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ORIGINAL ARTICLE

Reproductive status of *Tribolium castaneum* (Coleoptera: Tenebrionidae) affects its response to infection by *Steinernema feltiae* (Rhabditida: Steinernematidae)

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Abstract. Gender-specific reproductive roles are important factors determining sexual dimorphism. Here, we investigate the effects of sex-based differences and reproductive status on the defence of *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) against infection by *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae). Female and male beetles, either virgin or post-copulation, were exposed individually to nematodes. Individuals were then sampled every 12 h, dissected, and checked for the presence of nematodes; we also measured their phenoloxidase (PO) activity. Reproductive status affected resistance to nematodes and PO activity as infected virgin individuals had a higher PO activity and lower mortality than reproducing individuals, with no differences between sexes. Mortality also increased with time, while PO activity did not change. Parasite load was related to reproductive status and sex, with reproducing females with the highest parasite loads in all treatments, and virgin males with more nematodes than sexually active males. Our results indicate that the costs of reproduction impair the immunological system of *T. castaneum* similarly in both sexes. It is possible, however, that other components of the immunological system that we did not measure, such as lysozyme activity, are impaired by infection with *S. feltiae* in a sex-specific way.

INTRODUCTION

Infection by parasites has serious fitness consequences for hosts, who may thus experience strong selective pressure to defend themselves (Schmid-Hempel, 2011). Therefore, a significant component of the life history strategy of most organisms is their investment in defence (Schmid-Hempel & Ebert, 2003), often in the form of an immune system. However, the immune system is costly to both maintain and use (e.g., Kraaijeveld & Godfray, 1997; Schmid-Hempel & Ebert, 2003, Schmid-Hempel, 2011) and trade-offs are likely to arise that constrain its evolution.

Many studies indicate that there is a trade-off between immune function and reproductive effort (e.g. Adamo et al., 2001) and trade-offs between immunity and reproduction are a central concept in explanations of sexual selection (Zuk & McKean, 1996; French et al., 2007). In fact, a reduction in immune function due to reproductive activity is documented for several species (Siva-Jothy et al., 1998; Adamo et al., 2001). There is, however, no evidence of reproductive activities resulting in a reduction in the immune response in two species of damselflies (Córdoba-Aguilar et al., 2011) or of a trade-off between reproduction and the response to the parasite (*Serratia marcescens*) in *Acheta domesticus* (Nava-Sánchez et al., 2015).

In promiscuous species, such the study species, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae), sexes are expected to allocate resources in different ways. Male fitness is usually limited by the number of females fertilized, while female fitness is limited by the number of offspring produced. Males therefore increase fitness by increasing mating rates, while females gain fitness through increased longevity and resistance to parasites, a phenomenon known as Bateman's principle (Bateman, 1948; Rolff, 2002). Therefore, males are often more susceptible to parasites than females, in both vertebrates (e.g., Zuk, 1990; Poulin, 1996; Zuk & McKean, 1996; Moore & Wilson, 2002) and invertebrates (e.g., Gray, 1998; Wedekind & Jakobsen, 1998; Adamo et al., 2001; Schwarzenbach et al., 2005; Córdoba-Aguilar & Munguía-Steyer, 2013). In vertebrates this pattern is usually attributed to the immunosuppressive influence of testosterone (Alexander & Stimson, 1988; Zuk, 1990). Insects lack testosterone, but instead, the production of juvenile hormone after copulation can down-regulate the expression of phenoloxidase (Rolff & Siva-Jothy, 2002).

Phenoloxidase is one of the most important immune responses in many insects. It is a key enzyme in the melanization cascade, which determines their resistance to different pathogens and is also involved in hardening of the shells of insects' eggs. Activity of this enzyme is often used to estimate immune function in insects (reviewed in Cerenius et al., 2008; González-Santoyo & Córdoba-Aguilar, 2012). Phenoloxidase activity (and melanization in general) has profound fitness consequences in several pathogen-host systems, such as parasitoids and *Drosophila melanogaster* (Kraaijeveld & Godfray, 1997) and *Mnais costalis* and *Hoplorhynchus polyhamatus* (Siva-Jothy et al., 2001). There is also evidence of trade-offs between phenoloxidase and other fitness traits such as development time and body mass (Cotter et al., 2004) or survival (Kraaijeveld & Godfray, 1997). In contrast, reproductive activity in the damselflies, *Argia anceps* and *Hetaerina americana,* does not appear to have any influence on PO activity (Córdoba-Aguilar et al., 2011).

The red flour beetle (*Tribolium castaneum*) is a highly promiscuous species that is sexually dimorphic in size (Sokoloff, 1974) and in its immune response (Freitak et al., 2012). It is a major pest of stored food products, causing substantial losses to global grain harvests (Rossi et al., 2010). Entomopathogenic nematodes (EPN; Rhabditida: Steinernematidae and Heterorhabditidae) can be used as biological control agents against *T. castaneum* as they are commercially available (Georgis et al., 2006) and do not infest vertebrates (Bathon, 1996). They are obligate parasites of insects that go through a free-living dauer (infective) juvenile (IJ) stage. IJs invade their hosts via natural body openings, such as the mouth, anus and spiracles, and, once in the haemocoel, they release their bacterial symbionts (*Xenorhabdus* in *Steinernema* and *Photorhabdus* in *Heterorhabditis*), which kill the host within a few days (Hirao, 2010). Currently, infection by *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae), the parasite used in this study, is only lethal for larvae and pupae of *T. castaneum*; with the highest mortality recorded for adults ca. 40% (Ramos-Rodríguez et al., 2006). This difference may indicate that adult individuals allocate more resources to immune defence against either the nematodes or their bacterial symbionts. Thus, the *S. feltiae-T. castaneum* system is ideal for investigating questions regarding sex-specific changes in resource allocation in hosts and the effect of these changes on host immunological responses. It also presents an opportunity to determine if hosts' reproductive efforts would change the effectiveness of nematodes as biological control factors.

Consequently, in this study we investigated the influence of sex and reproductive status (virgin versus reproducing) of *T. castaneum* beetles on their response to infection by *S. feltiae.* Towards this end, we measured both parasite load and phenoloxidase activity in infected and control beetles.

MATERIALS AND METHODS

Experimental design

The beetles in this study were kindly provided by B. Milutinović (see CR-01 in Milutinović et al., 2013). The strain is kept outbred at a constant temperature of 30°C (the "normal" temperature in this study) in constant darkness, and fed ad libitum on a medium composed of organic wheat flour and yeast (9 : 1 ratio). *Tribolium castaneum* beetles do not need additional water sources as they absorb humidity from the substrate (Sokoloff, 1974). The beetles were kept in plastic boxes with lids that had ventilation holes covered with steel mesh; the humidity in the culture was 70% RH. Experimental animals were reared under laboratory conditions for approximately 35 generations and kept outbred. A commercial strain of *Steinernema feltie*, e-nema, was kindly provided by R.-U. Ehlers.

In *T. castaneum*, parasite load can be measured by dissecting animals and phenoloxidase activity assessed only by using fresh samples. For this reason, these measurements were made on separate groups. Furthermore, a preliminary study indicated that, due to the small size of the beetles, the collection of haemolymph from numerous samples in a short time can only be done using whole animals.

We randomly chose 500 pupae of *T. castaneum* from the stock culture. Sex determination in this species is easiest at the pupal stage, so the experimental animals were divided into females and males as pupae and then allowed to mature in standard culture conditions. Five days after maturation, adults were divided into two groups: in the first, individual beetles were kept separate, while in the second group, one male was paired with one female and allowed to mate. After one week, the couples were separated, all beetles were weighed (Mettler Toledo Microbalance), and both virgin and reproducing individuals were divided into two groups: control and nematode-exposed. A dose of 60 IJs/beetle was established in a preliminary study as high enough to ensure infection (i.e. allow IJs to enter into the beetle's body). The beetles were infected by placing them in Eppendorf tubes filled with 1 ml of wet sand. The beetles were starved for the duration of the experiment, 48 h, as in the preliminary study as no further infection by nematodes was recorded after that time. Infection was carried out at 25°C, a temperature optimal for *S. feltiae* (Hirao et al., 2010) and still within the range of optimal temperatures for *T. castaneum* (Bucher, 2009).

From infected animals in each reproductive status group (female and male virgin, female and male post-reproduction), 20 individuals were sampled after 12, 24, 36 and 48 h, and washed with Ringer's solution on a small sieve to remove any nematodes from the body surfaces. Then, 10 individuals were dissected and checked for the presence of nematodes, while the remaining 10 were frozen for the phenoloxidase measurements. Likewise, 10 of the control animals were sampled for phenoloxidase after the same time intervals and the remaining 10 were left in order to determine naturally occurring mortality.

Phenoloxidase assay

In total, 240 animals were checked for phenoloxidase. Our preliminary study showed that freezing did not change the measurements of phenoloxidase activity. Each animal was homogenized whole, diluted in 100 µl of Ringer's solution and centrifuged at 4°C for 10 min. Next, 10 µl of each sample was placed in a 96-well plate. Four pseudo-replications were performed for each individual in order to detect artefactual readings during spectrophotometer measurements. We added 90 µl of TRIS/Ca^{2+} (0.1 M) and 10 µl of L-DOPA (3 mg ml^{-1} in distilled water, Sigma-Aldrich Co., St. Louis, MO, USA) to each well, then incubated the samples in darkness at room temperature. Spectrophotometer measurements (wavelength 490 nm, micro ELISA Reader Expert Plus, ASYS Hitach GmbH, Austria) were made after 5, 10, 15 and 30 min to estimate when phenoloxidase activity stabilized. For all measurements, phenoloxidase values stopped changing after 30

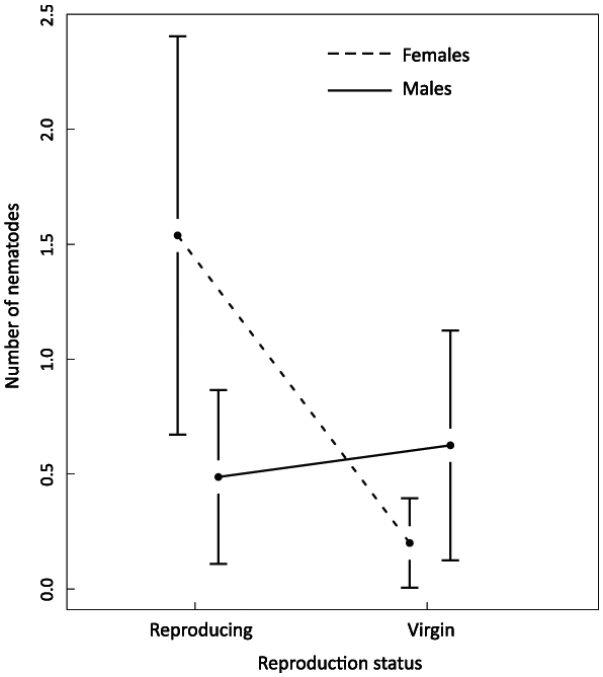

Fig. 1. The number of nematodes recorded in reproducing and virgin individuals of *T. castaneum* infected with *S. feltiae*. Mean and standard errors are shown.

min, so these values were used for further analyses. From each of four pseudo-replications of a given sample, the average phenoloxidase activity in units of absorbance was calculated. Changes in phenoloxidase activity were calculated as follows:

$$PO_a = \frac{PO_{30} - PO_5}{25} \qquad (1)$$

where PO_5 is phenoloxidase activity after 5 min, PO_{30} is phenoloxidase activity after 30 min, and PO_a is phenoloxidase activity in [units (of absorbance)$^{-1}$*min^{-1}].

Protein assay

The amount of protein was determined using the BCA (Sigma-Aldrich Co., St. Louis, MO, USA) method. A 10-µl aliquot of each sample was mixed with 200 µl of a 1 : 50 mixture of copper (II) sulphate and bicinchronic acid solution (Sigma-Aldrich), then incubated for 30 min at room temperature in darkness. Absorbance (wavelength 570 nm) was measured. A standard curve was developed using a serial dilution of bovine serum albumin (BSA, Sigma-Aldrich, 4 mg ml^{-1}). From this curve, the protein concentration in each sample was determined based on its absorbance.

Normalization of phenoloxidase activity

After averaging absorbance for all pseudo-replications of a given sample, values of phenoloxidase activity were normalized for protein content, using the following equation:

Table 1. Results of generalized linear models for number of IJ nematodes in *T. castaneum* beetles exposed to infection by *S. feltiae* (Poisson distribution). Factors marked in bold are statistically significant.

Factor	Estimate	Standard Error	z value	p
Intercept	**0.232**	**0.255**	**0.910**	**0.363**
Reproductive status	**–2.04**	**0.377**	**–5.418**	**<0.001**
Sex	–1.15	0.263	–4.37	<0.001
Time	0.006	0.007	0.921	0.357
Reproductive status × sex	**2.29**	**0.484**	**4.73**	**<0.001**

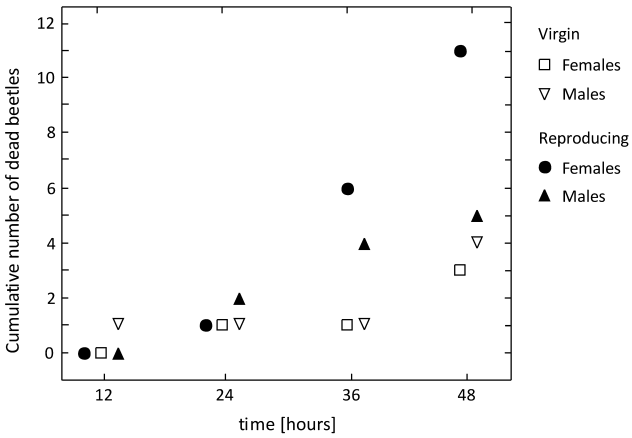

Fig. 2. The number of dead *T. castenum* recorded over time after infection with *S. feltiae*. No dead beetles were recorded in the control group (not exposed to nematodes) during the experiment; thus, the data presented are only for infected beetles. Cumulative number of dead beetles are shown.

$$PO = \frac{PO_a}{P} \qquad (2)$$

where P is protein concentration in mg and PO is phenoloxidase activity in [units (of absorbance)$^{-1}$*mg protein^{-1}*min^{-1}].

Statistical analyses

The number of nematodes present was analyzed only for infected beetles. The tests used were dependent on the data distribution in each case: generalized linear models (GLM) for nematode number (Poisson distribution), GLM for survival (binomial distribution) and GLM for PO activity (Gaussian distribution). In the control group all animals survived. In the case of PO activity, data were log (ln) transformed to obtain a normal distribution and body mass was used as a covariate. Statistical analyses were done using R software (R Development Core Team, 2012).

RESULTS

The number of nematodes infecting a beetle was small and depended on reproductive status ($p < 0.001$) and sex ($p < 0.001$). We also detected significant interactions between sex and reproductive status ($p < 0.001$), with reproducing females infected with the highest number of nematodes, whereas virgin males had a higher parasite load (Fig. 1; Table 1). Time had no effect on parasite load ($p = 0.363$). The factors that affected the proportion of dead beetles in the infected group were reproductive status ($p = 0.033$), and time ($p = 0.0003$), with mortality higher in reproducing animals and increasing with time (Fig. 2; Table 2).

In the case of PO activity, the interaction between infection and reproductive status was significant ($p = 0.007$), but no main factor was statistically significant. Infected virgin

Table 2. Results of generalized linear models of the mortality of *T. castaneum* beetles exposed to infection by *S. feltiae* (binomial distribution). Factors marked in bold are statistically significant.

Factor	Estimate	Standard Error	z value	p
Intercept	22.7	1292	0.018	0.986
Infection	–18.8	1292	–0.015	0.988
Reproductive status	**1.10**	**0.516**	**2.14**	**0.033**
Sex	0.602	0.498	1.21	0.227
Time	**–0.081**	**0.022**	**–3.59**	**< 0.001**

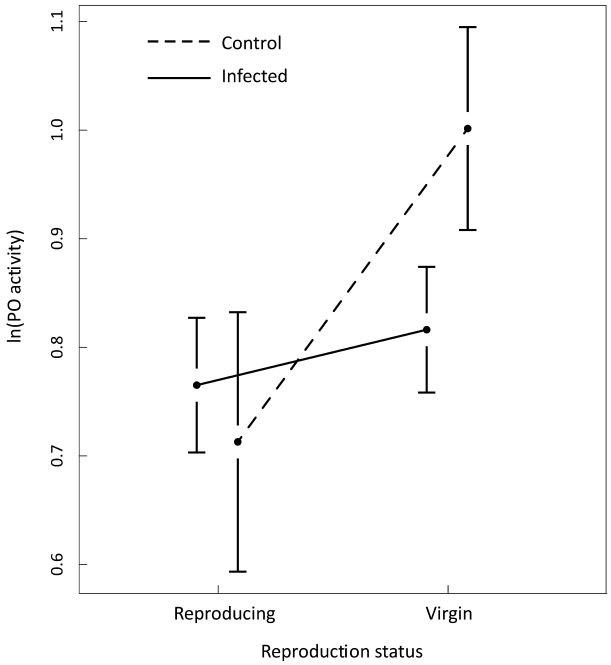

Fig. 3. The phenoloxidase (PO) activity (units of absorbance^{-1}*mg protein^{-1}*min^{-1}) recorded in reproducing and virgin *T. castanuem* following infection with *S. feltiae*. Mean and standard errors are shown.

beetles had the highest PO activity, but infection decreased PO activity in reproducing animals (Fig. 3, Table 3).

DISCUSSION

Our study confirmed that reproduction may impair the activity of phenoloxidase, as is stated by Rolff & Siva-Jothy (2002). We recorded the highest activity in virgin beetles and the lowest in reproducing animals, indicating that reproduction decreased the amount of resources available for the immunological response. Likewise, virgin females contained the lowest number of nematodes of all the treatment groups. At the same time, reproducing beetles infected with *S. feltiae* suffered a higher mortality than virgins. Our results thus seem to indicate that reproduction incurs costs that can decrease the effectiveness of the immunological response, while also increasing the cost of that response in reproducing individuals.

This finding seems to confirm the existence of a trade-off between reproductive activity and immune activity. At the same time, however, we did not find strong support for the assumptions of Bateman's Principle, which states that it is the female sex that should invest more in parasite resistance (Bateman, 1948; Rolff, 2002). There are many studies on invertebrates in which males are more susceptible to parasites. For example, Gray (1998) reports a higher mortality in male house crickets (*Acheta domestica*, L. 1758) after experimental infection with *Serratia liquefaciens* and, similarly, Wedekind & Jakobsen (1998) show that male copepods, *Macrocyclopus albidus* (Jurine, 1820), are more likely to be infected by helminths. Likewise, Adamo et al. (2001) demonstrate a similar effect in *Gryllus texensis* exposed to *S. marcescens.*

Table 3. Results of generalized linear models of the phenoloxidase (PO) activity recorded for *T. castaneum* beetles exposed to infection by *S. feltiae* (normal distribution). Data were log (ln) transformed. Factors marked in bold are statistically significant.

Factor	Estimate	Standard Error	t value	p
Intercept	**0.674**	**0.068**	**9.88**	**<0.001**
Infection	–0.052	0.061	–0.857	0.392
Reproductive status	0.051	0.060	0.844	0.400
Sex	0.057	0.043	1.33	0.186
Time	0.002	0.002	1.28	0.202
Reproductive status × infection	**0.238**	**0.086**	**2.75**	**0.006**

At first, the results of our phenoloxidase assays also seem to conflict with many reports in the literature: we did not record differences in phenoloxidase activity between the sexes. There are a number of studies on sexual dimorphism in the immune response (specifically, phenoloxidase activity), most of which record a significantly higher phenoloxidase activity in females. For example, a meta-analysis by Nunn et al. (2009) includes 11 studies on phenoloxidase, of which 10 report female-biased phenoloxidase and only one a male bias. Likewise, our results also conflict with the existing literature in reporting a reproduction-based difference in phenoloxidase activity, as there are also studies that report no such differences between groups of different reproductive status. For example, Schwarzenbach et al. (2005) report that sexes of the yellow dung fly (*Scathophaga stercoraria*, L. 1758) differ in hemolymph phenoloxidase activity only significantly associated with age, not copulation or egg laying.

An explanation for this may be found using optimal resource allocation models. As the resources available to an organism are limited, they should be invested in a way that maximizes fitness (so-called "Allocation Principle" see: Perrin & Sibly, 1993). Their allocation to reproduction will decrease the amount available for maintenance (including immunological defence) (Cichoń, 1997). However, neither reproduction nor the immune response are simple processes. Reproduction imposes costs at every stage of reproductive activity (that is, gamete production, courting, mating, guarding and parental care) and all of these may also pose a significant cost for males (see, for example, Scharf et al., 2012). Likewise, an insect's immunological system consists of a range of mechanisms from humoral (for example, production of reactive oxygen species and antibacterial peptides) to cellular (such as encapsulation and phagocytosis) (Gillespie et al., 1997; Lavine & Strand, 2002). It is the interplay between all these elements, mediated by the mating system and the environment, which shapes the reproduction-based differences between sexes in their susceptibility to parasite infection. For example, Stoehr & Kokko (2006) propose that as the strength of sexual selection on males increases, so too should the magnitude of the sex-based difference in immune response. However, in exceptional cases males may still invest the same amount of resources as females, or even more, in the immune response, depending on the effect of parasites on condition and/or on the relationship between condition and reproduction (Stoehr & Kokko, 2006).

We therefore propose that the lack of a difference in mortality between the sexes may be the result of decreased sexual selection on the males in our laboratory culture; under these circumstances, both sexes may eventually invest similar amounts of resources in their immune response. At the same time, the distribution of the costs of the different stages of reproductive activity and investment in particular parts of the immune system may differ greatly between the sexes, which may be the cause of the recorded patterns in the particular traits investigated. One should also keep in mind that we checked mortality only after 48 h. As *T. castaneum* is a long lived animal, differences in survival between sexes might only become apparent after a longer period of time.

Our results indicate that using virgin instead of mated animals is not recommended, as the dynamics of infection and mortality may be influenced by the costs of reproduction. Results that are generated with virgin individuals are unlikely to resemble real-world phenomena, particularly if the study organism is a highly promiscuous species and is likely to experience significant costs of reproduction throughout its adult life. Additionally, we have shown that prolonged exposure to *S. feltiae* may be lethal even for adult *T. castaneum* despite the fact that they are not the primary infection targets for this commercially used entomopathogenic nematode.

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COMPETING INTERESTS. The authors declare no competing financial interests. P.K. developed the concept and designed the research, D.M and M.G. performed the experiment, S.M.D. did statistical analyses, D.M., P.K. and J.H. wrote the paper, J.H. designed phenoloxidase measurements.

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