RESEARCH ARTICLE

Mating with large males decreases the immune defence of females in *Drosophila melanogaster*

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Abstract

Mating has been widely reported to be a costly event for females. Studies indicate that female cost of mating in terms of fecundity and survivorship can be affected by their mates, leading to antagonistic coevolution between the sexes. However, as of now, there is no evidence that the female cost of mating in terms of immune defence is affected by their mates. We assess the effect of different sized males on antibacterial immune defence and reproductive fitness of their mates. We used a large outbred population of *Drososphila melanogaster* as the host and *Serratia marcescens* as the pathogen. We generated three different male phenotypes: small, medium and large, by manipulating larval densities. Compared to females mating with small males, those mating with large males had higher bacterial loads and lower fecundity. There was no significant effect of male phenotype on the fraction of females mated or copulation duration (an indicator of ejaculate investment). Thus, our study is the first clear demonstration that male phenotype can affect the cost of mating to females in terms of their antibacterial immune defence. Mating with large males imposes an additional cost of mating to females in terms of reduced immune defence. The observed results are very likely due to qualitative/quantitative differences in the ejaculates of the three different types of males. If the phenotypic variation that we observed in males in our study is mirrored by genetic variation, then, it can potentially lead to antagonistic coevolution of the sexes over immune defence.

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Introduction

Several studies have shown that in promiscuous species like *Drosophila*, where males provide no nuptial gifts or parental care, mating carries a fitness cost to females, with the cost most often being quantified in the currency of survivorship and fecundity. Mating multiply can potentially reduce the survivorship and fecundity of females (Fowler and Partridge 1989; Holland and Rice 1998; Brown *et al*. 2004; Kuijper *et al*. 2006). Along with fecundity and survivorship, immunity is another very important contributor to total fitness. Given the energetic constraints, maintenance as well as deployment of immune response can extract a fitness cost. Evolutionary trade-offs of immune defence with other life-history traits like fecundity (Fellowes *et al*. 1999; McKean *et al*. 2008), survivorship (Moret and Schmid-Hempel 2000; Ye *et al.* 2009), larval competitive ability (Kraaijeveld and Godfray 1997), and body size (Fellowes *et al.* 1999) have been well documented. While the immunosuppressive effect of mating has long been proposed, experimental results have been fairly inconsistent (Sheldon and Verhulst 1996; Siva-Jothy *et al*. 1998; Rolff and Siva-Jothy 2002; Fedorka *et al*. 2004; Schwarzenbach *et al*. 2005; Shoemaker *et al*. 2006). However, in a recent study (Short and Lazzaro 2010), it has clearly been shown that mating can reduce immunocompetence of an organism in a pathogen specific way. Additionally, mating can affect various components of the immune system (Siva-Jothy *et al.* 1998; Rolff and Siva-Jothy 2002; Fedorka *et al.* 2004). It is to be noted that the cost of mating sustained by the females is a by-product of male–male competition and is not a necessary outcome of all mating systems. Infact, under strict monogamy, the reproductive interests of males and females are expected to coincide and hence the cost of reproduction are expected to be low compared to promiscuous systems. Such cost of mating, in terms of reduced survivorship, fecundity, immunocompetence etc. can potentially lead to rapid antagonistic coevolution between sexes, provided females vary in the cost that

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they pay and males vary in the cost that they impose on females.

Interestingly, several studies have shown that females vary in the cost that they pay for mating in terms of survivorship (Wigby and Chapman 2004), fecundity (Linder and Rice 2005) and immunocompetence (Short and Lazzaro 2010). Further, the fitness costs of mating experienced by the females are primarily mediated through the accessory gland proteins (Acps) that are transferred by the males during mating (Chapman *et al.* 1995). These proteins act to increase male fitness (Chen 1984; Chen *et al*. 1988; Kalb *et al*. 1993; Herndon and Wolfner 1995; Heifetz *et al*. 2000) and are hence under positive selection in males (Swanson *et al.* 2001). Additionally, fitness costs to females can accrue due to persistent male harassment quite independent of mating (Lew *et al.* 2006). There is ample evidence to show the variation in the cost of mating experienced by the females is affected by the phenotype of males that they mate with. In a classic experiment, Rice (1996) found that males allowed to adapt to a static female phenotype evolved to impose a greater mortality cost on females compared to control males (Rice 1996), suggesting ample genetic variation among the males in their ability to 'harm' females. Further, this difference in female mortality was evident even with a single mating, indicating that the difference in mortality was probably due to components of the ejaculate and not behavioural. Studies addressing the effect of male phenotype on female cost of mating have found that females mated to large males have reduced fecundity and survivorship compared to females mated to small males (Pitnick 1991; Pitnick and Garcia-Gonzalez 2002; Friberg and Arnqvist 2003).

Unlike the effects of male phenotype on female fecundity and survivorship, there is little information about the effect of males on female immune defence. A recent study found that male genotype had no significant effect on the cost of mating paid by females in terms of immune defence (Short and Lazzaro 2010). However, the extent of variation in the relevant traits (say, quality/quantity of ejaculate, male body size etc.) across the male genotypes used in this study is not clear. To summarize, while it is established that there is a significant effect of males on the cost of mating experienced by females in terms of survivorship and fecundity, there is no evidence yet that males affect the female cost of mating in terms of immune defence.

The aim of the present study was to evaluate the effect of different male phenotypes on anti-bacterial immune defence and reproductive fitness of females. We used a large, outbred population of *D. melanogaster* as the host and *S. marcescens,* a gram negative bacterium, as the infecting agent. Phenotypic variation in males was created by growing the larvae at three densities (50, 150 and 250 eggs per vial). Larval density can potentially affect a host of adult traits but the most obvious effect is on adult body weight/size. Hence, we measured dry body weight as a proxy for male phenotype. We assayed overall female immune defence by assessing their bacterial load 24 h postinfection (McKean and Nunney 2001; Lazzaro *et al*. 2004, 2006; Short and Lazzaro 2010). Further, we designed our experiment to assess the effect of differences in male ejaculate, rather than differences in male behaviour, on the female cost of mating in terms of immune defence.

Materials and methods

Fly stock and maintenance

For this study we used a large, outbred laboratory population of *D. melanogaster* called LH. This population was maintained on a 14-day discrete generation cycle, at 25°C and 12 : 12 h light : dark cycle on cornmeal–molasses food. For details of the maintenance protocol, see Chippindale and Rice (2001).

Microorganism and infection protocol

The microorganism used for all the experiments was *S. marcescens* (strain ATCC 13880). Previous studies in our laboratory have shown that this strain grows equally well in males and females and is pathogenic, causing about 80% mortality in males and females over a 10-day period (Imroze and Prasad 2011). On the evening before infection, a fresh culture was inoculated in Luria broth (LB) and allowed it to grow overnight to an optical density (OD) of 1.0. The resulting population of bacteria was centrifuged and made into a slurry. Infection was carried out with the help of a 0.1 mm dissecting pin (Fine Science Tools, Foster City, USA) that had been dipped in the bacterial slurry. Flies under light CO_2 anesthesia were infected at the lateral side of the thorax and sham infection was carried out as a control, injecting flies with dissecting pins dipped in *Drosophila* Ringer solution.

Generation of experimental males and females

The entire experiment was replicated thrice, resulting in three independent blocks. All females used in the experiment came from vials with an egg density of 150 per vial. They were isolated as virgins and held in groups of 15 per vial until the day of experiment as described below.

Phenotypic variation in body size among males was created by manipulating egg density in the rearing vials. Body size is known to decrease with increasing egg and larval density (Atkinson 1979; Wilkinson 1987). About 2000 adult flies were transferred into a cage and kept for two days with a Petri plate containing cornmeal–molasses–agar medium supplemented with live yeast paste. Flies were then allowed to lay eggs for 6 h on a fresh food plate. Using a fine brush, eggs were transferred from the oviposition plate to a vial containing ~6 mL of standard food. We used three rearing densities: 50, 150 and 250 eggs per vial. Within each egg density, we set up enough replicate vials to accommodate ~2500 eggs. We will use the term 'large' for males derived from lowest egg density vials (50 eggs per vial), 'medium' for males from medium egg density vials (150 eggs per vial) and 'small' for

males from high egg density vials (250 eggs per vial). Males emerging in these vials were isolated as described below.

Dry body weight of males

While larval density is expected to affect various adult traits, we assayed dry body weight of the males as a proxy for male phenotype. A subset of the collected males from all three types of vial were flash frozen on the 12th day post oviposition (since this is the day they would normally interact with the females) and divided into eight groups of five males each. The flies were dried at 70°C for 24 h and each group of five flies was weighed.

Setting up single mating exposure

Very young (<4 h post eclosion) adult males and females were collected on the 10th day post oviposition, coinciding with peak eclosion, and held separately as virgins in groups of 15 in standard cornmeal–molasses food vials. For each block, we collected 30 such vials of females. Additionally, for each block, we collected 10 vials each of the three types of males (small, medium and large). On the 12th day post oviposition, each vial of 15 females was randomly designated to receive either 'small', 'medium' or 'large' males as their mates. Then, 15 standard females were paired with 15 males without using anesthesia in cornmeal–molasses food vials. Ten such vials were set up in each treatment × block combination. We ensured that the flies mated only once by separating the pairs after 40 min under light CO_2 anesthesia. We also observed the vials to quantify copulation duration (CD, the duration for which a pair remains in copula) at 25°C under uniform overhead illumination. In each of the vials, we recorded the number of mating pairs observed in 1 min intervals for entire period of the experiment. From these data we calculated the mean time to begin copulation and the mean time to end of copulation for each vial. The difference between the two values yielded the copulation duration. Thus from each vial we obtained one value of copulation duration. In each block, CD evaluation was carried out in all the 10 replicate vials of each treatment.

Effect of male type on immunity and fecundity

Females were given single mating exposure as described above. Males were discarded and females remained in the food vial for 2 days, after which we assayed (i) reproductive output; and (ii) bacterial load of females from each of the three mating treatments.

(i) Reproductive fitness evaluation: From each vial, five females (out of 15) were randomly chosen and transferred individually to test tubes (12 mm × 75 mm) containing corn meal–molasses medium. The females were discarded after 18 h and the eggs were incubated under standard conditions. The number of progeny emerging from each test tube was counted after 13 days and averaged across the five females from each vial to obtain average progeny number. For Blocks 1 and 2, reproductive fitness was evaluated from all 10 vials from each treatment. For block 3, reproductive fitness could be evaluated from only five of the 10 vials in all the three treatments due to problems in handling the oviposition tubes. In the same experiment, the number of females laying fertile eggs in oviposition tubes was also noted as an estimate of the proportion of females that actually mated.

(ii) Bacterial load estimation: Eight out of 10 vials were chosen at random. They were further divided into infection group (five vials) and sham-infection group (three vials). Infection/sham-infection was performed on six randomly chosen females from each vial following the protocol described above. After infection/sham infection, flies were put back in a fresh food vial and held for 24 h. On the next day, females were subjected to light CO_2 anaesthesia followed by homogenization in groups of three with a micropestle (Tarson, Kolkata, India) in 300 μL of LB. Hence, from each vial, we had two homogenates. From each homogenate, 50 μL aliquot of 1 : 100 dilution was spread on a LB agar plate and all the plates were incubated overnight at 37°C. Resultant colonies were checked visually for the colony morphology and also the characteristic colour to ensure that they were *S. marcescens* (Lazzaro *et al.* 2006). The numbers of bacterial colonies were assumed to be negatively correlated with the strength of immune response mounted by the female. Thus, from each vial of each treatment we obtained two values of bacterial load which were used as the units of analysis.

Data analysis

Data from the dry body weight, CD and reproductive fitness assays were analysed using a two factor ANOVA treating male size as the fixed factor crossed with random blocks. Data from the bacterial load experiment were analysed using a nested ANOVA treating male size as a fixed factor, block as a random factor along with the replicate plate nested within male size. This was because, the six females used to assay bacterial load came from the same vial and had hence experienced the same environment. Data from the bacterial load experiment and reproductive fitness assay were subjected to a natural log transformation before analysis. We used a Wilcoxon rank sum test to analyse the proportion of females that mated with the three types of males within each block since these data were highly non-normally distributed.

Results

Dry body weight decreased with increasing larval density (Figure 1; Table 1). Analysis of data from within each block using a one way ANOVA indicated a significant effect of male type (all $P < 0.01$). Each of the male types (small, medium and large) was significantly different from the others in its mean dry body weight in all the blocks (Tukey's HSD, all $P < 0.01$). CD did not significantly differ among different

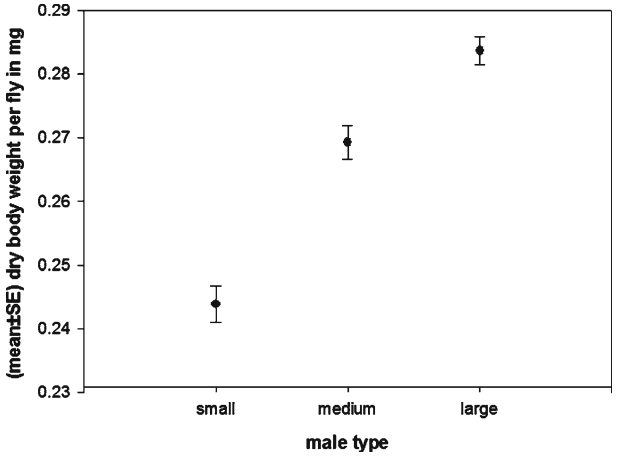

Figure 1. Dry body weight of males (average across the blocks). All the male types were significantly different from each other in their mean dry body weight.

types of males (Figure 2A; Table 1). This was true even when the blocks were analysed separately (one way ANOVA, all $P > 0.05$).

The number of females inseminated by small, medium or large males did not significantly differ (Figure 2B Wilcoxon rank sum test, all $P > 0.05$) in any of the blocks, which indicates that females did not exhibit any difference in receptivity towards different types of males. ANOVA results indicated that male type and block had a significant effect on the bacterial load of females (figure 3; table 2). Since females from different blocks varied significantly in terms of their bacterial load, we looked for consistency of the results within each block by subjecting the data from each block to a nested ANOVA. We found that within each block, there was a significant effect of male type on mean bacterial load carried by

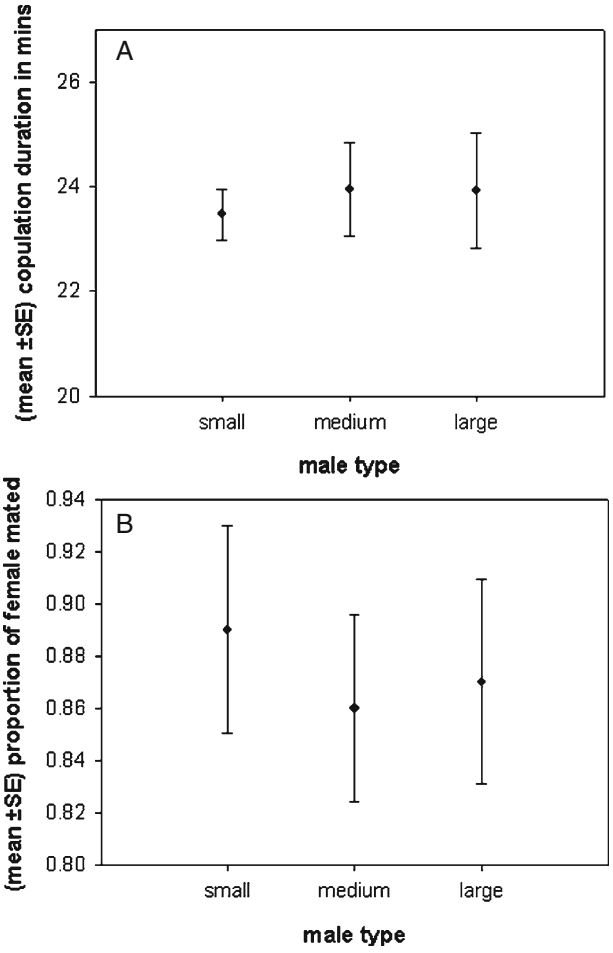

Figure 2. (A) Effect of male body size on CD (average across the blocks). CD was not significantly different among the three types of males. (B) Effect of male size on proportion of females mated (average across the blocks). The number of females inseminated by small, medium or large males did not significantly differ from each other.

Table 1. Dry body weight, copulation duration of different male types and effect of male type on female fecundity.

	df	SS	*F*	*P*
Dry body weight				
Male type	2	0.0.015	133.142	<0.0001
Block	2	0.001	7.0923	0.002
Male type*block	4	0.001	4.563	0.003
Copulation duration				
Male type	2	8.371	0.858	0.429
Block	2	269.206	27.595	<0.0001
Male type*block	4	3.76	0.193	0.9413
Fecundity				
Male type	2	0.286	14.743	<0.0001
Block	2	0.119	6.167	0.004
Male type*block	4	0.025	0.663	0.619

Summary of two way ANOVA on dry body weight, copulation duration and fecundity of females with male type as the fixed factor crossed with random blocks.

females. Multiple comparisons indicated that in blocks 1 and 3, females mated to small males had significantly lower mean bacterial load (Figure 3) compared to those mated to medium and large males (Tukey's HSD, all $P < 0.015$). Bacterial load did not significantly differ between females mated to medium and large males (Tukey's HSD, all $P > 0.05$). In block 2, bacterial load of females mated to medium males was significantly different than of those mated to small males but there was no significant difference between the bacterial load of females mated to large and small males (Tukey's HSD, $P > 0.05$). However, the general trend of the means (i.e. females mated to small males having lower bacterial load compared to females mated to large males) was similar to that seen in the other two blocks (Figure 3). The variation in the result was primarily due to the fact that females mated to medium males in block 2 had higher bacterial loads than any other type of female in the experiment. When we restricted the analysis in block 2 to data from females mated to small

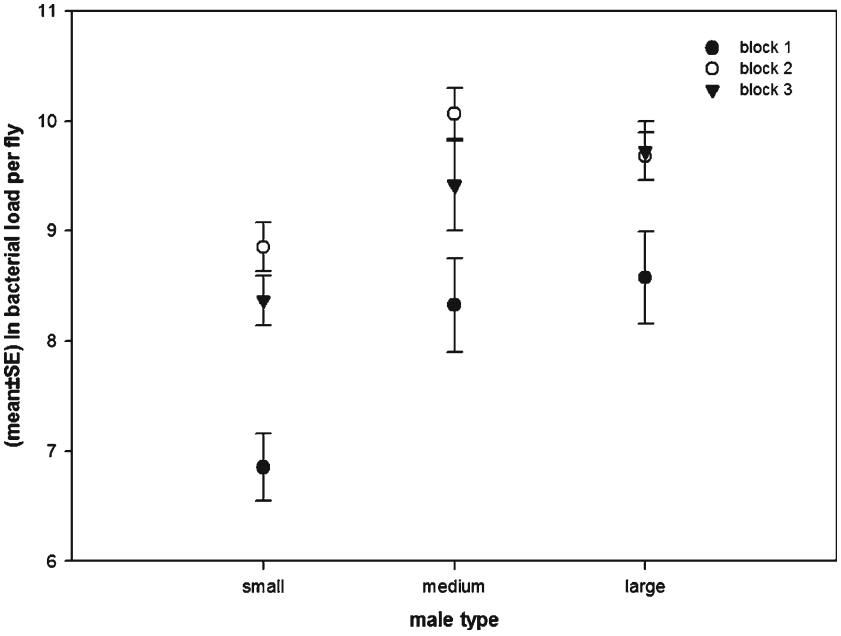

Figure 3. Effect of male size on bacterial load of females. Females mated to small males had significantly lower mean bacterial load compared to those mated to medium and large males.

males or large males only, we still found a significant effect of male type (nested ANOVA, df = 1, $F = 5.67$, $P < 0.038$) indicating that even in block 2, females mated to small males still had lower bacterial loads than females mated to large males. In fact, similar analysis in the other two blocks (with data from females mated to small and large males only) showed significant effect of male type within each of the blocks (nested ANOVA, all $P < 0.007$). To summarize, we found that bacterial load of females mated to small males was consistently lower than the bacterial load of females mated to large males. Sham infected flies failed to produce colonies in LB agar plates.

Reproductive fitness was found to be different in females mated to different types of males (Figure 4; Table 1). Although the females from the different blocks varied significantly in their fecundity, the general trend was highly consistent across the blocks. Separate one way ANOVA indicated a significant effect of male type on female fecundity within each block (all $P < 0.01$). In all the blocks, females mated to large males produced significantly fewer progeny than those mated to medium and small males (Tukey's HSD, all $P < 0.05$). However, number of progeny did not differ between females mated to medium and small males (Tukey's HSD, all $P > 0.05$).

Table 2. Effect of male type on female immune defence.

Factor	df	SS	*F*	*P*
Male type	2	33.677	18.328	<0.0001
Block	2	43.994	23.943	<0.0001
Male type* block	4	2.322	0.632	0.641
Plate (male type)	12	15.887	1.441	0.169

Summary of nested ANOVA on bacterial load with male type as the fixed factor, block as the random factor and replicate plates nested within male type.

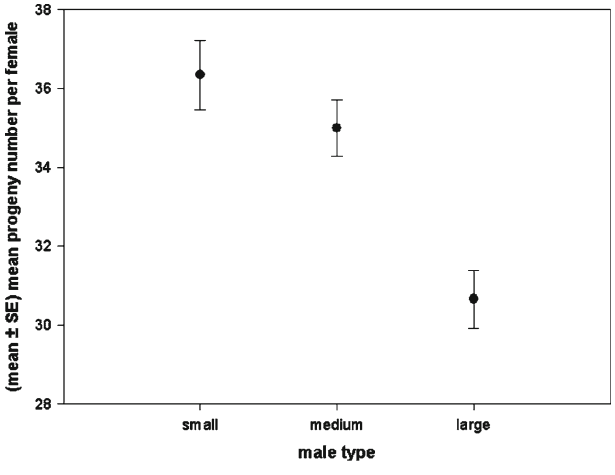

Figure 4. Effect of male size on fecundity of females (average across the blocks). Females mated to large males produced significantly fewer progeny than those mated to medium and small males.

Discussion

The primary objective of this study was to assess the effect of variation in male body size (used as a measure of male phenotypic variation) on two major components of female fitness—immune defence and fecundity. We clearly demonstrate that with a single mating exposure, immune defence of females mated to small males was significantly higher than that of the females mated to medium or large males. Additionally, fecundity of females mated to large males was significantly lower than that of the females mated to medium and small males. Taken together, these results indicate that, compared to mating with small males, mating with large males clearly imposes a higher fitness cost to females in terms of both fecundity and immune defence. While reduced fertility of females upon mating with large males has previously been reported (Pitnick 1991; Pitnick and Garcia-Gonzalez 2002; Friberg and Arnqvist 2003), our study is the first clear demonstration that male phenotype can affect the cost of mating to females in terms of their immune defence.

Many studies have documented the cost of mating to females and found that the cost is largely due to seminal fluid proteins transferred by the males during mating (Chapman *et al*. 1995). Specifically, sex peptides have been identified as a major cause for the observed mating cost in terms of fertility and lifespan (Wigby and Chapman 2005). Moreover, persistent courtship or coercion (male harassment) can also reduce subsequent female fitness (Lew *et al.* 2006). In our study, the male–female interaction was extremely brief and limited to a single mating. Hence the observed effect of male phenotype on female fitness can be largely attributed to an interaction between male ejaculate and female physiology, rather than courtship related behaviour.

One potential explanation for our results could be the differences in the proportion of females that mated with the three different types of males within the stipulated duration of interaction. While the effects of mating on female immune response have varied greatly across studies, a recent comprehensive study (Short and Lazzaro 2010) suggests that mating can decrease female immune response depending on the pathogen used. If one assumes that mating does reduce immune response in our experimental system and that a greater fraction of females were mated by larger males compared to smaller males, then the observed pattern of results follow. However, we reject this hypothesis since we found no significant difference between the fraction of females mated by small, medium and large males (see results section).

Given the above arguments, we conclude that the difference in female immune defence and fecundity observed in our experiment (Figure 3 & 4) is largely due to differences in the male ejaculate quantity and/or quality. CD has often been used as an index of male ejaculate investment (Friberg 2006; Bretman *et al.* 2009). In *Drosophila*, mating typically lasts for about 20 min and sperm transfer is completed during the first 6–8 min (Gilchrist and Partridge 2000). Hence a variation in CD is likely to indicate a variation in the amount of Acps transferred during mating. We found no significant difference in CD across the three types of males (Figure 2A; Table 1). This would seem to indicate that the three types of males transferred similar quantities of Acps. However, it is quite possible that the small males have a lower rate of Acp transfer due to their small size and therefore females mated to small males received a lower quantity of Acps despite there being no difference in CD. Additionally, the environmental manipulation that we used (varying larval density) to generate the three types of males might have led to other physiological changes in the males (in addition to changes in adult size) that might affect the quality of the ejaculate. However we did not assay either the qualitative or quantitative changes that might have occurred in the males used in our study and are hence unable to comment on this possibility.

Our results about the effects of male body size on female fecundity agree with those of previous studies (Pitnick 1991; Pitnick and Garcia-Gonzalez 2002; Friberg and Arnqvist 2003) in that females mating with larger males suffer reduced fecundity. While reduced CD during mating with larger males has been suggested as a cause of such a decline in fecundity (Pitnick 1991), we found no evidence in support of this suggestion. Other studies have documented the negative effect of male harassment, independent of mating, on female fecundity (Lew *et al.* 2006). However, our study clearly shows that negative effect of larger males on female fecundity can be manifested even with a single mating.

Our results on the effect of males on female immune defence vary from those of a recent study (Short and Lazzaro 2010) which investigated the effect of female genotype, male genotype and their interaction on the cost of mating to females in terms of antibacterial activity. The study found that while female genotype had a significant effect on the cost of mating, male genotype had no significant effect on female immunity costs. The difference in the results of the two studies is probably due to the magnitude of the variation among the males in the relevant traits influencing female cost of mating. Genetic variation identified in terms of different genotypes/strains/isofemale lines (Short and Lazzaro 2010) need not necessarily translate into variation in the relevant traits. Phenotypic manipulation, as was done in our experiments, can often dramatically increase the observed variation. For example, we know that the males from the three treatments in our experiment varied significantly in terms of their body weight. It is also possible that they varied in other relevant traits that were not measured. Hence, it is not surprising that we observed an influence of male phenotype on female fitness in our experiment.

Our study also has important implications in terms of the varying cost of mating to females and the consequent possibility of male–female coevolution. Previous studies have shown that mating carries a fitness cost to females in terms of both fecundity and lifespan (Fowler and Partridge 1989; Yanagi and Miyatake 2003; Kemp and Rutowski 2004),

and that this cost is increased by mating with large males (Pitnick 1991; Pitnick and Garcia-Gonzalez 2002; Friberg and Arnqvist 2003). Additionally, considerable genetic variation for 'male-induced harm' and 'female resistance to male-induced harm' exists in populations of *Drosophila* (Rice 1996; Linder and Rice 2005; Lew *et al.* 2006), leading to antagonistic co-evolution (intersexual conflict) of the sexes. This is confirmed by laboratory selection studies in which the 'harming' and 'resistance' traits rapidly evolve in response to manipulation of levels of intersexual conflict (Rice 1996; Holland and Rice 1999; Wigby and Chapman 2004). Our results show that females suffer an additional cost when mating with large males in terms of reduced immune defence. It has been suggested that males might promote female investment towards fecundity at the cost of immunity, thus promoting intersexual conflict over this trait (Fedorka *et al.* 2007; Lawniczak *et al.* 2007). While our results do suggest that immune defence can potentially be under intersexual conflict, they do not agree with the mechanism of altered resource allocation. Our results suggest a generalized female 'harm' while mating with large males rather than a reallocation of resources to fecundity over immunity.

Our results come from a specific model system (LH population of *D. melanogaster* with *S. marcescens*) and it is quite likely that in the wild, complex interactions involving male, female, pathogen and environment affect the cost of mating in terms of female immune defence. However, our results are likely to be quite general. This is because (i) *Drosophila*, in the wild, is often found under suboptimal larval densities (Atkinson 1979; Nunney 1990; Thomas 1993). The major consequence of this is a variation in adult body size. (ii) Being a natural pathogen to wide range of hosts, *Serratia* has also been isolated from *D. melanogaster* (Flyg *et al.* 1980; Flyg and Xanthopoulos 1983; Cox and Gilmore 2007) and it would not be surprising to find similar effects of other naturally occurring pathogenic bacteria on female immune defence. (iii) The reduction in female immune defence observed in our study was very likely a by-product of male–female antagonistic interactions. Such antagonistic interactions that lead to female 'harm' seem to be widespread, having been reported from varied populations (Pitnick 1991; Pitnick and Garcia-Gonzalez 2002; Friberg and Arnqvist 2003; Linder and Rice 2005), indicating the potential for widespread antagonistic coevolution. However, it is to be noted, that we used only phenotypic manipulation in our study to generate variation in males. If the kind of variation that we generated among males phenotypically, can in principle be mirrored by genetic variation, then, it can have major consequences for male–female coevolution. However, as of now, no such genetic variation has been identified.

To summarize, our study found that both fecundity and immune defence of females mated to small males was significantly higher than those of the females mated to large males implying a significant effect of male phenotype on the cost of mating suffered by females. This effect was, very likely, mediated through a qualitative/quantitative difference in the male ejaculates and can potentially lead to intersexual antagonistic co-evolution.

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