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Original Article

Male *Drosophila melanogaster* adjust ejaculate size based on female mating status, fecundity, and age

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In contrast to early predictions, it is now widely accepted that males incur substantive costs from ejaculate production. Hence, males are predicted to allocate their reproductive investments, including ejaculate size, relative to the risk of sperm competition and to female quality. The study of sperm allocation, however, has been technically challenging with nonvirgin females because sperm from different males must be discriminated within the female reproductive tract. To date, such investigations have thus largely been restricted to species that transfer sperm in spermatophores or for which females can be fitted with a harness to capture the incoming ejaculate. In this study, we examined sperm allocation using male *Drosophila melanogaster* that express a fluorescently labeled protein in sperm heads, allowing us to quantify sperm numbers from different males within the female reproductive tract. We found that male *D. melanogaster* deliver significantly more sperm to mated, large or young females compared with virgins, small or old females, respectively, whereas copulation duration was only significantly longer with large than with small females. These results provide further evidence for costly ejaculate production and consequent prudent allocation of sperm. *Key words:* ejaculate size, body size, female reproductive value, sperm competition, sperm number. *[Behav Ecol 22:184–191 (2011)]*

Whereas males are generally considered to be promiscuous and nondiscriminating in mate choice, numerous studies of diverse taxa have revealed that males often exhibit a greater propensity to mate with higher quality females (reviewed in Andersson 1994; Amundsen 2000; Bonduriansky 2001). Theoretical and empirical studies have further established the common occurrence of postcopulatory cryptic male choice, manifested as adjustments in the composition of ejaculates (reviewed in Wedell et al. 2002; Cameron et al. 2007; Parker and Pizzari 2010). The tailoring of ejaculates has been demonstrated to be stunningly sophisticated, including the strategic modulation of sperm numbers, sperm velocity, sperm viability, and/or seminal fluid protein composition (e.g., Cornwallis and Birkhead 2007; Thomas and Simmons 2007; Wigby et al. 2009).

Were sperm "cheap," as was once the prevailing dogma, then there would be no selection for ejaculate tailoring. However, although a single sperm may be energetically cheap (Trivers 1972; but see Pitnick and Markow 1994a, 1994b; Pitnick et al. 1995), spermatogenesis and ejaculates are now widely regarded as having substantive costs (reviewed in Dewsbury 1982; Olsson et al. 1997). Consequently, selection theoretically favors strategic allocation of ejaculates with regard to the perceived risk of sperm competition (i.e., competition between ejaculates from different males for fertilization; Parker 1970), with more sperm transferred to females as this risk increases (Parker 1970, 1990b, 1998; Wedell et al. 2002; Parker and Pizzari 2010). This is because, all other things being equal, males transferring more sperm than rival males will, on average, fertilize a greater proportion of a female's eggs (Martin et al. 1974; Martin and Dziuk 1977; Parker 1982, 1990a, 1990b). Numerous empirical studies support these predictions (Gage 1991; Simmons et al. 1993; Wedell and Cook 1999; Martin and Hosken 2002; Pizzari et al. 2003; delBarco-Trillo and Ferkin 2004; Pound and Gage 2004; but see Simmons and Kvarnemo 1997; Schaus and Sakaluk 2001; Ramm and Stockley 2009).

Optimal sperm allocation models also indicate that males should always gain by delivering more sperm to females of superior quality and/or fecundity, irrespective of their remaining sperm reserves (Galvani and Johnstone 1998). Indeed, males of various taxa adjust ejaculates to female quality (Parker et al. 1996; Gage 1998; Simmons 2001; Wedell et al. 2002; Engqvist and Sauer 2003; Pizzari et al. 2003; Cornwallis and Birkhead 2007; Rönn et al. 2008; Xu and Wang 2009). It is important to note that sperm competition risk and female quality will positively covary to the extent that highly fecund females remate faster and more frequently than females of poor quality (e.g., Simmons and Kvarnemo 1997; Gage 1998; Wedell and Cook 1999; but see Pitnick et al. 2001; Pitnick and García-González 2002).

Males may assess relative sperm competition risk and/or female quality using a variety of potential cues including 1) the presence of rival males before or during copulation (e.g., Gage 1991; delBarco-Trillo and Ferkin 2004; Bretman et al. 2010; Ingleby et al. 2010), 2) female mating status (e.g., Lewis and Iannini 1995; Martin and Hosken 2002; Friberg 2006; Thomas 2011), and 3) physiological correlates of female condition and fecundity such as body size, age, or the cuticular hydrocarbon profile (e.g., Engqvist and Sauer 2001, 2003) as female fecundity typically covaries positively with body size and negatively with age (e.g., Honěk 1993; Bonduriansky and Brassil 2005; Barnes et al. 2008; Xu and Wang 2009).

Some investigations of cryptic male choice have quantified ejaculate mass or the number of sperm transferred (e.g., Gwynne and Simmons 1990; Cook and Gage 1995; Wedell 1998; Wedell and Cook 1999; Teng and Zhang 2009), whereas other studies have relied on indirect assays such as copulation

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duration (e.g., Dickinson 1986; Martin and Hosken 2002; Siva-Jothy and Stutt 2003; Friberg 2006). This latter approach has been used by necessity in many cases where nonvirgin females are included, due to the inability to discriminate among sperm from different males, and used for convenience in other cases due to the labor-intensive nature of removing and counting sperm from the female reproductive tract. It is important to note, however, that copulation duration may not always reliably estimate the number of sperm transferred (e.g., Gilchrist and Partridge 2000).

In the fruit fly *Drosophila melanogaster*, females mate repeatedly across their life span and before sperm reserves are depleted, thus instigating sperm competition between overlapping ejaculates (Gromko, Gilbert, and Richmond 1984). Mating costs for males are substantial, resulting from vigorous courtship (Partridge and Farquhar 1981; Cordts and Partridge 1996), gamete production (Lefevre and Jonsson 1962; Gromko, Newport, and Kortier 1984; Pitnick 1996), and immunosuppression (McKean and Nunney 2001), so they should be particularly prudent in the allocation of their limited resources. Several empirical studies provide evidence for male mate choice in *D. melanogaster*. For example, males copulate longer in situations of perceived sperm competition and thus appear to increase their reproductive success (Bretman et al. 2009, 2010). Males also increase courtship intensity and copulation duration when females have, or are perceived to have, mated previously (e.g., Singh RS and Singh BN 2004; Friberg 2006). This form of male discrimination has been experimentally shown to be based on changes to the hydrocarbon profile after female mating (e.g., Tomkins and Hall 1981; Everaerts et al. 2010). Exposure to rival males prior to copulation also increases the amount of certain seminal proteins transferred (i.e., the sex peptide and ovulin; Wigby et al. 2009). Additionally, male *D. melanogaster* selectively bias courtship and copulation toward large females, particularly when sperm-depleted (Byrne and Rice 2006), and it is well established that female fecundity increases with body size (e.g., Robertson 1957; Lefranc and Bundgaard 2000; Byrne and Rice 2006).

In contrast, much less is known about cryptic male choice relative to female age, largely because studies on sperm competition in *D. melanogaster* typically use females that are less than 1 week old (e.g., Gromko and Pyle 1978; Letsinger and Gromko 1985; Clark and Begun 1998; Manier et al. 2010). Older females, however, probably contribute a relatively high proportion to natural populations, given that individuals captured in the field can survive for over a hundred days in the laboratory (Linnen et al. 2001), and females presumably are receptive for up to 2 months (Mack et al. 2003). Nonetheless, old females have a higher risk of mortality and lay significantly fewer eggs (e.g., Barnes et al. 2008), thereby lowering their mean reproductive value. Males are thus predicted to discriminate against them, as shown by a decrease in male courtship intensity as females senesce (Cook R and Cook A 1975).

Overall, studies indicate that male *D. melanogaster* allocate their mating efforts relative to the risk of sperm competition and/or the reproductive value of their mates, but it remains unclear whether they also modulate their ejaculates accordingly. Whereas strategic allocation of seminal fluid proteins has been recently documented (Wigby et al. 2009), there has been speculation (most recently by Wigby et al. 2009; Bretman et al. 2010) but no empirical evidence for the tailoring of sperm numbers. Particularly in insects, where sperm transfer during copulation cannot be intercepted by a harness for quantification of sperm as in other internal fertilizers (e.g., Pizzari et al. 2003), the number of sperm can only be determined by dissection of females. This, however, poses a particular challenge of quantifying sperm transfer when a female has been previously inseminated. Consequently, tests of ejaculate tailoring relative to female mating status or nonvirgin female quality in internally fertilizing invertebrates have been restricted to species that deliver ejaculates in spermatophores (e.g., Cook and Gage 1995; Wedell and Cook 1999; Teng and Zhang 2009). Our knowledge about sperm tailoring in insects is thus still meager, and in fruit flies, it is lacking completely. This is rather surprising given that fruit flies are such a widely used model system in reproductive and evolutionary biology and that much research focuses on understanding the factors underlying sperm precedence.

In this study, we used *D. melanogaster* that were genetically engineered to produce fluorescently tagged sperm heads (Manier et al. 2010), which facilitated direct quantification of male sperm allocation to nonvirgin females. We examined whether male *D. melanogaster* strategically allocate sperm, using within-male pairwise comparisons of sperm numbers delivered to females along 3 different quality axes: mating status (virgin vs. nonvirgin), body size (i.e., fecundity), and age.

MATERIAL AND METHODS

Study animals

The experimental strain of *D. melanogaster* is a large outbred population (LH_m) maintained in a population cage of approximately 1000 individuals at 24 °C and 12:12 h light:dark on standard cornmeal–molasses–agar–yeast medium. Unless otherwise noted below, flies for experiments were bottle reared, collected as virgins on the day of eclosion using CO_2, then aged in single-sex vials containing standard medium and live yeast. All females and standard "competitor" males (in the case of experiments with nonvirgin females) were LH_m wild type. All "experimental" males were from a line genetically engineered to produce sperm that were tagged with a red fluorescent protein (DsRed-Monomer), then backcrossed for 6 generations to the LH_m strain (for details on the fly strains and the genetic transformation methods, see Manier et al. 2010). Sperm from these males could be easily visualized for counting within the female reproductive tract under a fluorescent microscope and unambiguously discriminated from the sperm of competitor males.

Experimental design

Three experiments were performed to investigate ejaculate tailoring by males. Each experiment consisted of within-male pairwise comparisons ($N = 50$ males) of sperm numbers transferred to females differing in 1) mating status (i.e., virgin or nonvirgin), 2) body size, or 3) age, and all experiments followed the same general protocol. Two days after eclosion of experimental males, we mated 50 males once to a nonexperimental, 2-day-old virgin female of average size in individual vials, after which the female was discarded (this initial mating was simply used to ensure that the experimental matings were not confounded by potential virgin effects; see Bjork et al. 2007). The following day, we paired 25 of these males each to a single female of one treatment group (e.g., young) and the other 25 males each to a female of the corresponding second treatment group (e.g., old). We measured the duration of copulation and, 90 min after the start of mating (no females remated during this interval), removed the female, froze her in liquid nitrogen and stored at −70 °C until analysis. The male remained in the original vial and was paired to a new female of the alternate condition on the following day. After this copulation, we froze both the male and female, again 90 min after the start of mating. The period of 90 min allows the sperm to migrate from the bursa copulatrix

to the female's sperm storage organs (i.e., spermathecae and seminal receptacle) to facilitate quantification, but it minimizes the risk of female sperm ejection before freezing (Manier et al. 2010; all females were confirmed to not have ejected sperm). Subsequently, we dissected the female reproductive tract into a drop of phosphate-buffered saline on a microscope slide and unfolded the seminal receptacle before covering the specimen with a coverslip and sealing it with paper cement. Under a fluorescent microscope at a magnification of ×400, we counted all sperm in the lower female reproductive tract, including the bursa, seminal receptacle, and the 2 spermathecae with ducts. Additionally, we measured thorax length for all males and females to the nearest 0.01 mm using the reticule of a dissection microscope (magnification ×80).

Except for virgin and old female treatments (see below), all females mated once with a competitor male 4 days before the experimental mating. This interval was chosen to maximize the likelihood of female remating while minimizing female aging for the experimental mating, particularly for virgin and young females. All eclosed progenies produced by females during this interval were quantified to determine female fecundity.

Virgin-versus-mated female experiment

We used females at 6 days of age that were reared under identical conditions, except that the nonvirgins were mated once to a competitor male 2 days after eclosion.

Small-versus-large female experiment

Females of 2 distinct body size classes were generated by transferring first-instar larvae to vials at densities of 300 and 15 individuals, respectively, within 3 h of hatching. Larval density has previously been shown to influence larval development and adult body size through different levels of competition for nutrients (e.g., Pitnick and García-González 2002; Byrne and Rice 2006; Amitin and Pitnick 2007). Using thorax length as a reliable measure of adult body size (Robertson and Reeve 1952), females were found to be significantly larger from the low-density than from the high-density rearing condition (thorax length: mean ± standard error [SE] = 1.11 ± 0.01 vs. 0.90 ± 0.01 mm, $F_{1,98} = 369.2$, $P < 0.0001$). After collecting virgin females on the day of eclosion, we maintained both treatment groups under identical conditions. Unlike the other experiments, the preexperimental (virgin) mating of these females was 4 rather than 2 days after eclosing to ensure that the small females, which exhibited slower larval development (12–13 days compared with 8–9 days), were sexually mature (e.g., Pitnick and Markow 1994a). Nonetheless, the period between the first and the test mating remained 4 days as in the other experiments.

Young-versus-old female experiment

Females were 6 or 18 days old, respectively, on the day of the test mating. In *D. melanogaster*, this period has been associated with a decline in productivity but not in viability (Barnes et al. 2008), which allowed us to maximize the age difference (and associated effects) while minimizing the risk of high mortality during the experiment. All females mated once with nonexperimental males 2 days after eclosing as described above. We mated the old females again to nonexperimental males 8 and 14 days after eclosing to avoid the unnaturally long sexual abstinence, although the 6 days between mating opportunities approximates a more typical remating interval in *D. melanogaster* and depletes most of the stored sperm (e.g., Pyle and Gromko 1978; Singh RS and Singh BN 2004). The mating of old females on day 14 coincided with the first mating of the young females (day 2), 4 days before the experiment.

Analyses

We performed all statistical analyses using the statistical software package R version 2.11.1 (R Development Core Team 2010). We tested for within-male differences in the number of sperm transferred or copulation duration, respectively, using linear mixed-effects models (henceforth simply "LME") with "male" as random factor and "treatment," "mating order," "female size," and "male size" as fixed effects, except for the second experiment where female size was the treatment and thus omitted as a covariate. In the analyses on sperm number, we included "copulation duration" as an additional fixed effect in the initial models to account for the possibility that differences in sperm transfer may be confounded by variance in the copulation duration (see INTRODUCTION). After examining the results deriving from the full models, we performed stepwise model selection (backwards, based on Akaike's information criterion; Venables and Ripley 2002) to obtain the minimum adequate models. We also tested for correlations between sperm number and copulation duration in simplified models with only "male" as a random effect but not controlled for other fixed effects. Finally, we examined relative changes (rc) in sperm number and copulation duration between the first and second mating ($rc = (v_2 - v_1)/v_1$, with v as sperm number or copulation duration, respectively) to determine whether male investments changed significantly regardless of mating order.

Throughout this paper, we report effect sizes (r or partial r) with 95% noncentral confidence intervals (CIs) (Nakagawa and Cuthill 2007).

RESULTS

Virgin versus mated females

Males delivered a mean (±SE) of 728.5 ± 31.02 sperm to virgin females and 835.7 ± 29.25 sperm to previously mated females (Table 1, Figure 1a). From the first to the second mating, we found a significant relative change in sperm numbers with a consistent bias toward mated females (Figure 2a). However, the magnitude of this change within males was not related to male body size ($r = -0.09$ (95% CI: −0.35 to 0.19), $t = -0.65$, $P = 0.52$), suggesting that the degree of sperm allocation is independent of male size (and thus potentially male quality). Copulation duration did not differ between virgin and mated

Table 1

Results of the minimum adequate models on sperm number as the response variable after stepwise deletion of fixed effects from the full models, for the 3 experiments: A) virgin–mated females, B) small–large females, and C) young–old females

Variables	Partial r	95% CI	t	P
A) Mated–virgin				
Mating status	–0.49	–0.65 to –0.25	–3.91	0.0003
B) Small–large				
Size class	0.56	0.34 to 0.70	4.73	<0.0001
Mating order	–0.31	–0.53 to –0.04	–2.28	0.03
Male size	0.47	0.22 to 0.64	3.64	0.0007
C) Young–old				
Age class	0.58	0.39 to 0.74	4.87	<0.0001
Mating order	–0.25	–0.48 to 0.03	–1.81	0.08
Male size	0.41	0.37 to 0.72	3.14	0.003

"Male" as the random factor was significant for all experiments (all $P < 0.01$). Copulation duration had no significant effect on sperm number in all experiments (all $P > 0.23$) and so did not appear in any of the minimum adequate models.

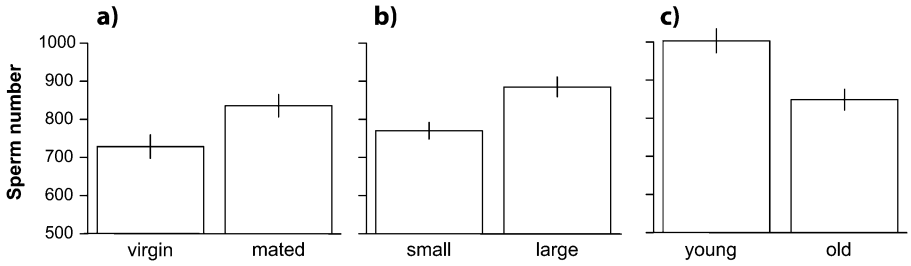

Figure 1
Mean (±SE) number of sperm delivered to females relative to female mating status (a), body size (b), and age (c).

females (28.1 ± 0.81 vs. 27.4 ± 0.97 min, respectively; Table 2, Figure 3a) and was not significantly associated with sperm number (simple LME without additional fixed effects: $r = 0.08$ (−0.20 to 0.34), $t = 0.55$, $P = 0.59$; Supplementary Figure S1a).

Small versus large females

Males transferred significantly more sperm to large compared with small females (884.9 ± 26.19 vs. 770.1 ± 22.00 sperm; Table 1, Figure 1b). The magnitude of the relative change in sperm numbers between the first and second mating (Figure 2b) increased with male body size, albeit not statistically significantly so ($r = 0.27$ (−0.01 to 0.49), $t = 1.82$, $P = 0.06$). Males also copulated significantly longer with large than with small females (26.3 ± 0.79 vs. 23.0 ± 0.62 min, respectively; Table 2, Figure 3b). Nonetheless, sperm number was not associated with copulation duration ($r = 0.20$ (−0.08 to 0.44), $t = 1.42$, $P = 0.16$; Supplementary Figure S1b).

Young versus old females

Males transferred significantly more sperm to young than to old females (1002.9 ± 31.30 vs. 848.54 ± 26.94 sperm; Table 1, Figure 1c), but the relative change between the first and second mating (Figure 2c) was independent of male size ($r = -0.09$ (−0.35 to 0.19), $t = -1.08$, $P = 0.53$). There was no significant difference in copulation duration between young and old females (24.6 ± 0.70 vs. 24.3 ± 0.70 min; Table 2, Figure 3c). Copulation duration also did not covary significantly with sperm number ($r = 0.15$ (−0.13 to 0.40), $t = 1.08$, $P = 0.29$; Supplementary Figure S1c).

Female fecundity

We determined female fecundity by the number of progenies produced during the 4 days preceding the experimental mating (except virgin females). Progenies of a few females could not be quantified reliably for various reasons (e.g., unknown numbers of flies escaped) such that sample sizes differed between treatments. Large females produced significantly more progenies than small females (88.3 ± 2.37 [$N = 49$] vs. 66.7 ± 2.78 progeny [$N = 47$]; $F_{1,94} = 35.07$, $P < 0.0001$), and young females produced more than old females (107.2 ± 4.13 [$N = 43$] vs. 91.9 ± 5.36 progenies [$N = 46$]; $F_{1,87} = 5.19$, $P = 0.025$). In LME models with "male" as a random factor and "treatment" as a fixed factor, the number of sperm transferred by males was independent of female productivity (prior to copulation) in both the experiment on female body size (partial $r = 0.03$ [−0.26 to 0.31], $t = 0.17$, $P = 0.87$) and that on female age (partial $r = 0.02$ [−0.28 to 0.32], $t = 0.11$, $P = 0.91$).

DISCUSSION

Consistent with theoretical predictions about optimal sperm expenditure relative to the level of sperm competition and female reproductive value, our 3 experiments showed that male *D. melanogaster* tailor their ejaculates with respect to female mating status, female body size, and female age. These results are consistent with previous reports on differential male mating efforts in this and other species (see below).

It is important to consider whether the treatment effects on sperm number were purely male driven or whether female-mediated processes (i.e., differential sperm uptake, retention, and/or storage) may have contributed to the patterns. For example, old females are thought to be less capable of storing sperm than younger females, thereby shifting the relative number of sperm between competing ejaculates (Mack et al. 2003). However, our protocol of freezing females prior to sperm ejection from the bursa (typically occurring 2–5 h after mating), and quantifying all sperm throughout the reproductive tract, precludes any influence by females beyond the sperm transfer (Manier et al. 2010). Although we cannot rule out female influence on sperm transfer (as has been experimentally demonstrated in the guppy, *Poecilia reticulata*; Pilastro et al. 2004), there is at present no evidence for female control over sperm transfer in *D. melanogaster*. Furthermore, because males often transfer more than twice the number of sperm ultimately stored by females (Manier et al. 2010), it seems more likely that sperm transfer is under male rather than

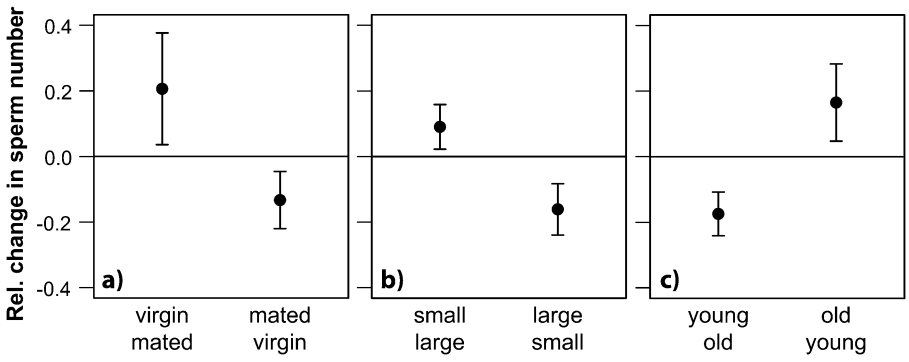

Figure 2
Relative change in numbers of sperm transferred by males between their first and second mating (first | second) for female mating status (a), body size (b), and age (c). The error bars depict the 95% CI around the mean values. All 95% CI exclude zero and indicate significant changes in the respective direction at $\alpha = 0.05$.

Table 2

Results of the minimum adequate models on copulation duration as the response variable after stepwise deletion of fixed effects from the full models, for the 3 experiments: A) virgin–mated females, B) small–large females, and C) young–old females

Variables	Partial r	95%CI	t	P
A) Mated–virgin				
Mating status	–0.07	–0.21 to 0.32	–1.51	0.14
Female size	–0.45	–0.53 to –0.05	–2.38	0.02
B) Small–large				
Size class	0.44	–0.18 to 0.61	3.40	0.001
C) Young–old				
Female size	0.23	0.05 to 0.46	1.65	0.11

"Male" as the random factor was significant ($P = 0.002$) for (A) but not the other experiments (both $P > 0.52$).

female control, with females potentially evolving sperm ejection as a strategy to influence the outcome of sperm competition or simply adjust sperm numbers to their storage capacity (Manier et al. 2010).

Ejaculates and female mating status

Males transferred significantly more sperm to mated than to virgin females. Theoretical models show that optimum ejaculate allocation largely depends on males' knowledge of the risk of sperm competition for a particular mating (e.g., Parker 1990b; Parker et al. 1997). In fact, males of several species are sensitive to the risk of sperm competition and deliver increased numbers of sperm when the probability of competition is high, for example, in the presence of rival males (e.g., Gage 1991; Gage and Barnard 1996; Bretman et al. 2010). Furthermore, discrimination between virgin and mated females has been demonstrated in various species (Cook and Gage 1995; Wedell 1998; Wedell and Cook 1999; Teng and Zhang 2009). In *D. melanogaster*, this discrimination is based at least in part on the cuticular hydrocarbon profile (Friberg 2006), which changes following copulation (e.g., Tomkins and Hall 1981; Everaerts et al. 2010). The pattern of sperm tailoring demonstrated here makes adaptive sense, given that males transfer more sperm than females are capable of storing, yet with previously mated females the extent of displacement of resident sperm, and hence second–male sperm precedence, increases with the number of sperm transferred (Manier et al. 2010).

In addition to sperm numbers, male *D. melanogaster* also adjust seminal fluid components to the risk of sperm competition. For example, Wigby et al. (2009) found increased transfer of sex peptide and ovulin in the presence of a competitor male compared with noncompetitive matings. Because at least sex peptide is bound to the sperm tail (Peng et al. 2005), sperm allocation might be the underlying mechanism of this differential allocation of sex peptide. However, it has not yet been established what proportion of sex peptide in ejaculates is bound to sperm as opposed to being in the seminal plasma. Nonetheless, the similar trends in Wigby et al. (2009) and our studies suggest that male adjustments to the mating environment involve a suite of ejaculate components rather than a single parameter.

Ejaculates and female reproductive value

We found that males delivered 15–18% more sperm to young or large (and significantly more productive) females than to old or small females, respectively. These results support theoretical predictions about optimal ejaculate investments relative to the female reproductive value (e.g., Galvani and Johnstone 1998) and reinforce conclusions of previous studies of other taxa (e.g., Goshima et al. 1996; Gage 1998; Sato and Goshima 2007; Xu and Wang 2009). It may be adaptive for males to invest more in matings with more fecund females. Consistent with this interpretation, female remating interval is believed to be determined, in part, by the number of sperm she has in storage (Gromko, Gilbert, and Richmond 1984). Alternatively, but not mutually exclusively, males may be responding adaptively to sperm competition risk if more fecund females (e.g., young and large) mate more frequently (e.g., Simmons and Kvarnemo 1997; Gage 1998; Wedell and Cook 1999).

Whereas the variation in female body size provides a relatively simple visual cue for males to assess a female's fecundity or remating potential (either by determining absolute female size or relative to their own size), it is less clear how males determine female age. The most likely mechanism is a change (or series of changes) in female pheromones (e.g., cuticular hydrocarbons) across the female life span. Although the postulated changes to female physiology and the requisite male sensitivity to them await experimental investigation, a recent study provides some evidence for alterations in several compounds of cuticular hydrocarbons between 2- and 6-day-old virgin females in *D. melanogaster* (Everaerts et al. 2010). The plausibility of such an age-determining mechanism is further supported by results from other species of Diptera and the use by biologists of relative abundance of specific cuticular hydrocarbons as an age-grading technique for field populations (e.g., Chen et al. 1990; Desena et al. 1999; Brei et al. 2004).

Copulation duration

In all 3 experiments, we found no significant effect of copulation duration on the number of sperm transferred. This result may not be surprising because, unlike other insects where the number of sperm transferred increases linearly with mating duration (Simmons 2001), male *D. melanogaster* transfer sperm relatively quickly between 6 and 10 min after the start of mating (Gilchrist and Partridge 2000), although copulations typically last 15–25 min (e.g., Gilchrist and Partridge 2000; Friberg 2006; Bretman et al. 2009; this study). Much of the copulation period after sperm transfer is devoted to the

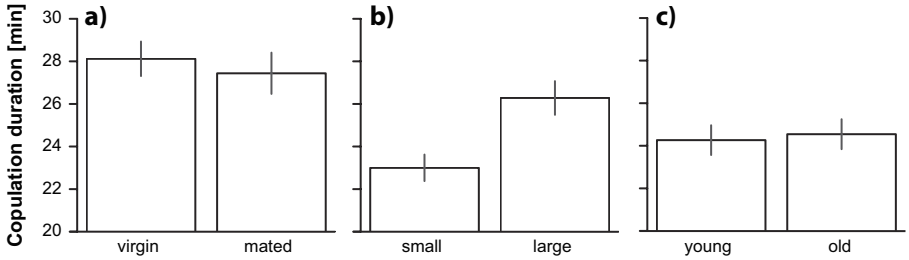

Figure 3
Mean (±SE) copulation duration relative to female mating status (a), body size (b), and age (c). Only the difference between small and large females was statistically significant ($P = 0.001$).

transfer of seminal fluid, including accessory gland proteins that have been shown to affect female sexual receptivity, egg laying rate, and sperm storage physiology (Wolfner 1997; Andersson et al. 2000; Wolfner 2002; Chapman and Davies 2004; Grillet et al. 2006). Our results indicate that, unless the pattern of sperm transfer throughout copulation is known, investigations using copulation duration as an index of sperm transfer should be interpreted with caution.

Unlike previous reports (e.g., Friberg 2006), we found no significant difference in copulation duration between virgin and mated females. The reason(s) for this discrepancy remains unclear. The only axis of female variation in the present study to elicit a significant treatment effect on copulation duration was female body size, with longer copulations with larger females. Byrne and Rice (2006) have reported a similar result, particularly if males were sperm-depleted. In our study, males were most probably not under a risk of sperm depletion as they mated only once on each of 3 consecutive days, whereas they can successfully inseminate multiple females within a day (e.g., Hihara 1981). It is possible that small females are particularly disfavored due to their considerably low fecundity. It also seems plausible that a male inseminating a small female might reach the point where the relatively small female reproductive tract allows no further delivery of ejaculatory material and the males uncouple sooner than when mating with a large female.

Conclusions

We document sophisticated modulation of sperm numbers relative to female mating status, body size, and age in *D. melanogaster*. To date, studies of sperm allocation in internally inseminating invertebrates have been restricted to species that deliver sperm in spermatophores or to virgins for investigation of ejaculate tailoring in response to variation in female condition. Using males that produce fluorescently labeled sperm, however, we were able to directly quantify this phenomenon in an insect in which males do not produce spermatophores. Our results extend previous studies on *D. melanogaster* that have reported adjustments in male reproductive investments in response to female quality or sperm competition, and they provide further evidence for sperm expenditure relative to anticipated fecundity payoffs, given that sperm production is likely to be costly and limited.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/.

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