Consequences of sperm displacement for female dung flies, *Scatophaga stercoraria*

P. Stockley¹* and L. W. Simmons²

¹Population Biology Research Group, School of Biological Sciences, Nicholson Building, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK (stockley@liv.ac.uk)

²Department of Zoology, The University of Western Australia, Nedlands, WA 6907, Australia

Displacement of stored sperm during copulation occurs in many insects. This process provides direct benefits for males via increased fertilization success, but the fitness consequences of sperm displacement for females are less clear. Here we investigate potential benefits of sperm displacement for female yellow dung flies, *Scatophaga stercoraria*. We find no evidence that female dung flies gain direct benefits from displacement of previously stored sperm in terms of increased fertility or fecundity. There was no difference in the relative survival rate, development time, size or fluctuating asymmetry of offspring produced by females that had previously stored sperm displaced before oviposition and those that did not. Females using previously stored sperm to fertilize their eggs produced significantly higher ratios of male to female offspring. These novel findings have important implications for understanding the evolutionary dynamics of male–female interactions in sperm competition.

Keywords: coevolution; fertility; sex ratio; sperm displacement; sperm storage

1. INTRODUCTION

Female insects often copulate more than once to fertilize their eggs, which, in many species, leads to displacement of stored sperm (reviewed in Simmons & Siva-Jothy 1998). Sperm displacement is beneficial for copulating males, as it increases success in sperm competition (Parker 1970a). It is less clear, however, whether females benefit from the replacement of previously stored sperm. Although females are often active in the process of sperm displacement (Eberhard 1996), costs associated with female remating behaviour in some species suggest sexual conflict in relation to sperm competition (reviewed in Stockley 1997). Investigation of potential benefits of sperm displacement for females is therefore important both for explaining the function of female multiple mating behaviour and for advancing our understanding of the evolutionary dynamics of male–female interactions in sperm competition.

The process of sperm displacement is particularly well documented in the yellow dung fly, *Scatophaga stercoraria* (Parker 1970b; Parker *et al.* 1990; Parker & Simmons 1991; Simmons *et al.* 1998). After a typical copulation of 35 minutes, around 80% of ova are fertilized by the last male to mate (Parker 1970b). Because the proportion of eggs fertilized by the female's last mate remains constant in successive egg batches, it appears that displaced sperm are lost from storage, and those remaining mix randomly within the female's sperm storage organs (Parker 1970b; see also Parker *et al.* 1990; Parker & Simmons 1991). New analyses indicate that female dung flies are actively involved in the process of sperm displacement (Simmons

et al. 1998). Sperm are deposited in the female's bursa copulatrix during copulation, from where they traverse long, narrow spermathecal ducts to reach three (occasionally four) spermathecae, which are the sites of sperm displacement, storage and utilization. Given the distance that sperm must travel to reach the spermathecae, it is unlikely that ejaculate transport and displacement could be facilitated without the active participation of the female. Similar patterns of female involvement in sperm transport have been described for other dipterans (Gilbert 1981; Otronen & Siva-Jothy 1991; Arthur *et al.* 1998), and implicated in *S. stercoraria* (Ward 1993). However, the question of whether females actually benefit from sperm displacement has not yet been addressed.

There are at least two types of benefit that female dung flies might gain from displacement of stored sperm when they arrive to oviposit at the dung. The first are direct benefits, for example, sperm that have been retained in storage during the period of ova maturation may exhibit reduced fertility (Khalifa 1950). Alternatively or additionally, females may gain indirect benefits from sperm displacement in terms of offspring fitness. There exist several possibilities for females to gain indirect (or genetic) benefits via sperm displacement. For example, by choosing to copulate with a male of superior genetic quality to her previous mate, a female may obtain 'good genes' for her offspring (Thornhill 1983). Opportunities for mate choice are limited within the mating system of *S. stercoraria* because females are quickly seized by searching males on arrival at the dung (Parker 1970c). Although females may potentially influence the quality of male by which they are first captured (Borgia 1981), it is unlikely that they could gain genetic benefits from sequential selection of mates. A further possibility

*Author for correspondence.

concerning indirect or genetic benefits of sperm displacement, which has not yet been explored, is that the use of stored sperm for fertilization might lead to a reduction of offspring fitness. This could occur, for example, if there is progressive damage to the DNA of sperm in storage, resulting in heritable deleterious mutations being passed on to the offspring. Sperm lose motility and can even disintegrate when female secretory products fail to reach the sperm storage organs (Davey & Webster 1967; Villavaso 1975), which illustrates the fact that sperm health can deteriorate with time. Moreover, Tsubaki & Yamagishi's (1991) study of melon flies, *Dacus cucurbitae*, implicated mortality of first-male sperm as the cause of increased second-male fertilization success with increased interval between copulations.

A third possibility is that female dung flies do not benefit from displacement of stored sperm, or that they incur costs of remating. Their involvement in sperm displacement may therefore result from male manipulation of female-mediated mechanisms that function primarily in the context of storage and transport of sperm for fertilization.

Here, we aim to investigate whether female dung flies gain short-term benefits from the process of sperm displacement. We look for evidence of differences in fertility between females using previously stored sperm for fertilization and those using sperm received immediately before oviposition. By using the same males to mate twice with experimental females, and thus to displace their own sperm, we investigate for the first time the potential fitness consequences for offspring associated with long-term sperm storage and displacement.

2. METHODS

(a) *Rearing flies*

Dung flies were reared from eggs collected at Woodpark Farm, Wirral, Cheshire, UK. Small batches of eggs and dung were taken from cow pats across the area, and kept on moist filter paper in Petri dishes. The eggs were maintained at 20 °C for approximately 48 h, by which time they had all hatched. Larvae were transferred to 100-ml plastic pots containing 20 g of fresh cow dung. Jars were secured with metal lids pierced with small air holes and were maintained at 20 °C for about three weeks until adult flies began to emerge. On the first day of emergence, each fly was transferred to a separate 100-ml jar containing two small cotton wool balls—one soaked in water and the other in sugar solution. The flies were maintained at constant temperature (20 °C) on a 12:12 h light/dark cycle and fed with 10–15 live *Drosophila melanogaster* twice weekly. Fresh water was provided by regularly remoistening the cotton wool balls. Feeding was maintained for six weeks to allow ample time for full sexual maturation of both sexes (Foster 1967).

(b) *Sperm displacement experiment*

The experiment was done at 20 °C in a constant temperature room where the flies were also housed between treatments. Initially, all females were allowed a single copulation, each with a different male. Males were first transferred to clean 100-ml jars containing a smear of fresh cow dung, and a female introduced to each. Pairs were observed until copulation was completed (males were replaced if the pair had failed to copulate within 30 min). Care was taken to remove females

quickly after copulation to prevent oviposition. All individuals were maintained as described above (fed *ad libitum* with *Drosophila*) for a further three weeks after the first copulation, and half of the females were then selected at random to receive a second copulation with the same male. Second copulations were done as above. After completion of the second round of copulations, each female (from both treatments) was provided with a large smear of fresh cow dung on moist filter paper in which to oviposit. After 24 h, dung smears containing ova were transferred to Petri dishes, moistened, and maintained at 20 °C until all larvae had hatched (*ca.* 48 h). Numbers of hatched and unhatched eggs were counted for each female.

(c) *Rearing and measurement of offspring*

Twenty larvae were selected at random from those produced by each female and placed together in separate, marked, 100-ml plastic pots containing 20 g of fresh cow dung. The dung was first mixed thoroughly and weighed to provide a homogenous mixture of identical quality and weight per capita for each group of larvae. A ratio of 1 g of dung per larvae was chosen to ensure that moderate levels of competition would occur between the developing larvae at 20 °C (Sigurjónsdóttir 1980, 1984). Where females had produced fewer than 20 larvae, smaller numbers were transferred to pots containing an equivalent per capita mixture of dung (e.g. ten larvae transferred into 10 g of dung). The larvae of each female were therefore reared under identical conditions of density and dung quality. Jars were secured with metal lids pierced with small air holes and maintained at 20 °C for around three weeks. Development time for each individual was calculated as the interval between egg laying and emergence. As emergence began, each jar was monitored daily over a period of two weeks, and the number and sex of any adult flies noted. Emerging flies were killed by freezing at –5 °C. Right hind-legs were removed and tibiae measured to the nearest 0.05 mm at ×25 magnification. Hind tibia length is a good measure of overall adult size, which correlates well with other measures of body size (Sigurjónsdóttir 1984).

Fluctuating asymmetry (FA) was measured as an additional indicator of offspring fitness. FA represents small random deviations from perfect bilateral symmetry and is an indicator of developmental instability that can arise from genomic stress (Parsons 1992; Møller & Swaddle 1997). Thus, if sperm ageing resulted in an accumulation of genetic mutations, we might expect genomic stress, and therefore developmental instability, to be greater in the offspring of females that did not have their sperm stores displaced before oviposition. FA has also been shown to influence both predatory and mating success in *S. stercorearia* (Liggett *et al.* 1993; Swaddle 1997). The wings of each individual were removed for asymmetry measurements and adhered to a microscope slide with clear tape. The length of the posterior cross-vein was measured on each of the right and left wings. Wing-vein measurements were made using a phase-contrast microscope image at ×200 magnification. The image was calibrated onto the centre of a video monitor and measurements were taken directly from the screen. Asymmetry measurements were not taken from individuals with wings that had been damaged or folded during the process of mounting. The properties of FA measurements were assessed following the procedures of Swaddle *et al.* (1994). A mixed-model analysis of variance (ANOVA) showed that the variance in wing-vein FA measures was significantly greater than the measurement error ($F_{19,38}=11.40$, $p=0.000$). There was no evidence of directional asymmetry in the wing veins because the frequency distribution

Table 1. Measures of offspring fitness (mean $\pm$ s.e.) for females ovipositing with and without sperm displacement

measure of offspring fitness	treatment	
	no displacement	displacement
% offspring emergence	66.81 $\pm$ 3.21	58.85 $\pm$ 4.20
mean male offspring development time (days)	26.77 $\pm$ 0.36	26.38 $\pm$ 0.33
mean female offspring development time (days)	26.08 $\pm$ 0.40	25.10 $\pm$ 0.40
mean male offspring HTL ³ (mm ³)	30.43 $\pm$ 1.08	32.71 $\pm$ 1.15
mean female offspring HTL ³ (mm ³)	17.27 $\pm$ 0.41	17.35 $\pm$ 0.48
mean offspring wing asymmetry ($\times 10^{-2}$ mm)	1.42 $\pm$ 0.71	1.56 $\pm$ 1.27

of right minus left values did not differ from a normal distribution with a mean of zero (one-sample *t*-test, sample mean = -0.002 , $t_{326} = -1.75$, n.s.; skewness = -0.53 , $Z = 0.16$, $p = 0.88$; kurtosis = 0.98 , $Z = 0.20$, $p = 0.84$). We calculated the absolute value of right minus left for comparisons between treatments.

3. RESULTS

Nineteen females received only one copulation three weeks before oviposition and 20 received a second copulation with the same male, and thus had previously stored sperm displaced immediately before oviposition. Females in the two treatment groups did not differ significantly with respect to body size, nor was there any difference in the size of males with which they copulated (females, $t = -1.24$, d.f. = 36, $p = 0.22$; males $t = -1.61$, d.f. = 36, $p = 0.12$). Mean female hind tibia length cubed (HTL³) $\pm$ s.e. was 19.12 ± 0.63 mm³ ($n = 38$, range 10.65–25.93 mm³), and mean male HTL³ $\pm$ s.e. was 27.64 ± 1.29 mm³ ($n = 38$, range 12.49–39.30 mm³).

(a) Female fertility and fecundity

No differences in fertility or fecundity were detected between females that had stored sperm displaced and those that did not. There were no significant differences in either the total number of ova laid by females in the two treatment groups or in the percentage of ova that hatched. The mean number of ova laid ranged between 12 and 74 with a mean $\pm$ s.e. of 48.16 ± 3.14 for females without sperm displacement and 40.40 ± 13.04 for females with sperm displacement ($t = 1.81$, d.f. = 37, $p = 0.08$). The proportion of ova that hatched ranged between 0 and 100% (five females laid clutches that failed to hatch), with a mean of $70.58 \pm 6.52\%$ for females without sperm displacement and $70.18 \pm 8.64\%$ for females with sperm displacement ($t = 0.37$, d.f. = 37, $p = 0.71$).

(b) Offspring fitness

No differences in measures of offspring fitness were found between females that had stored sperm displaced and those that did not (table 1). There was no significant difference in percentage offspring survival measured as the proportion of larvae that emerged as adults ($t = 1.52$, d.f. = 32, $p = 0.14$). Previous work has shown that there are sexual differences in body size and development time (Simmons & Ward 1991). Thus, to partition the effects of sperm displacement treatment and offspring sex on these

fitness parameters, we performed a single-factor (mating treatment) repeated-measures (offspring sex) ANOVA using the mean values of male and female offspring size and development time for each female. Although sons and daughters differed significantly in body size and development time, we found no significant variance in these fitness parameters that could be attributed to sperm displacement (mean offspring body size: sperm displacement treatment, $F_{1,28} = 1.05$, $p = 0.31$; offspring sex, $F_{1,28} = 400$, $p = 0.000$; mean offspring development time: sperm displacement treatment, $F_{1,30} = 1.56$, $p = 0.22$; offspring sex, $F_{1,30} = 53.85$, $p = 0.000$; table 1). There was no difference in the mean FA measurements of male and female offspring within clutches (Wilcoxon signed-rank, $n = 31$, $p = 0.54$) and so we pooled the sexes for this analysis. There was no significant variance in offspring FA that could be attributed to sperm displacement (Mann–Whitney $U = 144$, $p = 1$; table 1).

(c) Offspring sex ratio

The mean sex ratio of offspring produced by females that did not experience sperm displacement was significantly more male-biased than those of females receiving fresh sperm ($t = 2.17$, d.f. = 32, $p = 0.038$). Females whose stored sperm was replaced produced offspring of approximately equal sex ratios, whereas those using previously stored sperm to fertilize their eggs produced around three times more male than female offspring (figure 1).

4. DISCUSSION

Displacement of stored sperm did not influence female fertility or fecundity. These results are consistent with previous studies of *S. stercoraria*, in which singly mated females produced at least four normal egg batches over a six-to-eight week period with no significant drop in fertility (Parker 1970b). Effects of remating on female fertility and fecundity are variable among insects (reviewed in Ridley 1988), which may be explained to some extent by variation in male sperm-allocation strategies. For example, female mosquitoes, *Culex molestus*, show no reduction in fertility within at least two months of a single copulation (Kitzmilller & Laven 1958). In contrast, female *Drosophila* regularly experience sperm limitation consistent with a male strategy of submaximal insemination to increase the number of females mated (Markow 1985; Pitnick 1991). Sperm leakage from the female storage organs has also been documented in *Drosophila*

Figure 1. Mean ($\pm$ s.e.) ratio of male to female offspring for females ovipositing with and without sperm displacement.

melanogaster, with more than half of stored sperm being lost in the first 48 hours after mating (Gilbert 1981). We found no evidence of similar effects in *S. stercorearia*. Female dung flies are apparently capable of maintaining viable stored sperm for prolonged periods, and receive enough sperm from a single copulation to fertilize at least four clutches (Parker 1970*b*). Although a reduction in female fertility or fecundity after sperm displacement would be a more surprising observation than an increase, such effects have been reported previously in insects (Barrett 1977). That no similar effect was found here for *S. stercorearia* indicates that females do not experience fertility costs in association with sperm displacement. Longer-term costs such as potential reductions in female longevity (Chapman *et al.* 1995) were not examined in our study.

No measures of offspring fitness were related to sperm displacement before fertilization. There were no differences in relative survival rates, development time, size or fluctuating asymmetry of offspring produced by females that had previously stored sperm displaced before oviposition and those that did not. These results indicate that female dung flies experience neither benefits nor costs of sperm displacement in relation to offspring viability and fitness. It is unlikely therefore that heritable deleterious mutations are associated with sperm ageing and/or exposure to oxidative damage, as described for vertebrates (Fraga *et al.* 1991). Other possible genetic benefits associated with remating cannot be ruled out by our experiment, which was designed to investigate benefits of sperm displacement *per se*. It is possible, for example, that stored sperm are typically displaced by males of superior genetic quality to the female's previous mate. We suggest, however, that heritable fitness advantages associated with male phenotype may be infrequent in natural populations of *S. stercorearia*. This is because male success in competition is mainly determined by body size, which is environmentally determined in the field (Sigurjónsdóttir 1984; low levels of heritable variation in body size can be

revealed under controlled laboratory conditions, see Simmons & Ward (1991)). Although Ward (1993) suggested that female dung flies could gain genetic benefits from post-copulatory choice of large males, Simmons *et al.* (1996) found no evidence for selective sperm use after controlling for the effects of sperm competition. The mechanism of sperm displacement in *S. stercorearia* may also preclude possible fitness benefits associated with mixtures of sperm from multiple males within the female reproductive tract (Keller & Reeve 1995; Zeh & Zeh 1996, 1997), as relatively few sperm will remain from a female's previous mates after sequential displacement. Nevertheless, the recent work of Ward (1998) suggests that even with ratios of sperm biased toward the females last mate, females can selectively use sperm of a particular phosphoglucose mutase genotype in an adaptive manner. Thus, cooperating in sperm displacement may prove beneficial for females.

Use of previously stored sperm for fertilization was associated with male-biased offspring sex ratios. Females that used previously stored sperm to fertilize their eggs produced significantly higher ratios of male to female offspring. This is an intriguing result that warrants further detailed investigation. Shifts towards the production of male progeny with depletion or reduced viability of sperm are well documented in the haplo-diploid Hymenoptera, in which males develop from unfertilized haploid eggs and females from fertilized diploid eggs (Godfray 1994). However, such shifts are not easy to explain in organisms where sex is chromosomally determined. Sex determination in insects is not well understood. It has been studied in *Drosophila*, where the primary factor determining the sex of an individual appears to be the ratio of X chromosomes to autosomes; the Y chromosome has no influence on sex determination (Hodgkin 1990; Slee & Bownes 1990). Increasing bias toward the production of male offspring could arise if there were biased mortality of sperm carrying X chromosomes. In at least three families of Diptera, sex determination involves X-chromosome elimination in the somatic nuclei during early cleavage divisions (Crouse 1960; Mori *et al.* 1979; Lauge 1985). In the coccid *Pseudaulacaspis pentagona*, ageing of female gametes predisposes them to chromosome elimination (Brown & Bennett 1957), so that offspring sex ratios become distorted toward an excess of males. Delayed fertilization has been shown to result in a predominance of males in a variety of taxa (reviewed in Werren & Charnov 1978). Whether ageing of male gametes similarly predisposes them to the loss of X chromosomes has not been examined in invertebrates, although the examples outlined above clearly illustrate a mechanism by which gamete age could account for the phenomenon that we have reported here. Moreover, studies of mammals also indicate that sperm ageing can result in a bias towards production of male progeny (Sapp & Martin-DeLeon 1992).

The question remains whether a male-biased offspring sex ratio represents a cost for females (the correction of which would offer a potential benefit of sperm displacement). Male-biased sex ratios may be advantageous under certain conditions. For example, many of the Hymenoptera respond to variation in local mate

competition by changing the sex ratio of their offspring; when conditions signal low mate competition for offspring, females increase the production of males (Godfray 1994). Again, it is easy to see how the genetics of sex determination facilitate such flexibility in haplo-diploid organisms. Nevertheless, evidence is accumulating that the females of species where sex is chromosomally determined can also facultatively adjust the sex ratio of their offspring at the time of fertilization (Svensson & Nilsson 1996; Gunnarsson & Andersson 1996; Komdeur *et al.* 1997). The theoretical models of Werren & Charnov (1978) predict that for species with overlapping generations in which there are differences in longevity between the sexes, females should always produce more offspring of the currently rare sex. It is at least possible that females use copulation as a cue to male availability, and produce more sons when they do not encounter males before oviposition. In our experiment, females that did not experience sperm displacement had obviously not encountered males before oviposition. Comparable conditions may occur in the field if natural sex-ratio fluctuations result in an occasional shortage of males, such that females arriving at the dung are not remated immediately before oviposition. Natural sex-ratio fluctuations may occur in adult populations because male *S. stercoraria* are subject to greater temperature-induced mortality (Ward & Simmons 1990) and could therefore become rare during the warmer periods of the reproductive season (see Parker (1970*d*) for seasonal patterns of male and female abundance). If biased sex ratios were beneficial, females might be selected to favour male-producing sperm over female-producing sperm at the time of fertilization. Such sperm selection would not result in reduced fertilization success for the last male to mate. However, a change to producing male offspring need not be an adaptive female response to male rarity. If the ageing of sperm in the females sperm stores affected the sex ratio, any benefits associated with producing an excess of males would come as an incidental by-product of sperm ageing and a mechanism of sperm selection need not be invoked.

In summary, we find no evidence that female dung flies gain direct benefits from sperm displacement in terms of increased fertility, or indirect benefits from increased offspring fitness. They may gain benefits by avoiding male-biased sex ratios associated with prolonged storage of sperm. We find no evidence for short-term costs associated with sperm displacement, which indicates that there is no obvious conflict with female interests. Further investigation is now required to establish possible long-term costs for females of sperm displacement, such as reduced longevity (Chapman *et al.* 1995), and the fitness consequences of sex-ratio distortion.

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