Female and male genetic contributions to post-mating immune defence in female *Drosophila melanogaster*

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Post-mating reduction in immune defence is common in female insects, and a trade-off between mating and immunity could affect the evolution of immunity. In this work, we tested the capacity of virgin and mated female *Drosophila melanogaster* to defend against infection by four bacterial pathogens. We found that female *D. melanogaster* suffer post-mating immunosuppression in a pathogen-dependent manner. The effect of mating was seen after infection with two bacterial pathogens (*Providencia rettgeri* and *Providencia alcalifaciens*), though not after infection with two other bacteria (*Enterococcus faecalis* and *Pseudomonas aeruginosa*). We then asked whether the evolution of post-mating immunosuppression is primarily a 'female' or 'male' trait by assaying for genetic variation among females for the degree of post-mating immune suppression they experience and among males for the level of post-mating immunosuppression they elicit in their mates. We also assayed for an interaction between male and female genotypes to test the specific hypothesis that the evolution of a trade-off between mating and immune defence in females might be being driven by sexual conflict. We found that females, but not males, harbour significant genetic variation for post-mating immunosuppression, and we did not detect an interaction between female and male genotypes. We thus conclude that post-mating immune depression is predominantly a 'female' trait, and find no evidence that it is evolving under sexual conflict.

Keywords: immune defence; mating and reproduction; *Drosophila melanogaster*; genetic variation; trade-off; sexual conflict

1. INTRODUCTION

Immune defence (defined as the combined ability of an organism to both actively fight and to tolerate an infection (Ayres & Schneider 2008)) is generally considered to be costly in that its maintenance and deployment often results in physiological and evolutionary trade-offs against other traits important for fitness, including longevity (Moret & Schmid-Hempel 2000; DeVeale *et al*. 2004; Ye *et al*. 2009), larval ability to compete for food (Kraaijeveld & Godfray 1997), body size (Fellowes *et al*. 1999), fertility (Ye *et al*. 2009) and fecundity (Fellowes *et al*. 1999; McKean *et al*. 2008). It has been proposed that mating may have immunosuppressive effects in females in order to allow limited resources to be shunted from immunological requirements to reproductive needs (Sheldon & Verhulst 1996). Examples of post-mating immune depression include a reduction in phenoloxidase activity after mating in the beetle *Tenebrio molitor* (Rolff & Siva-Jothy 2002), and decreased encapsulation ability correlated with increased oviposition in damselflies (Siva-Jothy *et al*. 1998). Additionally, mating causes decreased survival after infection with a pathogen in female *D. melanogaster* (Fedorka *et al*. 2007) but see (McKean & Nunney 2005; Wigby *et al*. 2008), and increased mating effort leads to decreased hemocyte number, lytic activity and encapsulation ability in the cricket *Allonemobius socius* (Fedorka *et al*. 2004). Post-mating immunosuppression may not be universal, as it

was not detected in yellow dung flies *Scathophaga stercoraria* (Schwarzenbach *et al*. 2005), and phenoloxidase activity and parasite resistance are even increased after mating in female *A. socius* and *Gryllus texensis*, respectively (Fedorka *et al*. 2004; Shoemaker *et al*. 2006). Determining how and why immune defence is altered by mating (or other fitness-related activities) is crucial to our understanding of how immune defence evolves, as well as how it functions at the whole-organism level.

The immune system of *D. melanogaster* is well understood, and extensive genetic analysis has revealed many of the genes involved in the function of the humoral and cellular immune response (reviewed in Lemaitre & Hoffmann 2007). This work has mainly focused on the function of the canonical immune system; but pleiotropic connections to mating (or other costly processes) have the potential to dramatically alter or limit the function and evolution of overall levels of defence (reviewed in Lawniczak *et al*. 2007). In the present study, we first determined whether mating affects the function of female immune defence. We used multiple pathogens in order to establish the generality of the phenomenon and to elucidate the potential importance of pathogen diversity on changes in defence owing to mating. We were also interested in determining the role that this trade-off could play in shaping the evolution of immune defence. We therefore assessed the level of genetic variation among females for the reduction in defence they experience after mating and among males for the level of post-mating immunosuppression they elicit in their mates. We also determined whether the change in defence

is dependent on the particular combination of male and female genotypes engaging in copulation. We measured genetic variation in both sexes because we were particularly interested in assessing the potential for ongoing sexual conflict in this system, as it has been suggested in the literature that the fitness of males and females may be affected differently depending on the level of immunosuppression females experience after mating, and that this could lead to sexual conflict (Fedorka *et al.* 2007; Lawniczak *et al.* 2007). This hypothesis could be provisionally supported by data we have collected, which suggest that the male ejaculate plays a role in reducing female defence (S. M. Short, M. F. Wolfner & B. P. Lazzaro 2010, unpublished data). Furthermore, components of the seminal fluid have been demonstrated to be involved in dynamic evolutionary interactions such as sexual conflict (Rice 1996), and multiple proteins in the male ejaculate of *D. melanogaster* have been shown to be rapidly evolving (Swanson *et al.* 2001; Mueller *et al.* 2005). If extant genetic variation in female immunosuppression was maintained by an ongoing intersexual interaction (e.g. sexual conflict) in our sampled population, we could expect to observe that the magnitude of post-mating immunosuppression is determined by the specific male and female genotypes participating in a mating (Gillespie & Turelli 1989). *D. melanogaster* possess significant genetic variation for immune defence (e.g. Lazzaro *et al.* 2004; Tinsley *et al.* 2006), and variation in the immunological cost incurred by mating is one potential source of that genetic variation.

2. MATERIAL AND METHODS

(a) Fly stocks and maintenance
We used the following lines of *D. melanogaster*: Canton S (a wild-type inbred strain), and 18 lines chosen randomly from the *Drosophila* Genetic Reference Panel (DGRP), a collection of inbred isofemale lines collected in Raleigh, NC (Ayroles *et al.* 2009). Each line is genetically distinct and the total set represents a 'snapshot' of naturally occurring genetic variation in this population at the time of sample. Nine DGRP lines (coded 1F–9F) were used to assay female variation. These were RAL-324, RAL-362, RAL-820, RAL-639, RAL-375, RAL-315, RAL-437, RAL-786 and RAL-486. Nine different DGRP lines (coded 1M–9M) were used to assay male variation. These were RAL-391, RAL-774, RAL-358, RAL-303, RAL-380, RAL-712, RAL-732, RAL-208 and RAL-360. Flies for all experiments were reared at $24°C$ on a 12 h light–dark cycle on standard glucose medium (12 g agar, 100 g glucose and 100 g Brewer's yeast per 1.2 l of water, plus 0.04% phosphoric acid and 0.4% propionic acid (final concentration) added to inhibit microbial growth in the food).

(b) Experimental design
To test the effect of mating on bacterial load and survival after infection with multiple pathogens, we conducted multiple experiments (one per pathogen) each in a complete block design, where both virgin and mated females were assayed for either bacterial load or survival in each replicate of the experiment. To test for genetic variation across lines from the DGRP, we used females from nine lines (coded 1F–9F) and males from nine additional lines (coded 1M–9M). The experiment was conducted in a manner similar to a lattice square design, with minor departures from

classical set-up owing to experimental contingencies. Bacterial load data for virgin and mated females were collected for all 81 pairwise crosses between all nine 'F' lines and all nine 'M' lines, with the entire experiment conducted in duplicate. Owing to the labour involved in assaying infection phenotypes in a crossing scheme of this scale, we opted to measure only the bacterial load phenotype in this part of the experiment. We feel that this is justified in that mated females sustain significantly higher bacterial loads and significantly higher mortality than virgins do after infection with *Providencia rettgeri* (figures 1*a* and 2*a*), so either phenotype is a reliable indicator of overall defence. Additionally, because of the magnitude of the experiment, data for all of the 81 pairwise crosses (comprising a single replicate of the entire experiment) were collected over 9 days. On each day, nine of the 81 pairwise combinations were observed, with females from each 'F' line mated to males from a single, randomly assigned 'M' line, such that all 'F' lines and all 'M' lines were used each day. At the end of the 9-day experiment, all 'F' lines had been paired to all 'M' lines once, with data for virgin and mated females from each of these 81 combinations recorded. The randomization scheme for this experiment was generated using the 'plan' procedure in SAS (SAS Institute, Cary, NC, USA). On any given day, we shuffled the order in which each 'F' line and each 'M' line was mated and infected using the 'sample' function in R (R Foundation for Statistical Computing, Vienna, Austria).

(c) Mating set-up
All matings were set up individually between a single virgin female and a single virgin male. All flies (males and females) were collected as virgins and aged 3 days post-eclosion with ad libitum access to food in groups of approximately 30. The day before matings were to be set up, virgin females were anaesthetized with CO_2 and placed in individual vials containing abundant media. They were then randomly allocated to 'virgin' or 'mated' treatment. Females were allowed to recover overnight. The next morning (within 3 h of incubator 'dawn'), unanaesthetized virgin males were aspirated into each vial assigned to the 'mated' treatment and each mating was individually observed. Matings lasting less than 15 min were not used for the experiments testing multiple pathogens, but this lower bound was reduced to a minimum of 10 min in the experiment to assess genetic variation. This was done because many mating pairs in this experiment copulated for shorter times than Canton S flies, possibly owing to natural variation in mating times. Lowering this boundary enabled inclusion of approximately 25 per cent of our final dataset, and therefore significantly increased our sample size. The number of 10 min matings were not equally distributed across genotypes (χ^2-test for the null hypothesis of equal distribution across lines for males: $\chi^2 = 228.73$, d.f. $= 8$, $p < 2.2 \times 10^{16}$ and females: $\chi^2 = 68.5$, d.f. $= 8$, $p < 9.7 \times 10^{12}$), but the average length of mating did not correlate with change in bacterial load (for all 81 genotype combinations: $r = -0.024$, $p = 0.83$), so we are confident that the inclusion of these shorter matings did not bias the results of our study. After mating, mated females were removed from the presence of males.

(d) Infection procedure
Two to three hours after mating cessation, mated females and their virgin counterparts were alternately anaesthetized in groups of 15 or fewer on CO_2 and pricked in the thorax
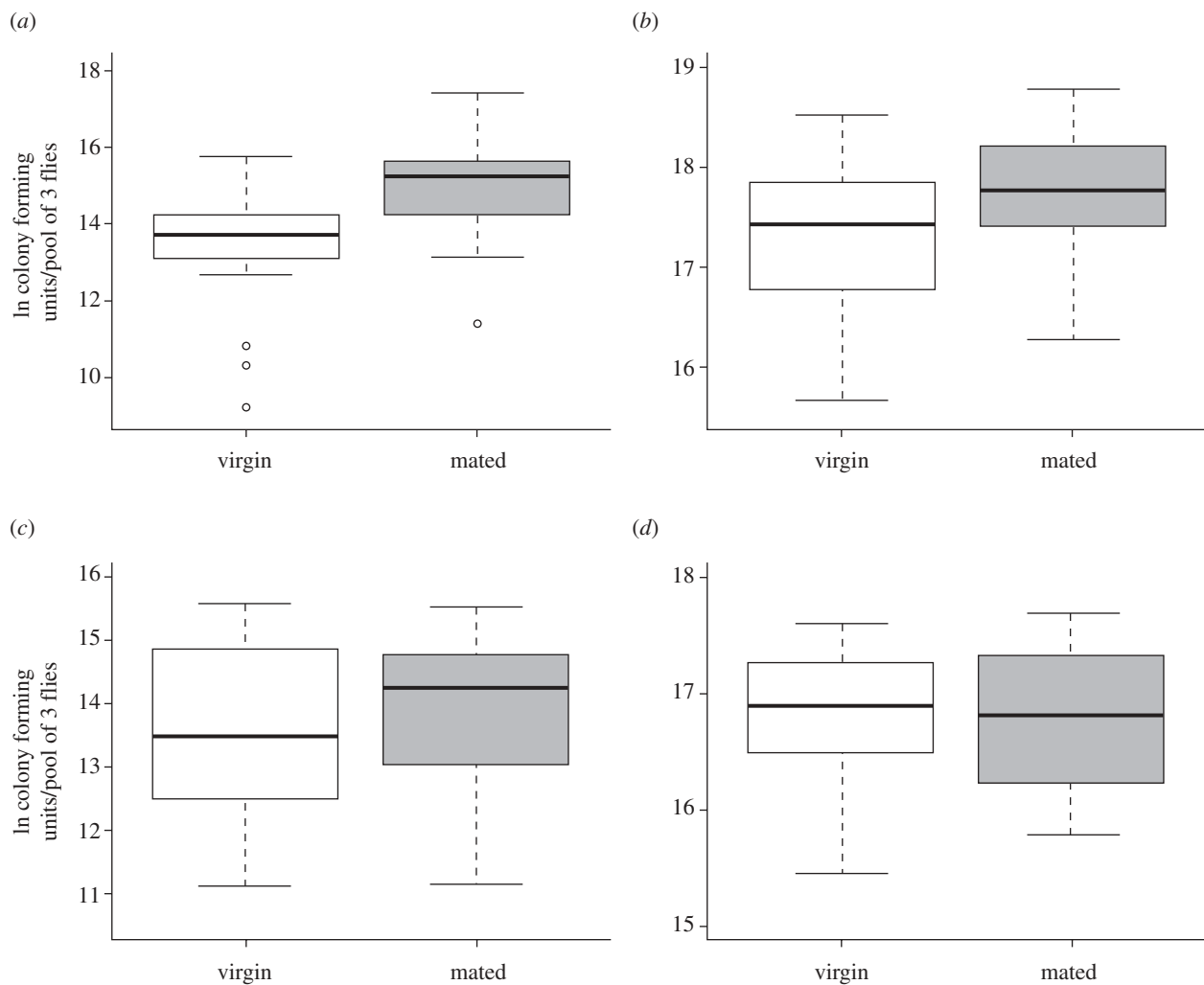
(a)

(b)

(c)

(d)

Figure 1. The effect of mating on female bacterial load after infection with four bacterial pathogens. Bacterial loads of wild-type (Canton S) females mated to wild-type (Canton S) males were significantly higher than those of virgin wild-type (Canton S) females after infection with (a) *P. rettgeri* ($F_{1,67} = 28.77$, $p < 0.0001$) and (b) *P. alcalifaciens* ($F_{1,77} = 9.86$, $p = 0.0024$), but not after infection with (c) *E. faecalis* ($F_{1,52} = 1.20$, $p = 0.279$) or (d) *Ps. aeruginosa* ($F_{1,32} = 0.17$, $p = 0.6804$). We infected virgin and mated females in parallel 2–3 h after mated females completed copulation. Total sample sizes were as follows: for *P. rettgeri*, $n_{mated} = 36$ and $n_{virgin} = 35$ for *P. alcalifaciens*, $n_{mated} = 43$ and $n_{virgin} = 38$, for *E. faecalis*, $n_{mated} = 28$ and $n_{virgin} = 28$, and for *Ps. aeruginosa*, $n_{mated} = 17$ and $n_{virgin} = 18$. Each data point consists of three pooled females, and data were collected over three replicates for each bacterial species with the exception of *Ps. aeruginosa*, for which only two replicates were collected. Uninfected controls (not shown) were sham-infected with a sterile needle and always yielded zero bacteria.

with a needle dipped in dilute bacterial culture (see below). Females were then placed in a vial containing media to recover. A subset of flies from each mating treatment was pricked with a sterile needle as a wounding control. Bacterial species used for infection were as follows: *P. rettgeri* (isolated from wild-caught *D. melanogaster* by B. Lazzaro in State College, PA, USA), *Providencia alcalifaciens* (isolated from wild-caught *D. melanogaster* by P. Juneja and S. Short in Ithaca, NY, USA), *Enterococcus faecalis* (isolated from wild-caught *D. melanogaster* by B. Lazzaro) and *Pseudomonas aeruginosa* (species type strain, PAO1) (table 1). *P. rettgeri*, *P. alcalifaciens* and *Ps. aeruginosa* are all Gram-negative bacteria, while *E. faecalis* is Gram-positive. All of these species are opportunistic pathogens with broad host ranges, and all have the ability to infect humans (Devriese *et al.* 2006; Manos & Belas 2006; Yaher & Parsek 2006). All bacterial cultures were grown overnight in Luria broth (LB) at 37°C from a single bacterial colony. Each overnight culture was then diluted with sterile LB to O.D.$_{600}$ = 1.0, with the exception of *E. faecalis*, which was diluted to O.D.$_{600}$ = 0.5. This

resulted in delivery of approximately 3×10^3 bacterial cells to each infected female with *P. rettgeri* and *P. alcalifaciens*, approximately 1×10^4 with *Ps. aeruginosa* and approximately 5×10^2 with *E. faecalis*.

(e) *Bacterial load assay*

To assay bacterial load, females were aged after infection with ad libitum access to food for either 24 ± 0.5 h (for the experiments represented in figures 1 and 2) or 26–28 h (for the genetic variation experiment). At this time, females were anaesthetized on CO_2 and homogenized in 500 µl LB in pools of three (figures 1 and 2) or five (genetic variation experiment). The homogenate was diluted 1 : 100 for *E. faecalis*, 1 : 1000 for *P. rettgeri*, and 1 : 10 000 for *P. alcalifaciens* and *Ps. aeruginosa* prior to plating. Fifty microlitres of each diluted homogenate was deposited in a spiral pattern on LB agar using a WASP II spiral plater (Microbiology International, Bethesda, MD, USA), and plates were incubated overnight at 37°C. We verified that the colonies on the plate were of the species used for infection by visual
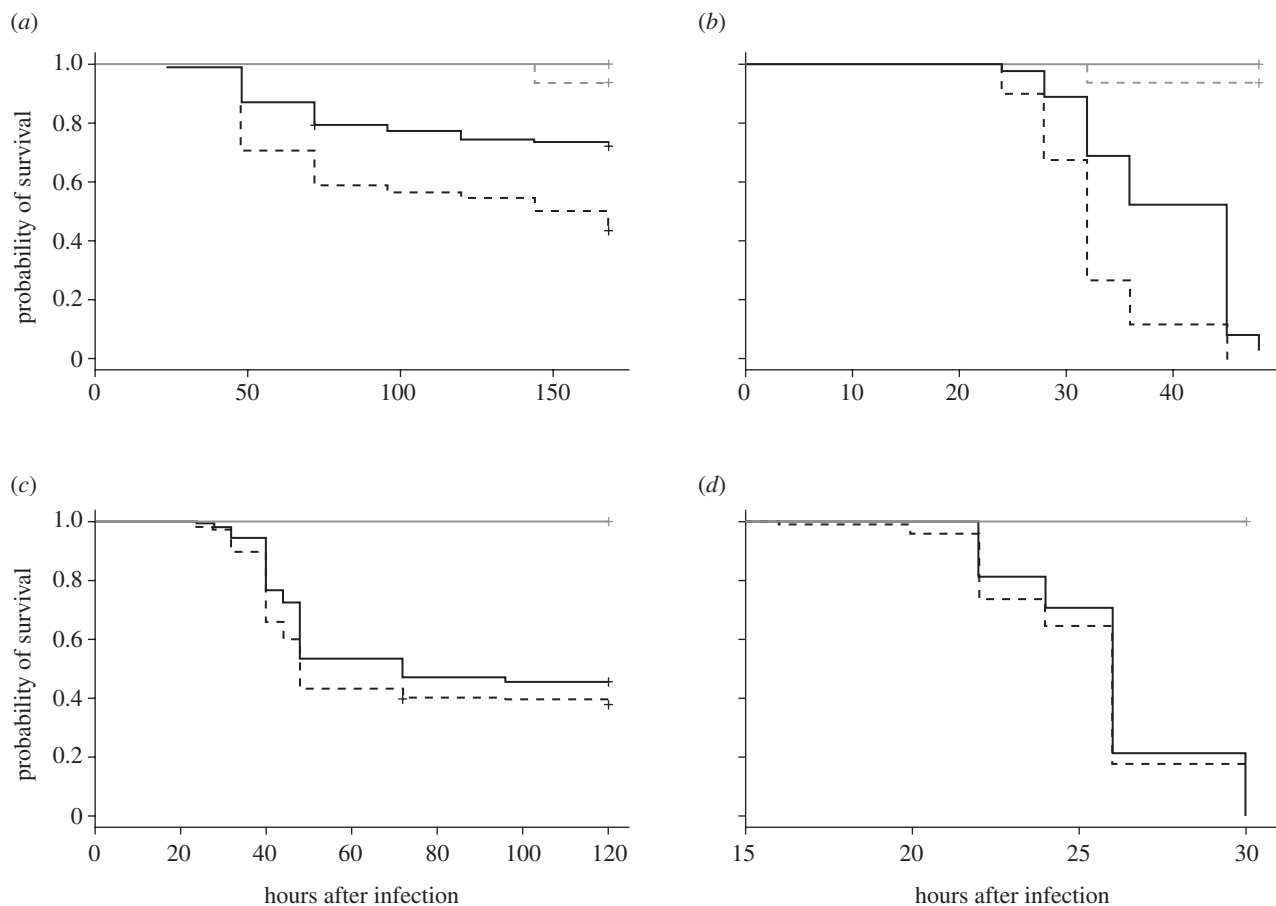
Figure 2. The effect of mating on female survival after infection with four bacterial pathogens. Survival over time of wild-type (Canton S) females mated to wild-type (Canton S) males was significantly lower than that of virgin wild-type (Canton S) females after infection with *P. rettgeri* (panel (*a*), $p < 0.0001$) and *P. alcalifaciens* (panel (*b*), $p < 0.0001$), but not after infection with *E. faecalis* (panel (*c*), $p = 0.0811$) or *P. aeruginosa* (panel (*d*), $p = 0.3466$). Survival curves were estimated using the Kaplan–Meier method. Significance values are for the effect of mating treatment in infected females and were determined by Cox regression analysis. We infected both mated and virgin females in parallel 2–3 h after mated females complete copulation. $N = 44–75$ infected females per mating status per replicate, and two to four replicates were performed for each survival experiment. Uninfected controls pierced with a sterile needle (lines shown in grey) showed negligible mortality for both mated (dashed grey line) and virgin (solid grey line) treatments.

Table 1. Pathogens that vary in biology and virulence were used for infection of virgin and mated female *D. melanogaster*. Per cent mortality is averaged across virgin and mated females, and natural pathogens are those that have been isolated from the hemolymph and/or thoracic muscle of wild-caught *D. melanogaster* (see §2 for details).

pathogen	virulence level	natural pathogen
P. rettgeri	moderate (approx. 40% mortality)	yes
P. alcalifaciens	high (approx. 98% mortality)	yes
E. faecalis	moderate (approx. 60% mortality)	yes
Ps. aeruginosa	high (100% mortality)	no (strain PAO1)

inspection and periodic analysis of 16S rDNA. 16S rDNA was amplified from randomly selected colonies throughout the experiment using the primers fD1 and rP2, which amplify the rDNA of most eubacteria (Weisburg *et al.* 1991). PCR product from these colonies as well as from positive control colonies taken from a pure freezer stock of each bacterial species was digested with *Stu*I and/or *Msp*I, and the digested products were run on a 1 per cent agarose gel. Digest patterns of colonies taken from infected females always matched those of the pure freezer stock. Bacterial colonies were counted using the ProtoCOL plate counting system (Microbiology International) associated with the spiral plater, allowing estimation of the number of viable bacteria present in each pool of homogenized females. These were the primary data used for analysis. Seven to 16 data points were collected per replicate per treatment for each experiment in figure 1. For the genetic variation experiment, two to five data points were collected per treatment per replicate for each pairwise combination, yielding a total of 4–10 data points per treatment for each pairwise combination, with the exception of 6F × 2M, for which only a single replicate was obtained. Six plates with zero colonies were excluded from analysis for the genetic variation experiment, as these zero counts could represent either an absence of bacteria in the flies or a technical error in the plating process. Since we cannot definitively say the flies contained zero bacterial cells, we chose to exclude these data points. The excluded data represent less than 0.5 per cent of the dataset

and eliminating these six data points has a negligible effect on the outcome of the analysis.

(f) Survival assay

To assay survival, females were placed in groups of approximately 10 after infection and observed either daily (for slower-acting pathogens like *P. rettgeri*), or at shorter intervals for the first 48 h after infection (for fast-acting pathogens like *Ps. aeruginosa*). Females from both virgin and mated treatments were put onto fresh food every other day. Survival was observed for 7 days after infection with *P. rettgeri* owing to its gradually induced mortality, but only for 5 days for *E. faecalis* since most mortality occurs in the first 48 h after infection with this bacterium. Survival for *P. alcalifaciens* and *Ps. aeruginosa* was observed for 48 h or until all flies were dead.

(g) Statistical analysis

To assess the effect of mating on bacterial load, we first performed a natural log transformation on bacterial load values for each bacterial pathogen to produce data that more closely fit a normal distribution. We then performed an ANOVA for each bacterial pathogen to determine the effect of mating status on bacterial load. Assumptions for the ANOVA were evaluated by running diagnostic plots (fitted values versus residuals, residual normal quantile–quantile plot) and visually assessing heteroskadicity and normality of residuals. In cases where residuals were found to be non-normal (verified by Shapiro–Wilk test), deviation from normality was due to a few outlier points. Removal of outlier points did not change the significance of mating status, and ANOVA results were therefore considered to be robust. These analyses were performed in SAS (SAS Institute).

To analyse our survival data, we used Cox regression analysis in SAS (SAS Institute) to determine the effect of mating status on survival over time. Event data were recorded for each fly (where an 'event' = death), and flies not dead by the last time point recorded were treated as censored data. Survival curves were generated using the Kaplan Meier method in R (R Foundation for Statistical Computing).

To determine the level of genetic variation between lines from the DGRP, we first performed a natural log transformation on the bacterial load data collected from females from each pairwise mating combination. We then performed an analysis of variance with Proc Mixed in SAS (SAS Institute) using the following mixed model:

$$Y_{ijkl} = \mu + \text{mating status}_i + \text{female genotype}_j$$
$$+ \text{male genotype}_k + \text{replicate experimental day}_l$$
$$+ \text{mating status}_i * \text{female genotype}_j$$
$$+ \text{mating status}_i * \text{male genotype}_k$$
$$+ \text{female genotype}_j * \text{male genotype}_k$$
$$+ \text{mating status}_i * \text{female genotype}_j * \text{male genotype}_k$$
$$+ \varepsilon_{ijkl}$$

where $Y = \ln(\text{bacterial load})$ data taken from all females, Mating status$_i$ $(i = 1,2)$ represents whether females were virgin or mated, female genotype$_j$ $(j = 1,9)$ represents the DGRP lines contributing females to crosses, male genotype$_k$ $(k = 1,9)$ represents the DGRP lines contributing males to crosses and replicate experimental day$_l$ $(l = 1,20)$ is a factor including all days over which the experiment was conducted. Each replicate required 9 days, and two replicates were

performed for a total of 18 days. Missing data were subsequently filled in over 2 additional days, resulting in d.f. = 19 for day in the model. The factor mating status$_i$ * female genotype$_j$ tests for genetic variation among females for post-mating immunosuppression, while mating status$_i$ * male genotype$_k$ tests for male genetic variation for the level of immunosuppression they elicit in their mates. Mating status$_i$ * female genotype$_j$ * male genotype$_k$ tests whether the effect of a particular male or female genotype on post-mating immunosuppression depends on the genotype of their mate.

Mean bacterial loads for each female and male genotype were obtained by finding the arithmetic mean of the log-transformed bacterial load data and back transforming it to obtain the geometric mean. We then calculated 95% confidence intervals (Sokal & Rohlf 1995).

3. RESULTS AND DISCUSSION

(a) Mating reduces female immune defence against two natural bacterial pathogens

It is yet unclear how ubiquitous post-mating reduction in immune defence is in insects. Many experiments testing potential trade-offs between immunity and defence have relied on indirect measures of immune quality (e.g. encapsulation ability, phenoloxidase activity or antimicrobial peptide gene expression) in the absence of actual infection (Siva-Jothy *et al.* 1998; Rolff & Siva-Jothy 2002; Fedorka *et al.* 2004; Lawniczak & Begun 2004; McGraw *et al.* 2004; Peng *et al.* 2005). While informative with regard to the potential mechanisms linking mating and the immune system, these assays do not directly measure changes in the ability of an organism to resist or tolerate an infection, and must be interpreted with care (Adamo 2004). Other studies have measured overall defence as a function of mating in the context of experimental infection (McKean & Nunney 2001, 2005; Shoemaker *et al.* 2006; Fedorka *et al.* 2007; Wigby *et al.* 2008). Three of the cited studies have been performed using female *D. melanogaster* (McKean & Nunney 2005; Fedorka *et al.* 2007; Wigby *et al.* 2008), but no clear consensus has emerged even from those as to whether females suffer a meaningful reduction in immune defence after mating. Two of these studies (McKean & Nunney 2005; Wigby *et al.* 2008) show no change owing to mating in the ability of females to clear non-pathogenic bacteria, while Fedorka *et al.* (2007) demonstrated that females infected with a pathogenic bacterium suffer higher mortality if they have mated. We hypothesized that the lack of consensus in this body of literature could be due to the effect of mating being dependent on the assay used to measure defence and/or the microbe used to test changes in defence (for example, pathogenic versus non-pathogenic infection). We, therefore, tested the effect of mating on female immune defence using two different assays (survival and systemic bacterial load) and four pathogens that differ in biology and pathogenicity (table 1).

We infected female *D. melanogaster* of the strain Canton S with each of the four bacterial pathogens in table 1, 2–3 h after mating cessation. We also infected virgin females in parallel to serve as a control comparison. We then assayed bacterial load (i.e. the number of colony forming units present in a fly) and survival in mated and

virgin females after infection with each bacterial species. Females pierced with a sterile needle yielded zero bacterial colonies and had negligible mortality. At 24 h after infection with *P. rettgeri* (figure 1*a*) or *P. alcalifaciens* (figure 1*b*), we observed significantly higher bacterial loads in mated females compared with virgin females (*P. rettgeri*: $p < 0.0001$, *P. alcalifaciens*: $p = 0.0024$). We also observed significantly reduced survival in mated females compared with their virgin counterparts after infection with either *P. rettgeri* (figure 2*a*, $p < 0.0001$) or *P. alcalifaciens* (figure 2*b*, $p < 0.0001$). In contrast, we observed no difference in bacterial load owing to mating after infection with either *E. faecalis* (figure 1*c*, $p = 0.279$) or *Ps. aeruginosa* (figure 1*d*, $p = 0.6804$), and no effect of mating on survival after infection with *E. faecalis* (figure 2*c*, $p = 0.0811$) or *Ps. aeruginosa* (figure 2*d*, $p = 0.3466$). Virgins infected with *E. faecalis* had a slightly (but not significantly) higher probability of survival at multiple time points (e.g. mean per cent survival at 5 days post infection for virgin $= 46.5\%$ and for mated $= 37.6\%$), but this effect was apparent in only two of the four replicates in the experiment (difference between treatments in two of the four replicates considered alone: $p = 0.0164$; in the other two replicates alone: $p = 0.8587$).

Our data show that mating results in reduced defence for females after infection with at least two pathogenic species of bacteria, both of which are pathogens of wild *D. melanogaster*. These results, coupled with previous findings showing no effect of mating in females after infection with a non-pathogenic bacterium (McKean & Nunney 2005; Wigby *et al.* 2008), suggest that, while general immune maintenance and immunocompetence are not impaired after mating, the ability of females to defend against pathogenic infection is hindered. Interestingly, and in contrast to our study, Fedorka *et al.* (2007) also used *Ps. aeruginosa* and *did* detect post-mating immunosuppression, suggesting that the magnitude of the effect may vary over bacterial strains, host genotypes or experimental conditions. Nevertheless, the total data suggest that the quality of immune defence in female *D. melanogaster* is frequently modulated by mating, an activity that is itself clearly essential to fitness.

(b) *Female, but not male,* D. melanogaster *harbour significant genetic variation for the effect of mating on immune resistance*

In order to gain insight into the evolution of the trade-off between mating and immunity, we assayed genetic variation among females for their resistance to infection after mating, genetic variation among males for their ability to alter female resistance and the degree to which the magnitude of post-mating immune depression depends on the specific male and female genotypes in mating pairs. We mated females from nine genetic lines of *D. melanogaster* to males from nine distinct genetic lines and, for all 81 pairwise crosses, assayed bacterial load after infection with *P. rettgeri* in both virgin and mated females. The entire experiment of 81 crosses was performed in duplicate, with two to five data points collected per cross in each replicate, where each data point is obtained from a pool of five females. We then performed an analysis of variance on the bacterial load data using the model in table 2.

Table 2. Analysis of variance for effects of male and female genotype on bacterial load in mated versus virgin females. The experiment was conducted over the course of multiple days. 'Experimental replicate day' refers to all days over which the experiment was conducted. 'Mating status' refers to mated females versus virgins.

factor	effect type	d.f.	F value	p-value
mating status	fixed	1	36.23	<0.0001
female genotype	fixed	8	26.42	<0.0001
male genotype	fixed	8	2.30	0.0193
experimental replicate day	random	19		
mating status * female genotype	fixed	8	4.32	<0.0001
mating status * male genotype	fixed	8	0.61	0.7730
female genotype * male genotype	fixed	64	0.078	0.0777
mating status * female genotype * male genotype	fixed	64	1.16	0.1905
residual error		1129		

Females are highly significantly genetically variable for the degree of post-mating immune depression that they experience (mating status * female genotype, $p < 0.0001$, table 2 and figure 3). The bacterial load of mated females relative to virgins ranged across female genotypes from a 4.1-fold increase to essentially no change (figure 3). To our surprise, however, we did not observe significant genetic variation in the ability of males to suppress female immune defence (mating status * male genotype, $p = 0.7730$, table 2 and figure 4). The bacterial load of mated females relative to virgins was relatively invariant across the male genotypes to which they mated, with the smallest change being a 1.4-fold increase and the largest a twofold increase. Such low levels of genetic variation among males were unexpected because female immune depression occurs only when the male ejaculate is intact with respect to sperm and accessory gland proteins (S. M. Short, M. F. Wolfner & B. P. Lazzaro 2010, unpublished data). Despite that, and despite the known adaptive evolution of some male ejaculate proteins, we fail to reject the null hypothesis that males are not variable for the magnitude of female immune modulation they elicit. We also found no evidence that female post-mating immune depression is determined by an interaction between the specific male and female genotypes engaged in a mating (mating status * female genotype * male genotype, $p = 0.1905$, table 2), casting further doubt on any hypothesis that this trait is evolving under sexually antagonistic coevolution.

Our observation that females are highly genetically variable for the degree of post-mating immunosuppression they experience is consistent with a potential evolutionary trade-off between mating (and/or consequent reproduction) and immune defence. However, since we did not directly assay fitness in this experiment, we cannot definitively assess the possibility of an evolutionary trade-off. If such a trade-off does exist, the genetic variability we observe could reflect antagonistic pleiotropy coupled with spatial or temporal
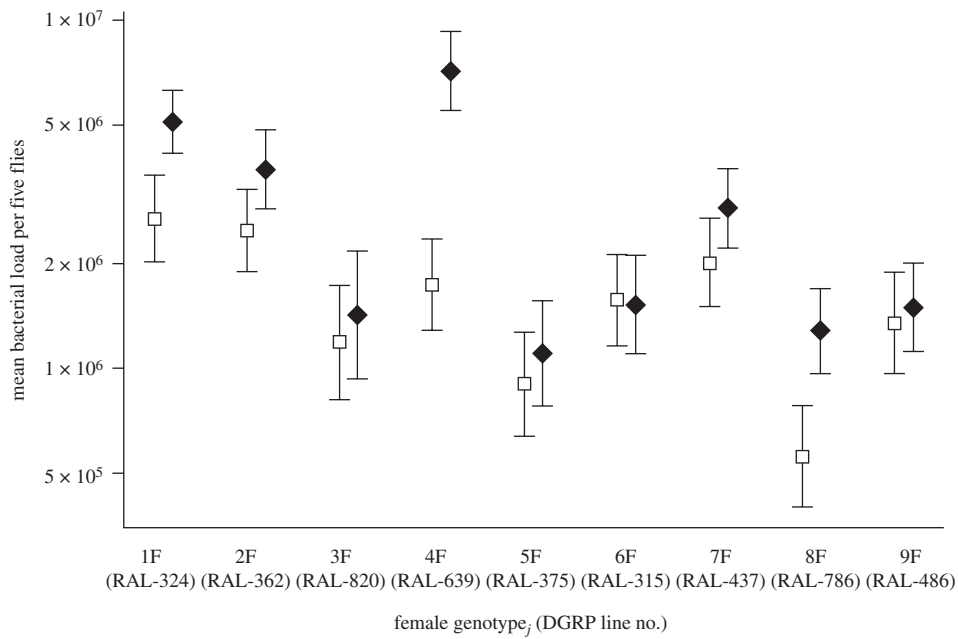
Figure 3. Variation in the effect of mating across female genotypes. We calculated corrected mean bacterial load for mated females (black diamonds) and virgin females (open squares) of each female genotype pooled across all male genotypes. For example, the black diamond for female 1F corresponds to the mean load sustained by 1F females after mating to males from genotypes 1M–9M, and the open square corresponds to loads sustained by virgin 1F females infected and plated alongside mated 1F females. Mean refers to geometric mean, and error bars represent a 95% confidence interval. The parenthetical numbers on the *x*-axis are the DGRP stock identity number.
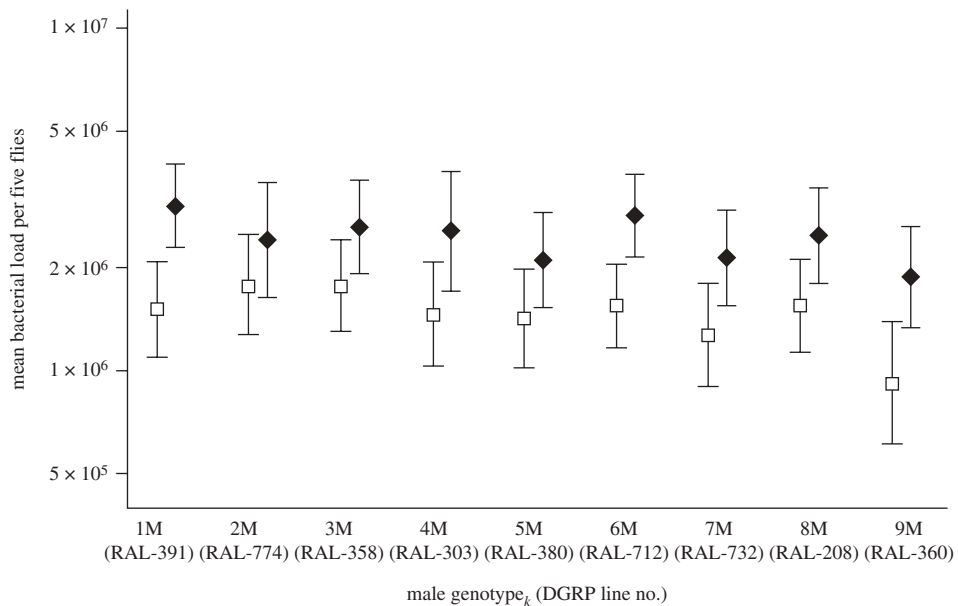

Figure 4. Variation in the effect of mating across male genotypes. We calculated corrected mean bacterial load for all females mated to each individual male genotype (black diamonds) and the mean bacterial load for the corresponding virgin controls from all female genotypes (open squares). For example, the black diamond for 1M corresponds to the mean of females from genotypes 1F–9F mated to 1M males, and the open square corresponds to the mean of virgin control females from lines 1F–9F infected and plated alongside the females mated to 1M males. Mean refers to geometric mean, and error bars represent a 95% confidence interval. The parenthetical numbers on the *x*-axis are the DGRP stock identity number.

environmental variation. In this scenario, conflicting selective pressures related to immunity and reproduction could lead to maintenance of genetic variation (Gillespie & Turelli 1989; Lazzaro & Little 2009). The observation reported here and in McKean & Nunney (2005), Fedorka *et al.* (2007), Wigby *et al.* (2008) that mating induces susceptibility to some infections more than to others suggests

that microbial heterogeneity might be one such example of environmental variation.

It has been hypothesized that ongoing sexual conflict could manifest in manipulation of female immune defence, such that males could potentially increase their fitness by reducing female immune defence in favour of reproduction (e.g. Lawniczak *et al.* 2007; Fedorka *et al.*

2007). However, the fact that we did not observe significant male genetic variation for post-mating female immunosuppression renders this hypothesis unlikely. Our data are not consistent with evolution of this trait being driven by ongoing sexual conflict.

4. CONCLUSIONS

In this work, we showed that female *D. melanogaster* become more susceptible to infection with two different natural bacterial pathogens after mating. Mated females sustained higher bacterial loads and lower survival compared with virgins. However, infection with two other pathogens was not more severe in mated females relative to virgins, revealing the mating effect to be pathogen-dependent. Wild females harbour substantial genetic variation for the magnitude of post-mating susceptibility they experience, but males harbour little if any genetic variability for the degree of immunosuppression they can drive. This effectively eliminates ongoing interlocus sexual conflict as a possible evolutionary scenario under which this trait could be evolving in the sampled population. It is much more likely that there is a physiological and perhaps evolutionary trade-off in females between reproduction and immune defence.

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