Copula in yellow dung flies (Scathophaga stercoraria): investigating sperm competition models by histological observation

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Abstract

While sperm competition has been extensively studied, the mechanisms involved are typically not well understood. Nevertheless, awareness of sperm competition mechanisms is currently recognised as being of fundamental importance for an understanding of many behavioural strategies. In the yellow dung fly, a model system for studies of sperm competition, second male sperm precedence appears to result from a combination of sperm displacement and sperm mixing. Displacement was until recently thought to be directly from the female’s sperm stores, the spermathecae (i.e. males were thought to ejaculate directly into these stores), and under male control. However, recent work indicates displacement is indirect (i.e. males do not ejaculate directly into the sperm stores) and that it is female-aided, although the evidence was not based on direct observation. Here, we used histological techniques to directly determine interactions during copula and sperm transfer. Our results are consistent with inference and clearly show that males ejaculate into the bursa copulatrix. Our data are also consistent with active female involvement in sperm displacement, which is indirect, and indicate the aedeagus may remove some spermatozoa from the bursa at the end of copula. In addition, evidence suggests females aid sperm transport to and from the spermathecae, possibly by muscular movement of a spermathecal invagination.

Keywords: Scatophaga; Functional anatomy; Sperm precedence; Mechanism; Sperm removal

1. Introduction

Since Parker’s (1970a) ground-breaking conceptualisation of sperm competition, the yellow dung fly (Scathophaga stercoraria (L.)) has been extensively investigated and has become a model system for sperm competition studies (e.g. Parker, 1970b; Simmons and Parker, 1992; Ward and Simmons, 1991; Ward 1993, 1998; Simmons et al., 1996; reviewed in Hosken, 1999). Many aspects of sperm competition are difficult to examine directly since they take place within the female. Therefore, models have been constructed which assume different mechanisms of sperm competition and generate different paternity outcomes. These are then checked against empirical paternity data, and the mechanism(s) of sperm competition inferred (Birkhead and Parker, 1997). Nevertheless, sperm competition mechanisms are largely unknown in most taxa, although the importance of understanding them is becoming increasingly clear since the adaptiveness of many reproductive processes, for example prolonged copulation, cannot be appreciated otherwise (Eberhard, 1996; Birkhead, 1998; Simmons and Siva-Jothy, 1998).

In yellow dung flies, males which copulate last typically fertilize about 80% of the subsequent clutch (i.e. P2, the proportion of eggs fertilized by the second male to mate in twice mated females, = 0.8) (Parker, 1970b). The mechanism of second (last) male sperm precedence involves sperm displacement, with second males displacing about 80% of previously stored sperm, since 1) the number of sperm stored by females does not appear to increase with successive copulations (Parker et al., 1990), and 2) P2 is strongly dependent on copula duration (Parker, 1970b). Parker and Simmons (1991) modelled sperm competition in yellow dung flies, treating the females’ three sperm storage organs, the spermathecae, as a single unit and they assumed that males...
directly displaced sperm from them (i.e. males ejaculate directly into the sperm stores). In their model, incoming sperm mix instantaneously with previously stored sperm within the spermathecae, and constant, random displacement of mixed sperm directly from these stores is assumed (Parker and Simmons, 1991). The model also assumes displacement is under direct male control and also that stored sperm is displaced directly from the female’s sperm stores at the same rate as incoming ejaculate, with sperm therefore moving both up and down the spermathecal ducts simultaneously.

While the average outcome of sperm competition in dungflies is neatly explained by the direct displacement model, it seems unlikely that it is an accurate description of copulatory events, if only because it treats the three spermathecae as a single unit (Parker and Simmons, 1991; Ward, 1993; Birkhead and Parker, 1997). Moreover, sperm storage in yellow dung flies appears to be influenced by male and female interactions (Otronen et al., 1997; Hellriegel and Bernasconi, 2000), and recent work by Simmons et al. (1999) using radio-labelled ejaculates provides strong evidence that direct displacement does not occur from the spermathecae. Simmons et al.’s (1999) results indicate that sperm movement to and from the sperm stores may be female mediated, as originally suggested by Ward (1993). However, they could only speculate on the mechanism involved and there have been no direct observations of sperm transfer to confirm their findings.

Studies of sperm movement in other taxa (e.g. Waage, 1979; Radwan and Witalinski, 1991; Gack and Peschke, 1994; Civetta, 1999) indicate that proposed mechanisms of sperm precedence can be either ruled out or more accurately inferred by direct observation. This in turn enables us to better understand behavioural strategies employed during sperm competition (Birkhead, 1998). The aim of the present study is to investigate sperm transfer in S. stercoraria using histological techniques in an attempt to better understand genitalic interactions, sperm movement and precedence, and hence the mechanism of sperm competition. At the same time, we test the validity of Simmons et al.’s (1999) recent conclusions.

2. Materials and methods

Flies collected from dung pats in fields near Fehraltorf, Switzerland were brought to the laboratory and allowed to lay eggs. From these clutches, virgin male and female flies were obtained (Ward and Simmons, 1991). Virgin females were fixed in Carnoy’s solution (Bancroft and Stevens, 1990) either without copulating (i.e. remained virgin), or were allowed to copulate (non-virgins). For copulations, one male and one female fly were placed into a small vial containing a small dung portion. Copulations proceeded for a set duration (5, 10, 15, 20, 25, 30 min) or to completion, at which times pairs were either immersed in liquid nitrogen and/or transferred to Carnoy’s fixative, or separated and the females immediately placed with new males and allowed to copulate (again for set durations or to completion) for a second time and treated as above. This protocol simulated take-over situations; when males interrupt copulations of rivals, and after a struggle, copulate with the female themselves (see Parker, 1978). In addition, some females copulated a second time one or two weeks later, and some pairs were not immersed in liquid nitrogen before fixing as this procedure may disrupt some tissue. However, immersion in liquid nitrogen has the advantage of stopping all movement virtually instantaneously, thus allowing genitalic interactions to be observed and sperm location within the female reproductive tract at a particular time to be assessed (see e.g. Eberhard and Pereira, 1997).

After fixing overnight, flies’ abdomens were removed, washed in n–butanol and left overnight in a butanol and paraffin wax mixture. Specimens were then transferred to pure wax (60°C) and, after several wax changes, were wax embedded. Sections were cut at 6–8 μm, stained with haematoxylin (either Harris’s or Mayer’s), counter stained with eosin, and slide mounted (Bancroft and Stevens, 1990). Specimens whose internal anatomy was measured were all oriented approximately the same way during embedding and we assumed this standardised internal organ orientation.

Slides were examined under a Wild M3C microscope fitted with a Donpisha 3CCD camera that relayed images to a PC running Bioscan Optimas software, which was used to make all measurements. The hind tibia lengths of all males and females were measured, and in females not immersed in liquid nitrogen the volume of spermathecae was calculated (internal and external using the formula for a prolate spheroid: \(\frac{2}{3} \pi a \cdot b \cdot W\); Abbott and Hearn, 1978), as was the length of the spermathecal invagination, a finger-like projection into the spermathecal lumen. We used non-frozen females to avoid possible tissue distortion caused by freezing. In addition to these measurements, fly anatomy, and, for pairs frozen in liquid nitrogen, genital interactions and the location of sperm within the female reproductive tract were noted. Genitals were said to be fully engaged when the aedeagus was still seen to closely align with the spermathecal duct openings, i.e. there was no indication of aedeagus withdrawal. In our analysis, the spermathecal duct was divided into three sections: the portion at the junction with the bursa, the middle segment, and the section at the junction with the spermathecae. Sperm were recorded as present or absent from these segments. We used logistic regression, a regression technique for dichotomous independent variables, to analyse these data. Sample sizes vary because of leg loss during freezing, damage to sec-
tions and because in some analyses only frozen or non-frozen specimens were used, as appropriate.

3. Results

The female reproductive tract is characterised by paired accessory glands, three spermathecae (a singlet and a doublet), each with its own narrow duct, and a large muscular bursa copulatrix which is met by the common oviduct antero-ventrally (Ward, 1993). In addition to its substantial musculature, the copulatory bursa is also heavily cuticularised, presumably to afford females some protection from the numerous spikes and spines on the males aedeagus. A full anatomical description is presented in Hosken et al. (1999). Here we concentrate on features relevant to sperm competition models.

During copula the aedeagus is deeply inserted into the bursa copulatrix, with the gonopore facing and almost abutting directly against the spermathecal duct openings, and it was at this point that free sperm entered the female tract (Figs. 1 and 2). Close aedeagus/spermathecal duct alignment is typical of many Diptera (e.g. Lachmann, 1997), and this congruence indicates the genital alignment described here is unlikely to have been altered by tissue-processing, and therefore accurately represents alignment during copula. However, some male abdominal movement sporadically occurs during copula, but there is no musculature that appears able to move the aedeagus alone (Figs. 2 and 5A). Interestingly, logistic regression indicated that genitals were more likely to be engaged after freezing and processing (i.e. as in Fig. 2)
in females whose first copula was shorter ($\beta = -0.1521$; Wald statistic=4.2; $P=0.039$; Fig. 3). This effect was independent of the duration of the second copula, or of male or female size, all of which had no significant effect on the likelihood of genitals remaining engaged (all $P>0.27$). It is noteworthy that the probability function is approximately linear and decreasing until just below a first copula duration of 40 minutes (i.e. a normal mean copulation duration, Parker, 1970b), and then flattens out.

Spermatozoa were always found in the spermathecae within five minutes of the commencement of the first copula of virgin females ($n=6$). Such rapid arrival of spermatozoa has been interpreted as evidence of female involvement in sperm storage in other flies, as calculated swimming speeds and distances travelled indicate arrival time is too rapid to be accounted for by swimming alone (Linley and Simmons, 1981). Spermatozoa were also always present in the spermathecae of females which had copulated for longer (5–30 minutes) or had copulated twice ($n=54$). However, sperm did not always appear to be evenly distributed either among females with copulations of similar durations (Fig. 4), or across a females’ spermathecae when copula was interrupted (see e.g. Fig. 2; Hosken et al., 1999). In addition, spermatozoa were not always present throughout a female’s reproductive tract (18 of 28 females frozen in copula, and 10 of 17 females with genitals still engaged, lacked sperm throughout their tracts. Sample sizes here indicate only specimens for which all sections could be examined). Specifically, segments, or in extreme cases the whole length, of a spermathecal duct was often devoid of sperm in spite of the fact that males were still coupled and ejaculating and sperm were in the spermathecae (Fig. 3). Uneven sperm distribution was not due to tissue processing as sperm were absent from all of some duct portions but not others, even when they appeared on the same slide. It was also not due to observations of different portions of the female tract as all sections/female were examined. Occasionally sperm could even be seen to be leaving the gonopore and the orientation of the sperm (inferred from the staining: sperm heads stain darker than tails) indicated they were moving away from the ducts back down the bursa (Fig. 2B). Additionally, sperm often occluded duct entrances to spermathecae during copula, although this was not always the case (Fig. 4: 30 spermatheca/duct junctions of females frozen in copula ($n=57$ females) were judged as being totally occluded, another 75 contained fewer or no sperm, six were damaged), and there was no obvious pattern to the distribution of sperm. Neither male size, female size,
relative size of males to females, or copula duration or delay was significantly associated with sperm presence or absence from portions of the ducts or the occlusion of the spermathecal entrances (analysed as sperm present throughout or absent in portions of the singlet or doublet ducts within females) (Logistic regression: \(-16.1 < \beta < 13.1\); all Wald statistic values < 0.52; all \(P\)-values > 0.47). There was strong evidence that males ejaculate in a continuous fashion: sperm were seen leaving the gonopore or within the ejaculatory duct of all frozen pairs that maintained genital contact, regardless of the copula duration (copula durations 5–30 mins; \(n=17\)). However, as stated above, in spite of obvious ejaculation and close aedeagus/spermathecal duct alignment, sperm were not always found in the spermathecal ducts. Interestingly, in the few cases where the direction of sperm movement/orientation within the spermathecal ducts could be inferred, there was no indication of bi-directional movement: sperm were all apparently aligned the same way, either towards or away from the spermathecae, even in females during their second copulation (\(n=4\)). Moreover, in these (and other) instances, sperm virtually totally occluded the ducts (28 of 37 females frozen during copula had at least one duct occluded; Fig. 4C), making it highly unlikely that an equal volume of sperm, or any fluid for that matter, could also be moving in an opposite direction within the duct at the same time.

Sperm were typically not found in the bursa copulatrix anteriorly of the spermathecal duct entrance (33 of 37 frozen pairs), but were often seen caudally to the spermathecal duct/bursa junction in the vestibular region of the bursa (\(n=36\) of 37 frozen pairs) (Fig. 2). Additionally, in a number of frozen specimens large quantities of sperm were noted externally within the males genital pouch and around the females rectum (13 of 37). During copula, large quantities of sperm were also typically found around an aedeagus projection caudo-ventrally to the spermathecal ducts and gonopore (27 of 37 frozen pairs: Fig. 2), or within crypts or folds in the cuticular lining of the dorsal surface of the bursa, also caudally of the spermathecal ducts. Interestingly, when the aedeagus is being withdrawn, the method of collapse drags the projection, around which large quantities of sperm was noted (a scoop-shaped section of the aedeagus; Fig. 2), through the copulatory bursa (Figs. 1 and 5). This mode of aedeagus withdrawal apparently removes some sperm from the bursa. However, the location of these spermatozoa, especially those sequestered within cuticular folds, plus the folded nature of the cuticle lining the female reproductive tract are likely to make mechanical removal difficult, and quantities of sperm were seen left behind by the scoop-like projection. In addition, varying amounts of sperm were noted in the bursa of females whose copula had naturally ceased (10 of 11 specimens).

Each spermatheca has a large finger-like invagination opposite the spermathecal duct entrance to the spermatheca (Fig. 4A). This invagination is filled with muscle that attaches to the oviduct (Fig. 4D), and muscle contractions could move it in and out in a piston-like manner, creating negative pressure in the spermatheca aiding sperm movement to storage. Supporting this hypothesis, the variation in the proportional depth to which this invagination projected differed significantly between virgin and non-virgin females (two-tailed variance test ratio; \(\nu_1=\nu_2=11\); \(S^2\) non-virgins=0.005; \(S^2\) virgins=0.001; \(F=6.032\); \(P<0.01\)). That is to say, there was more vari-
Discussion

Our examination of copula in the yellow dung fly clearly shows the site of ejaculation is the bursa copulatrix, at or very near the entrances of the spermathecal ducts. Thus our anatomical data indicate that the direct displacement model is not an accurate description of events during copula in yellow dung flies, as previously suggested (e.g. Parker and Simmons, 1991; Ward, 1993; Birkhead and Parker, 1997). Moreover, not only is there no evidence for direct displacement from the spermathecae, there is also no indication that equal volumes of sperm move, or could move, both ways in the ducts. Independently-derived data also indicate the bursa is the site of ejaculation (Simmons et al., 1999), further supporting the notion of indirect sperm displacement: that is, sperm are not deposited directly into the spermathecal organs as previously assumed. Additionally, a number of Simmons et al.’s (1999) assertions and assumptions are justified by our data. Simmons et al. (1999) suggested there is no aedeagus extension which can reach the sperm stores, we found none, and that the aedeagus and spermathecal ducts align during copula, as described in other flies (e.g. Lachmann, 1997), and as we noted here. This general concordance with the typical fly aedeagus/spermathecal duct alignment also indicates that genital alignment in our flies is unlikely to have been disturbed by processing. Simmons et al. (1999) also suggested that males do not introduce most of their sperm into the bursa at the commencement of copula, which agrees with our data (e.g. spermatozoa were still seen leaving the aedeagus after 30 minutes of copula), and that female sperm stores have a fixed internal volume, as we found. We also found a positive association between female size and total internal store volume, which is arguably expected since organs generally scale with body size (Calder, 1984; and see Parker et al., 1999; Gage, 1998), but also has implications for how female variance in P2 is viewed: for example, can variance in P2 be attributed to cryptic female choice or not (Pitnick and Brown, 2000).

Sperm movement from the bursa to the spermathecae appears to be intermittent and the anatomical data strongly suggest female involvement in sperm movement (cf. Ward, 1993; see also Otronen et al., 1997; Ward, 1998; Simmons et al., 1999; Hellriegel and Bernasconi, 2000). In addition to the bursa being the ejaculation site, several other lines of evidence support these conclusions. Firstly, the distribution of sperm within the female’s spermathecal ducts and spermathecae during copula was uneven. In many instances sperm were found in the spermathecae and could be seen leaving the aedeagus, but there was no sperm in the spermathecal ducts, and at times sperm could be seen to be moving out of the gonopore but away from the ducts in spite of the close aedeagus/duct alignment. This also indicates sperm motility is unlikely to be solely responsible for sperm movement to storage as sperm distribution in the female tract would presumably be more even otherwise, especially because males ejaculate continuously. Furthermore, anaesthesia of female dung flies after copula clearly alters sperm distribution patterns (Ward, 1998; Hellriegel and Bernasconi, 2000). Moreover, Linley and Simmons (1981) provide compelling evidence that sperm motility is unlikely to contribute to spermathecal filling in a range of Diptera, since sperm arrive in storage faster than they could swim (see also Clement and Potter, 1967), and Arthur et al. (1998) clearly show that without full female nervous involvement, sperm storage in Drosophila melanogaster is severely hampered. We also found sperm within the spermathecae soon after copula begins. The rapid arrival of sperm to storage sites is in agreement with the findings of Parker et al. (1990) who reported relatively high female fertility with copulations as short as five minutes. Finally, the nature of the duct/aedeagus alignment makes it difficult to imagine how males could force fluid both up and down the ducts simultaneously.

Other researchers have also suggested that female flies must aid sperm movement, and that bi-directional sperm movement is unlikely within narrow spermathecal ducts (e.g. Linley and Simmons, 1981; Ward, 1993; Birkhead and Parker, 1997; Simmons et al., 1999). Linley and Simmons (1981) suggested fluid is actively pumped out of dipteran spermathecae creating negative pressure (due in part to the thick-walled, fixed-volume sperm stores) which draws up sperm (and fluid) from the copulatory bursa, and Simmons et al. (1999) speculated that duct
contractions aid sperm movement. This second mechanism has also been proposed for a mosquito based on spermathecal duct morphology (Clement and Potter, 1967), and yellow dung flies have ducts containing essentially the same structures (Hosken et al., 1999). However, while we have no direct evidence for either of these mechanisms, one female contrivance likely to aid sperm movement in dung flies was found. The spermathecal invagination could potentially act like a hydraulic piston assisting sperm transit. This invagination is filled with muscle that attaches to the common oviduct. By moving the invagination females could create negative or positive pressure within the spermathecae moving fluid (and sperm) in or out. Consistent with the idea that females pump sperm up and down the ducts is the finding that variation in the proportional depth to which the invagination projected into the spermathecae was significantly greater in non-virgins than virgins. This mechanism seems especially plausible because of the chitinized nature of the spermathecae and their fixed volumes. Moreover, deformations of this nature are thought to aid sperm movement in a number of other insects (Happ and Happ, 1975; also see Eady, 1994), and spermathecal muscles have similarly been implicated in sperm storage in a number of taxa although the mechanics of the systems differ markedly. For example, Rodriguez (1994) showed that cutting the spermathecal muscle in a beetle altered sperm distribution and reduced fertility (and see e.g. King, 1962; Wilkes, 1965). We speculate that stretching of the female tract provides the stimulus for pumping since both copula and egg-laying require movement of sperm, and both stretch the female tract (Hosken et al., 1999), but note that other mechanisms may also be involved in sperm movement.

Interestingly, we occasionally noted what appeared to be uneven distributions of sperm across individuals’ spermathecae and within the ducts when copulations were interrupted (Ward, 1993; see also Hosken et al., 1999; Hellriegel and Bernasconi, 2000). This may have been due to filling order effects, the result of asynchronous female pumping to fill spermathecae, direct female manipulation of ejaculates, aedeagus/duct alignment or any combination of these factors. The assortative storage of sperm noted by Otronen et al. (1997) may also be explained by these mechanisms as copulas were also interrupted in their experiments. Importantly however, females can selectively utilize sperm to a degree based on relative PGM genotype (Ward, 1998), suggesting an even more active role in sperm storage/use than the anatomical data.

Our results also provide evidence that the strength of genital coupling is influenced by a female’s previous mating experience, with genitals staying fully engaged in females with shorter previous copulations. It thus appears that uncoupling occurs more quickly after longer first copulations, with the shape of the logistic curve indicating that after exceptionally long first copulations, full genital engagement is highly unlikely during second copulas. Why this occurs is unclear, but will be the subject of future investigation.

The discovery of a scoop-shaped section of the aedeagus which appears to remove some sperm from the bursa is of particular interest. Since females typically lay immediately after copula and fertilization occurs in the bursa, such sperm removal may be an additional means of sperm precedence, and analogous devices have been described in other insects (e.g. Waage, 1979; and see Simmons and Siva-Jothy, 1998). However, since fertilization appears to occur more anteriorly than the locations of the displaced sperm the evolutionary significance of direct removal remains unclear. Removal may be non-selective, simply being a by-product of the method of aedeagus collapse (e.g. Siva-Jothy et al., 1996), and sperm was found in the bursa of singly-mated females. Nevertheless, partial sperm removal together with the often cryptic location of sperm within the bursa may explain why Otronen et al. (1997) detected few sperm there. Similarly, partial sperm removal may partly explain why Simmons et al. (1999) found proportionally less second male ejaculate in the bursa than predicted. Occasional loss of ejaculate from the female tract, as noted by us, would also help to explain this discrepancy.

While a number of studies now unequivocally indicate that sperm displacement, and other reproductive processes, in flies involve male and female interactions (e.g. Arthur et al., 1998; Clark et al., 1999; Hosken and Ward, 1999; Otronen and Siva-Jothy, 1991; Rice, 1996; Ward, 1998), male yellow dung flies obviously influence the outcome of sperm competition: copulation duration greatly influences P2, and when allowed to copulate freely, males on average gain 80% of paternity in the subsequent clutch. However, the variability in P2 (e.g. Parker, 1970b; and see Simmons and Siva-Jothy, 1998) also requires explanation (Lewis and Austad, 1990), and active female involvement in sperm storage is one mechanism which could conceivably generate this variation (Hellriegel and Ward, 1998). Simmons et al. (1999) ask why females should assist sperm displacement? It may be that males cause female responses via their seminal fluids (as in Drosophila melanogaster and many other flies; Kubli, 1996; Chapman et al. 1995, 1998; Wolfner, 1997), and male dung flies have a large glandular secretory surface in their ejaculatory duct (Hosken et al., 1999). Additionally, male genital movement may stimulate egg-laying receptors causing females to begin sperm mobilization (for discussion of such mechanisms see Eberhard, 1996). Alternatively and/or additionally, it may pay females to increase the diversity of sperm in storage since doing so may allow them to adaptively manipulate offspring genotype or avoid genetically incompatible males (e.g. Ward, 1998; Tregenza and
Wedell, 1998; see also Hosken and Blanckenhorn, 1999).

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