



## ARTICLES

# Postcopulatory sexual selection in Mediterranean fruit flies: advantages for large and protein-fed males

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(Received 27 October 1998; initial acceptance 18 December 1998;  
final acceptance 24 March 1999; MS. number: 6034)

Previous laboratory studies of Mediterranean fruit flies, *Ceratitis capitata* (medflies), have identified large size and protein feeding as positive influences on the ability of males to secure copulations. In this study, we investigated whether large and protein-fed males experience additional advantages in terms of amount and distribution of sperm stored by mates. We also examined relationships between copula duration and sperm storage. Mates of large and protein-fed males were more likely to store sperm and to store more sperm than mates of small and protein-deprived males. Probability of sperm storage was associated with copula duration; all copulations lasting less than 100 min failed whereas 98% lasting longer than 100 min succeeded. Copulations involving sperm storage were longer if males were small or protein deprived or if the female was large, although there was no evidence of a relationship between copula duration and total sperm storage. Evidence from related studies suggests that variation in latency until sperm transfer, caused by size and diet, is a likely explanation for varying copula duration. Sperm tended to be stored asymmetrically between the female's two spermathecae, consistent with a mating system in which females maintain isolated populations of sperm from different males and later select between them. Storage was less asymmetric when large numbers of sperm were stored but there was little evidence that male size or diet affected this asymmetry. It is uncertain whether postcopulatory advantages of large and protein-fed male medflies arise from female preferences or male dominance through coercion or force.

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In most insect mating systems, males vary considerably in their ability to gain access to the limiting resource of ova (Alexander et al. 1997). Rather than arising from differential male ability in a single prerequisite activity, such as precopulatory courtship display, this variation may instead represent the net effects of male ability at overcoming a diverse suite of challenges. Activities preceding fertilization of ova may be divided into distinct phases and, after initial success, males may fail a subsequent challenge. For example, having successfully located or attracted a female, a male may then fail to copulate (Webb et al. 1984; Simmons 1988; Tuckerman et al. 1993). Recent studies have emphasized that courtship, and differential male success, may extend well beyond

the onset of copulation (Eberhard 1994, 1997; Brown 1997; Sakaluk 1997; Ward 1998). Males that find mates and copulate may fail, or have limited success, at later stages of sperm transfer and storage. In this study, we consider qualities of male Mediterranean fruit flies, *Ceratitis capitata* (medflies), that confer success at securing copulations and whether these qualities also confer success after copulation.

Some previous studies have implicated size and diet as important predictors of a male medfly's mating success. First, only males that obtain sufficient protein are able to participate in leks, the primary mating arena of this species (Yuval et al. 1998). Although data for wild flies in nature and simulated nature are conflicting (Arita & Kaneshiro 1988; Whittier et al. 1992), Orozco & Lopez (1993) found that, in simulated nature, large males from a laboratory strain mated more frequently than smaller rivals. Furthermore, some laboratory studies allowing

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males the opportunity to court females without the barrier of lek participation have found that large and protein-fed males copulate more frequently than small and protein-deprived males (Churchill-Stanland et al. 1986; Blay & Yuval 1997). Although variation in male ability to secure copulations has been partly explained by size and diet, there is large, and as yet unexplained, variation in postcopulatory success of male medflies.

First, as in some other insects (Webb et al. 1984; Choe 1995, 1997; Bloch et al. 1996), medfly copulations do not invariably culminate in sperm storage. Studies directly checking presence of sperm in spermathecae have reported failure in 2–5% (Wong et al. 1984) and 25% (Seo et al. 1990) of naturally terminating copulations. Other studies have reported failure to produce offspring after 7% (Whittier & Kaneshiro 1995) and 22% (Saul & McCombs 1993) of copulations, suggesting failure of sperm transfer or storage in these cases.

In addition to sometimes failing to have any sperm stored, male medflies vary dramatically in how many sperm are stored by mates; copulation may result in storage of fewer than 100 or more than 8000 sperm (Seo et al. 1990; Yuval et al. 1996). Total sperm storage may equate to postcopulatory success because females storing large amounts may be less prone to remate (Nakagawa et al. 1971; Eady 1995) or because there is less potential for dilution by rival ejaculates (Simmons 1986; Dickinson 1997). A male's postcopulatory success may also depend on how his sperm are distributed in storage. Some recent studies have suggested that female insects control sperm storage patterns, isolating different males' ejaculates to allocate offspring paternity (LaMunyon & Eisner 1993; Siva-Jothy & Hooper 1996; Otronen 1997a; Ward 1998). Consistent with this possibility for medflies, Yuval et al. (1996) found that one spermatheca typically contains many more sperm than the other. However, this sperm storage asymmetry (potential for ejaculate isolation) spanned the full range from all sperm in one spermatheca to an even distribution.

Male success in each component of postcopulatory sexual selection identified here (probability of sperm storage, total sperm storage, sperm storage pattern) has not been clearly related to specific male qualities. Although copula duration is known to depend on male size and diet (Churchill-Stanland et al. 1986; Field & Yuval 1999), how this translates to postcopulatory success is also poorly understood. We considered whether initial advantages for large and protein-fed males in securing copulations persist in postcopulatory sexual selection. Specifically, we focused on the following hypotheses: (1) male size and protein feeding are positive influences on the probability of sperm storage; (2) male size and protein feeding are positive influences on the amount of sperm stored by females; (3) degree of sperm storage asymmetry (potential for ejaculate isolation) is related to male size and diet; and (4) amount of sperm stored by females varies with copula duration.

## METHODS

### Origin and Maintenance of Flies

As in other medfly studies (Yuval et al. 1996; Field & Yuval 1999), we used flies of strain 'Sade' supplied as pupae by the Citrus Research Board laboratories, Bet Dagan, Israel. This laboratory strain differs from others in being routinely 'refreshed' with wild males collected in the same region as the original source flies (central coastal plains of Israel).

The pupae were kept at 24–26°C, 65–75% relative humidity and 14:10 h light:dark photoperiod in a room with constantly recirculating air. Adult flies from each batch of pupae emerged over 3–4 days, with peak emergence typically being on the second day. Only flies that emerged on the second day were used in experiments. Flies were sexed within 24 h of emergence after which males were maintained on two different diet regimes: 'protein fed' and 'protein deprived'. Protein-fed males had access to a mixture of sucrose and protein hydrolysates, with water supplied separately, whereas protein-deprived males had access to a 20% sucrose solution, also with water provided separately. All females were given the same diet as protein-fed males. Each batch of sexed adult flies was divided into three 5-litre cages: one with 80–100 females; one with 60–80 protein-fed males; and one with 60–80 protein-deprived males. These flies were kept in the same room as prior to emergence and were used in experiments on the fourth day after emerging as adults.

### Experiments

Testing procedures closely resembled those in previous studies (Seo et al. 1990; Blay & Yuval 1997; Field & Yuval 1999). On the day of testing, 2.5 h after the light phase began, we set up two 5-litre testing cages: one with 30 protein-fed males and the other with 30 protein-deprived males. We used an aspirator to transfer the flies. Next, we transferred 30 females to each cage of males and checked the cages for copulating pairs at 5-min intervals for 2 h. We removed copulating pairs from the testing cage by gently coaxing them into glass test-tubes which were then plugged with cotton wool. Because males sometimes spend more than 20 min mounted, but not yet copulating, we ensured that the male had clasped the female's ovipositor (the genital opening) with his surstyli (genital clamps) before scoring a pair as copulating (Eberhard & Pereira 1993). Copulating pairs were then checked at 5-min intervals for termination of copula.

Between 2 and 24 h after the flies mated, we determined the amount and distribution of sperm stored by mated females. After killing the fly by crushing the head and thorax with forceps, we removed the spermathecae directly into a drop of phosphate-buffered saline (PBS). Each spermatheca was then transferred to a 12- $\mu$ l drop of PBS on a haematocytometer stage. The spermatheca was broken apart with entomological pins and the sperm mass gently removed and teased apart. The drop was stirred for 1 min and a cover slip lowered into place.

**Table 1.** Logistic regressions investigating probability of copulating and probability of sperm storage in relation to male size and diet, and female size

Source	df	$\chi^2$	P
Probability of copulating (overall model significance: $\chi^2_{2,294}=16.84$ , $P<0.001$ )			
Male diet	1	10.38	0.001
Male size	1	9.85	0.002
Probability of sperm storage (overall model significance: $\chi^2_{6,178}=30.15$ , $P<0.001$ )			
Male diet	1	16.03	0.001
Male size	1	7.43	0.006
Female size	1	9.41	0.002
Male diet*male size	1	12.28	0.001
Male diet*female size	1	3.91	0.048
Male size*female size	1	8.81	0.003

Using the central and four corner haematocytometer grids, we counted the number of sperm in 5% of the total area at  $\times 400$  magnification under a phase-contrast microscope (Zeiss axioscop). If no sperm were detected in this area, we also checked the spermatheca remnants and scanned the haematocytometer stage before recording absence of sperm. We removed the right wing of each fly and, as an index of size, we measured the distance from alular notch to distal tip to the nearest 0.025 mm, using an ocular micrometer. Experiments were replicated five times, and the flies for each replicate came from a different batch (pupated at least 3 days apart).

### Statistical Analyses

To investigate probability of mating and probability of some sperm being stored, we used logistic regression and tested significance using likelihood ratio chi-square. Initially, all possible interactions between male diet, male size and female size were considered and replicate was included as a blocking effect. Next, highly nonsignificant ( $P>0.1$ ) interactions were removed stepwise from the highest order of interaction until significant effects ( $P<0.1$ ) were detected. When replicate was a highly nonsignificant effect at every stage, it was excluded to increase the power of remaining comparisons.

To explain some of the population variation in copula duration, total sperm storage and sperm storage asymmetry (difference between spermathecae as a percentage of total sperm storage), we used analysis of covariance (ANCOVA). When deriving models in ANCOVA, we used the same stepwise procedure as for logistic regressions. Residuals of each initial model suffered heteroscedasticity which was best resolved by square-root transformation ( $Y'=\sqrt{Y+0.5}$ ).

General population characters for copula duration, total sperm storage and sperm storage asymmetry are presented as backtransformed means with lower and upper 95% confidence limits ( $L_1$  and  $L_2$ , respectively; Sokal & Rohlf 1981). As an indication of effects of continuous variables, correcting for other effects, we present

**Table 2.** Analyses of covariance investigating copula duration, total sperm storage and sperm storage asymmetry

Source	df	F	P
Copula duration (overall model significance: $R^2=0.42$ , $F_{7,159}=15.60$ , $P<0.001$ )			
Replicate	4	18.00	0.001
Male diet	1	5.22	0.024
Male size	1	15.92	0.001
Female size	1	8.63	0.004
Total sperm storage (overall model significance: $R^2=0.30$ , $F_{7,159}=9.22$ , $P<0.001$ )			
Replicate	4	13.06	0.001
Male diet	1	4.52	0.035
Male size	1	10.72	0.001
Female size	1	0.24	0.628
Sperm storage asymmetry (overall model significance: $R^2=0.03$ , $F_{3,159}=1.36$ , $P=0.256$ )			
Male diet	1	3.04	0.083
Male size	1	0.17	0.676
Female size	1	0.14	0.713

standardized partial regression coefficients ( $b'$ ). To identify effects of male diet (a categorical variable), correcting for other effects, we present backtransformed least squares means (LSMs) and 95% confidence limits for the two diet regimes. All analyses were carried out using JMP 3.1 (SAS 1995).

## RESULTS

### Probability of Copulating

A male's probability of copulating within 2 h depended on his size and diet: a male that was large or protein fed was more likely to mate than a male that was small or protein deprived (Table 1, Fig. 1a). The lack of significant interaction between size and diet indicates that a male's vulnerability to the effects of protein deprivation on his ability to secure a copulation within 2 h did not depend on his size.

### Probability of Sperm Storage

Sperm were detected in spermathecae after 168 (94%) of 178 assessed copulations. A male's diet was an important predictor of whether sperm would be stored by mates (Table 1); females were less likely to store sperm of protein-deprived males than of protein-fed males. Effects of diet were limited to small males, however, as large males were comparatively unaffected (Fig. 1b). Female size was also an important effect, as large females were less likely to store sperm than were small females. Effects of male and female size on probability of sperm storage were interrelated: small males were more likely to have sperm stored if their mate was also small (less than the mean size; Fig. 1c).

For sperm storage to take place, a minimum copula duration of ca. 100 min seemed necessary, although longer copulation did not guarantee success; each of seven copulations lasting less than 100 min failed

whereas 168 of 171 copulations lasting more than 100 min succeeded (chi-square test of independence:  $\chi^2_1=122.41$ ,  $N=178$ ,  $P<0.001$ ). The shortest successful copulation lasted 110 min, and the longest unsuccessful copulation lasted 265 min.

### Copula Duration and Sperm Storage

Copulations involving sperm storage lasted an average of 197.5 min ( $N=168$ , range 110–335 min). Successful copulations were longer if the male was protein deprived (protein fed: LSM=194.4 min,  $L_1=187.1$ ,  $L_2=202.0$ ; protein deprived: LSM=208.0 min,  $L_1=199.6$ ,  $L_2=216.7$ ; Table 2), if the male was small ( $b'=-0.28$ ; Table 2, Fig. 2a) or if the female was large ( $b'=0.21$ ; Table 2, Fig. 2b).

Females stored an average of 3191.5 sperm ( $N=168$ , range 140–10 400 sperm). A male's size and diet were important predictors of how many of his sperm were stored by females, more sperm being stored if the male was large ( $b'=0.26$ ; Table 2, Fig. 3) or protein fed (protein fed: LSM=3639.2 sperm,  $L_1=3232.7$ ,  $L_2=4069.8$ ; protein deprived: LSM=3037.7 sperm,  $L_1=2701.8$ ,  $L_2=3393.3$ , Table 2). There was no evidence that total sperm storage was influenced by female size ( $b'=0.04$ ; Table 2). Additionally, there was no evidence of a relationship between copula duration and total amount of sperm stored (replicate:  $F_{1,166}=13.64$ ,  $P<0.001$ ; copula duration:  $F_{1,166}=1.46$ ,  $b'=-0.10$ ,  $P=0.229$ ).

### Sperm Storage Asymmetry

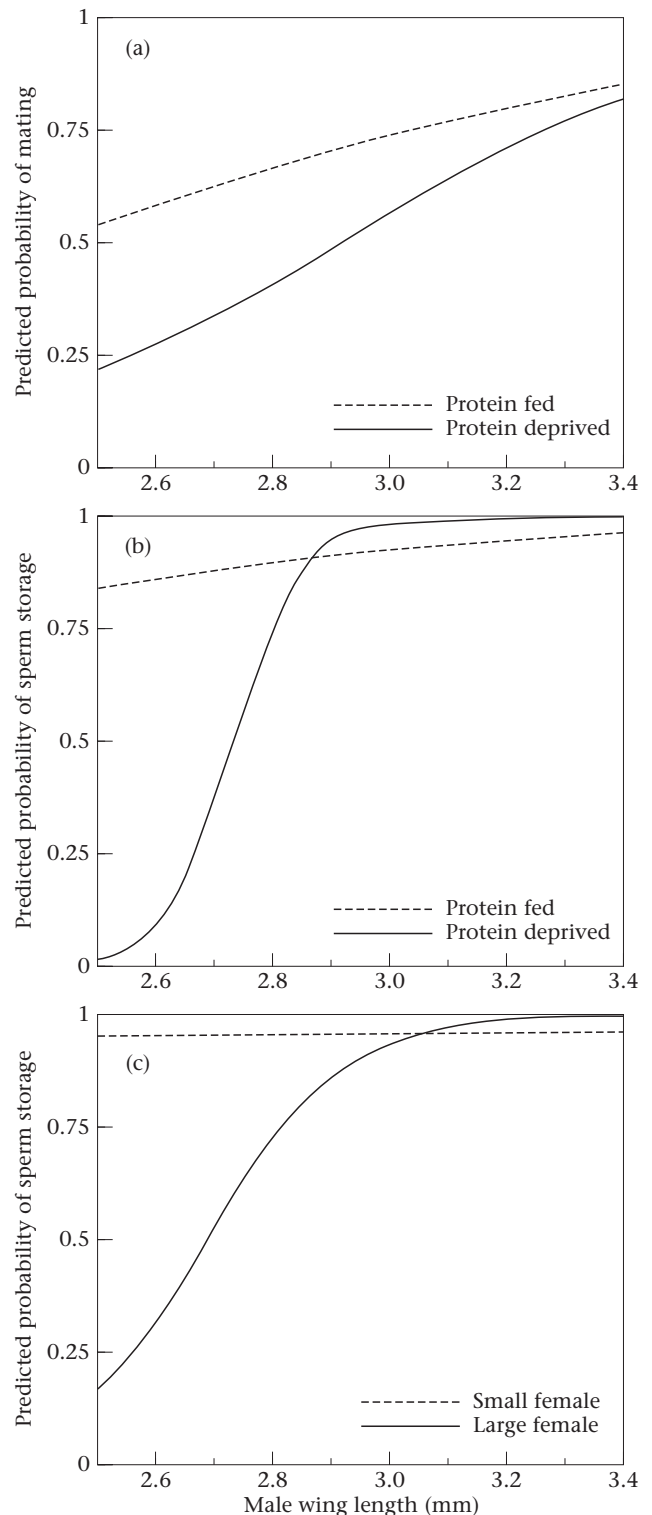
On average, sperm storage asymmetry was 23.6% ( $N=168$ , range 0–95.4%). An ANCOVA detected weak evidence of a relationship between male diet and storage asymmetry (protein fed: LSM=21.1%,  $L_1=17.5$ ,  $L_2=25.1$ ; protein deprived: LSM=26.8%,  $L_1=22.1$ ,  $L_2=31.9$ ; adjusted power=0.29), but there were no effects of male size ( $b'=0.04$ ) or female size ( $b'=-0.03$ ; Table 2).

Sperm storage asymmetry was negatively related to the total amount of sperm stored ( $R^2=0.06$ ,  $F_{1,166}=10.20$ ,  $P=0.002$ ; Fig. 4). Accordingly, to check whether the weak effects of male diet on sperm storage asymmetry operate via total sperm storage, we ran the original model (Table 2) with total sperm storage as an additional effect. The resulting model supports this pathway: total sperm storage remained highly significant ( $F_{1,159}=8.73$ ,  $P=0.004$ ), male size and female size remained highly nonsignificant (male size:  $F_{1,159}=1.10$ ,  $P=0.296$ ; female size:  $F_{1,159}=0.07$ ,  $P=0.794$ ), and the weak effects of male diet were negated ( $F_{1,159}=1.68$ ,  $P=0.197$ ).

## DISCUSSION

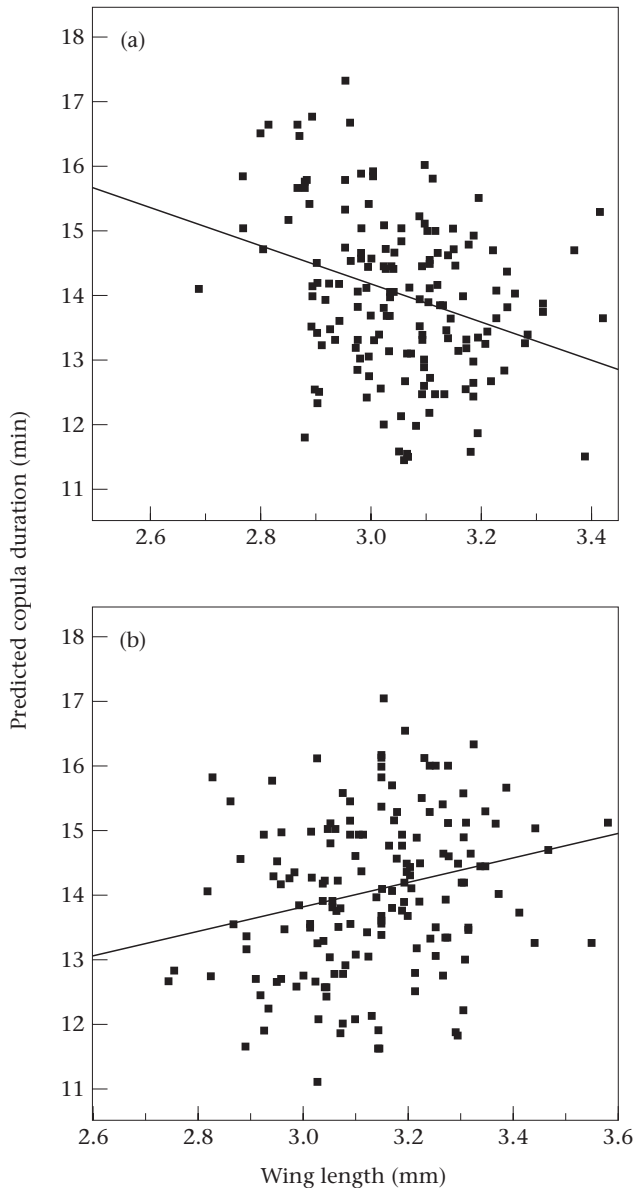
### Diet and Size as Predictors of Reproductive Success

Previous studies in simulated nature and in the laboratory have identified protein feeding and large size as positive influences on a male's chances of mating (Churchill-Stanland et al. 1986; Orozco & Lopez 1993;



**Figure 1.** Predicted probability of (a) male mating (within 2 h) and (b) sperm storage in relation to male size and diet. (c) Predicted probability of sperm storage in relation to male and female size.

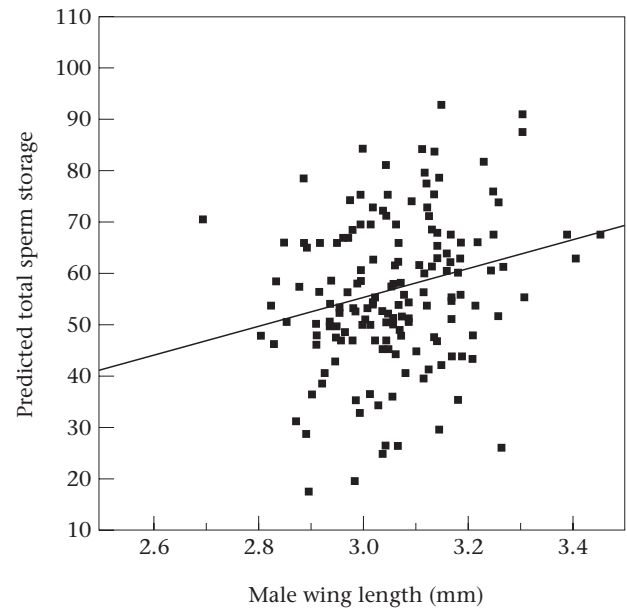
Blay & Yuval 1997). In our laboratory study we confirmed the greater mating propensity of large and protein-fed males, and then investigated how size and diet influence postcopulatory success. We found that, in addition to



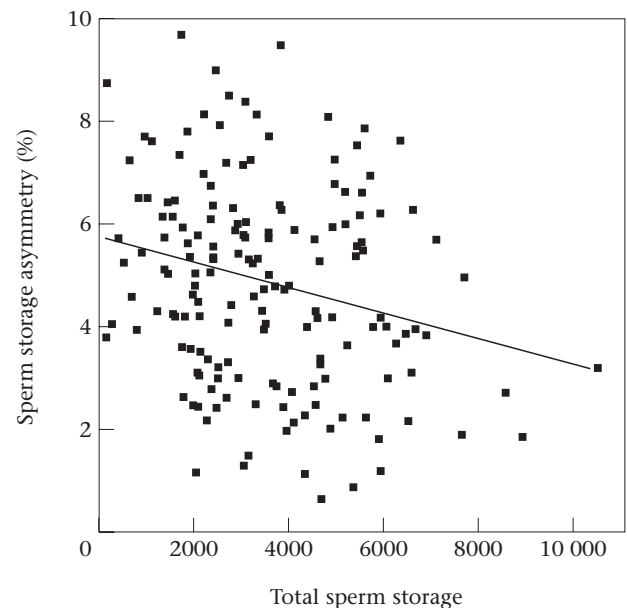
**Figure 2.** Partial regression plot describing the relationship between duration of successful copulation (square-root transformed) and (a) male size and (b) female size, correcting for effects of replicate, male diet and female (male) size.

advantages in securing copulations, large and protein-fed males were more likely to have sperm stored by mates. Additionally, large and protein-fed males tended to have more sperm stored by mates and may therefore fertilize more of each mate's eggs. Other studies have found that male activity level (Whittier et al. 1994) and symmetry of secondary sexual characters (Hunt et al. 1998) are also useful predictors of male copulatory success in medflies. Further research is needed to reconcile the relevance of these different male characters at each phase of the mating sequence and to determine whether the importance of each male character varies in relation to the laboratory or wild population studied.

At first sight, our results for total sperm storage appear discordant with those of Blay & Yuval (1997), who



**Figure 3.** Partial regression plot describing the relationship between total sperm storage (square-root transformed) and male size, correcting for effects of replicate, male diet and female size.



**Figure 4.** Regression plot describing the relationship between sperm storage asymmetry (square-root transformed) and total sperm storage.

concluded that female medflies store more sperm if they mate with protein-deprived males. However, Blay & Yuval (1997) did not account for the effects of male size, which also varied between the male diet regimes. Rather than being a direct effect of diet, this previous result very likely stems from mated protein-deprived males being, on average, larger than mated protein-fed males.

It seems more than coincidence that male success at (1) securing copulation, (2) securing sperm storage and

(3) having large quantities of sperm stored by mates are all enhanced by large size and protein feeding. This consistency in qualities defining male success at different phases suggests a close relationship between mechanisms underlying differential male success at these phases. One possibility is that females prefer certain qualities that are more pronounced in large and protein-fed males, and that courtship before, during and after intromission allows a stepwise screening of males for these qualities. Females are certainly able to exercise discrimination prior to mounting by simply decamping (Whittier et al. 1994). The behaviour of copulating male medflies is complex, including bouts of rubbing the female's abdomen with legs II and III, rhythmic genitalic pumping and abrupt pulling of the female's ovipositor (Eberhard & Pereira 1995). Similar behaviour in other insects has been interpreted as courtship functioning in 'cryptic female choice' (Eberhard 1994, 1997; Otronen 1997b). In medflies, sperm are transported to the spermathecae apparently largely by peristalsis of the spermathecal ducts (Dallai et al. 1993), suggesting a high degree of female control over this aspect of male success. Perhaps a male's quality is not always sufficiently apparent during pre-copulatory courtship and so female medflies have evolved an ability to delay accepting a male until after a more thorough postcopulatory assessment, and then to store an amount of sperm commensurate with the male's quality.

Other studies of medfly reproductive behaviour have been interpreted almost exclusively from a female's perspective, with differential male success given as evidence of female preferences (Arita & Kaneshiro 1988; Whittier et al. 1994; Whittier & Kaneshiro 1995). There are, however, viable alternatives to direct female choice that deserve consideration. In some insects, large size is positively related to fighting ability (Zuk & Simmons 1997) and protein feeding enhances energy reserves (Jacome et al. 1995); the success of large and protein-fed males may be partly attributable to superior strength and stamina conferring an ability to court frequently and, by coercion or force, to overcome female resistance to mounting, genital contact, intromission and sperm storage. Additionally, large and protein-fed males may produce larger ejaculates and this could partly explain their advantages in total sperm storage.

Discerning whether male or female influences underlie differential male success in postcopulatory sexual selection is further complicated by the possibility of 'passive female choice' in which females use resistance as a test of male vigour (Alexander et al. 1997; Arnqvist 1997). Our study was designed primarily to identify male qualities associated with postcopulatory success. Identifying the relative importance of male and female interests in the postcopulatory success of large and protein-fed males will require experimental procedures that carefully control each sex's influence.

### Variation in Copula Duration

Successful copulations could last as little as 110 min or as long as 335 min. There was, however, no evidence

of a relationship between copula duration and amount of sperm stored by females. Data presented by Seo et al. (1990) are consistent with ours; they found a similar number of sperm in spermathecae after copulations lasting between 70 and 190 min. In medflies, long copulations do not appear simply to reflect longer periods required for transferring larger quantities of sperm.

Large and protein-fed males copulated for shorter durations than small and protein-deprived males. This size and diet variation in copula duration may result from tactical decisions related to the utility of persistence or from differences in ability to complete postcopulatory tasks. In some arthropods, there is a postinsemination mate-guarding phase during which the male remains in copula to prevent the female remating, using his entire soma as a mating plug (Clark 1988; McLain 1989; Yuval et al. 1990; Alcock 1994; Dickinson 1997). However, Seo et al. (1990) interrupted copulations at intervals up to 150 min and found that sperm storage increased over this period (average copula duration in their study was 120 min), suggesting that postinsemination guarding is absent in medflies.

Nevertheless, the timing of sperm transfer is known to vary in medflies. Seo et al. (1990) reported large variation in the latency from first genitalic contact to sperm transfer; in their study, 13% of males had begun transferring sperm when interrupted after only 4 min in copula whereas 14% had not yet begun after 2 h. Additionally, Eberhard & Pereira (1995) reported considerable variation in latency from first genitalic contact to intromission. The same postcopulatory interactions that may limit a male's chances of sperm storage may also in part dictate the timing of sperm transfer and overall copula duration. Small and protein-deprived males may have prolonged copulations because they take longer to complete intromission. This may be because females take longer over decisions to accept these males. Alternatively, these comparatively weak males may be slower to overcome female resistance.

The findings that copulations were prolonged and probability of sperm storage reduced if females were large are difficult to interpret in terms of direct female choice; there is no obvious reason why large females should take longer than small females over mating decisions or discriminate more severely against small and protein-deprived males. Alternatively, this result does make sense in terms of female resistance to coerced or forced copulation; large females might exert more powerful resistance and sustain resistance for longer before successfully repelling the male or yielding to him.

In addition to these explanations based on sexual conflict, other possibilities should not be overlooked. One alternative is that, once intromission is complete, small and protein-deprived males take longer to pass their ejaculate to the female. For example, small males may have a narrower ejaculatory duct or small and protein-deprived males may be less able to exert pressure to propel the ejaculate. Also, effects of female size may be partly explained by sperm having a longer path to travel to the spermathecae in larger females.

## Sperm Storage Asymmetry and Ejaculate Isolation

Yuval et al. (1996) estimated sperm storage asymmetry in the two spermathecae by determining the percentage of total sperm that were in the more full one. Translating this to the measure we used reveals that their results are consistent with the 23.6% reported here: 28% for flies of the Sade strain and 26% for flies collected from nature.

In arctiid moths, *Utetheisa ornatrix*, damselflies, *Calopteryx splendens xanthostomus*, yellow dung flies, *Scatophaga stercoraria*, and dryomyzid flies, *Dryomyza anilis*, complex sperm storage organs have been associated with isolation of different males' ejaculates, possibly as an instrument of female choice (LaMunyon & Eisner 1993; Siva-Jothy & Hooper 1996; Otroneu 1997a; Ward 1998; but see Simmons et al. 1996). Our results are consistent with this possibility for medflies. By biasing sperm storage towards one of her two spermathecae, the female medfly appears to retain an option of accepting sperm from a second mate, biasing this male's contribution towards the less full spermatheca, and maintaining the two sperm populations in partial isolation for postcopulatory decisions of offspring paternity. Storage asymmetry, the potential for ejaculate isolation, was negatively associated with total amount of sperm stored. Consequently, qualities associated with total sperm storage, such as male diet, may be associated with variation in storage asymmetry. Reduction in storage asymmetry may be proximally explained by a reduced opportunity to accept sperm asymmetrically once one spermatheca nears capacity.

### Acknowledgments

We thank André Gilburn, Scott Field, Roy Kaspi and an anonymous referee for constructive comments on the manuscript. We also thank Hilary Voet for statistical advice and Siggy Mossinson for technical assistance. Flies were provided by Y. Rössler and R. Akiva of the Israel Citrus Board. This research was supported by a grant from the Israel Science Foundation to B.Y.

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