Journal of Animal Ecology

British Ecological Society

Journal of Animal Ecology 2016, **85,** 291–301 doi: 10.1111/1365-2656.12433

Immunosenescence and the ability to survive bacterial infection in the red flour beetle *Tribolium castaneum*

Imroze Khan*, Arun Prakash and Deepa Agashe*

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

Summary

1. In most animals, ageing is associated with a decline in immune function (immune senescence). However, different components of the immune system seem to age differentially, and many studies do not measure the ultimate fitness consequences of immune function after infection. Previous work shows that immune function may be traded off with other fitness components such as reproduction. It is possible that age alters the nature of these trade-offs, particularly in conjunction with factors such as gender and mating that can also affect investment in immune function.

2. We tested the impact of age, sex and mating on post-infection survivorship in *Tribolium castaneum* flour beetles, as well as the components of baseline constitutive innate immunity and external (secreted) immune function in uninfected individuals. We also tested whether the reproductive ability of uninfected females is traded off with immune function (baseline innate and external immunity) and post-infection survivorship across age groups.

3. We found that age, sex and mating significantly affected immune components and infection outcome, although the magnitude and nature of the impact varied in each case. We found that older beetles were more susceptible to infection by the pathogen *Bacillus thuringiensis* even though major components of the constitutive innate immune defence (antibacterial and phenoloxidase activity) remained unchanged or improved with age. Thus, these aspects of innate immunity cannot explain the observed decline in post-infection survival of older beetles. We did not find trade-offs between the reproductive ability of uninfected females and their immune function. In contrast to innate immunity, external immunity showed an overall decline with age but was also affected by sex and mating. Finally, we show that bacterial infection alters external immunity via complex interactions between age, sex and mating status.

4. Our work uncovers novel interactions between age, sex and mating that can determine the evolution and outcome of immunosenescence by affecting the time course of relative investment in different immune and fitness components.

Key-words: antibacterial activity, external defence, life history, phenoloxidase activity, reproductive senescence, sexual dimorphism

Introduction

In animals, ageing is usually accompanied by a general decline of immune function. Such age-related impairment of the immune system (immunosenescence) increases susceptibility to infections and contributes to inflammatory disease, leading to increased morbidity and mortality (DeVeale, Brummel & Seroude 2004; Shanley *et al.* 2009). However, different components of the immune system do not age consistently across species, individuals and life stages (e.g. reviewed in DeVeale, Brummel & Seroude 2004; Adamo 2004). Even in a well-studied model organism like *Drosophila melanogaster*, it is difficult to predict the relationship between various immune components and their combined impact on the ability to fight infections as a function of age. Some studies indicate an age-associated reduction in immune function: older flies had higher surface and internal pathogen loads (Ren *et al.* 2007), reduced haemocyte number and function (Horn, Leips & Starz-Gaiano 2014), reduced antimicrobial peptide production on infection with killed bacteria (Zerofsky *et al.*

*Correspondence author. E-mails: imrozek@ncbs.res.in (IK); dagashe@ncbs.res.in (DA)

2005) and were more likely to die from infection (Ramsden, Cheung & Seroude 2008). On the other hand, older flies up-regulated various immune-related genes (Zou *et al*. 2000; Pletcher *et al*. 2002; Landis *et al*. 2004). After infection with live bacteria, older flies also increased expression of antimicrobial peptide transcripts (Zerofsky *et al*. 2005) and had lower pathogen loads (Khan & Prasad 2013), suggesting an age-associated improvement in immune function. However, bacterial load after infection is not always correlated with survival (Corby-Harris *et al*. 2007). Finally, age had a variable impact on infection clearance across different fly lines, indicating genetic variation for immune senescence (Lesser, Paiusi & Leips 2006; Felix *et al*. 2012). While several genotypes improved their ability to clear bacterial infection with age, others either showed a decline or no change with age (Lesser, Paiusi & Leips 2006). A similarly complicated picture emerges across invertebrate species, for example in mosquitoes (Ariani *et al*. 2014) and especially in social insects in which community interactions and age-associated behavioural shifts add to the complexity of immune senescence outcomes (for a recent discussion, see Roberts & Hughes 2014).

What is the cause of the observed variation in components of immune function and their outcome with senescence? Broadly, variation in immune function has been attributed to its substantial costs, generating life-history and physiological trade-offs and leading to co-evolution between specific hosts and parasites (Sheldon & Verhulst 1996; Kraaijeveld & Godfray 1997; Rolff & Siva-Jothy 2003; Schmid-Hempel 2003; Schmid-Hempel & Ebert 2003; Siva-Jothy, Moret & Rolff 2005; Ardia, Parmentier & Vogel 2011). In many species, investment in reproduction is traded off with immune function (Richner, Christe & Oppliger 1995; Gustafsson *et al*. 1997; Siva-Jothy, Tsubaki & Hooper 1998; Verhulst, Dieleman & Parmentier 1999). If age alters the costs and trade-offs associated with each immune component, it might explain some of the variation in immune senescence. For instance, young individuals under strong selection to reproduce may maximize their reproductive investment by tightly regulating immunity and minimizing investment in costly immune functions. After reproductive senescence, weaker selection on immune regulation may result in an age-related immune activation due to misregulation. Different immune functions are also known to trade off with each other (Simmons & Roberts 2005; Cotter *et al*. 2008), and individual investment in different immune components may thus change with age. For instance, immune functions requiring higher energetic investment should show stronger trade-offs with reproductive fitness, and hence, starker contrasts between young and old animals. Finally, factors such as gender and reproductive status can determine the nature of trade-offs and investment in immune function (Bateman 1948; Trivers 1972). Several studies suggest that compared to females, males have lower immunocompetence (Kurtz *et al*. 2000; Kurtz & Sauer 2001) and reduced survival after infection (Moret & Schmid-Hempel 2000; Bedhomme *et al*. 2004; Joop *et al*. 2006; McNamara *et al*. 2013). Mating typically decreases immunity (Fowler & Partridge 1989), which may also differ in the sexes. For instance, in *Tenebrio* beetles, mating is associated with decreased phenoloxidase (PO) levels in females, but not in males (Rolff & Siva-Jothy 2002). Hence, immune senescence may change as a function of gender as well as mating status.

To begin to quantify the potentially complex interplay of age, sex, mating and immune function, we conducted experiments with the red flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae). In addition to the innate immune system, *Tribolium* beetles secrete defensive quinone compounds that inhibit the growth of microbes in their surroundings (for a recent discussion see Joop *et al*. 2014), and can be regarded as external (or secreted) immune function acting outside the body (reviewed in Otti, Tragust & Feldhaar 2014). These compounds are secreted by stink glands located on the thorax and abdomen and consist primarily of the potent antimicrobials benzoquinone and pentadecene (Li *et al*. 2013). Quinone production varies across *T. castaneum* genotypes (Prendeville & Stevens 2002), and in the closely related species *Tribolium confusum*, quinone production is highly heritable (Yezerski, Gilmor & Stevens 2004). Quinone biosynthesis depends on the availability of tyrosine, which is also the starting point of the PO cascade, a major component of innate immunity (Cerenius, Lee & Söderhäll 2008). This sets up the possibility of interactions between the innate immune system and external defence. In fact, recent work shows that RNAi-mediated knockdown of genes necessary for quinone biosynthesis reduces PO activity, indicating connected genetic networks (Li *et al*. 2013). A single beetle can store up to 50 μg quinones, representing ~0·05% of adult body weight (Sokoloff 1974; Unruh, Xu & Kramer 1998). Hence, quinone production may impose a large energetic burden on the animal and generate a phenotypic trade-off with investment in innate immunity. *Tribolium* beetles thus provide a unique opportunity to explore the evolution of these two modes of immune defence in conjunction with each other. For instance, recent work with burying beetles demonstrates trade-offs between internal and external immunity, showing that forced up-regulation of internal immunity reduced the externally secreted immune response (Cotter *et al*. 2013).

We describe a set of experiments to address five major questions about immune senescence using laboratory populations of *T. castaneum*. First, do age, sex and mating status affect beetle survival when challenged with the natural pathogen *Bacillus thuringiensis*? Secondly, is the observed variation in post-infection survival associated with changes in innate immune function (measured as baseline constitutive innate immunity without infection) as a function of beetle age, sex, and mating status? Thirdly, are the effects of age, sex and mating similar for

innate and external immune defence? Fourthly, does infection-induced internal immune function affect external immune defence? Finally, is there evidence for an age-specific trade-off between reproductive fitness of an individual and its immune function?

We find that baseline constitutive innate immunity and external defence in uninfected beetles show opposite patterns of change with age: while innate immune function improves with age, external defence decreases with age. However, the increase in the measured aspects of innate immunity fails to prevent higher mortality in infected old beetles. Importantly, we do not find evidence for trade-offs between reproductive fitness and innate immune function or external defence. Instead, interactions between age, sex and mating status alter both innate immunity and external defence. Finally, we find that bacterial infection affects external immunity of an individual depending on its age, sex and mating status. Our results demonstrate the importance of a joint assessment of internal immune traits and defensive secretions in an ageing animal. Our work thus provides new insights into functional immunity associated with senescence, highlighting the relevance of life-history parameters on post-infection survival.

Materials and methods

To ensure high genetic variability in experimental populations, we created a large outcrossed line of *T. castaneum* (>2000 adults/generation), allowing 20 breeding pairs each of six wild-caught lines (collected from across India) to oviposit for a week. We maintained this population on whole-wheat flour on a 45-day discrete generation cycle at 34 °C (±1 °C) for 8 months before starting our experiments. For all infections, we used *B. thuringiensis* DSM no. 2046 isolated from a Mediterranean flour moth (Roth *et al.* 2010), a natural pathogen of red flour beetles that decreases fitness and imposes significant mortality (Abdel-Razek *et al.* 1999; Hou, Fields & Taylor 2004). Female beetles from our population undergo reproductive senescence within 60 days post-pupation (Fig. S1). Hence, in our experiments, we used 17 and 77-day-old individuals (post-pupation) as 'young' and 'old' adults, respectively.

Since we could not carry out all experiments simultaneously, we generated four separate sets of experimental individuals (sets A, B, C and D). Set A was used to test the impact of age, gender and mating on post-infection survivorship and antibacterial activity. Set B was used to test the impact of age, gender and mating on external defence. Set C was used to test for the effect of bacterial infection on external defence, in conjunction with age, sex and mating status. Set D was used to test the impact of age and sex on PO activity of virgin beetles. Below, we give a brief summary of all experimental protocols; detailed methods are given in the supporting information.

To generate progeny for each age group, we allowed ~500 adults from the outbred line to oviposit in 500 g of wheat flour for 48 h and used the resulting offspring as experimental individuals. We housed male and female pupae separately in 1·5 mL microcentrifuge tubes (single sex tubes: 3 pupae/tube, 1 g flour, supplying fresh flour every 5 days) and held them as virgins until the 10th or 70th day post-pupation for 'young' or 'old' age groups. For sets A, B and C, we then randomly assigned adults within each age group to 'virgin' and 'mated' groups. For the virgin group, we held individuals in 48-well microplate filled with 0·5 g of flour for 7 days before subjecting them to further experiments. For the mated subset in sets A and B, we paired individuals with 10-day-old mates (post-pupation) in 48-well plate filled with 1 g of flour for 6 days, with an additional day for oviposition. In set C, within the mated group, individuals were paired with respective mates and held for 7 days before subjecting them to further experiments (without an additional day for oviposition). Mates were also held as virgins in similar rearing conditions as the experimental young males and females. Set D was comprised of only virgin beetles. Within a set, we subjected all adults across age, sex and mating status treatments to experiments on the same day. To quantify trade-offs between immune function and reproductive fitness, we first measured the number of offspring produced by each mated female in sets A and B (24 h oviposition in 1 g flour; eggs allowed to develop in 5 g flour for 21 days) and then assayed immune function and survival of each female.

To quantify post-infection survivorship, we infected beetles (septic injury method) between the head and thorax with a 0·1 mm minutien pin (Fine Science Tools) dipped into a slurry made from 2 mL overnight culture of *B. thuringiensis* [optical density (OD) of 1, measured at 600 nm; n = 15–21 beetles/sex/mating status/age group]. We carried out sham infection with a pin dipped in insect Ringer solution (n = 10 beetles/sex/mating status/age group). We recorded individual survival every 3 h until 24 h post-infection, and daily at 11 pm for the following 7 days. We quantified antibacterial activity of uninfected beetles using the protocol reported in Roth *et al.* (2010; modified from Faye & Wyatt 1980), measuring the zone of inhibition produced by whole-body beetle homogenate on a lawn of *B. thuringiensis* growing on nutrient agar plates (n = 17–19 beetles/sex/mating status/age group). We also tested antibacterial activity of virgin uninfected beetles against another entomopathogen *Serratia marcescens*, and a non-pathogenic strain of *Escherichia coli* (DH5) (n = 15–16 beetles/sex/age group/bacterial species). We measured the PO activity of virgin uninfected beetles across age groups (n = 10–11 beetles/sex/age group) as described in Li *et al.* (2013); we did not measure PO activity of mated beetles. Since antibacterial and PO activity assays were performed with uninfected beetles, they serve as a measure of changes in baseline constitutive innate immunity without any immune induction.

To measure external immune defence, we followed the protocol described by Prendeville & Stevens (2002), measuring the zone of inhibition produced by individual cold-shocked uninfected beetles embedded in a lawn of bacterial growth on nutrient agar plates (n = 17–18 beetles/sex/mating status/age group). The cold shock induces secretion of quinones from the stink glands. To measure the effect of prior infection on external defence, we randomly assigned young (or old) virgin beetles to one of three immune treatments (n = 17–18 beetles/sex/mating status/age group/immune treatment): (i) infection with low dose of live pathogen (pellet from 1 mL of 1 OD_{600} bacterial culture resuspended in 1 mL of insect Ringer solution), (ii) infection with heat-killed bacteria (heat-killed bacterial slurry prepared by heating 2 mL of 1 OD_{600} freshly grown culture at 90 °C for 20 min) and (iii) sham infection with insect Ringer solution. For each treatment, we used the septic injury method described above. Next day, for each treatment, we divided individuals randomly into virgin and mated subsets as described earlier. After holding the animals for another 6 days, we quantified their external immune defence as

described above. We estimated the impact of immune system activation by quantifying the difference in external defence between beetles infected with heat-killed bacteria and sham-infected beetles. The difference in external defence between beetles infected with live bacteria and sham-infected beetles served as a measure of the effect of infection. None of the treatments caused mortality within the experimental timeframe (between immune treatments and external immunity assay).

DATA ANALYSIS

We analysed post-infection survivorship data using a three-way proportional hazard survival analysis with age, mating status and sex as fixed factors [response variable was post-infection survival (in days), with animals still alive after 8 days noted as censored values]. To determine the factors affecting innate immunity and external defence, in each case we analysed data with a three-way ANOVA using age, sex and mating status as fixed factors. We tested for pairwise differences between treatment combinations after correcting for multiple comparisons, using Tukey's honest significant difference (HSD). PO activity data were not normally distributed (Shapiro–Wilk normality test); hence, we used a non-parametric Wilcoxon rank sum test to test for impacts of age and sex on PO activity.

The experimental design for testing the impact of prior infection on external defence was most complicated. We first analysed external defence in all treatments (sham, heat-killed and infected) with an ANOVA using age, sex, mating status and infection status as fixed factors. To better understand the complex multifactor interactions, we separately compared data for heat-killed vs. sham infection and live bacteria vs. sham infection treatments, and used Tukey's HSD for pairwise comparisons.

For each model described above, we checked whether all-way interaction terms of the full model were significant. If they were not significant, we tested reduced models, sequentially removing non-significant model terms and comparing models at each step to determine whether the reduced model performed better than the previous model. In all cases, the final reduced model explained as much variation as the full model (models compared using a log likelihood ratio test; $P > 0{\cdot}1$), and hence, we accepted the simplified model.

Results

FACTORS AFFECTING POST-INFECTION SURVIVORSHIP

Our first aim was to test the impact of age, sex and mating status on beetle survival after infection with *B. thuringiensis*. We did not find any mortality among sham-infected beetles of both age groups within the experimental window. Hence, we attributed age-related variation in post-infection survivorship solely to the difference in age-specific response to bacterial infection. As expected, we found a significant decline in post-infection survival with age (Fig. 1a), so that young individuals survived longer and were less susceptible to infection, whereas old mated females were most susceptible (Fig. 1a; Table 1). Although mating status and sex alone did not alter survival, these two factors showed a significant interaction

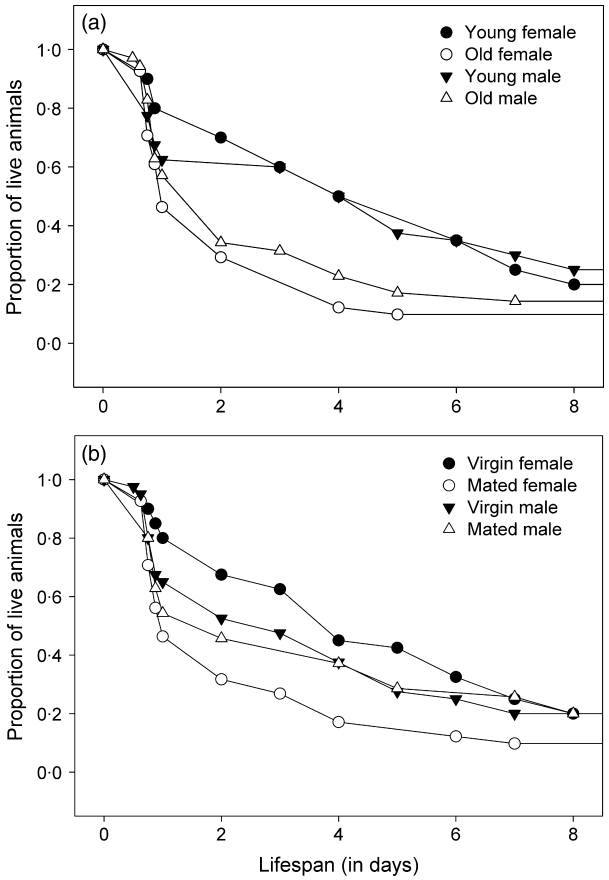

Fig. 1. Survival of *T. castaneum* adults following infection with *B. thuringiensis*. (a) Effect of age and sex on post-infection survivorship. (b) Effect of mating and sex on post-infection survivorship.

Table 1. Summary of the best reduced proportional hazards model to test the impact of age, sex and mating status on survival of beetles infected with *Bacillus thuringiensis*

Factor	d.f.	Chi-square	*P*
Mating	1	2·818	0·093
Sex	1	0·553	0·457
Age	1	10·418	**0·001**
Mating × Sex	1	3·85	**0·049**

Model terms with significant effects are highlighted in bold.

(see Table 1): mating reduced post-infection survival in females but not in males (Fig. 1b). Next, we tested whether aspects of innate immunity mirrored our results for post-infection survivorship.

FACTORS AFFECTING INNATE IMMUNE COMPONENTS

We conducted a full factorial experiment to determine the impact of age, sex and mating status on whole-body antibacterial activity of adult beetles against *B. thuringiensis*. We found that sex did not affect antibacterial activity (Fig. 2a; Table S1). Mating status and age interacted to

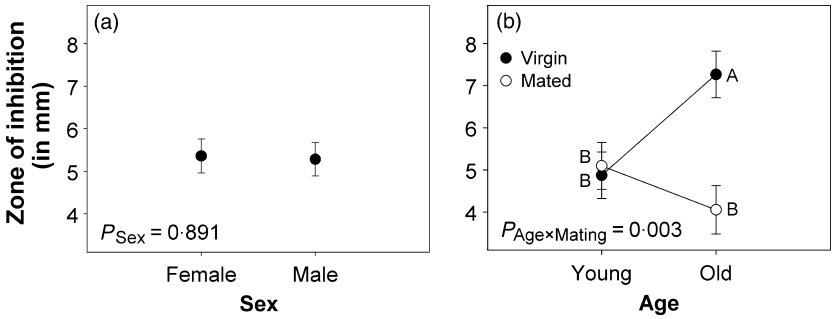

Fig. 2. Effect of (a) sex and (b) age × mating status interaction on antibacterial activity, measured as the mean diameter (±SE) of the zone of inhibition of bacterial growth on agar plates produced by whole-beetle homogenate. *P* values in panel a and b show the effect of sex and age × mating status interaction, respectively. In panel b, points not sharing common alphabets are significantly different from each other (determined using Tukey's HSD).

affect antibacterial activity: in virgins, antibacterial activity increased with age, but did not change significantly in mated individuals (Fig. 2b). We observed a similar age-related increase in antibacterial activity of virgins against two other bacteria, *S. marcescens* and *E. coli* (Fig. S2). Our results also suggest that mating imposes a significant cost only in older individuals by reducing antibacterial activity (Fig. 2b).

Given the age-related increase in antibacterial activity of virgin beetles, we tested whether they also exhibit an increase in PO activity, a major innate immune component. Similar to the results for antibacterial activity, we found that PO activity increased with age, with no evidence for sexual dimorphism (Fig. 3; Table S2). Together, these results indicate a general age-associated increase in innate immune components in virgins.

FACTORS AFFECTING EXTERNAL IMMUNE DEFENCE

We quantified the impact of age, sex and mating status on the external immune defence of experimental beetles.

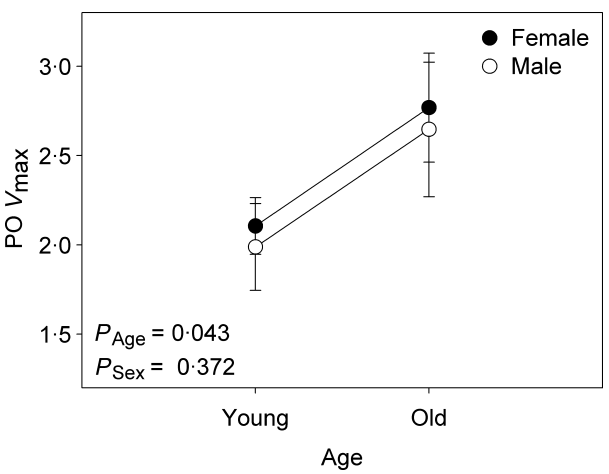

Fig. 3. Effect of beetle age on phenoloxidase activity of virgins measured as the average slope (±SE) of the linear part of the reaction quantifying the phenoloxidase enzyme activity.

Mating decreased external defence (Fig. 4a, Table S3), indicating that mating and/or associated physiological changes may impose a cost with respect to external immune defence. We also found a significant interaction age × sex on external defence (Fig. 4b). While females showed an age-related decline in external defence, males did not show significant age-specific variation in their response (Fig. 4b). Young females thus produced larger zones of inhibition than young males, but when old, males and females had similar external defence (Fig. 4b).

IMPACT OF PRIOR INFECTION ON EXTERNAL DEFENCE

Our experiment was designed to tease apart the effects of immune system activation vs. infection on external defensive secretions as a function of age, sex and mating status. Previous experiments have suggested that infection negatively affects major components of fitness such as reproductive ability and survivorship (Khan & Prasad 2011) and could thus limit investment in external immune defence. We found that by itself, infection did not alter external immune defence, whereas age, sex and mating status all had significant impacts (Fig. S3; Table 2). However, we also found multiple interactions between the different factors, indicating that their effects are not straightforward (Fig. 5; Table 2).

A significant interaction between infection and mating status (Table 2) revealed that mating reduced external defence in beetles from the heat-killed and sham infection treatments, but had no effect on individuals infected with live pathogens (Fig. S3). However, this interaction between infection and mating status further depended on sex and age, as evidenced by the three-way interaction between sex, infection and mating status, as well as a four-way interaction between all factors (Table 2). In young individuals that were sham-infected or infected with heat-killed bacteria, mating reduced external defence in females but not in males (Fig. 5a,b). In contrast, in young individuals infected with live bacteria, female external defence increased after mating, whereas male external

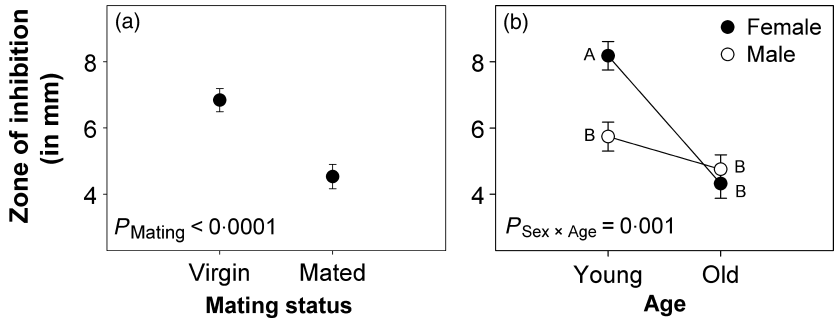

Fig. 4. Effect of (a) mating status and (b) age × sex interaction on external defence, measured as the mean diameter (±SE) of the zone of inhibition of bacterial growth on agar plates produced by cold-shocked beetles embedded in the agar plate. *P* values in panel a and b show the effect of mating status and age × sex interaction, respectively. In panel b, points not sharing common alphabets are significantly different from each other (determined using Tukey's HSD).

Table 2. Summary of four-way ANOVA for external defence with infection status (IS), age (A), sex (S) and mating status (M) as fixed factors

Factor	d.f.	SS	*F*-ratio	*P*
IS	2	6·721	0·649	0·52
A	**1**	**293·805**	**56·77**	**< 0·0001**
S	**1**	**394·024**	**76·135**	**< 0·0001**
M	**1**	**190·318**	**36·774**	**< 0·0001**
IS × A	2	8·219	0·794	0·453
IS × S	2	1·612	0·156	0·856
IS × M	**2**	**71·56**	**6·914**	**< 0·001**
A × S	1	10·867	2·099	0·148
A × M	1	8·216	1·588	0·208
S × M	1	1·237	0·239	0·625
IS × A × S	2	19·997	1·932	0·146
IS × A × M	2	7·498	0·724	0·485
IS × S × M	**2**	**111·721**	**10·794**	**< 0·0001**
A × S × M	**1**	**33·798**	**6·531**	**0·011**
IS × A × S × M	**2**	**77·001**	**7·439**	**0·001**
Error	414	2142·593		

Model terms with significant effects are highlighted in bold.

defence decreased after mating (Fig. 5c). Thus, when faced with a live pathogen, young females appear to increase their investment in external defence after mating, whereas males show the opposite trend. In contrast, in older individuals, females did not respond to infection treatments (whether sham, heat-killed or live; Fig. 5d–f) whereas males either did not respond (sham infection and infection with live bacteria; Fig. 5d,f) or reduced external defence (heat-killed treatment; Fig 5e). Thus, males and females show contrasting responses to mating and infection as they age.

To further understand these effects in light of the cost of immune system activation (sham-infected vs. heat-killed treatment) vs. infection with live bacteria (sham-infected vs. infected), we separately compared these treatment pairs. The analysis showed that the three-way interaction between age, sex and mating is only seen when comparing sham infection vs. heat-killed treatment and not sham infection vs. infection with live bacteria (Table S4). Thus, the combined effect of age and sex on the impact of mating is significant only when measuring the cost of immune activation in response to the heat-killed treatment (Fig. 5b,e; Table S4). In the heat-killed treatment, females show a mating-associated decline in external defence only when they are young. In contrast, mating imposes a cost only in older males (compare Fig. 5b,e). This also explains why we did not uncover an age × sex × mating interaction in the previous experiment with uninfected individuals and shows that immune activation can have large indirect impacts on individual external defence.

CORRELATION BETWEEN REPRODUCTIVE OUTPUT AND IMMUNE FUNCTION IN FEMALES

None of the measured immune components (post-infection survivorship, antibacterial activity or external defence) were correlated with reproductive fitness across age groups (post-infection survivorship: $R^2 = 0{\cdot}008$, d.f. = 1, $P = 0{\cdot}57$; antibacterial activity: $R^2 = 0{\cdot}07$, d.f. = 1, $P = 0{\cdot}111$; external defence: $R^2 = 0{\cdot}074$, d.f. = 1, $P = 0{\cdot}106$; see Fig. S4). It is worth noting that all females from the 'young' age group produced offspring, and only a small fraction (<2%) of old females did not produce any offspring (data not shown). Given this, we assumed that all females were inseminated and most likely, females that failed to produce offspring had an age-associated impairment in reproductive ability. However, we have not tested this explicitly and it is possible that a small fraction of old females did not mate successfully.

Discussion

We evaluated the impact of ageing on immune function and the ability to survive pathogenic bacterial infection. Our data clearly show that ageing reduces the ability to survive after a *B. thuringiensis* challenge, but antibacterial activity against *B. thuringiensis* and PO activity of virgins increase with age. A similar mismatch between age-associated change in the ability to survive bacterial infection and the aspects of antibacterial immunity was reported

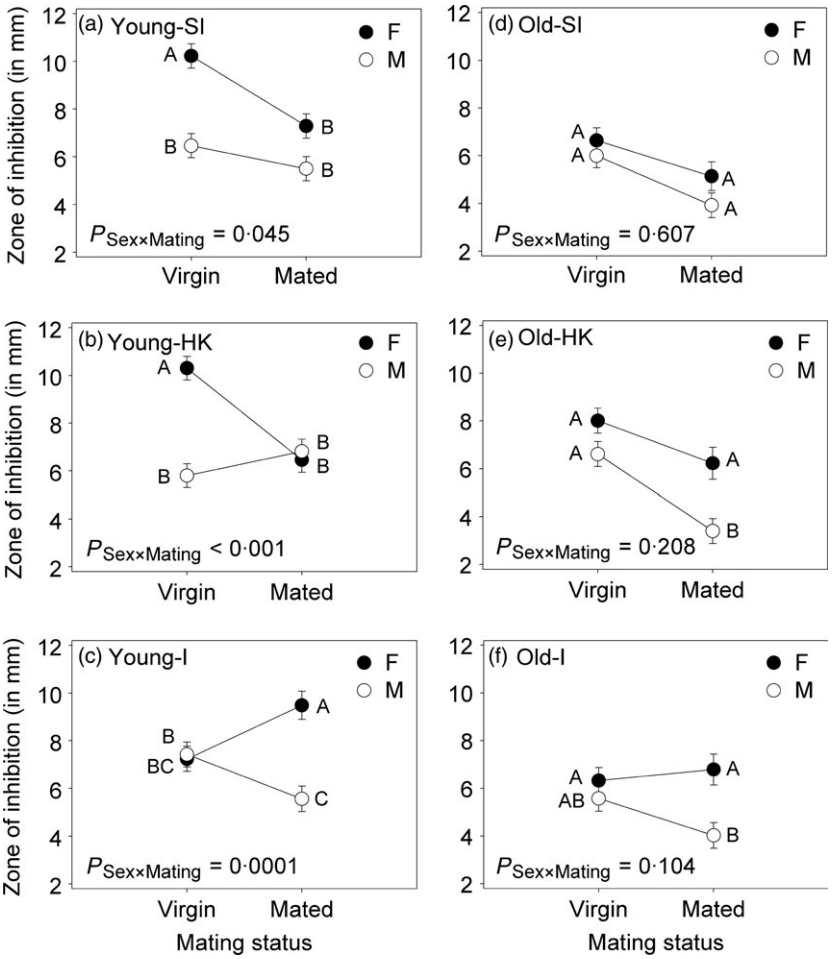

Fig. 5. Impact of *B. thuringiensis* infection on external defence under different age, sex and mating treatments. External defence was measured as described in Fig. 4. *P* values in panels show the effect of sex × mating status interaction. SI = Sham-infected individuals, HK = individuals infected with heat-killed bacteria, I = individuals infected with live bacteria, F = female, M = male. Points not sharing common alphabets are significantly different from each other (determined using Tukey's HSD).

previously in fruit flies (Ramsden, Cheung & Seroude 2008). Our data also show that mating status and age interact to affect antibacterial activity against *B. thuringiensis*, such that it improved with age in virgin beetles but remained unchanged in mated individuals. This pattern of age-related increase in antibacterial activity of virgins seems to be a general and non-specific phenomenon, being effective against multiple bacteria. Together, the age-related increase in antibacterial and PO activity of uninfected virgins may represent baseline levels of immune activation or chronic inflammatory-like responses with age reported in other species (Licastro *et al*. 2005). Overall, while our results suggest an age-related improvement in the baseline constitutive immune response in the absence of infection, this improvement did not translate into higher post-infection survivorship. However, we note that decline in post-infection survival with age can be mediated by other components of innate immunity that we did not measure, and future work may uncover such components.

VARIABLE IMPACTS OF AGEING ON IMMUNE FUNCTION AND POST-INFECTION SURVIVORSHIP

Why do older beetles succumb to pathogens more easily, despite an apparent increase in immune function? One reason may be that other aspects of senescence make older animals more vulnerable to infection. For instance, the ability to tolerate bacterial infection decreases with age (Ayres & Schneider 2008). Old individuals may thus rely on active resistance to bacterial infection, leading to selection for ever-increasing immune function with age. However, such an increase in immune expression may be constrained by substantial physiological costs (e.g. see Discussion on immunopathology below) or may trade off with other traits relevant for fitness (McKean & Nunney 2008), including other immune components (Cotter, Kruuk & Wilson 2004). Such trade-offs may potentially explain why older individuals are more susceptible to infection despite increased immune activation. However, in our experiments, we do not find evidence of a trade-off between reproductive fitness and components of immune function such as antibacterial activity for either age group. Thus, females that produced more offspring did not die faster after infection, and did not show a reduction in internal or external immune defence. Hangartner *et al*. (2013) also failed to find any trade-offs between reproduction and immune function (PO activity and resistance to microsporidian infection) in *T. castaneum* lines selected for divergent reproductive success. These results are in contrast to the expectation that an individual's reproductive investment may restrict the expression of

immune-related traits. However, we would like to stress three major limitations of our experiment. First, the trade-off may be mediated in the opposite direction to what we have measured: once infected, females that mount a stronger immune response have low reproductive fitness. Secondly, given that females were not resource limited, the impact of trade-off may be too weak to be detected. Finally, for logistic reasons, we measured 24 h fecundity as a fitness proxy, and it is possible that trade-offs may be apparent on longer time-scales. Hence, more detailed and manipulative experiments are necessary before we can write off immune function reproduction trade-offs in *T. castaneum*.

Up-regulation of immune function genes may also arise simply as a by-product of a general loss of gene regulation associated with senescence, and natural selection on old individuals may be too weak to effectively prevent misregulation. Such deregulated immune activation may increase the risk of immunopathology, that is damage by the host's own immune response against infection (Sadd & Siva-Jothy 2006). For example, rapid-acting and non-specific innate immune components such as PO can cause severe damage to host tissue and organs by producing reactive and cytotoxic intermediates (Urabe *et al.* 1994; Cerenius & Söderhäll 2004; Zhao *et al.* 2011). Older individuals may thus succumb to infection even if their ability to fight bacterial infection improves or remains unchanged with age. Although we found enhanced PO activity in ageing beetles, we did not test whether this leads to increased immunopathology in older beetles. Future work quantifying the degree of immunopathological damage as a function of age is necessary to explicitly test this idea.

Our results imply that two commonly measured immune traits (antibacterial and PO activity) are poor predictors of age-related post-infection survival. Previous observations by Corby-Harris *et al.* (2007) for different populations of *D. melanogaster* similarly indicated that measures of immune function and host ability to survive an infection are not positively correlated, even though both traits exhibit ample genetic variation. Thus, the lack of correlation between immune traits and their outcome may not be restricted to the context of ageing alone, but may be a more general phenomenon that arises due to a complicated set of interactions between life-history parameters, ecological factors and the evolutionary history of an organism.

A limitation of our experiments is that we could only measure immunity and susceptibility to bacterial infection for two age groups (17 and 77 days post-pupation). Hence, we are unable to determine the trajectory of age-related changes in immune function (e.g. whether immune components increase monotonically or as a step function). We also note that we did not measure cellular defence, an important component of host response immediately after infection. An earlier report suggests that the ability of circulating haemocytes to perform phagocytosis declines with age in scorpion flies, *Panorpa vulgaris* (Kurtz 2002). It is possible that a similar reduction in cellular defence may explain the age-related increase in susceptibility to bacterial infection. Finally, while we found that components of baseline constitutive immunity increased with age, ageing may reduce an individual's ability to activate the immune system upon infection; we could not test this possibility in our study. However, studies on fruit flies (e.g. Khan & Prasad 2013) and mealworms (*Tenebrio molitor*, closely related to *T. castaneum*; I Khan, unpublished data) that quantified bacterial load across age groups suggest that immunity generally improves with age, though the relationship varies across genotypes (Lesser, Paiusi & Leips 2006).

Contrary to innate immune function, we found that ageing reduced the ability to inhibit bacterial growth through external secretions, and even a prior infection did not alter the ageing of the external defence. However, in contrast to our finding, a previous study of *T. castaneum* reported that in both sexes, quinone levels in the storage glands increased after adult eclosion, peaked in 50-day-old individuals and was maintained until 80 days post-eclosion (Unruh, Xu & Kramer 1998). In a separate study of the closely related species *T. confusum*, Roth (1943) reported that older individuals accumulated larger volumes of secretory material in their abdominal reservoirs. However, these studies did not quantify the secretion of quinones outside the body; nor did they directly test the potency of the secretion in inhibiting bacterial growth. It is possible that although older individuals have higher quinone levels, the physiological ability to secrete the compounds, the rate of secretion or the potency of the secretion declines with age. Recent work by Pedrini *et al.* (2010) suggested that infection reduced the secretion of defensive compounds in *T. castaneum*, although both infected and uninfected beetles produced equivalent total amount of quinone. Ageing could perhaps alter the secretion system in a similar fashion, and this could explain the contrast between our results and previous studies (e.g. Unruh, Xu & Kramer 1998). Clearly, further studies are required to understand the relationship between potential defence (benzoquinone production) and realized defence through external secretions (ability to inhibit microbial growth).

SEX AND MATING STATUS AFFECT IMMUNE INFECTION AND POST-INFECTION SURVIVAL

Sexual dimorphism in immune function and its outcome may arise due to three major processes. First, such differences may be adaptive in light of different constraints on males and females (e.g. sex-specific physiological or evolutionary trade-offs). For instance, Rolff (2002) used Bateman's principle (Bateman 1948) to predict that female investment in innate immunity should be intrinsically higher than males because females gain direct benefits from increased life span, whereas males typically increase fitness via more matings. Alternatively, sex-specific

differences in the immune response may result from environmental factors (such as resource availability or sexual activity) that might differentially limit male and female fitness at various life stages (McKean & Nunney 2005). Finally, sexual dimorphism may arise as a result of sexual conflict regarding relative investment in offspring vs. self-defence (e.g. see review by Morrow & Innocenti 2012).

By itself, we observed a significant impact of sex only on external defence, so that females inhibited bacterial growth better than males through their defensive secretions. Similar patterns were found in externally secreted social immune defence of burying beetles, with females showing stronger antibacterial activity than males (Cotter & Kilner 2010) despite substantial fitness costs (Cotter *et al*. 2010). These patterns may be potentially adaptive if females gain greater direct (protecting self) or indirect benefits (protecting offspring against pathogens) from external defence compared to males. Further, *Tribolium* males tend to disperse more than females (Ritte & Lavie 1977), and hence, quinones secreted by females may benefit their progeny more than male quinones. We also found that young females have higher external defence than males and old females near reproductive senescence, suggesting that sex-specific, reproduction-mediated trade-offs may underlie the observed sexual dimorphism in external defence. Along with previous work (Unruh, Xu & Kramer 1998 and references therein), these results support Rolff's (2002) prediction about intrinsic sex-specific constraints driving dimorphism in immune function. On the other hand, the observed pattern of variation in external immune defence may also represent a secondary outcome of gender-specific senescence.

In contrast to external defence, we did not find a significant impact of sex on survival after bacterial infection, PO and antibacterial activity. This is consistent with recent studies on *D. melanogaster* showing that males and females do not differ in their ability to survive bacterial infection (Ramsden, Cheung & Seroude 2008) and have similar bacterial loads (Khan & Prasad 2013). These results are counter to the prediction by Rolff (2002) and support McKean & Nunney's (2005) hypothesis that neither sex has intrinsically superior immune function (also see above). Using fruit flies, McKean & Nunney (2005) demonstrated that while immunological sexual differences are absent when resources are abundant, males had better immune function under limiting resources and females had higher immunity when males were mating with multiple females. In our experiments, beetles were provided with abundant resources, which may have reduced the impact of any inherent immunological sex-specific differences. Thus, explicit experiments are necessary to distinguish between different hypotheses (described above) and to determine whether the observed patterns of sexual dimorphism in immune function are adaptive.

Overall, our results also highlight a general immunosuppressive role of mating, often in interaction with other factors. By itself, mating decreased antibacterial activity, as seen in previous examples of post-mating immune depression (See Sheldon & Verhulst 1996; Rolff & Siva-Jothy 2002). Mating also decreased external defence, similar to the results of a previous study by Hill & Tschinkel (1985) showing lower production of defensive compounds in mated individuals compared to virgins of the beetle *Zophobas atratus*. Despite these negative effects on immune function, mating status alone did not alter post-infection survival. In fact, mating status interacted with sex such that females paid a cost of mating while males did not.

Interestingly, beetles infected with live bacteria did not show any effect of mating because virgins reduce their external defence on infection whereas mated beetles increase external defence on infection. Furthermore, females drove this interaction, paying no cost of mating when infected. Our finding that external defence increased in mated females after infection with live bacteria may represent an adaptive response under pathogen attack, if increased investment in external defence secures a pathogen-free environment for their offspring. Alternatively, these patterns may arise as a result of sexual conflict (Morrow & Innocenti 2012), if after mating or infection, males benefit more than females from increased female external defence. However, we stress that currently there is no evidence for either hypothesis in *T. castaneum*.

CONCLUSION

Immune senescence is an intriguing phenomenon that bears on many aspects of evolutionary biology and ecology, particularly life-history theory. Although it seems straightforward that immune function should decrease with age, our work along with other studies points to a more complicated picture. Various immune components are up- or down-regulated not only as a function of age, but also depend on changes in mating status and gender. Perhaps as a result, the net impact of each component does not always predict an individual's ability to survive an immune challenge. We did not find trade-offs between different immune components and reproduction, suggesting that simplistic life-history models may also fail to explain the discordance between immune function and susceptibility to infection observed across taxa. Our results suggest that intricate relationships between various life-history and ecological parameters may drive immune senescence and investment in immune components, and we hope that our observations motivate further experimental work to confirm and understand these patterns. We suggest that experimental manipulation of specific immune components across age, stage and life-history classes will shed more light on the complex problem of immune senescence.

Acknowledgements

We thank Caroline Zanchii, Jens Rolff, Laasya Samhita and Saurabh Mahajan for critical comments on previous drafts of the manuscript. We

thank Nagaraj Guru Prasad for donation of bacterial strains. We acknowledge funding and support from a SERB-DST Young Investigator Grant to IK, a DST INSPIRE Faculty fellowship to DA and the National Center for Biological Sciences, India. The authors are not aware of any affiliations, memberships, funding or financial holdings that can potentially affect the objectivity of this research article.

Data accessibility

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.s34n8 (Khan, Prakash & Agashe 2015).

References

Abdel-Razek, A.S., Salama, H.S., White, N.D.G. & Morris, O.N. (1999) Effect of *Bacillus thuringiensis* on feeding and energy use by *Plodia interpunctella* (Lepidoptera: Pyralidae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae). *The Canadian Entomologist*, **131**, 433–440.

Adamo, S.A. (2004) How should behavioural ecologists interpret measurements of immunity? *Animal Behavior*, **68**, 1443–1449.

Ardia, D.R., Parmentier, H.K. & Vogel, L.A. (2011) The role of constraints and limitation in driving individual variation in immune response. *Functional Ecology*, **25**, 61–73.

Ariani, C.V., Juneja, P., Smith, S., Tinsley, M.C. & Jiggins, F.M. (2014) Vector competence of *Aedes aegypti* mosquitoes for filarial nematodes is affected by age and nutrient limitation. *Experimental Gerontology*, **61C**, 47–53.

Ayres, J.S. & Schneider, D.S. (2008) Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nature Reviews Immunology*, **8**, 889–895.

Bateman, A.J. (1948) Intra-sexual selection in *Drosophila*. *Heredity*, **2**, 349–368.

Bedhomme, S., Agnew, P., Sidobre, C. & Michalakis, Y. (2004) Virulence reaction norms across a food gradient. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **271**, 739–744.

Cerenius, L., Lee, B.L. & Söderhäll, K. (2008) The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*, **29**, 263–271.

Cerenius, L. & Söderhäll, K. (2004) The prophenoloxidase-activating system in invertebrates. *Immunological Reviews*, **198**, 116–126.

Corby-Harris, V., Pontaroli, A.C., Shimkets, L.J., Bennetzen, J.L., Habel, K.E. & Promislow, D.E. (2007) Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. *Applied and Environmental Microbiology*, **73**, 3470–3479.

Cotter, S.C. & Kilner, R.M. (2010) Sexual division of antibacterial resource defence in breeding burying beetles, *Nicrophorus vespilloides*. *Journal of Animal Ecology*, **79**, 35–43.

Cotter, S.C., Kruuk, L.E.B. & Wilson, K. (2004) Costs of resistance: genetic correlations and potential trade-offs in an insect immune System. *Journal of Evolutionary Biology*, **17**, 421–429.

Cotter, S.C., Myatt, J.P., Benskin, C.M.H. & Wilson, K. (2008) Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. *Journal of Evolutionary Biology*, **21**, 1744–1754.

Cotter, S.C., Topham, E.A., Price, A.J.P. & Kilner, R.M. (2010) Fitness costs associated with mounting a social immune response. *Ecology Letters*, **13**, 1114–1123.

Cotter, S.C., Littlefair, J.E., Grantham, P.J. & Kilner, R.M. (2013) A direct trade-off between personal and social immunity. *Journal of Animal Ecology*, **82**, 846–853.

DeVeale, B., Brummel, T. & Seroude, L. (2004) Immunity and aging: the enemy within? *Aging Cell*, **3**, 195–208.

Faye, I. & Wyatt, G.R. (1980) The synthesis of antibacterial proteins in isolated fat body from Cecropia silkmoth pupae. *Experientia*, **36**, 1325–1326.

Felix, T.M., Hughes, K.A., Stone, E.A., Drnevich, J.M. & Leips, J. (2012) Age-specific variation in immune response in *Drosophila melanogaster* has a genetic basis. *Genetics*, **191**, 989–1002.

Fowler, K. & Partridge, L. (1989) A cost of mating in female fruitflies. *Nature*, **338**, 760–761.

Gustafsson, L., Nordling, D., Andersson, M.S., Sheldon, B.C. & Qvarnström, A. (1997) Infectious diseases, reproductive effort and the cost of reproduction in birds. *Infection, Polymorphism and Evolution* (eds W.D. Hamilton & J.C. Howard), pp. 53–61. Springer, Netherlands.

Hangartner, S., Sbilordo, S.H., Michalczyk, Ł., Gage, M.J. & Martin, O.Y. (2013) Are there genetic trade-offs between immune and reproductive investments in *Tribolium castaneum*? *Infection, Genetics and Evolution*, **19**, 45–50.

Hill, C.S. & Tschinkel, W.R. (1985) Defensive secretion production in the tenebrionid beetle, *Zophobas atratus*. *Journal of Chemical Ecology*, **11**, 1083–1092.

Horn, L., Leips, J. & Starz-Gaiano, M. (2014) Phagocytic ability declines with age in adult *Drosophila* hemocytes. *Aging Cell*, **13**, 719–28.

Hou, X., Fields, P. & Taylor, W. (2004) Combination of protein-rich pea flour and pea extract with insecticides and enzyme inhibitors for control of stored-product beetles. *The Canadian Entomologist*, **136**, 581–590.

Joop, G., Mitschke, A., Rolff, J. & Siva-Jothy, M.T. (2006) Immune function and parasite resistance in male and polymorphic female *Coenagrion puella*. *BMC Evolutionary Biology*, **6**, 19.

Joop, G., Roth, O., Schmid-Hempel, P. & Kurtz, J. (2014) Experimental evolution of external immune defences in the red flour beetle. *Journal of Evolutionary Biology*, **27**, 1562–71.

Khan, I., Prakash, A. & Agashe, D. (2015) Data from: Immunosenescence and the ability to survive bacterial infection in the red flour beetle *Tribolium castaneum*. *Dryad Digital Repository*, http://dx.doi.org/10.5061/dryad.s34n8

Khan, I. & Prasad, N.G. (2011) Sex-specific effect of bacterial infection on components of adult fitness in *Drosophila melanogaster*. *Journal of Evolutionary Biology Research*, **3**, 79–86.

Khan, I. & Prasad, N.G. (2013) The aging of the immune response in *Drosophila melanogaster*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, **68**, 129–135.

Kraaijeveld, A.R. & Godfray, H.C.J. (1997) Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Nature*, **389**, 278–280.

Kurtz, J. (2002) Phagocytosis by invertebrate hemocytes: causes of individual variation in *Panorpa vulgaris* scorpionflies. *Microscopy Research and Technique*, **57**, 456–468.

Kurtz, J. & Sauer, K.P. (2001) Gender differences in phenoloxidase activity of *Panorpa vulgaris* hemocytes. *Journal of Invertebrate Pathology*, **78**, 53–55.

Kurtz, J., Wiesner, A., Götz, P. & Sauer, K.P. (2000) Gender differences and individual variation in the immune system of the scorpionfly *Panorpa vulgaris* (Insecta: Mecoptera). *Developmental & Comparative Immunology*, **24**, 1–12.

Landis, G.N., Abdueva, D., Skvortsov, D., Yang, J., Rabin, B.E., Carrick, J. *et al.* (2004) Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 7663–7668.

Lesser, K.J., Paiusi, I.C. & Leips, J. (2006) Naturally occurring genetic variation in the age-specific immune response of *Drosophila melanogaster*. *Aging Cell*, **5**, 293–295.

Li, J., Lehmann, S., Weißbecker, B., Naharros, I.O., Schütz, S., Joop, G. *et al.* (2013) Odoriferous defensive stink gland transcriptome to identify novel genes necessary for quinone synthesis in the Red Flour Beetle *Tribolium castaneum*. *PLoS Genetics*, **9**, e1003596.

Licastro, F., Candore, G., Lio, D., Porcellini, E., Colonna-Romano, G., Franceschi, C. *et al.* (2005) Innate immunity and inflammation in ageing: a key for understanding age-related diseases. *Immunity and Ageing*, **2**, 8–22.

McKean, K.A. & Nunney, L. (2005) Bateman's principle and immunity: phenotypically plastic reproductive strategies predict changes in immunological sex differences. *Evolution*, **59**, 1510–1517.

McKean, K.A. & Nunney, L. (2008) Sexual selection and immune function in *Drosophila melanogaster*. *Evolution*, **62**, 386–400.

McNamara, K.B., Lieshout, E., Jones, T.M. & Simmons, L.W. (2013) Age-dependent trade-offs between immunity and male, but not female, reproduction. *Journal of Animal Ecology*, **82**, 235–244.

Moret, Y. & Schmid-Hempel, P. (2000) Survival for immunity: the price of immune system activation for bumblebee workers. *Science*, **290**, 1166–116.

Morrow, E.H. & Innocenti, P. (2012) Female postmating immune responses, immune system evolution and immunogenic males. *Biological Reviews*, **87**, 631–638.

Otti, O., Tragust, S. & Feldhaar, H. (2014) Unifying external and internal immune defences. *Trends in Ecology & Evolution*, **29**, 625–634.

Pedrini, N., Villaverde, M.L., Fuse, C.B., Bello, G.D. & Juarez, M.P. (2010) *Beauveria bassiana* infection alters colony development and defensive secretions of the beetles *Tribolium castaneum* and *Ulomoides dermestoides* (Coleoptera: Tenebrionidae). *Journal of Economic Entomology*, **103**, 1094–1099.

Pletcher, S.D., Macdonald, S.J., Marguerie, R., Certa, U., Stearns, S.C., Goldstein, D.B. *et al.* (2002) Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Current Biology*, **12**, 712–723.

Prendeville, H.R. & Stevens, L. (2002) Microbe inhibition by *Tribolium* flour beetles varies with beetle species, strain, sex, and microbe group. *Journal of Chemical Ecology*, **28**, 1183–1190.

Ramsden, S., Cheung, Y.Y. & Seroude, L. (2008) Functional analysis of the *Drosophila* immune response during aging. *Aging Cell*, **7**, 225–236.

Ren, C., Webster, P., Finkel, S.E. & Tower, J. (2007) Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. *Cell Metabolism*, **6**, 144–152.

Richner, H., Christe, P. & Oppliger, A. (1995) Paternal investment affects prevalence of malaria. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 1192–1194.

Ritte, U. & Lavie, B. (1977) The genetic basis of dispersal behavior in the flour beetle *Tribolium castaneum*. *Canadian Journal of Genetics and Cytology*, **19**, 717–722.

Roberts, K.E. & Hughes, W.O. (2014) Immunosenescence and resistance to parasite infection in the honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology*, **121**, 1–6.

Rolff, J. (2002) Bateman's principle and immunity. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **269**, 867–872.

Rolff, J. & Siva-Jothy, M.T. (2002) Copulation corrupts immunity: a mechanism for a cost of mating in insects. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 9916–9918.

Rolff, J. & Siva-Jothy, M.T. (2003) Invertebrate ecological immunology. *Science*, **301**, 472–475.

Roth, L.M. (1943) Studies on the gaseous secretion of *Tribolium confusum* Duval II. The odoriferous glands of *Tribolium confusum*. *Annals of the Entomological Society of America*, **36**, 397–424.

Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P. *et al.* (2010) Paternally derived immune priming for offspring in the red flour beetle, *Tribolium castaneum*. *Journal of Animal Ecology*, **79**, 403–413.

Sadd, B.M. & Siva-Jothy, M.T. (2006) Self-harm caused by an insect's innate immunity. *Proceedings of the Royal Society B: Biological Sciences*, **273**, 2571–2574.

Schmid-Hempel, P. (2003) Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **270**, 357–366.

Schmid-Hempel, P. & Ebert, D. (2003) On the evolutionary ecology of specific immune defence. *Trends in Ecology & Evolution*, **18**, 27–32.

Shanley, D.P., Aw, D., Manley, N.R. & Palmer, D.B. (2009) An evolutionary perspective on the mechanisms of immunosenescence. *Trends in Immunology*, **30**, 374–381.

Sheldon, B.C. & Verhulst, S. (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution*, **11**, 317–321.

Simmons, L.W. & Roberts, B. (2005) Bacterial immunity traded for sperm viability in male crickets. *Science*, **309**, 2031–2031.

Siva-Jothy, M.T., Moret, Y. & Rolff, J. (2005) Insect immunity: an evolutionary ecology perspective. *Advances in Insect Physiology*, **32**, 1–48.

Siva-Jothy, M. T., Tsubaki, Y. & Hooper, R. E. (1998) Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. *Physiological Entomology*, **23**, 274–277.

Sokoloff, A. (1974) *The Biology of Tribolium, with Special Emphasis on Genetic Aspects, Vol. 2*. Oxford University Press, Oxford, UK.

Trivers R. (1972). Parental investment and sexual selection. *Sexual Selection and the Descent of Man* (ed. B. Campbell), pp. 136–179. Aldine, Chicago, Illinois, USA.

Unruh, L.M., Xu, R. & Kramer, K.J. (1998) Benzoquinone levels as a function of age and gender of the red flour beetle, *Tribolium castaneum*. *Insect Biochemistry and Molecular Biology*, **28**, 969–977.

Urabe, K., Aroca, P., Tsukamoto, K., Mascagna, D., Palumbo, A., Prota, G. *et al.* (1994) The inherent cytotoxicity of melanin precursors: a revision. *Biochimica Biophysica Acta (BBA) – Molecular Cell Research*, **1221**, 272–278.

Verhulst, S., Dieleman, S.J. & Parmentier, H.K. (1999) A tradeoff between immunocompetence and sexual ornamentation in domestic fowl. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 4478–4481.

Yezerski, A., Gilmor, T.P. & Stevens, L. (2004) Genetic analysis of benzoquinone production in *Tribolium confusum*. *Journal of Chemical Ecology*, **30**, 1035–1044.

Zerofsky, M., Harel, E., Silverman, N. & Tatar, M. (2005) Aging of the innate immune response in *Drosophila melanogaster*. *Aging Cell*, **4**, 103–108.

Zhao, P.C., Lu, Z.Q., Strand, M.R. & Jiang, H.B. (2011) Antiviral, antiparasitic, and cytotoxic effects of 5,6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response. *Insect Biochemistry and Molecular Biology*, **41**, 645–652.

Zou, S., Meadows, S., Sharp, L., Jan, L.Y. & Jan, Y.N. (2000) Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 13726–13731.

Received 21 January 2015; accepted 14 July 2015
Handling Editor: Sheena Cotter

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Reproductive senescence of female beetles measured as the mean total number of progeny (±SE) produced by each female (24 h oviposition period) within 60 days post pupation.

Fig. S2. Antibacterial (AB) activity against *S. marcescens* and *E. coli*, measured as the mean diameter (±SE) of the zone of inhibition of bacterial growth on agar plates produced by whole-beetle homogenate.

Fig. S3. Comparison of the effect of (A) Age (B) Sex and (C) Mating status on external defence between different immune treatments (SI = sham infection; HK = infection with heat-killed bacteria; I = infection with live bacteria).

Fig. S4. Summary of experiments showing a lack of tradeoffs between reproductive fitness of females (measured as the number of progeny produced by each female) and their A. post-infection lifespan, B. antibacterial activity, and C. external immune defence across age groups (Young-Y, Old-O).

Table S1. Summary of the best reduced model (ANOVA) for antibacterial activity with age, sex and mating status as fixed factors.

Table S2. Summary of Wilcoxon rank sum test for the effect of age and sex on PO activity in virgin beetles.

Table S3. Summary of three-way ANOVA (best reduced model) for external defence, with age, sex and mating status as fixed factors.

Table S4. Summary of four-way ANOVA for external defence in SI vs. HK and SI vs. I with infection status (IS), age (A), sex (S) and mating status (M) as fixed factors.

Data S1. Materials and methods.