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The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap principle

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The immunocompetence handicap hypothesis postulates that secondary sexual traits are honest signals of mate quality because the hormones (e.g. testosterone) needed to develop secondary sexual traits have immunosuppressive effects. The best support for predictions arising from the immunocompetence handicap hypothesis so far comes from studies of insects, although they lack male-specific hormones such as testosterone. In our previous studies, we found that female mealworm beetles prefer pheromones of immunocompetent males. Here, we tested how juvenile hormone (JH) affects male investment in secondary sexual characteristics and immune functions in the mealworm beetle, *Tenebrio molitor*. We injected male mealworm beetles with JH (type III) and found that injection increased the attractiveness of male pheromones but simultaneously suppressed immune functions (phenoloxidase activity and encapsulation). Our results suggest that JH, which is involved in the control of reproduction and morphogenesis, also plays a central role in the regulation of a trade-off between the immune system and sexual advertisement in insects. Thus, the results reflect a general mechanism by which the immunocompetence handicap hypothesis may work in insects.

Keywords: immunocompetence handicap hypothesis; immune function; juvenile hormone; pheromones; sexual selection; *Tenebrio molitor*

1. INTRODUCTION

Males of many species have extravagant morphological and behavioural secondary sexual traits, and female choice of mates is often based on these traits (Andersson 1994). Hamilton & Zuk (1982) suggested that resistance to parasites may be revealed through secondary sexual characteristics. The expression of male ornaments has been found to be negatively correlated with parasite load in many studies (i.e. males with large ornaments have low parasite loads or good parasite resistance) (Clayton 1991; Hamilton & Poulin 1997). The mechanism behind this correlation is, however, unclear, and it has been a subject of intense scientific debate. It has been postulated that sexual ornaments that are used as quality indicators must be costly to produce or maintain, because otherwise all males would develop large ornaments (reviewed in Kotiaho 2001). The immunocompetence handicap hypothesis (Folstad & Karter 1992) is one possible mechanistic explanation of these costs. It suggests that the character-quality correlation is maintained by a 'double-edged sword' effect of testosterone (or other such hormone): high concentrations enhance the expression of sexual characteristics, but also suppress immune functions either directly or consequently (Folstad & Karter 1992). Hence, only individuals of high quality can perform well with elevated steroid hormone levels, making the testosterone-dependent traits honest signals of quality. However, the hypothesis may include the possibility that the immunosuppression is adaptive (Wedekind & Folstad 1994).

Most invertebrates, and all insects, lack male-specific hormones such as testosterone (Nijhout 1994). However, the results of recent studies in insects have suggested that secondary sexual characteristics indicate a male's immunocompetence (the ability of an individual to resist and control pathogens) to females (Rantala *et al.* 2000; Ryder & Siva-Jothy 2000; Siva-Jothy 2000; Rantala *et al.* 2002; Rantala & Kortet 2003*a*,*b*). For example, it has been found in the mealworm beetle, *Tenebrio molitor* L., that females prefer pheromones from males with high immunocompetence (Rantala *et al.* 2002) and that both pheromone production and immune functions are condition dependent (Rantala *et al.* 2003). Furthermore, it has been found that infection by a tapeworm, *Hymenolepis diminuta*, reduces the attractiveness of male pheromones (Worden *et al.* 2000). However, the mechanism that mediates the suggested relationship between attractiveness of pheromones and immune functions is not known. A possible hormone that could regulate or be involved in the trade-off between immunocompetence and sexual ornaments in insects is juvenile hormone (JH).

Juvenile hormone is secreted by the corpora allata and is involved in the control of reproduction and morphogenesis in all insects (e.g. Nijhout 1994). There is some evidence that JH also influences immune function. For example, high titres of JH have been implicated in humoral immunosuppression in insects (Hiruma & Riddiford 1993). In addition, Rolff & Siva-Jothy (2002) found that JH mediated mating-related immunosuppression in both sexes of *T. molitor*. Studies in cockroaches have suggested that JH type III is associated with sex pheromone production (e.g. Sréng *et al.* 1999). Thus, JH might control the trade-off between immunocompetence and

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pheromone production, but the relationship between sexual advertisement and immunocompetence has not been examined by manipulating JH levels.

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. Kön-ing & Schmid-Hempel 1995; Rantala *et al.* 2000; Ryder & Siva-Jothy 2000; Siva-Jothy 2000; Rantala *et al.* 2002; Koskimäki *et al.* 2003). Encapsulation is a cellular response through which insects defend themselves against multicellular pathogens such as nematodes and parasitoids (Gillespie *et al.* 1997), but it also plays a role in defence against viruses (Washburn *et al.* 1996). In the cellular response, circulating cells in the haemocoel recognize an object as foreign and form a capsule surrounding it that melanizes and hardens. This results in the death of the intruder by asphyxiation (Fisher 1963) or through the production of necrotizing compounds (Nappi *et al.* 1995). The humoral system, on the other hand, comprises myriad soluble proteins and enzyme cascades which play important roles in recognizing, signalling and attacking foreign targets (Leonard *et al.* 1985) and it may function in co-ordinating the cellular responses (Pech & Strand 1995). One enzyme thought to be important in the immune response of insects against bacterial infection is lysozyme, which hydrolyses β-1,4 linkages in the peptidoglycan of bacterial cell walls (Götz & Trenczek 1991).

In this study, we manipulated JH levels in *T. molitor* to investigate whether or not the administration of extra JH increased the attractiveness of males to virgin females and whether it suppressed male immune functions and reduced male survival. To assess immunocompetence, we used three measures, the encapsulation response against a novel antigen, and phenoloxidase (PO) and lytic activity of haemolymph.

2. MATERIAL AND METHODS

(a) *Insects*

The beetles were taken from a laboratory stock population originating from a commercial supplier and maintained at the University of Jyväskylä by the authors. We collected pupae daily from a large bulk laboratory stock and 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 to ensure virginity. Beetles of each sex were randomly allocated to the treatments when aged between 10 and 14 days. Before the experiments, we weighed the fresh body mass of each beetle to the nearest 0.1 mg so that beetles used in the treatments could be equally matched in weight.

(b) *Juvenile hormone administration*

We injected 5 μg of JH type III (Sigma, St Quentin Fallavier, France) in 5 μl of Ringer: acetone (9 : 1) solution between the 2nd and 3rd sternite region using a 10 μl Hamilton syringe (30 G). Control males received 5 μl of Ringer: acetone solution only, but otherwise both groups were treated identically. Before injection, the beetles were anaesthetized on ice and immobilized. The inoculated beetles were kept under standard conditions in individual plastic film roll canisters.

(c) *Pheromone collection and preference tests*

To collect pheromones from males, we placed each male in a small (diameter 37 mm) Petri dish containing a filter paper disc (diameter 35 mm) for 48 h (Rantala *et al.* 2002). To test female preference for pheromones from different males, we presented the filter paper discs containing pheromones (above) from two randomly paired males ($n = 31$ weight-matched male pairs). The time after collecting pheromones and use of the discs was equal for each male pair and all the discs were used within 2 hours after removal of the males. The arena for female choice trials consisted of a 20 cm diameter glass dish inverted over 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 minutes 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 minutes, during which the female's movements were recorded using computer-aided time-take. Female preference was measured as the total time that the female spent on each filter disc (see Rantala *et al.* 2002).

(d) *Encapsulation rate assay*

Three hours after JH administration, we inserted a 2 mm-long piece of nylon monofilament (diameter 0.18 mm) through a puncture in the pleural membrane between the second and third sternite (see Rantala *et al.* 2002). The male's immune system was allowed to react to this object for 3 h, while the insects ($n = 40$) were kept individually in film roll canisters at constant room temperature (28 ± 1 °C). The implant was then removed and dried. The encapsulation rate was measured using the method of Rantala *et al.* (2002). The removed monofilament was examined under a light microscope and photographed using digital video from three different angles. The pictures were then analysed by using an image analysis program (Image Pro, Media Cybernetics Inc., Carlsbad, CA). The degree of encapsulation was analysed as the grey value of reflecting light from the 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 reflected the highest encapsulation rate (total black). Previously it has been shown that the repeatability of the measurement is very high (repeatability, $R = 0.997$; Krebs 1989; Rantala *et al.* 2002).

(e) *Phenoloxidase activity assay*

Three hours after JH administration, the neck of each male ($n = 40$) was cut with scissors and 2 μl haemolymph was collected from the wound with a plastic micropipette (Rantala *et al.* 2002). The haemolymph was then mixed with 99 μl of phosphate-buffered saline solution (pH 7.4). Samples were immediately frozen at −25 °C to disrupt the haemocyte membranes. After thawing, the samples and 200 μl of 10 mM L-DOPA were pipetted into the wells of a 96-well plastic microplate (Cliniplate, Labsystems, Finland). Absorbance at 492 nm was then measured spectrophotometrically with a plate reader (Multiskan Plus, Labsystems, Finland) at 20 °C at 1-minute intervals for 30 minutes. Lytic activity was expressed as the total change in optical density.

(f) *Lysozyme activity assay*

Three hours after JH administration, the neck of each male ($n = 50$) was cut with scissors and 4 μl haemolymph was collected from the wound with a plastic micropipette. The haemolymph was then mixed with 46 μl of phosphate-buffered saline

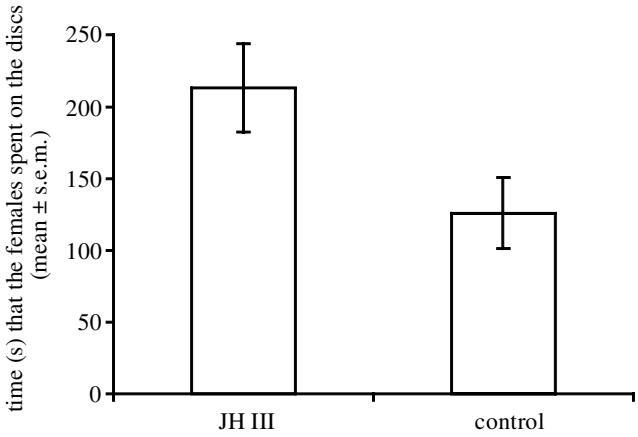

Figure 1. Female preference for pheromones of males after juvenile hormone or control treatment (mean ± s.e.m.).

(pH 6.4). We assayed lysozyme activity of haemolymph against *Micrococcus lysodeikticus* turbidometrically using methods similar to those described by Rantala and Kortet (2003*a*,*b*). We mixed 200 μl of freeze-dried *M. lysodeikticus* buffered (pH 6.4) solution (0.35 mg/ml) with 50 μl buffered haemolymph in a plastic multicuvette (Cliniplate, Labsystems, Finland). The optical density of the mixture at 492 nm was then measured at 20 °C at 1-minute intervals for 30 minutes with a plate reader (Multiskan Plus, Labsystems, Finland). Lytic activity was expressed as the total change in optical density.

(g) *Juvenile hormone and survival*

To estimate the possible energetic costs of JH treatment, we divided pairs of males (4–10 days old) into two treatment groups as described above ($n = 72$), and followed their survival when given only water. After treatment we gave one drop of water daily on the sand bottom of the film roll canisters using a Pasteur pipette and checked survival daily until all the beetles had died. The results were expressed as days of survival after treatment.

(h) *Statistics*

The time females spent on the filter paper discs and the survival time of the males were not normally distributed or could not be transformed, so the non-parametric Wilcoxon signed rank sum test was used to compare groups. The distributions of the parameters encapsulation rate, PO activity and lytic activity were normal, so Student's *t*-tests for paired samples were used.

3. RESULTS

(a) *Juvenile hormone treatment and pheromones*

Treatment with JH positively affected the attractiveness of male pheromones: females spent significantly more time on the filter paper discs from the males which received juvenile hormone than on the discs from control males (Wilcoxon test, $n = 31$; $Z = 1.97$; $p = 0.049$; figure 1).

(b) *Juvenile hormone treatment and male immune function*

The JH treatment group had a lower encapsulation rate than the control group ($t = -2.08$, d.f. $= 38$, $p = 0.044$; figure 2). Likewise, PO activity was significantly lower in males receiving JH compared with control males ($t = 2.24$, d.f. $= 25.14$, $p = 0.035$; figure 3). However, JH treatment did not affect the lytic activity of male haemolymph ($t = -0.096$, d.f. $= 50$, $p = 0.924$).

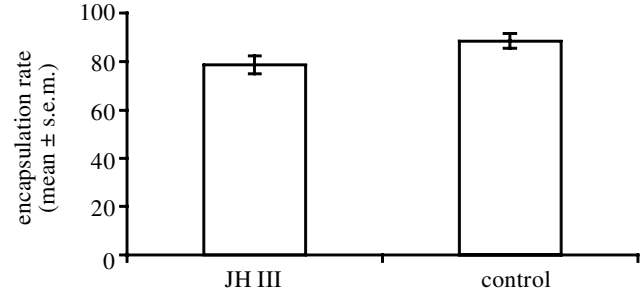

Figure 2. Encapsulaton rate (darkness value of implant) in males after juvenile hormone or control treatment (mean ± s.e.m.).

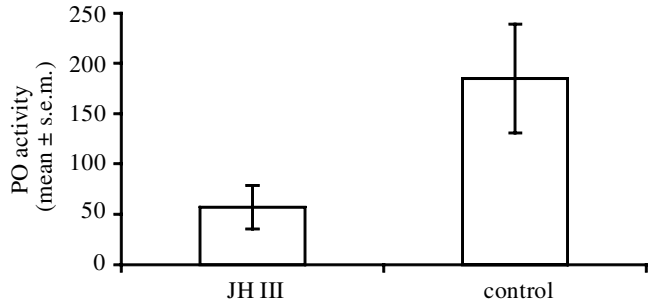

Figure 3. Phenoloxidase (PO) activity (change in absorbance) in males after juvenile hormone or control treatment (mean ± s.e.m.).

(c) *Juvenile hormone and survival*

Treatment with JH did not have a statistically significant effect on the survival of males. The average survival time of the males receiving JH was 21.8 days and the average survival time of control males was 22.4 days (Wilcoxon test; $n = 36$; $Z = 0.69$; $p = 0.488$).

4. DISCUSSION

We found that females spent more time on pheromone-containing filter paper discs from males receiving JH than on those from males that received only control solution. Thus, it seems that JH increases the attractiveness of male scent. Our results are consistent with those of previous studies on cockroaches, which showed that the corpora allata regulate pheromone production via JH secretion (Sréng *et al.* 1999).

While increasing pheromone production, JH reduced both the encapsulation rate and PO activity in males. This supports the results of Rolff & Siva-Jothy (2002), who suggested that JH is linked to mating-related lowering of insect immunity. Lowered PO activity is likely to have important immunological consequences in insects (see Rizki & Rizki 1990; Wilson *et al.* 2001) and it is known to reduce refractoriness to parasites (see Nigam *et al.* 1997; Shiao *et al.* 2001). However, JH treatment did not affect the lytic activity of haemolymph. Thus, it could be possible that JH regulates melanization through affecting PO levels but not other aspects of immunity.

Treatment with JH did not have any effect on male survival, in contrast to the results of a study by Herman & Tatar (2001), in which JH shortened the lifetime of monarch butterflies. The effect of JH treatment might have been so short-lasting that the beetle males were able to compensate for the effect of JH treatment after a few days. On the other hand, it is possible that in our experiment the beetles did not confront such pathogens and parasites as would necessitate an effective encapsulation response. Thus, we cannot be certain that there are no energetic or survival costs of having high concentrations of JH in the wild, where parasitic infections are pervasive.

Our previous finding of female preference for the pheromones of males with high immunocompetence seems contradictory to the current results, but the way JH ensures the honesty of pheromone signals is likely to be condition dependent. Thus, only the males in good condition can perform well with elevated JH levels needed to produce a lot of pheromones, which leads to a positive correlation between immune function and sexual advertisement (Rantala *et al.* 2002). The 'good genes' which females might acquire by choosing highly ornamented males might be genes affecting immunocompetence directly or genes having indirect effects on the immune system through body condition (Siva-Jothy & Skarstein 1998). However, measurement of the natural variation in JH concentrations in males and comparisons with immune functions remain for further studies.

Our results indicate that JH has an impact on the allocation of resources associated with immune function and pheromone production, i.e. sexual advertisement. To our knowledge, this is the first study that provides a general mechanism to explain the correlation between immune function and sexual ornament, including pheromones in insects. Thus, our results support the idea that the immunocompetence handicap hypothesis could also work in insects even though they lack male-specific hormones. The present results also suggest that some other non-male-specific hormones may affect the relationship between the immune system and sexual ornaments in a wide variety of animal taxa, and that focusing only on testosterone should be rejected in studies about the immunocompetence handicap hypothesis.

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