

Reproductive trade-offs from mating with a successful male: the case of the tephritid fly *Anastrepha obliqua*

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Received: 10 January 2007 / Revised: 3 February 2008 / Accepted: 6 February 2008 / Published online: 5 March 2008
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Abstract In lekking species, females may become sperm-limited when mating with sexually successful males, and this may be exacerbated by a poor male diet. Polygynous males may also be limited by the amount of accessory gland products (AGPs) they can transmit to females, which in turn may influence the females' refractory period and longevity. Here, we tested the effect of male mating history, larval and adult diet on copula duration, mating intervals, female fecundity, fertilisation success, life span and likelihood to remate using sexually successful males of the lekking tephritid fly *Anastrepha obliqua*. Flies originated from either a native or exotic host fruit and were protein-fed or deprived. Male diet and larval host influenced copula duration, while the time elapsed between matings was affected by the interaction of mating order and male adult diet. Female fecundity was not influenced by female position in mating order or protein inclusion into the male diet. However, mating order and male larval diet influenced female fertilisation success. Importantly, as males mated

successively they were less able to induce a refractory period on females, as the last females to mate with a male were more likely to remate and had slightly longer life spans than the first females to mate with males. These results might be attributed to a decrease in male AGPs with increasing male mating frequency. We discuss the role of conditional expression of male mating frequency with respect to *A. obliqua*'s life history, the trade-off that females face when mating with a successful male, the effect of larval diet on adult sexual performance and the possibility for sexual conflict to occur due to high male mating rates and fitness costs to females.

Keywords Mating behaviour · Sperm depletion · Refractory period · Larval host · Diet · Tephritidae

Introduction

The expense of sperm production has often been assumed to be negligible in comparison with that of egg production. Nevertheless, spermatogenesis and spermiogenesis are not cost-free (Dewsbury 1982; Wedell et al. 2002). Males may be limited in the amount of sperm they can transfer to females during copulation (Smith et al. 1990; Arnqvist and Danielsson 1999; Marcotte et al. 2005). As males can keep copulating despite being sperm depleted (Damiens and Bovin 2005), male mating history can be an important factor determining female fitness. For example, in the European corn borer, *Ostrinia nubilalis* (Hübner), females mated to males that had recently copulated exhibited reduced fertility (Royer and McNeill 1993), and in the turnip moth, *Agrotis segetum* Schiff, there is a negative correlation between female fertilisation success and the number of previous copulations by the male (Svensson

Communicated by G. Wilkinson

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et al. 1998). Lekking species may be particularly susceptible to sperm depletion, as in some cases males can monopolise up to 80% of available matings (Höglund and Alatalo 1995). Females mating with successful or high-status males that dominate mating opportunities face the possibility of having their reproductive success limited by obtaining insufficient ejaculates (Wedell et al. 2002; Montrose et al. 2004). Indeed, in the lekking sandfly *Lutzomyia longipalpis* (Lutz and Neiva), females are less fertile when mated with sperm-depleted males (Jones 2001).

Males transfer not only sperm but also accessory gland products (AGPs) that may be expensive to produce, and the amount or quality of these substances may decrease with increasing male mating frequency (Svärd and Wiklund 1986; Rogers et al. 2005). Accessory gland products transferred during mating are known to affect a number of behaviours in insects, of which a reduction in female receptivity and an increase in oviposition are the most common (reviewed in Chen 1984; Cordero 1995; Gillott 2003). If males have mated multiply, they may not be able to transmit enough AGPs to reduce females' refractory period. Likewise, the size of the ejaculate can influence the propensity of females to remate. In the Queensland fruit fly *Bactrocera tryoni* (Frogg), female receptivity is mediated by AGPs (Radhakrishnan and Taylor 2007), whereas in the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), short-term female receptivity is inhibited by sperm loads (Nakagawa et al. 1971; Mossinson and Yuval 2003). Receptivity returns typically in females that have laid eggs or have mated to accessory gland-depleted (i.e., multiply mated) males (Gillott 2003). For example, in the coffee leaf-miner *Leucoptera coffeella* (Guérin-Mèneville), females that mated with males that had previously mated three times were more likely to remate probably due to a smaller spermatophore being transferred (Michereff et al. 2004). A male's ejaculate can also influence female longevity. Female *Drosophila melanogaster* L. that mate repeatedly have shorter life spans due to the transfer of toxic seminal fluids from males (Chapman et al. 1995), whereas in the variable field cricket *Gryllus lineaticeps* Stal., female lifespan is augmented by AGPs (Wagner et al. 2001).

Male expenditure in the ejaculate is constrained in part by resource availability (Simmons 2001), which may begin in the early stages of development as larvae and/or as adults. Depletion of the ejaculate may be especially critical when males lack adequate protein sources or when they have developed in a nutritionally limiting host. For example, host quality has been shown to affect sperm numbers in Indian meal moths *Plodia interpunctella* Hübner (Gage and Cook 1994), while constraints in adult diet can decrease testis mass in *Drosophila grimshawi* (Oldenberg) (Droney 1998). Protein inclusion into the diet

has been shown to affect mating competitiveness in tephritids (Blay and Yuval 1997; Aluja et al. 2001a), and it may also affect the quantity of the ejaculate (Taylor and Yuval 1999).

Here, we were interested in studying the reproductive consequences for females mating with highly successful *Anastrepha obliqua* (Macquart) males, a likely candidate to exhibit ejaculate depletion on the basis of its lekking mating system and extended calling activity. Males call (wing fanning), emitting a pheromone that attracts females from 7:00 to 10:00 A.M. and from 4:00 to 6:00 P.M. (Aluja and Birke 1993). This long period of calling increases their opportunity for copulating as does the relatively short copula duration (on average 42.62 ± 18.14 min) compared to other species within the genus *Anastrepha* which sometimes mate up to for several hours (Aluja et al. 2000). Notably, in semi-natural field cage studies, males have copulated up to five times in 1 day (Aluja et al. 2001a). Shortly after mating, female tephritid flies oviposit into unripe host fruit where the larval stages develop. Specifically, *A. obliqua* females are polyphagous, and they oviposit on a variety of hosts, among them several native tropical plum species (*Spondias mombin* L., *Spondias purpurea* L., *Spondias radlkoferi* Donn. Sm.) and the exotic mango (*Mangifera indica* L.) (Norrbom 2004). Adult diet can influence sexual maturity (Aluja et al. 2001b), and a protein-supplemented diet has been shown to increase mating competitiveness (Aluja et al. 2001a), although its effect on sperm transfer is not known. Females that remate (up to 20%), do so on average 7 days after their initial copula when mated to protein-deprived males, while females that copulate with protein-fed males remate on average 23 days after copulating (Trujillo 1998). While the function of AGPs is unknown in *A. obliqua*, they could potentially inhibit female remating as they do in the Queensland fruit fly (Radhakrishnan and Taylor 2007). In *C. capitata*, accessory gland injections increase oviposition and affect female olfactory behaviour (Jang 1995).

In this study, we perform two sets of experiments to investigate the effect of male mating history of sexually successful *A. obliqua* from two larval host types (exotic or native) and two adult diets (protein-fed or protein-deprived) on female fecundity and fertilisation success. We also explore the effects that larval host and male adult diet could have on mating probability, copula duration and mating intervals. We chose the most successful males from each of the four categories so as to obtain females that had copulated with the same male over a short period of time and which could potentially be limited in the amount of ejaculate they received. Based on the above, we predicted that females would progressively suffer a loss in fertilisation success as a result of copulating with males that had already mated on multiple occasions the same day. In the

second experiment, we examined the effect of male mating history on female longevity and remating behaviour. We predicted that females would be more likely to remate and would remate faster as male mating history increased. Likewise, we predicted that longevity would be influenced by mating order and by whether or not the female had remated.

Materials and methods

Effect of male mating history, adult and larval diet on female fecundity and fertilisation success

A. obliqua pupae stemmed from wild tropical plums *S. mombin* (native host) and mangoes *M. indica* (cv ‘Criollo’) (exotic host) collected in the Soconusco Region, Chiapas, Mexico. Pupae were individually weighed on an analytical balance (Ohaus Model AP2105, Pine Brook, NJ, USA). To reduce variability due to size differences, only pupae weighing 16 to 21 mg were used (pupae weighed between 11 and 26 mg, considering both tropical plums and mangoes). Pupae from different hosts were kept 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 either of sucrose and yeast hydrolysate enzymatic as a source of protein (ICN Biochemicals, Aurora) in a (3:1) mixture (protein-fed males) or sucrose (protein-deprived males) soaked into paper napkin strips. Females were all fed a (3:1) mixture of sucrose (J. T. Baker, Mallinckrodt Baker S.A. de C.V., Xalostoc, Edo. de Mexico, Mexico) and protein. To identify individuals, flies were randomly marked with different colours of paint (Politec, Distribuidora Rodin S. A. de C.V., Tlahuac, Mexico D.F.) on the pronotum (Aluja et al. 2001a).

Adults were observed on average at 17 days of age (± 2.1 days for the native host; ± 3.5 for the exotic host). One day before observations began, ten females from each host were placed in separate Plexiglas cages (30×30×30 cm). The following day, five protein-fed or five protein-deprived males from each host were placed inside the cages with the females from the same host as the male. Cotton balls soaked with water were provided, but no food was available. For individuals reared on the native host, a total of ten cages with protein-fed males and four cages with protein-deprived males were observed. For the exotic host, six cages with protein-fed males and five cages with protein-deprived males were observed.

Observations took place from 6:30 A.M. to 6:30 P.M., or until the last copulation ended. All copulations and their duration were recorded. Mated females were captured immediately after copula termination, placed in plastic

containers (13×13×25 cm) and replaced with virgin females of the same age and that shared the exact same handling history as the ones being replaced. At the end of the day, the females that had mated with the most successful male were selected and placed in individual containers labelled according to the mating order. All cages contained an agar sphere (Bacteriological Agar, BD Bioxon™, Becton Dickinson de México, Cuatitlán Izcalli, Edo. de México, México) wrapped in Parafilm “M” (American National Can™ Chicago, IL, USA) that was hung with a paper clip from the roof of the container so as to provide an ovipositing substrate for the females (Díaz-Fleischer and Aluja 2003a). If two males in one cage were equally successful, the male that had completed the series of copulations the fastest was chosen.

Oviposition substrates were changed daily around noon until all females died. All eggs were counted, and 30 of them were placed daily over a black, moistened cloth placed inside a petri dish (“hatching chamber”; Jácome et al. 1995). Six days later, eggs were checked for hatching under a stereomicroscope. We note that in the case of 55 females (out of 111), fecundity and fertilisation success were only registered until females reached a median age of 75 days (61–93 days) due to extensive wing damage that prevented the females from flying to reach the oviposition unit. However, this period covers most of the females’ reproductive life as the peak in oviposition activity is from age 15 to 30 days of age (Díaz-Fleischer and Aluja 2003a).

Effect of male mating history on female remating behaviour and longevity

Infested tropical plums (*S. mombin*) were obtained from the surroundings of Xalapa, Veracruz, Mexico, and mangoes stemmed from the Soconusco Region, Chiapas. All fruit was placed in plastic containers with vermiculite as a medium for pupation. When adults emerged, they were separated by sex and fed sucrose plus protein (details in the previous section). At 14 to 17 days of age, five males were placed with ten females in a Plexiglas cage (30×30×30 cm). Females that mated were replaced with virgin females of the same age. Observations took place from 6:30 A.M. to 6:30 P.M. Seven or 23 days after their initial copulation, each mated female was placed with three virgin 14 to 17-day-old males. All copulations were recorded, females were then set aside, and their lifespan was registered. A total of six replicates were carried out.

Statistical analyses

The number of copulations that males obtained was log transformed and analyzed by analysis of variance according to larval host and adult diet. As no protein-deprived male mated with more than five females, variables such as

copula duration, intermating intervals, fecundity and fertilisation success could only be analyzed using the first five females to mate with males. Copula duration and the intervals between successive copulations were analyzed each with a linear mixed model. Males were considered as a random factor and male diet, larval host and mating order as fixed variables (Pinheiro and Bates 2000). To meet normality assumptions, the intervals between copulas were log transformed. Analyses were performed on R (version 2.3). The effect of mating order, larval diet and male adult diet on female fecundity and fertilisation success were analyzed by multiple regression using case resampling bootstraps ($R=9,999$) as data did not meet normality assumptions (Cook and Weisberg 1999). This analysis was performed on Arc (version 1.06) software.

Probability of remating was assessed using multiple logistic regression with significance tested using likelihood ratio tests. Female longevity according to male mating order was analyzed using a Cox Proportional Hazards Test on survival, with significance tested using likelihood ratio tests. The effect of remating (or failure to do so) on female longevity was also analyzed by a Cox Proportional Hazards Test. Statistical analyses were performed using JMP (version 5.0.1.2).

Results

Effect of male mating history, adult and larval diet on female fecundity and fertilisation success

Protein-fed males copulated significantly more times than protein-deprived males, up to nine and five times in 1 day, respectively ($F=7.592$, $df=1,22$ $P=0.01$; Fig. 1). Host origin or the interaction with adult diet had no effect on

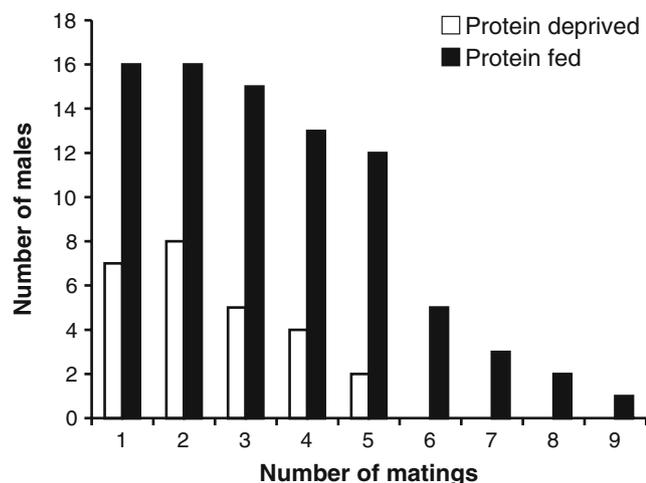


Fig. 1 Number of copulations obtained by protein-fed and protein-deprived males

the number of copulations obtained by males ($F=0.707$, $df=1.21$ $P=0.410$ host origin, $F=0.855$, $df=1.21$ $P=0.366$, $N=24$ host \times diet). The most successful males from the native host copulated a mean (\pm SE) of 3.333 ± 0.667 times in 1 day for protein-deprived males, and 4.700 ± 0.615 times for protein-fed males. Males from the exotic host copulated a mean of 3.400 ± 0.678 times for protein-deprived males, and 6 ± 0.516 times for protein fed males.

Copula duration did not vary with successive copulas, but was significantly influenced by the interaction between larval host and adult diet (Table 1). Copula duration for protein-deprived males reared on the exotic host was the longest, while copula duration for protein-deprived males reared on their native host was the shortest (Fig. 2). There were no significant differences in the length of copulation of protein-fed males reared on both hosts (Fig. 2). There was also a statistically significant interaction between male adult diet and male mating history on the time elapsed between matings (Table 2). Protein-fed males slightly increased the latency to mate as they mated with consecutive females, while protein-deprived males seemed to require shorter latencies to start mating as they achieved increasing numbers of matings. However, this significant effect is probably due to a small sample size, as few protein-deprived males mated with four or five females.

Females stemming from the native host laid an average (\pm SE) of 172.545 ± 25.626 eggs, while females from the exotic host laid on average 217.956 ± 47.149 eggs. However, male mating history, larval host or male diet had no statistically significant effect on female fecundity (Table 3). Male mating history and larval host did have a significant effect on lifetime female fertilisation success, while male adult diet had a non-significant effect (Table 3). First females laid a larger proportion of fertile eggs than subsequent females to mate with the same male, and overall, females reared on their native host exhibited higher fertilisation success than females reared on the exotic host (Fig. 3). Although non-significant, results suggest that females mated to protein-fed males laid more fertile eggs than females mated to protein-deprived males (a total of 4,199 eggs for protein-fed males vs. 1,278 eggs for protein-deprived males).

Effect of male mating history on female remating behaviour and longevity

Males mated up to six consecutive times in a single day, however, only 8.01% of all females remated either at 7 or 23 days after their initial copulation. There was a significant increase in the number of females that remated as male mating history increased ($G=6.505$, $df=1$, $P=0.0108$, $N=213$) and as more time elapsed between the first and subsequent mating (7 or 23 days; $G=5.308$, $df=1$, $P=0.0212$; Fig. 4). Also, there was a significant effect of male

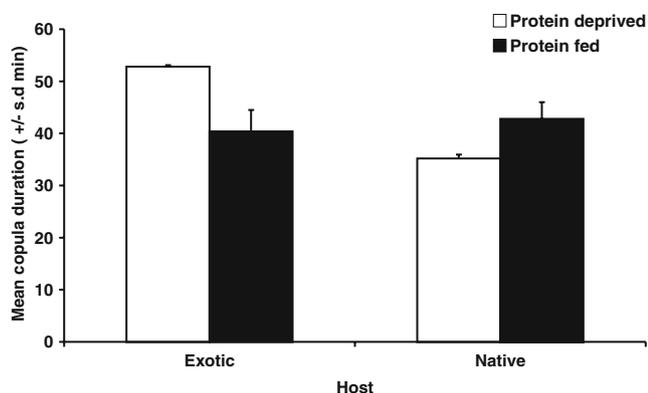
Table 1 Linear mixed model for copula duration according to larval host (native vs. exotic), adult diet (protein-fed vs. protein-deprived) and mating history (mating order=first, second, third, fourth or fifth copulation), $N=23$ males

	NumDF	denDF	F	p
Intercept	1	70	577.70	<0.0001
Larval host	1	19	0.801	0.382
Adult diet	1	19	0.705	0.411
Mating order	1	70	2.350	0.130
Host × diet	1	19	5.859	0.026
Host × order	1	70	0.001	0.979
Diet × order	1	70	0.741	0.392

mating history on female longevity (Cox Proportional Hazards, $G=3.844$, $P=0.050$, $df=1$, $N=213$). That is, female lifespan was slightly longer as male mating history increased (Table 4). However, there was no significant effect of remating on female longevity ($G=0.311$, $df=1$, $P=0.577$).

Discussion

In accordance with our predictions, male mating history influenced female fertilisation success and female likelihood to remate and longevity. Furthermore, male adult diet influenced male mating frequency and mating intervals. Consistent with previous studies on other tephritids (Blay and Yuval 1997; Aluja et al. 2001a; Perez-Staples et al. 2007), we found that inclusion of protein into the adult diet increased male mating frequency. In addition, results related to female fertilisation success suggest that protein-fed males are able to induce higher fertilisation success than protein-deprived males, as females mated to protein-fed males laid more fertile eggs than females mated to protein-deprived males, although this was non-significant ($P=0.059$).

**Fig. 2** Copula duration (back-transformed predicted mean±SD) according to larval host (native or exotic) and male adult diet (protein-fed or protein-deprived)**Table 2** Linear mixed model for the time intervals between matings according to larval host (native vs. exotic), adult diet (protein-fed vs. protein-deprived) and mating history (order=time between first and second, second and third, third and fourth, and fourth and fifth mating), $N=23$ males

	NumDF	denDF	F	p
Intercept	1	50	1,825.52	<0.0001
Larval host	1	18	0.172	0.683
Adult diet	1	18	0.019	0.892
Mating order	1	50	0.000	0.990
Host × diet	1	18	1.394	0.253
Host × order	1	50	0.268	0.607
Diet × order	1	50	5.134	0.028

We also discovered an interesting effect of larval diet (host-plant) on male sexual performance, as the interaction between larval and male adult diet significantly influenced copula duration. Protein-deprived males stemming from the native host *S. mombin* exhibited the shortest copulations while those reared in the exotic host *M. indica* exhibited the longest. Although copula duration does not explain the total amount of sperm stored by females (Perez-Staples and Aluja 2006), it is likely that males will be at an advantage in obtaining further copulations if they can mate quickly and effectively inseminate females. In previous studies with *A. ludens* (Díaz-Fleischer and Aluja 2003b) and with a third trophic level, *Anastrepha* larval-prepupal parasitoids, (Eben et al. 2000), a deleterious effect of the exotic host on larval and adult performance has also been found. The finding here that the longest copulation duration was recorded in males stemming from the exotic host confirms our previous studies indicating that larval host influences adult sexual performance. Further studies are needed to dissect what factors in the host chemistry may influence key fitness components of the adults.

Table 3 Bootstrap regression with 9,999 replications of female fecundity and fertilisation success according to male mating history (mating order, first, second, third, fourth or fifth to mate with a male), male adult diet (protein-fed or protein-deprived) and larval host (native or exotic), $N=98$ females

	Observed estimate	Bootstrap S.E.	Boot p value
Female fecundity			
Intercept	88.476	68.982	0.207
Larval host	44.853	57.248	0.425
Adult diet	66.579	71.907	0.300
Mating order	27.00	20.580	0.191
Female fertilisation success			
Intercept	0.54	0.102	0.000
Larval host	-0.113	0.009	0.046
Adult diet	-0.110	0.008	0.059
Mating order	-0.005	0.003	0.008

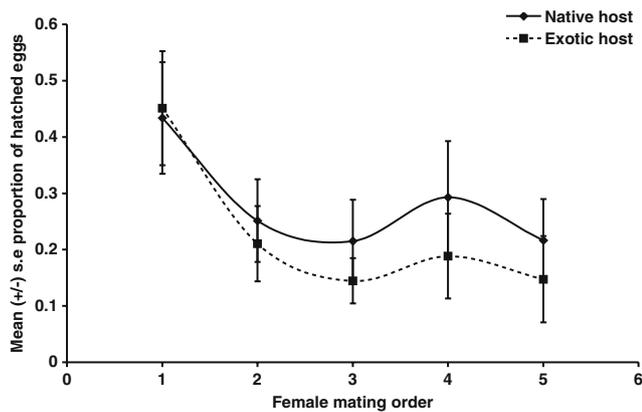


Fig. 3 Mean (\pm SE) proportion of eggs fertilized according to male mating history and larval diet. Female mating order refers to the first, second, third, fourth or fifth female to mate with a male in a single day

We found no effect of male mating history on lifetime fecundity. However, female fertilisation success decreased with mating order. This is consistent with what has been found in other species such as the seed beetle *Callosobruchus maculatus* (F.) and the New Zealand leafroller *Cnephasia jactatana* Walker (Savalli and Fox 1999; Jiménez-Pérez and Wang 2004). Similarly, in the Mediterranean fruit fly, females produce fewer offspring when mated to non-virgin males even when these had up to 5 days to replenish their sperm supplies (Whittier and Kaneshiro 1991). Females could be mating with potentially sperm-depleted males, or alternatively, females may not be able to use sperm stores efficiently to fertilize all eggs. Recently, we found that *A. obliqua* males mating up to three times in a single day do not exhaust their sperm reserves and can allocate similar amounts of sperm among consecutive females (Perez-Staples and Aluja 2006). However, even if females receive a full sperm load, sperm quality in terms of longevity and mobility may vary. Other seminal substances may also vary in quality, such that the last females to mate with a highly successful male in any given day may very well receive a lower quality ejaculate with consequences on her ability to lay fertile eggs.

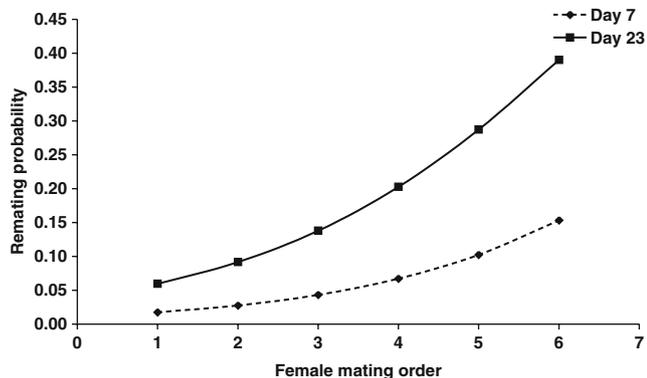


Fig. 4 Probability of females remating according to her mating order (first, second, third, fourth, fifth, or sixth to mate with a male) and time elapsed since the first mating (7 or 23 days)

Table 4 Female lifespan according to male mating history

Mating order	Sample size	Mean longevity (days)	Standard error	Min	Max
1st	71	66.81	3.46	23	141
2nd	7	88.28	13.73	44	131
3rd	71	70.39	3.56	23	151
4th	41	79.21	5.21	24	149
5th	18	79.66	5.40	38	124
6th	5	94.8	12.36	57	133

Thus, when females arrive at a lek site, there may be sexual conflict over mate choice. Successful males may manipulate females into mating with them. That is, high male mating rates are clearly selected, but at a great cost to females who might then mate with temporarily ejaculate-depleted males and thus suffer an overall fitness loss. Other costs to females mating with previously mated males include wasted time or increased risk in finding another mate for remating.

Females mating with previously mated males were also more likely to remate. Likewise, in the blowfly, the seed beetle and the obliquebanded leafroller, multiply mated males are less effective at inducing the refractory period of females (Smith et al. 1990; Savalli and Fox 1999; Marcotte et al. 2006). The potential inhibitory effect of the ejaculate may also decrease with time, as females were more likely to remate at 23 days than 7 days after mating. At both time periods, this was more evident for females who had mated with recently mated males than for females mated with virgin males. Again, sexual conflict over female mating rates may ensue. As receptivity returns for females, the opportunities to receive additional direct (e.g., sperm or nutrients) or indirect (genetic) benefits may increase for females, while males may have increased risk of sperm competition (Arnqvist and Rowe 2005).

Females mating with a previously mated male also benefited from an increased lifespan, although it only explained a small amount of the variation in longevity ($R^2=0.025$). As *A. obliqua* is polyphagous, females that remate (replenishing their sperm supply) and live longer could benefit from ovipositing in additional hosts after the ephemeral native host has stopped fruiting. Females that were the sixth to mate with a male lived a mean of 28 days more compared to females mated to virgin males. In stark contrast, the lifespan of female Mediterranean fruit flies and guava fruit flies increased by 6 and 32 days, respectively, when mated to virgin males compared to females mated with previously mated males (Whittier and Kaneshiro 1991; Pérez-Staples and Aluja 2004). In the case of *Anastrepha striata*, trophallaxis (i.e. transfer of substances that may be nutritive) with males before mating might explain the increase in lifespan when copulating with virgin males, while for *A. obliqua*, this opposite difference in longevity

can probably be attributed to females receiving less seminal substances as male mating history increases. In fact, in *D. melanogaster*, AGPs have been shown to be toxic, negatively affecting female lifespan (Chapman et al. 1995). However, *A. obliqua* females that mated twice and received two loads of ejaculate did not have lower lifespans than singly mated females. Thus, the purported roles and effects of the ejaculate on female lifespan seem to be dissimilar within Diptera.

Male mating frequency may be constrained by the amount and quality of the ejaculate that can be transmitted to consecutive females. An adequate larval environment to develop in and the ability to obtain a high quality adult diet can be key factors determining male mating and fertilisation success. Here, we have shown that females that mated with previously mated males suffer a cost in terms of reduced fertilisation success but are more likely to remate than females mated with less experienced males. Also, female life span increased when mated with previously mated males, and this could be advantageous as female likelihood to remate also increases with time. Thus, females may face a trade-off when mating with highly successful males at lek sites: they may become sperm-limited but may also live longer and be less constrained in remating opportunities.

Acknowledgments We sincerely thank six anonymous referees and two associate editors for many insightful comments and suggestions for improvement. We are also very grateful to Carlos Cordero (Instituto de Ecología, UNAM) for helpful suggestions and discussion throughout the project as well as for comments on the manuscript. Melissa Galicia and Lizbeth González provided critical technical assistance throughout the study. We thank Francisco Díaz-Fleischer, the technicians in Desarrollo de Métodos in the Moscafrut program, Tapachula, Chiapas, Mexico, and Martin Pale (Instituto de Ecología, A.C.) for helping us obtain pupae. We also thank Roberto Munguía-Steyer for statistical advice and Larissa Guillén and Nicoletta Righini (Instituto de Ecología, A.C.) for their general assistance. Financial support was provided by the Mexican Campaña Nacional Contra las Moscas de la Fruta (Secretaría de Agricultura, Ganadería, Desarrollo Rural y Pesca-Instituto Interamericano de Cooperación para la Agricultura) and a competitive grant from the Mexican Consejo Nacional de Ciencia y Tecnología (Project CONACYT-SEP-2004-C01-46846). This is part of the PhD dissertation of DPS, directed by MA and supported by CONACYT through a fellowship to DPS. The experiments performed here comply with the current laws of Mexico.

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