Sperm allocation and cost of mating in a tropical tephritid fruit fly

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Abstract

Males that copulate repeatedly may suffer from reduced sperm stores. However, few studies have addressed sperm depletion from both the female and male perspective. Here, we show that male Anastrepha obliqua (Diptera: Tephritidae) do not ejaculate all available sperm and are left with mature sperm in the seminal vesicles even after copulating as often as three times in half a day. Ejaculate size was not related to male mating history; time elapsed since the last mating, copulation duration, female thorax length or head width. Larval host origin did not affect the number of sperm stored by females. More sperm was found in the ventral receptacle compared to sperm stored in the three spermathecae. Males apparently do not suffer a cost of mating in terms of longevity, although we cannot rule out other fitness costs. Sperm production in this species may not be as costly as it is for other species. Results suggest that males strategically allocate similar numbers of sperm among successive mates without exhausting sperm reserves for future encounters. We discuss the role that differential sperm storage may have in mediating sperm competition and tie our results to the unique natural history of A. obliqua.

Keywords: Anastrepha obliqua; Tephritidae; Sperm depletion; Sperm storage; Ventral receptacle; Longevity

1. Introduction

Given that costs of sperm production are often not trivial (Dewsbury, 1982; Olsson et al., 1997), males should maximize the amount of sperm allocated to females while reserving enough sperm for future matings (Wedell et al., 2002). However, evidence suggests that males copulating with different females in rapid succession often suffer from sperm depletion (Preston et al., 2001; Wedell and Ritchie, 2004; Torres-Villa and Jennions, 2005). For example, in the Australian blowfly (Lucilia cuprina) (Wiedemann), and the red flour beetle (Tribolium castanum) (Herbst), sperm numbers stored by females decrease with successive matings (Smith et al., 1990; Bloch-Qazi et al., 1996). Thus, male mating history can influence the number of sperm transferred and investment in the first ejaculate can influence resources available for subsequent ejaculates (Engqvist and Sauer, 2003). Males may be selected to ejaculate more during the first mating due to the risk of failing to find subsequent mates (Reinhold et al., 2002) or may be instead selected to economize sperm by allocating a limited supply of sperm to many females. For example, studies on the medfly Ceratitis capitata (Wiedemann) suggest that female tephritids can practice sperm economy and can minimize wastage of sperm (Tug and Yuval, 2005). In addition, Pitnick and Markow (1994) showed that males of Drosophila nanopoltera Wheeler, Drosophila pachea Patterson and Wheeler, and Drosophila wassermanni Pitnick and Heed partition sperm among mates economizing among their frequent copulations (Pitnick and Markow, 1994). However, few studies have demonstrated ejaculate adjustments by individual males across successive matings. Therefore, this study examines a number of factors that might lead to such a pattern, including male mating history, copulation duration, time between matings, larval host origin and female size. These factors might be important because energy and time spent mating as well as nutritional resources accrued as larvae may lead to physiological constraints for sperm production (e.g., Gage and Cook, 1994). Also, males may allocate sperm differentially depending on female size if it is associated with fecundity. As there may be trade-offs between reproduction and survival (e.g., Cordero, 2000; Hunt...
et al., 2004), we also explore if male mating history is associated with male longevity.

Females may influence sperm storage (Bishop, 1996; Oronen et al., 1997) particularly in species with multiple storage organs (Siva-Jothy, 1987; Pitnick et al., 1999; Lewis and Jutkiewicz, 1998; Ward, 1998; Hellriegel and Bernasconi, 2000; Bernasconi et al., 2002; Fritz, 2004). In Anastrepha suspensa (Loew) and Ceratitis capitata, both tephritid flies, females have been shown to store sperm asymmetrically between spermathecae or between the spermathecae and the ventral receptacle (Taylor and Yuval, 1999; Fritz 2004; Twig and Yuval, 2005). Differential storage of sperm may lead to post-copulatory selection and possible cryptic female choice of these sperm (Eberhard, 1996).

Some studies have found a cost of mating or courtship on male longevity (Cordts and Partidge, 1996; Gems and Riddle, 1996; Kotiaho and Simmons, 2003). The trade-off between male investment per mating and the allocation of resources to survival may be especially critical when larval resources are not adequate (Cordero, 2000; Awmack and Leather, 2002). For example, in the polygynous butterfly, Callophrys xami (Reakirt), resource-limited virgin males live longer than resource limited non-virgin males (Cordero, 2000) and in the Indian meal moth, Plodia interpunctella Hübnner, sperm numbers decrease when larval diet is not adequate (Gage and Cook, 1994).

Here, we investigated whether males of the tephritid fly Anastrepha obliqua (Macquart) allocate sperm differentially among successive females, and whether sperm is stored equally in all storage organs. We considered whether male mating history, copulation duration, the intervals between matings, larval host origin and female size, influenced sperm quantities in the female storage organs (spermathecae and ventral receptacle). We predicted that less sperm would be found in females as male mating history increased and as the intervals between matings became longer. Since spermatogenesis in Anastrepha serpentina (Wiedemann) begins before adults emerge (Martínez et al. 1995) we predicted that individuals that had developed in hosts with lower resources would have developed as larvae, even if previous generations were from a different host (i.e., originally plum, then reared in mango). Pupae from different hosts were maintained separately. When adults emerged, they were separated by sex and host origin and placed in Plexiglas cages (30 × 30 × 30 cm). Males were offered water ad libitum. Food consisted of a mixture of sucrose and hydrolyzed yeast (3:1) (J. T. Baker, Mallinckrodt Baker S.A. de C.V., Xalostoc, Edo. de Mex., Mexico) (Jacome et al., 1995). To identify individuals, flies were marked with a spot of paint (Politec, Distribuidora Rodin S. A. de C.V., Tlahuac, Mexico D.F.) on the pronotum (Aluja et al., 2001).

Adults were observed when sexually mature (15–20 days of age). One day before observations began, 10 females were randomly placed in Plexiglas cages (30 × 30 × 30 cm). Individuals from different hosts were observed in separate cages. The following day, five males were placed inside the cage with the females. Flies had access to water (soaked cotton balls) but no food. Observations took place from 9:00 am until at least one male from each cage had copulated three times. All copulations and their duration were recorded. Mated females were captured immediately after copulating, placed in plastic containers (13 × 13 × 25 cm), and replaced with virgin females of the suitable exotic host (Mangifera indica L.) where larvae accrue fewer resources (Toledo and Lara, 1996; Diaz-Fleischer and Aluja, 2003).

A. obliqua males court and attract females by emitting a pheromone and wing fanning (calling) from the underside of leaves of non-host and host trees. They have a bimodal pattern of calling activity, doing so mostly in leks in the morning and afternoon (Aluja and Birke, 1993; Aluja et al., 2000). Males can copulate up to three times per day in field cages with an over-supply of virgin females (Aluja et al., 2001). Copulations last on average (+ SE) 47.1 ± 0.9 min (Aluja et al., 2001), and approximately 20% of females remate at least once (Trujillo Rodriguez, 1998). Females have four storage organs: two spermathecae on one side and a singleton on the opposite side of the reproductive tract (Martinez and Hernandez-Ortiz, 1997), and a ventral receptacle. Fritz and Turner (2002) have shown that in the closely related A. suspensa, the ventral receptacle stores sperm and has osmoregulatory functions that could influence sperm storage. In Ceratitis capitata, the ventral receptacle serves as a fertilization chamber, storing small quantities of sperm and also receiving sperm from the spermathecae (Twig and Yuval, 2005).

2. Methods

A. obliqua pupae were collected from infested mangoes (M. indica) (cultivar Criollo) and plums (S. mombin and S. purpurea) from the surroundings of Xalapa, Veracruz, Mexico or from the first or second-generation descendants of these flies raised on mangoes (cultivar Oro or Tommy) in the laboratory. In all cases, larval host origin was classified according to the host from which individuals had developed as larvae, even if previous generations were from a different host (i.e., originally plum, then reared in mango). Pupae from different hosts were maintained separately. When adults emerged, they were separated by sex and host origin and placed in Plexiglas cages (30 × 30 × 30 cm). Males were offered food and water ad libitum. Food consisted of a mixture of sucrose and hydrolyzed yeast (3:1) (J. T. Baker, Mallinckrodt Baker S.A. de C.V., Xalostoc, Edo. de Mex., Mexico) (Jacome et al., 1995). To identify individuals, flies were marked with a spot of paint (Politec, Distribuidora Rodin S. A. de C.V., Tlahuac, Mexico D.F.) on the pronotum (Aluja et al., 2001).
same age. Observations were repeated until we obtained 33 females from each mating regime (first, second or third to mate with a male) \((N = 99)\). A sub-sample of males that had mated with one, two or three females were also set aside for immediate dissection \((N = 70)\). Forty-eight virgin males that were not exposed to females were also dissected.

To determine male longevity according to mating order and larval host origin, we set aside males from the same observation cages that were not dissected. Mating status (virgin, mated once, twice or three times) and host origin \((Spondias\ or\ Mangifera)\) were recorded. Males from each observation cage were held together in plastic containers \((13 \times 13 \times 25\, \text{cm})\) and fed sucrose plus hydrolyzed yeast \((3:1)\) until they died. Densities varied from one to five males in each cage. Date of death was noted.

The day following mating observations, the three females that had mated with the most successful male from each cage were chosen and were anaesthetized with ethyl acetate. Female reproductive tracts were removed and placed in a cavity slide with a drop of modified tris buffer solution following Taylor et al. (2000). The ventral receptacle and spermathecae were dissected and placed separately in slides \((26 \times 76\, \text{mm})\) with 12 \(\mu\text{l}\) of modified tris buffer solution. Each of the four structures (ventral receptacle and three spermathecae) was broken apart with entomological pins glued to thin wooden sticks. The drop was stirred quickly for one minute. A 18 \(\times\) 18 mm coverslip was then placed on top of each of the storage organs and secured on each corner with a drop of transparent nail polish (Taylor et al., 2000).

Immediately after copulation, virgin males or males allowed to mate up to three times were anaesthetized with ethyl acetate and their reproductive tracts dissected. The seminal vesicles of each male were placed on a slide with 12 \(\mu\text{l}\) of modified tris buffer solution and broken apart. The drop was stirred vigorously for 1 min and then covered with a 18 \(\times\) 18 mm coverslip. Slides were kept in a warm and dry room until spermatozoids could be counted. A number was assigned to each slide without any relevant information from the subject so that all counts were done blind.

Spermatozoids were counted at 200 \(\times\) with a phase contrast microscope (Nikon Eclipse 50i). The whole slide was covered by counting all sperm in 36 fields (6 rows by 6 columns), corresponding to a total of 10.22\% of the whole area. To obtain the total amount of sperm for each storage organ, the total area of the cover slip \((324\, \text{mm}^2)\), was divided by 33.12 mm², which corresponds to the area of the 36 fields which were covered, thus obtaining a conversion factor of 9.78 which was used to multiply the sperm counts for each storage organ. Dissected flies were stored in 70\% alcohol. To measure flies, they were placed under a stereomicroscope connected to an image analyzer (Olympus SZX12). Head width and thorax length were used as size indices (Sivinski, 1993; Norry et al., 1999; Rodriguero et al., 2002) and were measured using Image-Pro Plus® image analysis version 4.5.1.22 (Media Cybernetics Inc., Silver Spring, MD., USA).

2.1. Statistical analyses

Number of sperm stored by females was analyzed using a linear mixed model (Crawley, 2002; Pinheiro and Bates, 2000) in S-Plus 2000 (version 4, MathSoft Inc., Cambridge, Mass., USA). Repeated measures were controlled by fitting male identity as a random effect. As three measurements were taken from each female (sperm from the doublet and single spermathecae and the ventral receptacle), females were nested within males and fitted as random effects as well. Sperm from the spermathecae could be from the single spermathecae or from the double spermathecae. As we could not distinguish between the two double spermathecae they were given the same code. Fixed effects included the three structures (ventral receptacle, single spermathecae and doublet spermathecae), three mating orders (first, second and third female to mate with a male), two hosts \((Spondias\ or\ Mangifera)\), copulation duration, interval length between matings for males, female head width and thorax length. Sperm counts were transformed to logarithm (base 10) plus one to normalize the data. All possible interactions were tested. Model selection was performed by likelihood ratio tests and by comparing akaike information criterion (AIC) values (Crawley, 2002; Pinheiro and Bates, 2000).

To examine patterns in ejaculate allocation and quantify how consistent males were among ejaculates transferred in each mating, we used repeatability statistics derived from a one-way ANOVA (Lessells and Boag, 1987; Pitnick and Markow, 1994).

Sperm stored in the seminal vesicles of males was analyzed using a backward stepwise linear regression using mating history, larval host origin, male head width and thorax length as independent variables (Crawley, 2002). To further explore differences in sperm reserves between virgin and non-virgin males, mated males were pooled and compared to virgin males using a Mann–Whitney \(U\)-test as data did not meet assumptions of parametric models.

Male longevity was analyzed using a linear mixed model. Because different densities of males may have an effect on longevity (Sivinski, 1993), the cage in which males were housed, was used as a random effect, thus taking into account varying densities of 1–5 males. Fixed effects included mating status and larval host origin. Numbers in text represent medians, ranges, and quartiles \((Q_{25} = 25\%\,\text{ percentile}, \, Q_{75} = 75\%\,\text{ percentile})\).

3. Results

Females stored a median of 2122 sperm in total \((\text{range} \ 0–11,483, \ Q_{25} = 616.14, \ Q_{75} = 3960.90, \ N = 99)\). Mating order \((P = 0.154)\), copulation duration \((P = 0.379)\), intervals between matings \((P = 0.924)\), larval host origin \((P = 0.334)\), female head width \((P = 0.723)\), female thorax
length ($P = 0.901$), and their interactions were excluded from the global model explaining sperm numbers found in females via AIC model selection. The minimum adequate model included mating order and female sperm storage structures. Sperm storage structures were significant in predicting the number of sperm that females stored (Table 1). Contrasts between structures revealed significant differences in the number of sperm stored between the ventral receptacle and the single spermathecae ($t = 10.28$, $P < 0.001$), and between the ventral receptacle and the doublet spermathecae ($t = 11.94$, $P < 0.001$), but no differences between the single spermathecae and the doublet spermathecae ($t = -0.07$, $P = 0.94$) (Fig. 1). Of total sperm stored, 57.20% of sperm was stored in the ventral receptacle for the first females to mate with a male, 50.06% for the second female and 48.05% for the third female. Repeatability statistics revealed that males were consistent in the amount of sperm transferred in each mating ($F_{32;66} = 2.02$, $r = 0.255$, $P = 0.008$).

The backward stepwise linear regression revealed that mating history, larval host origin, male thorax length, head width or their interactions were not important predictors of sperm stored by males in the seminal vesicles (minimal model, $\beta = 0.150$, $R^2 = 0.02$, $F_{1;16} = 2.69$, $P = 0.104$). Significantly more sperm were observed in the seminal vesicles of virgin males compared to mated males (Mann–Whitney U-test, $U = 2049.5$, $P = 0.038$). Virgin males had a median of 20 sperm (range 0–567, $Q_{25} = 7.5$, $Q_{75} = 51.5$, $n = 48$), while non-virgin males had a median of 10 sperm (range 0 to 1252, $Q_{25} = 0$, $Q_{75} = 29$, $n = 70$) left after copulating. Finally, we found that there was no statistically significant difference in the longevity of males according to mating status or larval host (Table 2).

### Table 1
Linear mixed model showing ANOVA values of sperm stored by females

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>df error</th>
<th>$F$-value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>286</td>
<td>301.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Structure</td>
<td>2</td>
<td>286</td>
<td>79.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Order</td>
<td>1</td>
<td>63</td>
<td>2.16</td>
<td>0.146</td>
</tr>
</tbody>
</table>

The variance of random effects was 0.23 for males and 0.50 for females nested in males. The total number of sperm were transformed to logarithms (log10 + 1). All other fixed effects were excluded from the model (host, copulation duration, intervals between matings, female head width and thorax length) as well as non-significant interactions.

### Table 2
Linear mixed model for the relationship between male longevity and mating status and host

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>Square medians</th>
<th>$F$-value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating status</td>
<td>3</td>
<td>1545.5</td>
<td>1.533</td>
<td>0.209</td>
</tr>
<tr>
<td>Host</td>
<td>1</td>
<td>1486.0</td>
<td>0.442</td>
<td>0.507</td>
</tr>
<tr>
<td>Mating status $\times$ Host</td>
<td>3</td>
<td>6889.169</td>
<td>2.051</td>
<td>0.110</td>
</tr>
<tr>
<td>Residuals</td>
<td>129</td>
<td>3359.658</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Random effects are the plastic containers where males were housed.

Fig. 1. Median (25%, 75% quartiles) number of sperm stored by females according to structure and male mating history. 1st, 2nd and 3rd indicate the first, second or third female to mate consecutively with a male. Structures are the ventral receptacle, single spermathecae and doublet spermathecae. Different letters indicate significant differences ($P < 0.05$).
4. Discussion

Multiple mating by females forces males to ejaculate enough sperm to reduce the risk of sperm competition while at the same time economizing sperm for future matings. However, as noted by Wedell et al. (2002), males may be constrained in the amount of sperm that they can produce, and as a consequence, fewer sperm will be transferred to females when mating consecutively. Results of our study reveal that sperm reserves in *A. obliqua* males do not drop significantly with successive matings on the same day. Males did not exhaust their sperm stores when mating, and were consistently able to allocate similar quantities to different females in rapid succession. Finally, male longevity was not affected by the amount of copulations males obtained. Although other fitness costs remain to be evaluated, the latter is an indication that in the case of *A. obliqua*, the cost of mating for males is apparently low.

Results of the repeatability analysis indicate that *A. obliqua* males are consistent in the amount of sperm delivered to consecutive females. Males do not exhaust their reserves because sperm were found in the seminal vesicles immediately after copulating. Even if males copulated up to three times in one day, mature spermatoozoa that can be readily ejaculated, were still present in this seldomly studied sperm storage structure. This indicates that regardless of the intervals between matings, males are not being completely depleted of mature sperm stores and may reserve sperm for further matings. Such is the case in the orb-weaving spider *Tetragnatha versicolor* Waleknaer, were males release only half of the sperm contained within their palps (Danielson-Francois and Bukowski, 2005), whereas parasitoid wasp males *Trichogramma evanesceens* Westwood keep copulating even though they are depleted of sperm stores (Damiens and Bovin, 2005). Here, males seem to be optimizing sperm use, partitioning it strategically between successive females. Male’s allocation strategy may be related to the risk of sperm competition (e.g., Gage, 1991; Simmons, 2001; Mallard and Barnard, 2003). However, in *A. obliqua*, this risk may be somewhat limited. Ejaculates are unlikely to compete within females as they oviposit shortly after mating before they can remate. Females are unreceptive for 7 days after their initial copulation if they have mated with a protein-deprived male, and up to 23 days if females have mated with a protein-fed male, and only 10–20% of females actually remate (Trujillo Rodriguez, 1998), leaving a relatively short window for sperm competition to take place.

No relationship between female body size or copulation duration was found in the number of sperm stored in females. Furthermore, male size did not explain the amount of sperm ejaculated by males. This is somewhat surprising, as it may be beneficial for males to tailor sperm numbers according to female size if larger females are more fecund (Engqvist and Sauer, 2003) or have larger storage organs. However, we do not know whether female size is related to fecundity in *A. obliqua*, as is the case in *A. suspensa*, were female thorax length is correlated with maximum fecundity (Sivinski, 1993). Similar findings have been reported in the golden egg bug (*Phyllomorpha laciniata* Vill) where no apparent relationship exists between male or female size and copulation duration on ejaculate size (Garcia-Gonzalez and Gomendio, 2004). Also, in the Australian redback spider (*Latropectus hasselti* Thorell), the red flour beetle (*Tribolium castaneum* [Herbst]), the medfly (*Ceratitis capitata*) and the Queens-land fruit fly (*Bactrocera tryoni* [Froggatt]) there appears to be no link between copulation duration and sperm transfer (Snow and Andrade, 2004; Bloch-Qazi et al., 1996; Taylor et al., 2000; Harmer et al., 2006). Yet, in the Caribbean fruit fly (*A. suspensa*), although male or female body size was not correlated to the quantity of sperm, copulation duration did have a positive relation with total sperm stored (Fritz, 2004). The lack of a significant relationship between copulation duration and sperm storage in *A. obliqua*, suggests that time spent during copulation may have other functions aside from sperm transfer, such as stimulating the female and transferring accessory gland products.

Our finding that more sperm is stored in the ventral receptacle compared to the spermathecae contrasts with what has been found in *A. suspensa*, *C. capitata* and recently *B. tryoni* (Fritz, 2004; Twig and Yuval, 2005). Specifically, in *C. capitata* females only store 15% of the ejaculate in the ventral receptacle (Twig and Yuval, 2005). Also, in *C. capitata*, sperm are stored asymmetrically between the two spermathecae (Taylor and Yuval, 1999), while in *A. suspensa*, there are more spermatooza stored in the paired spermathecae than in the singleton (Fritz, 2004). In contrast with these tephritids, in the case of *A. obliqua* we found no significant differences in the number of sperm stored between the paired spermathecae and the singleton. Sperm was found in the ventral receptacle 24 h after copulation had occurred, confirming that this is an important sperm storage site. In *C. capitata*, sperm numbers in the ventral receptacle remain constant up to 18 days after the initial copulation, while sperm numbers in the spermathecae do decrease (Twig and Yuval, 2005). For *A. obliqua* the ventral receptacle may be an important storage site aside from being the fertilization chamber. Importantly, a single ganglion innervates this area, suggesting that females may be able to manipulate ejaculates through cryptic female choice (Fritz, 2002, Twig and Yuval, 2005).

Apparently, there was no cost of mating for males at least in terms of longevity, although this does not exclude other potential mating costs to males. Likewise, in *A. suspensa*, male lifespan did not differ when males had access to females or when they remained virgin throughout their lives (Sivinski, 1993). Also, there was no significant difference in longevity according to larval host origin. We note however, that these results should be taken with caution as our experimental design could have clouded a
larval host effect by rearing flies stemming originally from Spondias in M. indica, especially if there are genetic differences between populations from each host. M. indica have toxic resorcinols which Spondias lack (Aguilar-Ortigoza, 2003) suggesting that this is a less benign medium for larval development compared to Spondias. Consistent with this, Diaz-Fleischer and Aluja (2003) reported high mortality of A. ludens (Loew) larvae developing in unripe mangoes. Furthermore, demographic parameters of A. obliqua adults reared in Mangifera are lower compared to adults reared in Spondias (Toledo and Lara, 1996). We also found no effect of larval host origin on sperm numbers; however, this could have been masked by the high protein diet provided to adults.

Ejaculate size will ultimately depend on the mating opportunities that males will have throughout the mating season (Wedell et al., 2002; Bussière et al., 2005). In A. obliqua, males have many opportunities to mate with different females during a single day given their bimodal calling period that provides them with a long time window during which they are able to attract and mate with females. However, as the native host of A. obliqua (fruits of Spondias [Anacardiaceae] trees) matures quickly and synchronously (Diaz-Fleischer and Aluja, 2003), females have access to a very short oviposition window. Therefore, in order to be successful, males need to inseminate many females quickly and efficiently. To be able to achieve this, they not only need to produce large amounts of sperm, but also be capable of efficiently partitioning the numbers of sperm transferred to females in consecutive copulations. That is exactly what we found to be the case here. A very similar phenomenon occurs in the cases of D. nannoptera, D. pachea, and to a lower extent D. wassermanti that exploit highly ephemeral necrotic caeti as oviposition substrates. As reported by Pitnick and Markow (1994), males of these species consistently partition the amount of ejaculate allocated to several females. Thus, it appears that host ephemerality represents an ecological condition that favours males that can consecutively ejaculate large quantities of sperm without becoming sperm depleted.

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