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Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*

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Summary

1. Pheromones are chemical signals that function not only as mate attractors, but may also relay important information to prospective mates. In order for the information to be reliable, the signal must be costly to produce and this is likely to result in condition dependent expression of the signal.

2. We present results from two experiments on the grain beetle *Tenebrio molitor* examining phenotypic condition dependence of pheromones and patterns of female preference for pheromones. We also analysed condition dependence of two measures of immunocompetence: encapsulation response and phenoloxidase activity.

3. By manipulating the nutritional condition of the males we found that the attractiveness of the male pheromones to virgin females was condition-dependent, indicating that the production of the pheromones is affected by the condition of the male. We also found that the phenoloxidase activity of the males was affected by the nutritional condition of the male but that encapsulation rate was not.

4. Our results show that pheromones are condition-dependent signals, the quantity of which females use in their mate choice.

Key-words: Immunocompetence, odours, sexual ornaments, sexual selection

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Introduction

Indicator models of sexual selection predict that male sexual ornaments develop condition-dependent expression thus allowing females to evaluate male quality as a mate (Zahavi 1975; Zahavi 1977; Nur & Hasson 1984; Andersson 1986; Pomiankowski 1988; Grafen 1990; Iwasa & Pomiankowski 1994; Rowe & Houle 1996). Male quality can be defined as the genetic potential of a male's offspring to survive (e.g. good genes) and reproduce (sexiness) (Kokko *et al*. 2002). Although evidence for indicator models of sexual selection has been found in many empirical studies (Milinski & Bakker 1990; Hill & Montgomerie 1994; Keyser & Hill 1999; David *et al*. 2000; Kotiaho 2000; Kotiaho, Simmons & Tomkins 2001; see also Johnstone 1995; Møller & Alatalo 1999; Jennions, Møller & Petrie 2001), research has focused almost exclusively on visual or acoustic signals, ignoring chemical signals such as pheromones.

Pheromones are a class of species-specific chemical compounds or molecules that are produced to communicate between members of the same species. Pheromones are transmitted through a medium such as air or water by diffusion and may be effective in very small concentrations. Pheromones function as mate attractors (Eisner & Meinwald 1995), but they may also relay other information to prospective mates. For example, pheromones may signal information about nuptial gifts (Dussourd *et al*. 1991), developmental stability (Thornhill 1992; Rikowski & Grammer 1999) or dominance status (Moore *et al*. 1997). Pheromones have also been suggested to function in kin recognition (Smith 1983; Simmons 1990). Because parasitic infections affect the information content of the pheromone signals (Carmichael, Moore & Bjostad 1993; Penn & Potts 1998; Worden, Parker & Pappas 2000), it is possible that pheromones function as condition dependent signals of mate quality.

The term immunocompetence is often used to refer to the ability of an individual's immune system to resist and control pathogens or parasites. The energetic expenditure of producing and maintaining components of the immune system may have a major effect on condition, thus creating a link between immune system and condition dependent sexual advertisement (Wedekind 1992; Wedekind & Folstad 1994). A trade-off is expected such that resources are used up by both

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the sexual advertisement and the immune system (Kotiaho 2001). Therefore, only individuals in good condition can mount a strong immune defence and produce an extravagant sexual ornament (Sheldon & Verhulst 1996; Westneat & Birkhead 1998).

In insects, one of the most informative ways to assay immunocompetence is to measure the cellular encapsulation response to a novel and standardized antigen such as a nylon monofilament (Köning & Schmid-Hempel 1995; Rantala *et al*. 2000; Ryder & Siva-Jothy 2000; Siva-Jothy 2000; Rantala *et al*. 2002; Rantala & Kortet 2003; Koskimäki *et al*. 2003). Encapsulation is a cellular immune response through which insects defend themselves against foreign particles (Salt 1970). During the encapsulation process, specialised haemocytes recognise invading particles as non-self and cause other haemocytes to aggregate and encapsulate the particle. A cascade of reactions involving the tyrosine-phenyloxidase pathway causes the melanization of the capsule and the death of the invading particle (Fisher 1963). Phenoloxidase (PO) is a key enzyme in the synthesis of melanin and the ability to produce melanin is an important aspect of the immune response (Gillespie, Kanost & Trenczek 1997). The encapsulation response also plays a role in defence against viruses (Washburn, Kirkpatrick & Vokman 1996).

The mealworm beetle *Tenebrio molitor* L. (Coleoptera, Tenebrionidae) is a cosmopolitan pest of stored grains, which has no conspicuous sexual dimorphism. However, sexes produce pheromones that attract members of the opposite sex (Happ 1969; August 1971; Tanaka *et al*. 1986). The pheromones of the male have been observed to stimulate female locomotor activity, to promote the aggregation of females in the vicinity of the male and to enhance copulatory behaviour (August 1971; Tanaka *et al*. 1986; Hurd & Parry 1991). *Tenebrio molitor* females prefer pheromones from males which have high immunocompetence (Rantala *et al*. 2002) and infection of *T. molitor* by a tapeworm, *Hymenolepis diminuta*, reduces the attractiveness of male pheromones (Worden *et al.* 2000).

The aim of this study was to determine whether *T. molitor* females exhibit mate preference according to the quantity of the male pheromones and whether pheromones are dependent on male nutritional condition. We also determined the condition dependence of male immunocompetence. To assess immunocompetence, we used the encapsulation response against a novel antigen and haemolymph phenoloxidase (PO) activity.

Materials and methods

STUDY POPULATION

The beetles used in our study were from a large laboratory stock population originating from a commercial supplier and maintained at the University of Jyväskylä. From this population we collected pupae daily and sexed them by the morphology of the eighth abdominal segment (Bhattacharya, Ameel & Waldbaer 1970). Newly emerged adults were placed individually in plastic film roll canisters with excess of fresh apple. For the experiment we chose only healthy adults (those with both antennae and no obvious morphological defects) of average weight (mean ± SD = 130·4 ± 16·3 mg).

FEMALE PREFERENCE OF PHEROMONES IN RELATION TO MALE CONDITION

At the age of 6 days, we measured the body mass of 80 males to the nearest 0·1 mg. After measuring body mass, males were assigned to 40 pairs such that the body mass within the pair did not differ (paired samples *t*-test, $t = 0{\cdot}61$, d.f. = 39, $P = 0{\cdot}55$). Next, males from each pair were randomly assigned to two food ratio treatments: constant food (*ad libitum* apple and water) or no food (*ad libitum* water only). After 7 days of food manipulation, we measured the body mass of the males and placed each on a small Petri dish containing a filter paper disc (diameter 37 mm) for 48 h (see also Rantala *et al*. 2002).

To test female preference for pheromones of males in good and poor nutritional condition, we presented females with a choice of two filter paper discs from a pair of the males; one from a male in the constant food treatment and the other from a male in the no food treatment. Prior to female choice trials both filter papers were exposed to apple to ensure that apple odour could not confound female preference. The arena for female choice trials consisted of a glass bowl (diameter 20 cm) inverted over a sheet of filter paper. The two filter paper discs containing the male pheromones were placed at opposite sides of the arena, at a distance of 8 cm from the centre (Fig. 1a). At the centre of the arena a virgin female was placed under a small Petri dish and allowed to settle for 8 min. At the start of the trial we removed the small Petri dish that restricted the female and placed the glass bowl over the arena. Each trial lasted for 10 min, during which we observed the movements of the female. Female preference was measured as the total time the female spent on the manipulated filter paper discs. All observations were conducted under red lighting.

FEMALE PREFERENCE OF PHEROMONES IN RELATION TO QUANTITY OF THE PHEROMONES AND MALE IDENTITY

In this second experiment we largely followed the methodology described for the first experiment. At the age of 6 days, we measured the body mass of 40 males to the nearest 0·1 mg. Males were assigned into 20 pairs such that the body mass within the pair did not differ (paired samples *t*-test, $t = 0{\cdot}74$, d.f. = 19, $P = 0{\cdot}47$).

To collect different quantities of pheromones from each male, we placed males on a small Petri dish with a half of a filter paper disc (diameter 37 mm) for 24 h.

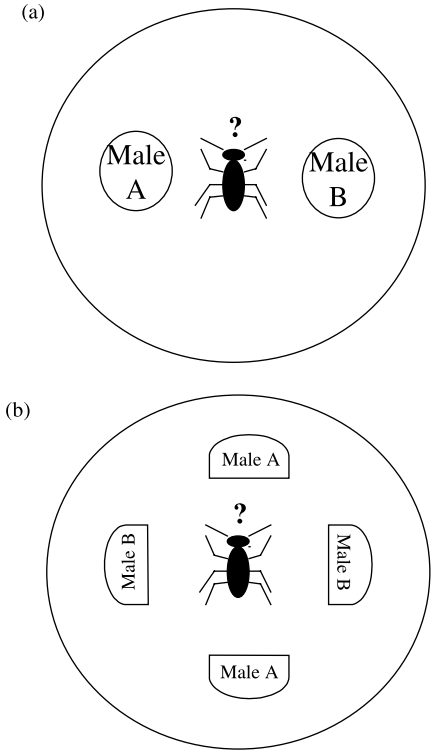

Fig. 1. (a) Experimental set up to test female preference of pheromones in relation to male condition. (b) Experimental set up to test female preference of pheromones in relation to quantity of the pheromones and male identity.

After 24 h we added the other half of the filter paper disc and left the males for another 24 h. We expected that the different exposure time (24 vs. 48 h) translated into different quantities of pheromone on the halves of the filter paper discs. To obtain differences in the quality of the pheromones we relied on the existence of natural variation in pheromone quality between males. By presenting 40 females with a simultaneous choice of two quantities of pheromone from two different males we were able to separate the effects of quantity of the pheromone and male identity on female preference. Each female was used only once but each male was used in two trials. The female preference experiment was conducted using the methodology described above but now with four halves of filter paper discs (Fig. 1b).

CONDITION DEPENDENCE OF IMMUNE FUNCTION

After collection of the pheromones in the 'female preference of pheromones in relation to male condition' experiment (see above), we implanted a 2-mm long piece of nylon monofilament (diameter 0·1 mm, rubbed with sandpaper) through a puncture in the pleural membrane between the second and third sternite. The immune system of the males was allowed to react to this implant for 3 h, during which males were kept individually in white film roll canisters at constant temperature (28 ± 1 °C). After 3 h all of the implants were removed and dried except from 3 males, from which we were unable to recover the implant. Implants were examined under a light microscope and recorded on digital video from three angles. The still video images were then analysed using image analysis (Image Pro-program, Silver Spring, USA). As a measure of the encapsulation rate we used the average grey value of the three video images, taken from different angles. The degree of encapsulation was analysed as grey values of the reflecting light from implants. The repeatability of this method of estimating encapsulation rate is very high (Rantala *et al*. 2002). Because a smaller grey value indicates higher encapsulation rate, figures would be counter intuitive. For this reason we transformed the data such that the darkest grey values correspond to the highest encapsulation rate. The transformation was done by subtracting the observed grey values from the control grey value (clear implant).

Before removing the implant, the neck of each male was cut with scissors and 1 μL haemolymph was collected into a plastic micropipette. The haemolymph was then mixed with 99 μL of phosphate buffered saline solution (pH 7·4). Samples were frozen at –25 °C in the freezer to disrupt the haemocyte membrane. After thawing, we added the sample and 200 μL of 10 mM L-DOPA into the wells on a 96 well plastic microplate (Labsystems Cliniplate, Finland). The mixture absorbance at 492 nm was then measured spectrophotometrically with a plate reader at 20 °C in one minute intervals for 30 min The phenoloxidase enzyme activity was expressed as the maximum rate of the reaction (Rantala *et al*. 2002).

STATISTICAL ANALYSIS

Prior to analysis we tested all variables for normality with the Kolmogorov–Smirnov test. To fulfil normality assumptions some variables were transformed with a natural logarithm (ln). Only the distribution of phenoloxidase activity differed from normality even after transformation and for this variable the analyses were carried out with nonparametric alternatives. All other tests were carried out with parametric statistics.

Results

FEMALE PREFERENCE OF PHEROMONES IN RELATION TO MALE CONDITION

Food treatment was effective in manipulating male condition as it had a clear effect on the body mass of the males. Males in the constant food treatment maintained their body mass while males in the no food treatment on average lost 6·3% of their body mass (repeated measures ANOVA interaction between treatment and the repeated measure $F_{1,78} = 6{\cdot}36$, $P = 0{\cdot}014$; Fig. 2).

Female preference of the pheromones was dependent on the nutritional condition of the males. Females

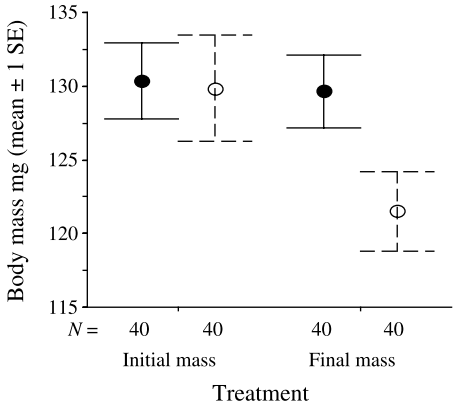

Fig. 2. Mean ± 1 SE body mass of males before and after the food treatments. Black symbols and solid line represent males in the constant food treatment, and open symbols and dashed line males in the no food treatment.

spent significantly more time on the filter paper discs from constant food males than on discs from no food males (paired sample *t*-test with ln transformed data, $t = -3{\cdot}89$, d.f. = 39, $P < 0{\cdot}001$; Fig. 3).

FEMALE PREFERENCE OF PHEROMONES IN RELATION TO PHEROMONE QUANTITY AND MALE IDENTITY

We used mixed model analysis of variance to analyse the female preference for quantity of pheromones and male identity (individual). The quantity was entered as a fixed two level factor and male identity was entered as a random factor. The female preference, i.e. time spent on each filter paper disc, was ln transformed prior to analysis. The interaction between quantity and male identity was not significant ($F_{39,80} = 1{\cdot}09$, $P = 0{\cdot}370$) and was removed from the analysis (see Snedecor & Cochran 1989). In the final model we had only the main effects, and both pheromone quantity and male identity had a significant effect on female preference (Table 1).

Table 1. Mixed model analysis of variance for female preference of pheromone quantity and male identity. Male identity is entered as a random factor

Source	SS type III	d.f.	MS	*F*	Significance
Quantity	41·36	1	41·36	22·89	0·000
Individual	134·13	39	3·44	1·90	0·004
Error	215·00	119	1·81		

CONDITION DEPENDENCE OF IMMUNE FUNCTION

We found that phenoloxidase activity was dependent on the nutritional condition of the males; in the constant food group phenoloxidase activity was 2·6 times higher than in the no food group (Wilcoxon, $Z = -2{\cdot}33$, $n = 40$, $P = 0{\cdot}020$; Fig. 4). However, we found no effect of nutritional condition on the encapsulation rate of the males (paired samples *t*-test, $t = -0{\cdot}69$, d.f. = 36, $P = 0{\cdot}495$; Fig. 5). There were no correlations between male initial body mass and phenoloxidase activity or encapsulation rate in the constant food group (Spearman, $r = 0{\cdot}07$, $n = 40$, $P = 0{\cdot}658$ and Pearson, $r = 0{\cdot}09$, $n = 39$, $P = 0{\cdot}605$, respectively) or in the no food group

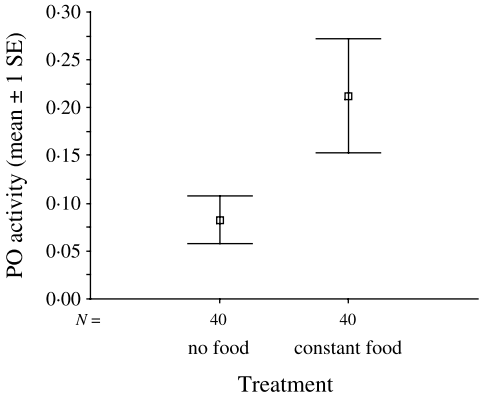

Fig. 4. Mean ± 1 SE phenoloxidation (PO) activity of males in the constant food and the no food treatments.

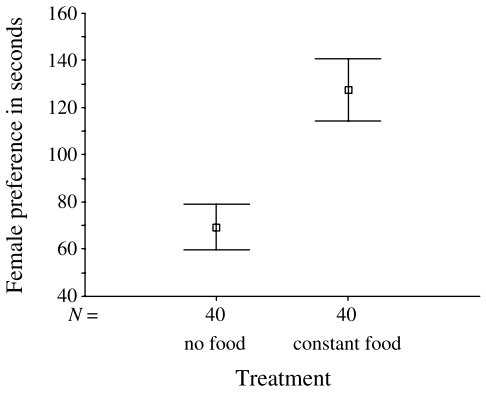

Fig. 3. Mean ± 1 SE female preference of the pheromones of males from the no food and the constant food treatments.

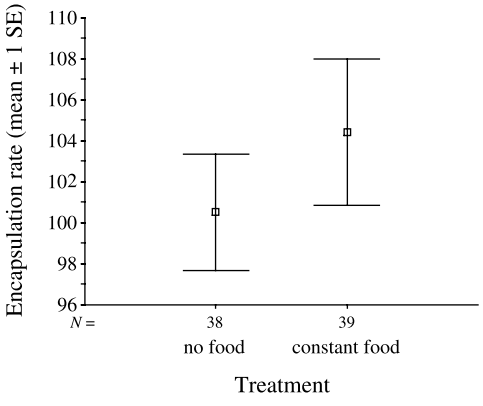

Fig. 5. Mean ± 1 SE encapsulation rate of males in the constant food and the no food treatments.

(Spearman, $r = 0{\cdot}08$, $n = 40$, $P = 0{\cdot}604$ and Pearson, $r = -0{\cdot}19$, $n = 38$, $P = 0{\cdot}248$, respectively).

Discussion

Our experiments revealed that the pheromone mediated attractiveness of males and immunocompetence in terms of phenoloxidase activity were condition-dependent such that both were down regulated by nutritional stress. This suggests that there should exist a trade-off between allocation of resources into the production of pheromones and immunocompetence. The dependence of the production of pheromones and immunocompetence on nutritional condition suggests that the production of pheromones and maintaining immunocompetence may be costly in terms of energy expenditure. However, to show that such energy expenditure is in fact evolutionarily costly, one of two things must be true (Kotiaho 2001): either the spent energy can not be recovered, resulting in decreased fitness, or the spent energy can be recovered but the recovery of the energy comes with a reduction in fitness. These requirements have not been realised in most studies claiming support for costs via energy expenditure (Kotiaho 2001).

Earlier it was found that females prefer pheromones of males with high immunocompetence (Rantala *et al*. 2002). This result suggests a lack of trade-off between production of pheromones and immunocompetence. One explanation may be that males in good condition can afford to invest in both. However, correlations like this have several interpretations (Reznick 1985; Grafen 1990; Folstad & Karter 1992; Sheldon & Verhulst 1996; Westneat & Birkhead 1998) and the positive relationship between the production of pheromones and immunocompetence may also result if males with good immunocompetence acquire less parasites and pathogens and are therefore able to maintain good body condition and produce high amounts of pheromones. Worden *et al.* (2000) found that experimental parasite infection reduces the attractiveness of males. This finding lends support to the existence of a trade-off in resource allocation between the production of pheromones and immunocompetence. Together with our finding of condition dependence of both the production of pheromones and immunocompetence, it seems likely that the mechanism behind the reduction of pheromone attractiveness is indeed a trade-off in resource allocation. Our results are consistent with studies by Clark, DeBano & Moore (1997), who found that sex pheromones in cockroach, *Nauphotea cinerea*, are condition dependent.

In our experiment on female preference in relation to quantity and individual, we found that female preference was affected by both. However, as we were not measuring the qualitative differences in the pheromones but were relying on female preference as an indicator, it is possible that our individual effect arises if different males produce pheromones at different rate. Different rates of pheromone production would translate into different quantities of the pheromone and thus could account for our finding that pheromones from different males are attractive to females to varying degrees. Previously, August (1971) found that the attractiveness of male pheromones is dose dependent at low concentrations, but that at high concentrations male pheromones become repellent to females. However, since August (1971) used a mixture of male extracts from many males, the repelling effect of high concentrations may be an artefact arising from interactions between the pheromones from several individuals.

Surprisingly, although we found that the short-term nutritional stress reduced the phenoloxidation activity of the haemolymph, it did not affect the encapsulation response. However, the phenoloxidase enzyme functions as a catalyzer in the formation of melanin during the encapsulation, and therefore it is likely that there is a time-lag in the effect of nutritional stress on encapsulation response. It may be that this time-lag is longer than our short-term experiment. Another possibility is that encapsulation may be such an important determinant of fitness that males cannot afford to down-regulate the investment in the potential to conduct the encapsulation response against foreign antigens.

Our finding that nutritional stress reduces the immunocompetence in *T. molitor* is consistent with previous studies in other insects. For example, in *Drosophila melanogaster* there is a nutrient-mediated effect on the genetic ability of the individual to respond with melanin production to immune insult (Sang & Burnet 1963), in *Anopheles gambiae* (Diptera: Culicidae) larval nutrition affects the ability of adults to respond to synthetic immune challenge (Suwanchaichinda & Paskewitz 1998), and in *Drosophila melanogaster* nutritional status affects resistance to parasitoids (Vass & Napi 1998). However, the opposite effect has been demonstrated in noctuids, where increased nutritional intake enhanced the negative effects of baculoviruses (Hoover *et al*. 1998). In this study, we did not find a correlation between male body mass and immune function, in contrast to Rantala *et al*. (2002). This was probably due to low variation in body mass, because the males used in this experiment were selected to be similar in their body mass.

In summary, our experiments verified the hypotheses that production of pheromones are condition dependent sexual traits (Penn & Potts 1998), and demonstrated that females use the quantity of pheromones in their mate choice. In light of our study, it seems that pheromones may function as reliable sexual traits for female choice.

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