

Do pheromones reveal male immunocompetence?

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Pheromones function not only as mate attractors, but they may also relay important information to prospective mates. It has been shown that vertebrates can distinguish, via olfactory mechanisms, major histocompatibility complex types in their prospective mates. However, whether pheromones can transmit information about immunocompetence is unknown. Here, we show that female mealworm beetles (*Tenebrio molitor*) prefer pheromones from males with better immunocompetence, indicated by a faster encapsulation rate against a novel antigen, and higher levels of phenoloxidase in haemolymph. Thus, the present study indicates that pheromones could transmit information about males' parasite resistance ability and may work as a reliable sexual ornament for female choice.

Keywords: immunocompetence; pheromones; sexual selection; *Tenebrio molitor*

1. INTRODUCTION

Pheromones are airborne chemical signals that are released by an individual into the environment and which affect the physiology or behaviour of other members of the same species (Beauchamp *et al.* 1976). Pheromones are one of the most commonly used social signals among organisms (Arnold & Houck 1982; Birch & Haynes 1982). Organisms as diverse as bacteria, nematodes, crustaceans, insects, amphibians, reptiles and mammals (including humans) have been shown to use them as signals during many different types of social encounters (Birch & Haynes 1982; Moore 1997; Moore *et al.* 1997; Stern & McClintock 1998; Penn & Potts 1998).

Theoretical and empirical studies have indicated that male secondary sexual traits may convey reliable information concerning their ability to resist pathogens and parasites (Hamilton & Zuk 1982; Andersson 1994). This is suggested to be the result of a trade-off between sexual trait expression and immune function (Folstad & Karter 1992; Sheldon & Verhulst 1996). For example, if resources must be diverted away from the immune system in order to maximize expression of a trait, males may suffer increased susceptibility to pathogenic infections (Folstad & Karter 1992; Sheldon & Verhulst 1996). Recent studies in insects support the idea that secondary sexual characters do indicate male immunocompetence to females (Rantala *et al.* 2000; Ryder & Siva-Jothy 2000; Siva-Jothy 2000). It has been suggested that pheromones might also function as an honest indicator of a male's resistance to parasites (Penn & Potts 1998) and it has been shown that vertebrates can distinguish, via an olfactory mechanism, major histocompatibility complex (MHC) types in their prospective mates (e.g. Penn & Potts 1999). However, to our knowledge, no studies have been published that establish whether pheromones transmit information about actual male immunocompetence.

The term immunocompetence is used to refer to the

ability of an individual's immune system to resist and control pathogens and parasites. In insects, one of the most informative ways to assay immunocompetence is to measure the magnitude of the cellular encapsulation response to a novel and standardized antigen such as a nylon monofilament (e.g. Köning & Schmid-Hempel 1995; Rantala *et al.* 2000; Ryder & Siva-Jothy 2000; Siva-Jothy 2000). A major humoral immune effector system in insects responsible for resistance to parasites is the phenoloxidase (PO) cascade (Rowler *et al.* 1986). PO is expressed and regulated in response to the presence of foreign materials or pathogens in the haemocoel (Söderhall 1982). The activation of this enzyme results in the melanization and death of the pathogen (Nappi *et al.* 1996). To assess immunocompetence we used two measures, the encapsulation response against a novel antigen and haemolymph PO activity.

The mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) is a cosmopolitan pest of stored grains, which lives as a larva for one to two years before maturing into an adult beetle. There is no obvious sexual dimorphism in this species. However, each sex produces distinct pheromones that attract members of the opposite sex (Happ 1969; August 1971; Tanaka *et al.* 1986). The pheromone of the male has been observed to stimulate the female's locomotor activity, to promote the aggregation of females in the vicinity of the male and to enhance the copulatory behaviour itself (August 1971; Tanaka *et al.* 1986; Hurd & Parry 1991). A previous study with *T. molitor* showed that infection by a tapeworm, *Hymenolepis diminuta*, reduces the attractiveness of male pheromones (Worden *et al.* 2000). This suggests that parasite-mediated sexual selection may occur in this species (Worden *et al.* 2000). However, it is not known whether pheromones transmit any information concerning male immunological quality *per se*. The aim of this study was to determine whether females prefer the pheromones of males with high immunocompetence. To test preference, we used a filter paper method similar to that used earlier in the studies with *T. molitor* (see Worden *et al.* 2000).

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2. MATERIAL AND METHODS

(a) *Insects*

The beetles were from a laboratory stock population originating from a commercial supplier (Fauntar, Vantaa) and maintained at the University of Jyväskylä by M.J.R. We collected pupae daily from a large bulk laboratory stock. We determined the sex of each pupa by examining the developing genitalia on the eighth abdominal segment. Shortly after emergence, the beetles were placed in individual plastic film-roll canisters with an excess of apple. Sexes were physically isolated from other individuals, in order to ensure virginity. Before the experiment, we weighed the fresh body mass of beetles to the nearest 0.1 mg.

(b) *Pheromone collection and preference tests*

To collect pheromones from males, we placed each male on a small (*ca.* 37 mm diameter) Petri dish containing a filter paper disc for 48 h on days 7–9 following their emergence. To test the female preference for pheromones from different males, we presented the filter paper discs containing pheromones (described above) from two randomly chosen males ($n = 82$ male pairs) together with a clean filter paper (control). The time after collecting pheromones and the use of the discs was exactly equal for each male pair and all the discs were used within 10 h after the removal of males from the disks. The arena for female choice trials consisted of a 20 cm diameter glass dish inverted over a filter paper. A virgin female beetle was placed under a small Petri dish in the centre of the circular arena to calm down for 8 min preceding the trial. At the start of the trial we removed the small Petri dish that restricted the female and placed the glass dish over the entire arena. Each trial lasted for 10 min, during which time the female's movements were videotaped under red-light illumination. Female preference was measured as the total time that a female spent on each filter disc. To statistically test differences in time which females used on each disc we performed non-parametric multiple comparisons to a Friedman test (Zar 1996).

To eliminate male weight effect on female choice we conducted another set of trials, where we presented discs containing pheromones (described above) from weight-matched males (at least 99% similarity in fresh body weight). Male pairs were chosen so that there was at least a 25% difference between males in their encapsulation rate. Discs were presented to two different females to assay the repeatability of female choice.

(c) *Measurements of immune functions*

After collection of the pheromones, we inserted a 2 mm long piece of nylon monofilament (diameter of 0.1 mm) through a puncture in the pleural membrane between the second and third sternite. The males' immune system was allowed to react to this object for 24 h, while insects were kept individually in film-roll canisters at constant room temperature (28 ± 1 °C). The implant was then removed and dried. We could not recover implants from 10 males. The encapsulation rate was measured using the method modified from Köning & Schmid-Hempel (1995). The removed monofilament was examined under a light microscope and recorded on digital video from three different angles. The pictures were then analysed using an image analysis program (Image Pro). The degree of encapsulation was analysed as a grey value of reflecting light from implants. As a measure of encapsulation rate, we used the average grey values of three video pictures. The scale was calibrated to indicate that the darkest grey received the highest encapsulation rate (total black).

To measure the repeatability of this method we re-scanned 16 randomly chosen implants and analysed them as above. The repeatability (R) of this method was high ($R = 0.997$; $F_{15,16} = 778.69$, $p < 0.001$) (Krebs 1989).

In the experiment for weight-matched males, we used nylon implants, which had been rubbed with fine-grained sandpaper (since this method proved to be more accurate). Three hours after inserting the implant, we took a haemolymph sample from males for PO activity measurements and removed the implant for image analysis.

Before removing the implant, the neck of each male was cut with scissors and 1 μ l of haemolymph was collected from the wound into a plastic micropipette. The haemolymph was then mixed with 99 μ l of phosphate buffered saline solution (pH 7.4). Samples were immediately frozen at -25 °C to disrupt the haemocyte membrane. After thawing, the sample and 200 μ l of 10 mM L-DOPA were pipetted into the wells on a 96-well plastic microplate (Cliniplate, Labsystems). The absorbance at 492 nm was then measured spectrophotometrically with a plate reader (Multiskan, Flow Laboratories) at 20 °C at 1 min intervals for 30 min. The enzyme activity was expressed as the maximum rate of the reaction.

3. RESULTS

There was a statistically significant positive relationship between different components of immune defence: encapsulation rate (cellular) and PO enzyme activity (humoural) (Spearman's $r = 0.26$, $p = 0.001$, $n = 154$). Furthermore, male fresh body weight was correlated with encapsulation rate (Spearman's $r = 0.24$, $p = 0.003$, $n = 154$) and PO enzyme activity (Spearman's $r = 0.27$, $p = 0.001$, $n = 164$).

(a) *Female preference for randomly chosen male pairs*

Females spent significantly more time on filter discs bearing pheromones from males with a high encapsulation rate than those discs from males with a low encapsulation rate or blank discs (clean filter discs) (Friedman ANOVA block design with each female choice trial as blocks, $n = 73$, $\chi^2 = 76.58$, d.f. = 2, $p < 0.001$) (figure 1a). Similar differences in preference were also found with PO (Friedman test, $n = 82$, $\chi^2 = 65.17$, d.f. = 2, $p < 0.001$) (figure 1b) and fresh body weight (Friedman test, $n = 82$, $\chi^2 = 63.88$, d.f. = 2, $p < 0.001$) (figure 1c). Thus, it seems certain females use pheromones to discriminate between males.

(b) *Female preference for weight-matched male pairs*

Again, females spent significantly more time on filter discs bearing pheromones from males with a high encapsulation rate than those discs from males with a low encapsulation rate (Wilcoxon test, $n = 38$; $Z = -3.14$, $p = 0.002$) (figure 2a). Similar differences in preference were also found with PO (Wilcoxon test, $n = 35$; $Z = -3.29$, $p = 0.001$) (figure 2b). In addition, we verified preference similarity for two different females for each male pair. The similarity of female preference was tested using a Kappa Measure of Agreement. The agreement of females was highly significant ($n = 38$, $\kappa = 0.50$; $p = 0.001$).

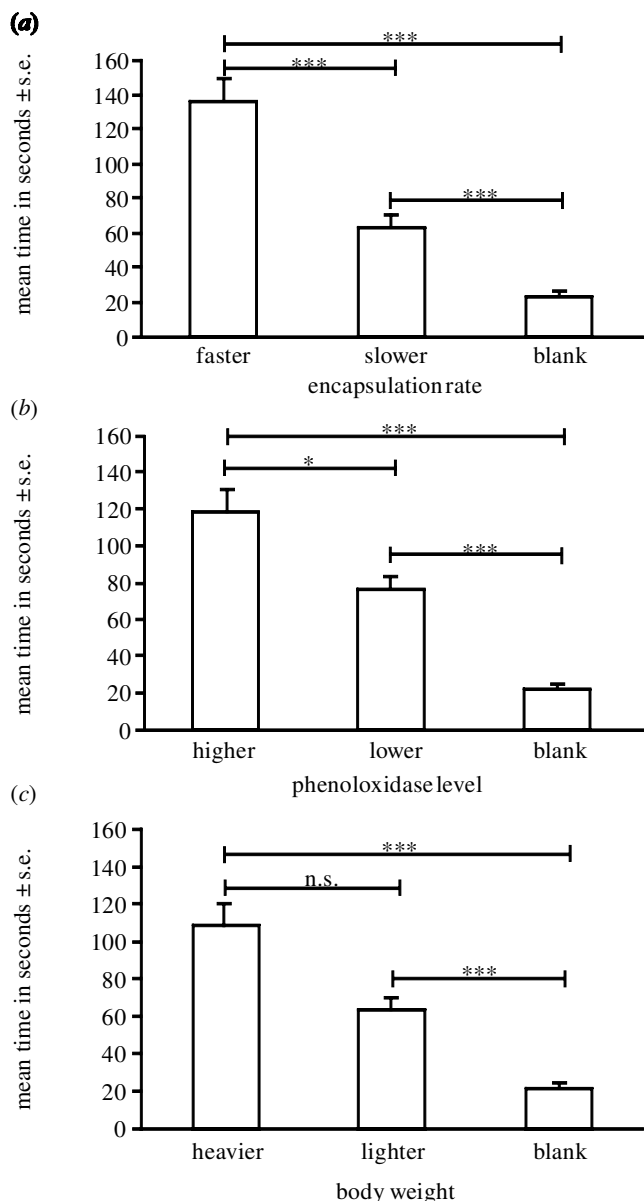


Figure 1. (a) The average time each female spent on filter discs of males with faster encapsulation rate (mean = 59.1, s.d. = 15.6), slower encapsulation rate (mean = 39.8, s.d. = 13.2) and a blank disc (clean filter disc) ($n = 73$ pairs). (b) The average time each female spent on filter discs of males with higher phenoloxidase levels (mean = 49.9, s.d. = 56.7), lower phenoloxidase levels (mean = 12.1, s.d. = 27.6) and a blank disc (clean filter discs) ($n = 82$ pairs). (c) The average time each female spent on filter discs from heavier males (mean = 127.0 mg, s.d. = 23.6), lighter males (mean = 102.5 mg, s.d. = 22.7) and a blank disc (clean filter discs) ($n = 82$ pairs).

4. DISCUSSION

It has been shown that pheromones function not only as mate attractors, but they may also relay important information to prospective mates. For example, females have been observed to prefer the pheromone of males with low fluctuating asymmetry (Thornhill 1991; Rikowski & Grammer 1999) and parasite burden (e.g. Penn & Potts 1998). Pheromones have also been shown to provide information on male aggressiveness (Moore & Moore 1999) and affect female fitness (Moore *et al.* 2001). How-

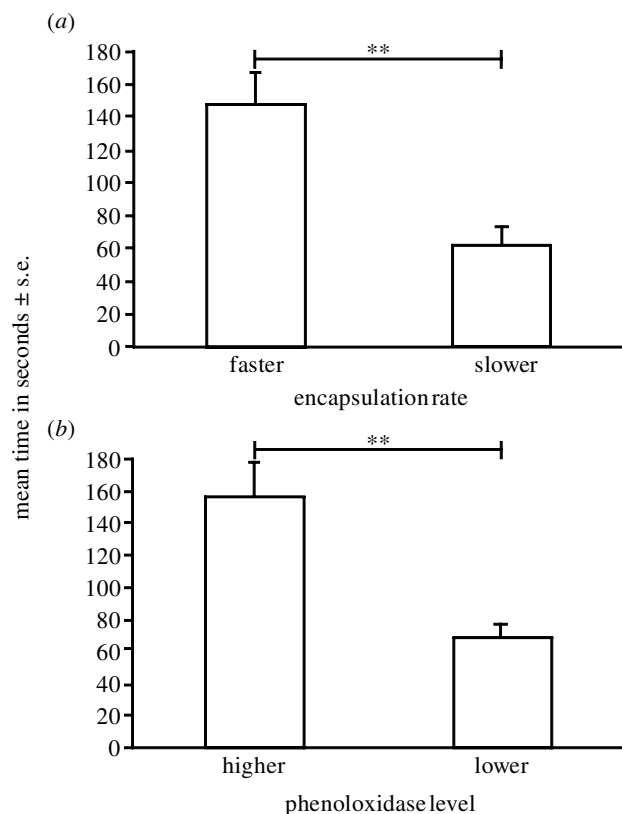


Figure 2. (a) The average time each female spent on filter discs from similar weight males with faster encapsulation rate (mean = 120.3, s.d. = 21.8) and slower encapsulation rate (mean = 84.17, s.d. = 21.8) ($n = 38$ pairs). (b) The average time each female spent on filter discs from similar weight males with higher phenoloxidase levels (mean = 171.6, s.d. = 113.6) and lower phenoloxidase levels (mean = 54.5, s.d. = 36.0) ($n = 35$ pairs).

ever, to our knowledge, this is the first study indicating that females prefer the scent of males with better immunocompetence. According to our results, it seems that pheromones could also transmit information to females about male immunocompetence.

Although studies about pheromones and immunocompetence are lacking, there are several studies in vertebrates, which have shown that they can distinguish, via olfactory mechanisms, MHC types in their prospective mates (e.g. Wedekind *et al.* 1995; Wedekind & Furi 1997; Reusch *et al.* 2001). MHC-dependent mating preference may function to produce disease-resistant, MHC-heterozygous offspring to reduce inbreeding, or both (Potts & Wakeland 1993; Brown & Eklund 1994; Apanius *et al.* 1997). However, it has not been shown that the odours caused by MHC genes function as pheromones (see Penn & Potts 1998). Furthermore, in contrast to vertebrates, invertebrates do not have such an adaptive immune response (e.g. Gillespie *et al.* 1997).

Recent studies in immunoeology have shown that maintenance and activation of the immune system in insects is a trade-off against other fitness components (Kraaijeveld & Godfray 1997; Moret & Schmid-Hempel 2000). According to the immunocompetence handicap hypothesis, sexual trait expression may also be constrained through a trade-off with immune functions (Folstad & Karter 1992; Sheldon & Verhulst 1996). Thus, it is poss-

ible that we may find a correlation between the attractiveness of male pheromones and male immunocompetence, because only males of high phenotypic quality are able to cope with the costs of maintaining both an efficient immune system and producing pheromones. Conversely, the pheromones preferred by females might be a by-product of immune functions (Penn & Potts 1998). However, in both such cases, the pheromones might work as an honest secondary sexual trait (see Grafen 1990). August (1971) showed that a female's preference for male pheromone is dose dependent and thus it is possible that males with better immunocompetence can simply produce more pheromones than those males with lower immunocompetence.

'Good-genes' models of sexual selection propose that male ornaments indicate underlying quality, which can be inherited by the offspring, thereby improving their viability (e.g. Andersson 1994). Interestingly, in studies with a species closely related to *T. molitor*, the red flour beetle (*Tribolium castaneum*), Boake (1985) found no correlation between progeny fitness and the attractiveness of male pheromones. However, she did not measure immunocompetence. Perhaps the 'good genes' which females acquire are the genes which affect resistance to parasites (see Hamilton & Zuk 1982). Support for this idea comes from recent studies in insects, which show that secondary sexual characters do indicate male parasite resistance (Rantala *et al.* 2000; Ryder & Siva-Jothy 2000; Siva-Jothy 2000), and the resistance to parasites is heritable (Kraaijeveld & Godfray 1997; Ryder & Siva-Jothy 2001). The present study indicates that females of *T. molitor* prefer the scent of males with high immunocompetence. By preferring the pheromones of males with high immunocompetence, females may benefit by increasing parasite resistance of their offspring.

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