Copulation enhances resistance against an entomopathogenic fungus in the mealworm beetle *Tenebrio molitor*

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SUMMARY

Ecological immunology is based upon the notion that activation and use of the immune system is costly and should thus be traded off against other energy-demanding aspects of life history. Most of the studies on insects that have examined the possibility that mating results in trade-offs with immunity have shown that mating has immunosuppressive effects. The connection between mating and immunity has traditionally been investigated using indirect measures of immunity. However, studies that have assessed the effects of mating on the resistance against real pathogens have had conflicting results. A previous study on *Tenebrio molitor* showed that copulation decreases phenoloxidase activity in the haemolymph, and concluded that copulation corrupts immunity in this species. In the present study we tested whether mating also affects the ability of *T. molitor* to resist the entomopathogenic fungus, *Beauveria bassiana*. Interestingly, we found that mating enhanced resistance against the fungal infection and that the effect was stronger on males than females. Furthermore, we found that male beetles were overall more susceptible to the fungal infection than were females, indicating an immunological sex difference in immunity. Our study highlights the importance of the use of real pathogens and parasites in immuno-ecological studies.

Key words: host resistance tests, immune defence, life history trade-offs, mating, sexual dimorphism.

INTRODUCTION

Immunity is considered costly and it is thus expected to be traded-off against other energy-demanding aspects of life history such as somatic maintenance, growth and reproduction (Sheldon and Verhulst, 1996; Schmid-Hempel, 2003). A number of studies on invertebrates that have examined the possibility that mating results in trade-offs with immunity have found that mating induced down-regulation of immune function (Siva-Jothy *et al*. 1998; Fedorka *et al*. 2004; McKean and Nunney, 2001; Rolff and Siva-Jothy, 2002). However, an individual's ability to fend off a disease can also increase after mating. For example, non-virgin female crickets, *Gryllus texensis*, have an enhanced ability to fend off a bacterial disease compared with virgin females (Shoemaker *et al*. 2006). In addition, in female *Drosophila melanogaster*, sperm and seminal proteins upregulate genes associated with antimicrobial defence (McGraw *et al*. 2004; Peng *et al*. 2005). In most cases the link between mating and immunity has been investigated using indirect measures of immunity such as haemocyte load (the amount of immunocytes in the haemolymph) and phenoloxidase activity (PO, which is the key enzyme in the synthesis of melanin). For example, the effects of mating on immunity in the mealworm beetle, *Tenebrio molitor*, have previously been investigated using assays measuring cellular immunity (Rolff and Siva-Jothy, 2002). Rolff and Siva-Jothy (2002) found significantly lower PO activities, but no effects on haemocyte load, in male and female beetles 24 h after mating compared with virgins. It was suggested that mating induces the release of juvenile hormone, which acts to down-regulate PO activity in this species (Rolff and Siva-Jothy, 2002). Recent studies on insects have not found any association between PO activity in the haemolymph and resistance against naturally occurring pathogens (Schwarzenbach and Ward, 2007). Likewise, it may not be that higher PO activity in the haemolymph also means a stronger encapsulation response to a parasite. For example, in the Autumnal Moth, *Epirrita autumnata*, starvation reduced the encapsulation response to a nylon monofilament, but increased PO activity (Yang *et al*. 2007).

Recent studies have shown that declines in one aspect of an animal's innate immunity should not be interpreted as impaired overall immunity and, if possible, the measurement of multiple immune parameters should be performed (Adamo, 2004*a*, *b*; Fedorka and Zuk, 2005). Declines in some immune parameters may indicate that another immunological defence mechanism is activated (Adamo, 2004*a*). In addition, a recent study by Fedorka *et al*. (2007) showed that the estimates of immunity based on immune gene expression did not reflect the actual

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ability of *Drosophila* females to fend off pathogens in the hours following mating. This suggested that the discrepancy between different measures of immunity could also be due to when different measures are taken. Assessing whether an activity such as mating has fitness costs such as reduced survival can be measured using host resistance tests, in which the likelihood of survival against an infection is measured (see Adamo, 2004*a* and the references therein). The aim of this study was to test whether copulation has an effect on the resistance against real pathogens in *T. molitor*. We thus tested whether mating affects the ability of *T. molitor* to resist a natural pathogen, the entomopathogenic fungus *Beauveria bassiana*.

MATERIALS AND METHODS

We established a *T. molitor* laboratory population from a commercially produced stock obtained from Imazon Company, Sweden. Stock larvae and adults were reared in plastic pots (0·34 m × 0·28 m × 0·23 m) containing wheat bran and apple, and were maintained in a controlled environment (in 12L:12D, 25±1 °C). Pupae were collected 3–4 times a week and sexed visually by assessing the morphology of the 8th abdominal segment (see Bhattacharya *et al.* 1970). Pupae of each sex were kept in separate plastic dishes until emergence. The dishes containing the pupae were checked daily for the emergence of any adults. After collection, to ensure virginity all adults were housed individually in plastic film roll canisters. All beetles were fed with fresh apple. Beetles used for the experiment were 7–14 days old. Before the experiment, each beetle was weighed to the nearest 0·1 mg.

For the experiment, mated beetles were produced by choosing a random virgin male and a random virgin female, then allowing them to mate once on a plastic Petri disc. The control beetles (virgins) were not allowed to mate but were otherwise treated similarly. Resistance to the entomopathogenic fungus *Beauveria bassiana* (strain Mycotrol) was tested by exposing both mated (155 males and 169 females) and virgin (139 males and 149 females) beetles to the fungus by dipping them in a LD$_{50}$ ($1·42 \times 10^6$ spores/ml) spore solution for 5 sec. The fungal spores were dissolved in distilled water (for detailed methods see Rantala and Roff (2007)). A second set of beetles were dipped in distilled water (150 mated males and 170 mated females; 150 virgin males and 150 virgin females). Both treatments were exposed to disease simultaneously within 3 h after mating treatments.

After treatment each beetle was placed back into the film roll canister from which it was originally kept. All canisters were then placed into a cabinet at room temperature with natural light and fed with fresh apple. Mortality was checked daily for 15 days. After 15 days mortality caused by fungal infection is minimal and mortality is not different from natural mortality (authors' personal observations). Thus, beetles that survived more than 15 days were considered as having survived. We constructed a Cox survival regression model to reveal the predictors in the experiment which best explained measured variance in mortality. Mating status, fungus exposure and sex were used as categorical covariates, and weight was included as a continuous covariate in the model.

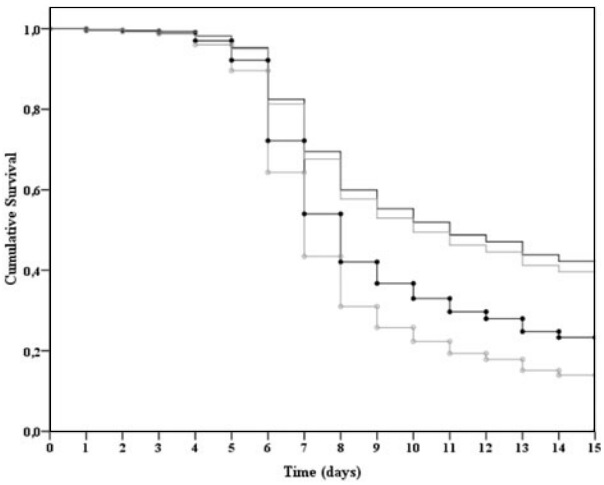

Fig. 1. Survival of infected male and female beetles (mated ♂: black line (●), virgin ♂: grey line (○), mated ♀: black line, virgin ♀: grey line).

RESULTS

Fungus exposure reduced survival (odds ratio = 2·073, Wald = 150·836, D.F. = 1, $P < 0·001$). Thus, further analyses were conducted separately for fungus exposure and control treatments. When infected with *B. bassiana* survival was affected by mating status (odds ratio = 0·848, Wald = 4·104, D.F. = 1, $P = 0·043$) and sex (odds ratio = 0·521, Wald = 61·245, D.F. = 1, $P < 0·001$). Mated beetles had significantly lower mortality than virgin beetles (Fig. 1). For infected females the effect of mating was smaller than it was for infected males. In addition, both virgin (odds ratio = 0·473, Wald = 36·714, D.F. = 1, $P < 0·001$) and mated (odds ratio = 0·568, Wald = 24·931, D.F. = 1, $P < 0·001$) female beetles had higher survival after fungal exposure treatment than male beetles. Without infection survival was affected only by sex (odds ratio = 0·856, Wald = 3·751, D.F. = 1, $P = 0·053$).

DISCUSSION

In our experiment we found that mated beetles were more resistant to the *B. bassiana* – fungal infection than were virgin beetles. For infected females the effect of mating on survival was smaller than it was for infected males probably because the base level of their immunity is much lower than females. In contrast to our results, previous studies on damselflies, *Matrona basilaris japonica* (Siva-Jothy *et al.* 1998), ground crickets, *Allonemobius socius* (Fedorka *et al.* 2004), fruit flies, *Drosophila melanogaster* (McKean and Nunney, 2001) and on mealworm beetles, *Tenebrio molitor* (Rolff and Siva-Jothy, 2002) that have investigated the link between mating and immune defence have shown that mating induces immunosuppression. However, there is conflicting evidence in *Drosophila* with no difference in the ability of female flies maintained with males in their immune responses to *Escherichia coli* bacteria compared with virgin females only kept with other females (McKean and Nunney, 2005). In addition, non-virgin female crickets, *Gryllus texensis*, were more resistant to infection by the bacterium *Serratia marcescens* than virgin females (Shoemaker *et al.* 2006). Enhanced immunity in mated individuals could be an adaptive response to reduce the risk of sexually transmitted diseases and other microbes (McGraw *et al.* 2004). In addition, sperm and seminal proteins may be seen as non-self molecules that trigger the immune response and, in fact, male sperm and accessory gland proteins have been shown to upregulate genes associated with antimicrobial defence in female *D. melanogaster* (McGraw *et al.* 2004). Sexually transmitted diseases in insects can be common, highly pathogenic, and have the ability to sterilize their host (Knell and Webberley, 2004). Thus, it seems that depending on the species it may be adaptive for females to upregulate their immune function, despite possible energetic costs.

In male drumming wolf spiders and fruit flies immunosuppression was found to be a consequence of either increased courtship or an integrated effect of both mating and courtship (McKean and Nunney, 2001; Ahtiainen *et al.* 2005), whereas in male crickets the number of matings was not found to affect immunity (Gershman, 2008). Interestingly, the reduction in the lifespan of mated soil nematodes was found to be caused by additional sperm production and not by the physiological activity of mating (Van Voorhies, 1992). The costs of courtship and mating will vary between species, and it has been suggested that individuals could moderate their mating frequency to minimize any costs to their immunity (Gershman, 2008). In our study the beetles were allowed to mate only once. Hence, the costs associated with courtship and mating may have been small.

In the present study, the likelihood of dying from the infection was smaller for females (both virgin and mated) than it was for males, which indicates an immunological sex difference in the ability to survive fungal infection in this species. Among many vertebrates, males tend to show a greater susceptibility to parasitic infections and also tend to have reduced immune responses compared with females (Zuk and McKean, 1996). Among invertebrates the situation has been found to be more complicated (see Rantala and Roff, 2007) and alternative hypotheses besides the Bateman's principle, which is traditionally used to explain the observed immunological sex differences, have recently been suggested (McKean and Nunney, 2005; Rantala and Roff, 2007).

The beetles in our study received food *ad libitum* so they were not forced to re-allocate resources from other life-history components to immunity. Immunity is considered costly and it is thus expected to be traded-off against other energy demanding life-history components like reproduction (Sheldon and Verhulst, 1996; Schmid-Hempel, 2003). When food is limited, increased energy intake is not possible and a large allocation to immune defence reduces the energy available to other life-history factors (Moret and Schmid-Hempel, 2000). In a benign environment such as the laboratory, stressed individuals may be able to eat more to compensate for any increased energy demands (see Tyler *et al.* 2006). Animals may also manipulate their resource allocation to low priority fitness components before reducing their energetic commitment to high-priority fitness components like immune defence (Rigby and Moret, 2000). However, a trade-off between mating and immune defence does not always disappear under conditions of excess food. In a study on fruit flies food availability did not explain the observed immunosuppression in mated male flies (McKean and Nunney, 2001). Repeating the study comparing starved and well-fed beetles would thus be of great interest for future work.

One could think that our study would have been biased by the fact that some males failed to mate. Low-quality males with low immunity might be more frequently rejected by females and dropped from the study (see Rantala *et al.* 2002, 2003*a*, *b*), raising the average immunity of males retained in the study. However, we found that only 13 males out of 318 (4%) were not able to mate which is too small to bias the result. Furthermore, it is important to note that females may fail to mate due to the low quality of their partners, not their own quality, so the set of females retained in our study were not biased by this factor.

A previous study on *Tenebrio molitor* suggested that copulation corrupts immunity because, after mating, PO activity in the haemolymph was reduced after mating (Rolff and Siva-Jothy, 2002). Instead, no effect of mating on haemocyte load was found. However, a decline in only one parameter of immunity, such as PO activity, following mating gives very little information about an individual's overall immune response. In addition, PO activity has not been shown to correlate well with resistance against naturally occurring pathogens (Shoemaker *et al.* 2006; Schwarzenbach and Ward, 2007) and studies using *Drosophila* mutants that lack prophenoloxidase-activating enzyme suggests that PO does not play a role in resistance against bacterial or fungal infections (Leclerc *et al.* 2006). Thus, it seems that PO may not be a good proxy for disease resistance. Our experiment found that non-virgin individuals were more resistant to a fungal disease than were virgin individuals. We thus suggest that until the discrepancy between the different measures of the immune system is solved, a cautionary approach is needed and studies should be repeated in many different populations before any generalizations are made.

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REFERENCES

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

Adamo, S. A. (2004*b*). Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology* **50**, 209–216.

Ahtiainen, J. J., Alatalo, R. V., Kortet, R. and Rantala, M. J. (2005). A trade-off between immune function and sexual signalling in a natural population of the drumming wolf spider *Hygrolycosa rubrofasciata*. *Journal of Evolutionary Biology* **18**, 985–991.

Bhattacharya, A. K., Ameel, J. J. and Waldbaer, G. P. (1970). A method for sexing living pupal and adult yellow mealworms. *Annals of the Entomological Society of America* **63**, 1783.

Fedorka, K. M., Linder, J. E., Winterhalter, W. and Promislow D. (2007). Post-mating disparity between potential and realized immune response in *Drosophila melanogaster*. *Proceedings of the Royal Society of London, B* **274**, 1211–1217.

Fedorka, K. M., Zuk, M. and Mousseau, T. A. (2004). Immune suppression and cost of reproduction in the ground cricket, *Allonemobius socius*. *Evolution* **58**, 2478–2485.

Fedorka, K. M. and Zuk, M. (2005). Sexual conflict and female immune suppression in the cricket, *Allonemobius socius*. *Journal of Evolutionary Biology* **18**, 1515–1522.

Gershman, S. N. (2008). Sex-specific differences in immunological costs of multiple mating in *Gryllus vocalis* field crickets. *Behavioural Ecology* **19**, 810–815.

Knell, R. J. and Webberley, K. M. (2004). Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. *Biological Reviews* **79**, 557–581.

Leclerc, V., Pelte, N., El Chamy, L., Martinelli, C., Ligoxygakis, P., Hoffmann, J. A. and Reichhart, J.-M. (2006). Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. *EMBO Reports* **7**, 231–235.

McGraw, L. A., Gibson, G., Clark, A. G. and Wolfner, M. F. (2004). Genes regulated by mating, sperm, or seminal proteins in mated female *Drosophila melanogaster*. *Current Biology* **14**, 1509–1514.

McKean, K. A. and Nunney, L. (2001). Increased sexual activity reduces male immune function in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences, USA* **98**, 7904–7909.

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

Moret, Y. and Schmid-Hempel, P. (2000). Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**, 1166–1168.

Peng, J., Zipperlen, P. and Kubli, E. (2005). Drosophila sex-peptide stimulates female innate immune system after mating via Toll and Imd pathways. *Current Biology* **15**, 1690–1694.

Rantala, M. J., Jokinen, I, Kortet, R, Vainikka, A. and Suhonen, J. (2002). Do pheromones reveal male immunocompetence? *Proceedings of the Royal Society of London, B* **269**, 1681–1685.

Rantala, M. J., Kortet, R. & Vainikka, A. (2003*a*). The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap principle. *Proceedings of the Royal Society of London, B* **270**, 2257–2261.

Rantala, M. J., Kortet, R., Kotiaho, J. S., Vainikka, A. and Suhonen, J. (2003*b*). Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. *Functional Ecology* **17**, 534–540.

Rantala, M. J. and Roff, D. A. (2007). Inbreeding and extreme outbreeding cause sex differences in immune defence and life history traits in *Epirrita autumnata*. *Heredity* **98**, 329–336.

Rigby, M. C. and Moret, Y. (2000). Life-history trade-offs with immune defenses. In *Evolutionary Biology of Host-Parasite Relationships: Theory Meets Reality* (ed. Poulin, R., Morand, S. and Skorping, A.), pp. 129–142. Elsevier Science Amsterdam, The Netherlands.

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

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

Schwarzenbach, G. A. and Ward, P. I. (2007). Phenoloxidase activity and pathogen resistance in yellow dung flies *Scathophaga stercoraria*. *Journal of Evolutionary Biology* **20**, 2192–2199.

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

Shoemaker, K. L., Parsons, N. M. and Adamo, S. A. (2006). Mating enhances parasite resistance in the cricket *Gryllus texensis*. *Animal Behaviour* **71**, 371–380.

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

Tyler, R. E., Adams, S. and Mallon E. B. (2006). An immune response in the bumblebee, *Bombus terrestries* leads to increased food consumption. *BMC Physiology* **6**, 6.

Van Voorhies, W. A. (1992). Production of sperm reduces nematode lifespan. *Nature, London* **360**, 456–458.

Yang, S., Ruuhola T. and Rantala M. J. (2007). Impacts of starvation on immune defense and other life history traits of an outbreaking geometrid, *Epirrita autumnata*: a possible ultimate trigger of the crash phase of population cycle. *Annales Zoologici Fennici* **44**, 89–96.

Zuk, M. and McKean, K. A. (1996). Sex differences in parasite infections: Patterns and processes. *International Journal for Parasitology* **26**, 1009–1023.