaculate in the spermatheca. After eight ovipositions a median of 20 000 sperm (7.8% of a median ejaculate) were still stored by treatment 1 females. In contrast, the sperm content in multiply mated females varied between 42 000 and 1.287 million (median 309 000) after eight ovipositions.

(b) Mating and oviposition frequency

The mating interval of treatment 2 females was 4.50 ± 0.02 (± 1 s.e.) days with a range of 3.10 to 11.62 days ($n = 14$). The proportion of ovipositions preceded by a copulation was not different for the first four pods versus the last four pods ($\chi^2_1 = 0.773$, $p = 0.379$). This indicates that female age had no effect on mating frequency.

The oviposition interval in treatment 2 females was 4.33 ± 0.01 (1 s.e.; range = 3.30–5.49 days). Treatment 2 females did not differ in their laying frequency early and late in life. The inter-pod interval of the first four pods (median 2.88 days) was the same as that of pods 5 to 8 (median 2.71 days) (Wilcoxon matched pairs, $W = -41.00$, $p = 0.110$). Considering females with $n \geq 6$ pods, the oviposition frequency in treatment 1 females was 4.90 ± 0.01 (± 1 s.e.) days being not different from that of treatment 2 females (Mann–Whitney U -test, $U_{8,14} = 70.5$, $p = 0.152$).

(c) Variation in female fitness parameters

The total number of eggs was linearly related to the total number of pods:

$$\text{egg number} = 0.534 + 6.972 \times \text{number of pods} \\ (r^2 = 0.949).$$

Egg numbers per pod, fertilization and hatching success, and offspring sex ratio did not differ between the two treatments (table 2). Treatment 2 females produced pods with a mean offspring dry weight above those of treatment 1 females (table 2; figure 2). As the mean offspring dry weight was negatively correlated with hatchling

Table 2. *Linear mixed model for the relationship between several female fitness parameters, mating frequency and laying order, with female age at oviposition included as a covariate*

(Singly mated and multiply mated females were used to separate the influence of stored and, hence, aged versus freshly ejaculated sperm upon female fitness parameters. The factor *W* composite effects is the product of three of the parameters.)

trait	treatment (mating frequency)		repeated measures (laying order)		interaction (sperm age)		covariate (female age at oviposition)	
	<i>z</i> -ratio	<i>p</i>	<i>z</i> -ratio	<i>p</i>	<i>z</i> -ratio	<i>p</i>	<i>z</i> -ratio	<i>p</i>
eggs per pod	1.799	0.072	0.302	0.764	0.565	0.572	-1.902	0.057
hatching success	0.723	0.470	-0.699	0.485	0.074	0.941	0.007	0.994
fertilization success	0.032	0.974	-0.465	0.642	0.745	0.456	-0.227	0.820
offspring sex ratio (male %)	1.799	0.073	0.364	0.716	-1.407	0.160	1.296	0.195
mean offspring dry weight	3.602	0.000	-0.096	0.924	-1.128	0.259	1.813	0.070
dry weight of a randomly drawn hatchling	2.161	0.031	-0.413	0.680	-0.796	0.426	1.808	0.070
composite effects <i>W</i>	1.456	0.144	-0.455	0.653	0.294	0.772	0.799	0.424

number in treatment 1 females ($r = -0.251$, $n = 94$, $p = 0.015$) but not in treatment 2 females ($r = -0.139$, $n = 88$, $p = 0.197$) we used the dry weight of one randomly selected hatchling out of a pod. This, however, did not alter the previous result (table 2). None of the variation in the fitness parameters was significantly explained by laying order or the interaction of laying order $\times$ treatment (table 2).

4. DISCUSSION

Two processes can lead to a decline in the number of spermatozoa in the female's genital tract. First, spermatozoa decrease in number because of fertilization or processes relating to it, then called sperm use (Gromko 1984; Bressac & Chevrier 1998). Second, in a process called passive sperm loss (Birkhead & Biggins 1998) spermatozoa die due to either physiological ageing or because of active sperm ingestion by the female (e.g. Cunningham *et al.* 1971; Tsubaki & Yamagashi 1991). In the first case we should expect the decrease to be best explained by either the number of eggs laid or the number of eggs fertilized. In the second we should expect that the number of days since copulation best explains sperm loss and that the decline follows an exponential decay (Tsubaki & Yamagashi 1991; Birkhead & Petrie 1995; Sax *et al.* 1998; but see Bressac & Chevrier 1998). We quantified the decline in the number of spermatozoa in the genital tract of female *C. parallelus* and conclude that passive sperm loss takes place in that species. Long-term sperm storage by females is known in several grasshopper species from casual reports (Norris 1954; Pickford & Gillott 1971; Whitman 1986) as well as qualitative data (López-León *et al.* 1994; Reinhardt & Jentsch 1999).

In our study, there was no seasonal decline in the fertilization success in once-mated females. We regard this as evidence that decreasing sperm numbers do not lead to a decrease in the fertilization success. The occurrence of developmental arrests has been considered an expression of the accumulation of deleterious genetic material (Dungay 1913; Dharmarajan 1950; First *et al.* 1963; Thibault 1970). If this is indeed the case, we here provide evidence that no such accumulation takes place. We found

no evidence that embryos fertilized by older sperm tended to show more developmental arrests and, hence, a lower hatching success. This confirms negative findings by other workers (Byers & Muller 1952).

In a previous study on this species, we found no direct fitness benefits of female multiple mating and precopulatory sexual selection in terms of egg numbers per pod, hatching success and offspring dry weight (Reinhardt & Köhler 1999). In this study, one part of these previous results was not confirmed. Multiple mating was beneficial to females as they produced heavier offspring than singly mated ones. Despite food abundance in both treatments, multiply mated females may have been able to invest more into the offspring because they received more protein-rich spermatophores. In grasshoppers, the amino acids of the spermatophore proteins are directly incorporated into the oocytes (Friedel & Gillott 1977; Butlin *et al.* 1987; Pardo *et al.* 1994, 1995). A possible benefit of multiple mating is also shown by our finding that there was a trade-off between hatchling number and mean dry weight in once-mated but not multiply mated females. However, a combination of three of the fitness traits, probably the closest measure to overall fitness, was not negatively affected by either treatment, laying order or sperm age.

Apart from direct benefits, two further important reasons remain to explain multiple mating by females: remating for the uptake of fresh and perhaps more competitive sperm (Roche *et al.* 1968; Miller *et al.* 1969), and a gain of indirect benefits through postcopulatory mate choice (Brown 1997). We tested the first hypothesis and compared singly and multiply mated females. We expected that singly mated treatment females use older sperm for fertilization than multiply mated. Multiply mated females remated after 4.0 days and approximately for each egg pod. After that time only about one-half of the number of spermatozoa of a previous mating is available to the female, and recently ejaculated fresh sperm was abundant in the spermathecae of the females of our multiply mated treatment. Thus, even if sperm mixing (K. Reinhardt and J. Meister, unpublished data) rather than displacement (Parker & Smith 1975) is the mechanism of sperm competition in grasshoppers, treatment 2

Figure 2. Female fitness parameters in relation to laying order and mating treatment. Filled circles represent multiply mated females, open circles once-mated females. The interaction term is a measure of sperm age.

females use a very large proportion of fresh sperm for fertilization. Therefore, the main assumption for our design, a comparison of fresh and aged sperm, seems to hold true. We did not detect a seasonal decrease in fertilization success, yet we might expect a decrease in the quality of stored sperm and hence offspring. Fresh sperm is more motile, and more motile sperm has a higher velocity (Aitherton *et al.* 1979) and shows a greater fertilization capacity (Lillie 1919; Roche *et al.* 1968; Lanmann 1968; Miller *et al.* 1969; Lodge *et al.* 1971; Smith & Lodge 1987; Levitan *et al.* 1991; André & Lindegarth 1995). Bella *et al.* (1992) found slight evidence for sperm ageing in the competitive ability in *C. parallelus* by comparing the proportion of offspring fathered by the second male to

mate (P_2 -values) from homogametic and heterogametic matings, but no correlation of sperm age with fertilization success was detected. Although sperm ageing processes and consequences within males and females might be quite different (Lanmann 1968), there seems to be a similar effect of a decrease in sperm quality aged in the testes of human males. It was suspected that such aged sperm is less competitive and is therefore regularly ejaculated by male masturbation (Baker & Bellis 1993).

In situations where sex ratio unity is adaptive (Trivers & Willard 1973; Charnov 1982), a further fitness cost can arise when sperm aged in the female produces a biased offspring sex ratio (Sapp & Martin-DeLeon 1992; Corkery & Martin-DeLeon 1996). In general, information

about the effect of sperm age on offspring sex ratio is rare. In a bush cricket, Reinhold (1996) found that a seasonal variation in primary sex ratio was due to male age but not sperm age. In one study of *C. parallelus*, the primary sex ratio was about 63% male embryos whereby the sex ratio did not change with laying order (Bella *et al.* 1992). However, the authors concluded that preferential fertilization by non-X (male-determining) rather than differential death of XX (female-determining) sperm or embryos was more likely to contribute for the observed sex ratio difference. We did not find any effect of season or sperm age on the sex ratio although we had only the secondary sex ratio available. However, maternal investment can vary under expected sperm limitation. The results by López-León *et al.* (1994) in another species indicate that females tend to preferably fertilize the first and last egg pods and Bella *et al.* (1992) state that, in *C. parallelus*, the first egg pod is frequently small and shows poor survival. Yet, we could not find differential maternal investment, measured as hatchling dry weight, with regard to season, female age and sperm age. This confirms the study by Landa (1992) that seasonal variation should be absent in diapausing species.

In conclusion, sperm depletion does not occur in females of the grasshopper *C. parallelus*. Females have no (Reinhardt & Köhler 1999) or some (this study) material benefits from multiple mating. Neither decreasing sperm numbers nor increasing sperm age are costly for females in terms of a lower fitness. Females do not seem to alter their investment strategy after a long period without mating opportunity. Yet, females mate multiply, either because mating is not risky, not very costly (Reinhardt & Köhler 1999) or because there are indirect genetic benefits.

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