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Terminal investment in multiple sexual signals: immune-challenged males produce more attractive pheromones

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Summary

1. Trade-offs between current and future resource allocation can select for elevated reproductive effort in individuals facing mortality. Males are predicted to benefit from increasing investment in costly sexually selected signals after experiencing an acute life span reduction, although few examples of such facultative terminal investment are known.

2. In the mealworm beetle, *Tenebrio molitor*, males' odours become more attractive to females following a life-threatening immune challenge. However, the pheromones involved are unknown, hindering further insight into the proximate mechanisms and ultimate consequences of terminal investment.

3. Using chemical and behavioural analyses, we show that the cuticular hydrocarbons (CHCs) of *T. molitor* are sexually dimorphic and are used by females to locate and select males. Moreover, both male CHCs and glandular pheromones were affected by experimental immune challenge in a fashion that made them more attractive to females.

4. The results suggest that males terminally invest in both short- and medium-range pheromones when they perceive reduced future survival. Moreover, the constitutive and inducible aspects of male and female CHC production are consistent with sex-specific selection on the signalling and defensive functions of CHCs. The implications of terminal investment for 'dishonest' signalling and the efficacy of sexual selection are discussed.

Key-words: cuticular hydrocarbons, dishonest signalling, immunity, lipopolysaccharide, *Tenebrio molitor*

Introduction

Terminal investment in offspring production, in which individuals facing mortality produce more or better-provisioned offspring, is a widespread phenomenon that follows from life-history theory (e.g. Clutton-Brock 1984; Bonneaud *et al*. 2004; Isaac & Johnson 2005; Hanssen 2006; Velando, Drummond & Torres 2006; Creighton, Heflin & Belk 2009; Barribeau, Sok & Gerardo 2010). If there is a trade-off between current and future offspring production, individuals close to death should benefit from investing more in current reproduction than those with a longer expected life span. We similarly predict terminal investment in the costly sexual signals used by males to secure mating opportunities (Kokko 1997; Lindström *et al*. 2009). An increase in signalling effort with age has been documented in many taxa (e.g. Mountjoy & Lemon 1995; Cote *et al*. 2010; Lafaille, Bimbard & Greenfield 2010; Kuriwada & Kasuya 2011) and may represent terminal investment in some cases. Facultative increases in signalling by males experiencing an acute survival threat also imply terminal investment; putative examples include the augmented courtship activity of male *Drosophila nigrospiracula* parasitized by mites (Polak & Starmer 1998) and the heightened aggression and attractiveness of male *Peromyscus leucopus* mice harbouring botflies (Cramer & Cameron 2007).

Terminal investment in sexual advertisements has fascinating implications for sexual selection and the evolution of honest signalling, because strong signals from infected or dying individuals may confound the receiver's assessment of the sender's phenotypic and genetic quality (Kokko 1997; Lindström *et al*. 2009). Such terminal investment also appears to contradict a prominent hypothesis in sexual selection research: that sexual signals reveal the sender's ability to resist parasites (e.g. Hamilton & Zuk 1982; Folstad & Karter 1992; Sheldon & Verhulst 1996). Terminal investment in signalling has therefore been described as dishonest (e.g. Sadd *et al*. 2006) or manipulative (Kivleniece *et al*. 2010), because it

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might cause low-quality senders to produce misleadingly strong signals. Signal plasticity should also increase the residual variation in the relationship between senders' phenotypic quality and their signal quality, which may have far-reaching evolutionary consequences.

Good evidence for facultative terminal investment in signalling has been found in the mealworm beetle *Tenebrio molitor* (Fig. 1). Male odours adsorbed on filter paper are attractive to females in behavioural bioassays (e.g. Worden, Parker & Pappas 2000; Rantala *et al*. 2003b; Sadd *et al*. 2006; Vainikka *et al*. 2007), and the odours of experimentally immune-challenged males, which face a reduced future life span (Armitage *et al*. 2003; Moret 2006; Sadd & Siva-Jothy 2006; Kivleniece *et al*. 2010), are more attractive to females than controls (Sadd *et al*. 2006; Kivleniece *et al*. 2010; Krams *et al*. 2011). These results suggest that males terminally invest in an olfactory signal. However, key details of the system remain undiscovered, principally the identity of the chemical signal, impeding further investigation into the proximate mechanisms and ultimate consequences of terminal investment.

In *T. molitor*, males and females are each thought to produce a volatile, glandular sex pheromone (3-dodecenyl acetate and 4-methyl-1-nonanol respectively) that stimulates locomotion in the opposite sex (Tanaka *et al*. 1986; Bryning, Chambers & Wakefield 2005). Immune-challenged males might therefore up-regulate the production of volatile pheromones. However, additional olfactory signals are likely to be present in this species, and these may also have contributed to the observed terminal investment. Cuticular hydrocarbons (CHCs) are relatively non-volatile, waxy substances present on the body surface of most terrestrial arthropods that function in desiccation and parasite resistance as well as signalling and recognition (Howard & Blomquist 2005; Blomquist & Bagnères 2010). CHCs are thought to act as pheromones, i.e. substances that affect the behaviour and/or physiology of conspecifics, in many arthropod orders (Thomas & Simmons 2008; Holman *et al*. 2010b). Previous studies of chemical communication in *T. molitor* have used experimental designs that cannot determine the relative importance of volatile pheromones and CHCs (and in some cases also male behaviour) in attracting females (e.g. Tschinkel, Willson & Bern 1967; Happ 1969; Rantala *et al*. 2003b; Sadd *et al*. 2006; Kivleniece *et al*. 2010; Krams *et al*. 2011). Consequently, the roles of CHCs and volatile odours in mate choice and terminal investment are unknown.

In the present study, we used gas chromatography–mass spectrometry (GC–MS) to characterize the CHCs of male and female *T. molitor*. We also tested whether CHCs are affected by immune challenge, a necessary condition if they are to explain the observed link (Sadd *et al*. 2006; Kivleniece *et al*. 2010; Krams *et al*. 2011) between immune challenge and olfactory attractiveness. We then used CHC extracts in behavioural bioassays to establish whether CHCs function in mate recognition and sexual selection. We first examined whether males and females are preferentially attracted to the CHCs of the opposite sex, and tested whether some males' CHCs are consistently more attractive. We then presented the CHCs of immune-challenged and control males to females to test whether immune challenge affected the attractiveness of males' CHCs. The volatile odours of immune-challenged and control males were also presented to females in a Y-maze experiment.

Fig. 1. Mating *Tenebrio molitor* (photograph by Richard A. Naylor).

Materials and methods

INSECT CULTURES

Cultures were established from *c*. 5000 larval *T. molitor* obtained from Avifauna ApS and kept in plastic boxes (38 × 30 × 20 cm) with an oatmeal, wheat bran and yeast substrate and *ad libitum* fresh apple. Pupae were sieved from the stocks, then sexed by inspection of the developing genitalia on the eighth abdominal segment (Bhattacharya, Ameel & Waldbauer 1970); male and female pupae were stored in separate boxes lined with tissue. Eclosing adults were removed daily and housed in individual plastic cylinders (28 × 37 mm) containing tissue paper and fresh apple. We excluded beetles with visible abnormalities.

CUTICULAR HYDROCARBON EXTRACTION AND ANALYSIS

Cuticular hydrocarbons were collected from adults (freeze-killed 8–9 days post-eclosion) by immersing the beetle in 500 μL of pentane for ten minutes. The extract was left to evaporate overnight in a laminar flow cabinet. This protocol ensured that only the least volatile chemicals, principally CHCs, were left in the vial. We later used GC–MS (n = 97) to verify that the CHC extracts did not contain detectable levels of the glandular male and female sex pheromones, 3-dodecenyl acetate and 4-methyl-1-nonanol (Tanaka *et al*. 1986; Bryning, Chambers & Wakefield 2005). Even if trace amounts of these pheromones remained in the vial, the mass of pheromone presented to females in our bioassays would have been less than that of the smallest CHC peaks and therefore well below 1 μg, the minimum quantity reportedly detectable by female *T. molitor* (Bryning, Chambers & Wakefield 2005). Prior to use in choice trials, each CHC extract was re-diluted in 200 μL of pentane and vortexed. Extracts that were analysed by GC–MS were instead re-diluted in 600 μL of C_{22} in pentane

(1 ng μL^{-1}); the C_{22} was used as an internal standard to quantify the mass of CHCs present in the extract.

We analysed beetles' chemical profiles by injecting 2 μL of CHC extract into an Agilent Technologies 6890N gas chromatograph (capillary column: HP5MS 30 m × 250 μm × 0·25 μm; injector: *split–split less*; carrying gas: helium at 1 mL min^{-1}), using the following temperature programme: 70–250 °C at 30 °C per minute, then 250–320 °C at 3 °C per minute, then hold at 320 °C for 5 min. Compounds were identified on the basis of their mass spectra, produced by an Agilent 5975 inert mass selective detector (70 eV electron impact ionization) coupled with the gas chromatograph. CHC profiles were analysed using MSD ChemStation software (Agilent) to determine the identity and abundance of different peaks. Prior to principal component analysis (PCA), peak areas were scaled using the transformation $\ln(A_p/g(A_p))$, where A_p is the area of the peak and $g(A_p)$ is the geometric mean of all peak areas for that individual (Aitchison 1982). Samples were processed in a random order and analysed blind to immune status.

IMMUNE CHALLENGE WITH LIPOPOLYSACCHARIDE

Beetles (8–9 days post-eclosion) were randomly assigned to two treatment groups. The experimental group was subjected to an immune challenge: the pleural membrane between the 2nd and 3rd terminal abdominal sternites was pierced with a fine pin that had been dipped in lipopolysaccharide (LPS, a non-pathogenic substance derived from gram-negative bacteria that induces a harmful immune response in *T. molitor*; e.g. Moret 2006) dissolved in insect Ringer (2·5 mg mL^{-1}). The control group was handled identically, but was pierced with a new, Ringer-treated pin. Pins were cleaned in ethanol and flame-sterilized between uses. The treatments were blind such that neither the contents of the Ringer solutions nor the gender of the beetle was known (adults cannot be sexed without squeezing them to extrude the genitalia). Beetles were frozen for CHC extraction 24 h after treatment or used in Y-maze experiments 24 ± 2 h after treatment.

CUTICULAR HYDROCARBON CHOICE TRIALS

The behavioural bioassay was similar to that used in previous studies of *T. molitor* olfactory signalling (e.g. Worden, Parker & Pappas 2000; Rantala *et al.* 2003b; Sadd *et al.* 2006; Vainikka *et al.* 2007). We performed three-way choice trials, in which focal beetles (aged 8–9 days) were presented with two different CHC extracts and a pentane-only 'blank', which had the same odour as the background (highly pure, HPLC-grade pentane was used throughout). The set-up consisted of an inverted Petri dish 'arena' (diameter: 90 mm) lined with clean filter paper. Three circular pieces of filter paper (diameter: 25 mm) were placed in the arena equidistant from each other and from the centre; two of these received 50 μL of CHC extract (*c.* 25% of the CHCs present on a beetle), each from a different individual, and the blank received 50 μL of clean pentane. The solvent was left to evaporate for 10 min. The extracts and blanks had been labelled with a code unique to each trial, facilitating blind data recording. The focal beetle was placed in the centre of the arena under a plastic cover and left to settle for 10 min; each focal beetle and Petri dish was used only once.

After the settling period, the cover was removed, and the beetle's behaviour was recorded using Etholog software (Ottoni 2000) for 10 min. Behaviour was recorded as the time spent in contact with each of the filter paper discs; a beetle was judged to be in contact with a disc if its head and/or antennae were above it. All experimental procedures were performed under red light, which is invisible to *T. molitor* (Rantala *et al.* 2003b; Sadd *et al.* 2006). Statistical analyses were performed using R 2.13.0 (http://www.r-project.org/). Choice trials were analysed using generalized linear mixed models (GLMM; implemented in the lme4 package for R) with trial ID as a random factor and Poisson errors; factor levels were compared using contrasts. The response variable was the number of seconds in contact with the focal odour source, square-root transformed and rounded to the nearest integer; this transformation reduced right skew, removing the overdispersion present in models of the untransformed data.

EXPERIMENT 1: CHCS AND MATE CHOICE

In experiment 1a ($n = 30$), male beetles were presented with CHCs from one female and one male, as well as a blank; each pair of male and female extracts was used only once. In experiment 1b ($n = 30$), female choosers were similarly presented with male and female CHCs plus a blank. These experiments allowed us to test whether beetles show a sex-specific attraction to CHCs and whether conspecifics CHCs are preferred over nothing. In experiment 1c, male extracts were randomly grouped into pairs, and females were presented with CHC extracts from two males plus a blank. Each pair of male CHC extracts was used in three choice trials, using a new female each time, for a total of 60 trials ($n = 20$ male pairs). Experiment 1c allowed us to test whether some males' CHCs are consistently more attractive across different females.

EXPERIMENT 2: IMMUNE CHALLENGE AND THE ATTRACTIVENESS OF CHCS

In experiment 2a ($n = 30$), female beetles were presented with CHCs from an LPS-challenged male and a control (Ringer-treated) male, as well as a blank. In experiment 2b ($n = 30$), female choosers were presented with CHCs from challenged and unchallenged females in a similar manner. All beetles were used only once and were randomly assigned to treatments and trials. These experiments allowed us to test whether immune challenge affected the attractiveness of male CHCs to females and of female CHCs to other females.

EXPERIMENT 3: IMMUNE CHALLENGE AND THE ATTRACTIVENESS OF MALE VOLATILE PHEROMONES

To test whether male volatile odours (e.g. the sex pheromone reported by Bryning, Chambers & Wakefield 2005) are affected by immune challenge, female beetles were presented with an LPS-challenged male and a control (Ringer-treated) male in a standard Y-maze experiment ($n = 28$ trials). Males were placed in enclosures in the arms of the Y-maze (shown in Fig. S1), separated from the area where the female could walk by two wire meshes with a 12-mm gap between them. Males were therefore between 12 mm (if in the part of their enclosure closest to the female's area) and 38 mm (if on the far side) from the female's part of the maze, such that their weakly volatile CHCs could not be perceived by the female (Brandstaetter, Endler & Kleineidam 2008). At the start of each trial, the female was placed at the base of the Y-maze under a cover to settle for 10 min, before being released. The amount of time spent by the female in each arm of the maze was recorded over 10 min using Etholog software. Trials were performed blind to treatment under red light, males were randomly allocated to the arms of the maze and the apparatus was thoroughly cleaned with ethanol between trials. The data were analysed as for the CHC choice trials.

Results

CUTICULAR HYDROCARBONS ARE SEXUALLY DIMORPHIC AND REVEAL IMMUNE STATUS

The *T. molitor* cuticular hydrocarbon profile is composed of several classes of hydrocarbons, namely alkanes, alkenes, dienes, methylalkanes and dimethylalkanes, with chain lengths of 23–33 carbons (Fig. 2). After reducing the CHC data set by PCA, we found that PC2 (11·0% explained variance) was significantly higher in males than in females (GLM; $t_{95} = 11{\cdot}0$, $P < 0{\cdot}0001$). Additionally, PC3 (7·5%) was significantly higher in control beetles than in LPS-challenged beetles ($t_{94} = 2{\cdot}2$, $P = 0{\cdot}029$) and higher in males than in females ($t_{94} = 4{\cdot}3$, $P < 0{\cdot}0001$). The interaction between sex and treatment was not significant, although there was a trend for PC3 to be more strongly reduced by LPS treatment in males than in females ($P_{93} = 0{\cdot}15$). These results show that the cuticular hydrocarbon profile is sex specific and is affected by immune challenge, and provide some evidence that immune challenge had a greater effect on the CHC profile of males than females. PC1 was unaffected by sex ($P = 0{\cdot}58$) and treatment ($P = 0{\cdot}91$).

Male beetles had a substantially greater mass of CHCs than females (males: 84·3 ± 3·0 μg; females: 62·2 ± 4·5 μg; $t_{95} = 4{\cdot}1$, $P < 0{\cdot}0001$); males are marginally smaller than females (Rolff, Armitage & Coltman 2005), so this result shows that male cuticle has more CHCs per mm^2. The total mass of CHCs was unaffected by immune challenge ($P_{94} = 0{\cdot}54$). Furthermore, both sex and immune status affected the mean weighted retention time (MWRT; van Zweden, Dreier & d'Ettorre 2009; Holman, Dreier & d'Ettorre 2010a), a metric that describes the relative abundance of long-chain hydrocarbons in the chemical profile. Immune-challenged females had a significantly higher MWRT than control females (Fig. 3; $t_{48} = 2{\cdot}1$, $P = 0{\cdot}04$), but there was no treatment effect in males ($t_{45} = 0{\cdot}85$, $P = 0{\cdot}40$). Females also had a higher MWRT than males ($t_{95} = 2{\cdot}1$, $P = 0{\cdot}04$). These results suggest that females have proportionally more long-chain compounds than males and that immune challenge increased the relative abundance of long-chain CHCs in females but not in males.

To identify which hydrocarbon peaks were affected by treatment, we performed univariate tests (quasi-likelihood GLMs; $\alpha = 0{\cdot}05$) on peaks with strong loadings on PC2 and PC3, analysing male and female data separately. In males, the methylalkane 11-MeC_{23} comprised a significantly higher proportion of the profile in LPS-treated males than in control males, while the proportions of the alkene $C_{29:1}$ and the dienes $C_{29:2}$ and $C_{31:2}$ were reduced by immune challenge (Fig. 3; Table S1). Because proportional changes in each peak depend on the relative abundance of the other peaks, we also examined changes in the mass of each peak, estimated from the internal standard. The mass of most CHC peaks was non-significantly lower in immune-challenged males (Fig. 3; Table S1); the exception was 11-MeC_{23}, the mass of which was significantly higher in immune-challenged males. In females, the only peak whose proportion was significantly affected by treatment was $C_{31:1}$, which was lower in challenged females. When analysing the CHC mass data set, we found no peaks that were significantly affected by LPS treatment in females (Fig. 3; Table S1). We similarly tested all peaks with GLMs to identify those that differed significantly between males and females (using proportion data); 19 of 28 peaks differed significantly between the sexes (Fig. 2; Table S1). The most conspicuously male-specific CHCs were $C_{25:1}$ and C_{29}, while females had higher proportions of all methyl- and dimethylalkanes, and most other alkenes.

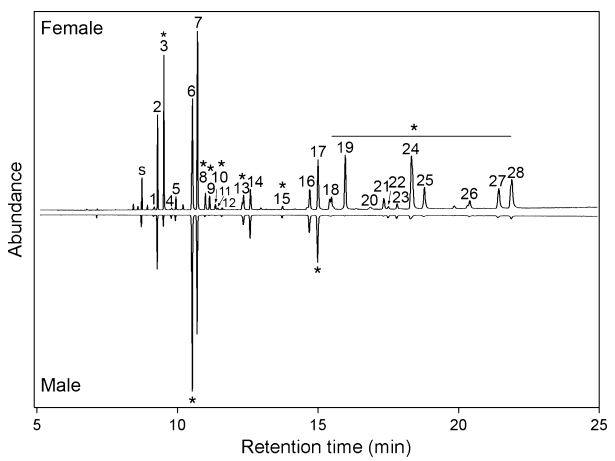

Fig. 2. The cuticular hydrocarbon (CHC) profile is sexually dimorphic. The male CHC profile has been inverted and scaled to the same height for comparison. Asterisks denote peaks that make up a significantly higher proportion of the CHC profile in the sex where they appear (GLM, d.f. = 94, $\alpha = 0{\cdot}05$). Peak identities are given in Fig. 3 and Table S1; 'S' is the internal standard, C_{22}.

EXPERIMENT 1: CHCS AND MATE CHOICE

Experiment 1a revealed that male beetles were significantly more attracted to male CHCs than to the odourless blank (Fig. 4a; $z = 2{\cdot}99$, $P = 0{\cdot}0028$). Males spent similar amounts of time in contact with male and female CHCs ($z = 0{\cdot}84$, $P = 0{\cdot}40$). In experiment 1b, female beetles were more attracted to male CHCs than female CHCs (Fig. 4a; $z = 5{\cdot}021$, $P < 0{\cdot}0001$) and preferred female CHCs to the blank ($z = 2{\cdot}71$, $P = 0{\cdot}007$). In summary, females showed a sex-specific preference for CHCs, while males were attracted to conspecific CHCs but did not show a significant preference for either sex.

In experiment 1c, females spent more time in contact with the male CHC extracts than the blank (GLMM with male pair ID and trial as random factors; $z = 6{\cdot}71$, $P < 0{\cdot}0001$), as expected. The repeated measures design of experiment 1c allowed us to test whether the CHCs of some males were consistently more attractive than the CHCs of others. When the blank data were excluded from the model and male ID was fitted as a random factor, male ID explained 24·6% of the variation in the female response, and the explanatory power of the model decreased strongly if male ID was removed

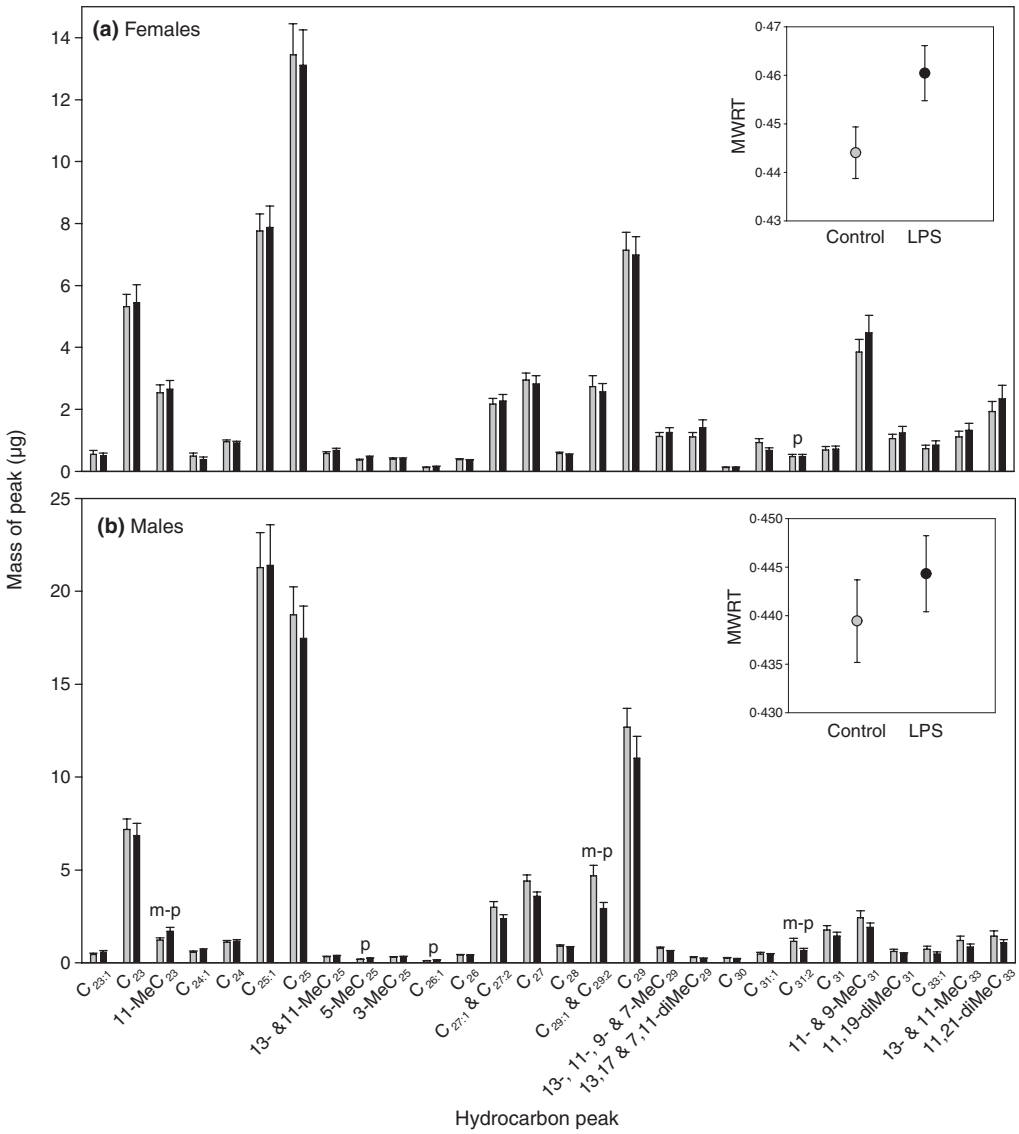

Fig. 3. The cuticular hydrocarbon profile was affected by lipopolysaccharide (LPS) immune challenge in both males and females. The insets show difference in mean weighted retention time between challenged and control individuals; higher values denote a greater proportion of long-chain hydrocarbons. The letters denote peaks whose proportion of the total profile (p) and/or absolute mass (m) was significantly affected by LPS treatment (GLM, d.f. = 48, $\alpha = 0{\cdot}05$). Black bars: LPS-treated individuals, grey bars: controls; data show the mean and standard error.

($\chi^2_1 = 815$, $P < 0{\cdot}0001$). This result suggests that the CHCs of some males consistently elicited a stronger female behavioural response than others.

EXPERIMENT 2: IMMUNE CHALLENGE AND THE ATTRACTIVENESS OF CHCS

In experiment 2a, female beetles showed a significant preference for the CHCs of immune-challenged males over control males (Fig. 4b; $z = 2{\cdot}14$, $P = 0{\cdot}032$). As expected, control males were significantly preferred to the odourless blank ($z = 5{\cdot}95$, $P < 0{\cdot}0001$). Experiment 2b found no differences among treatments: the CHCs of LPS-treated and control females were equally attractive to female beetles (Fig. 4b; $z = 0{\cdot}35$, $P = 0{\cdot}73$), but the female extracts were unexpectedly not preferred over the blank (control females vs. blanks: $z = 0{\cdot}68$, $P = 0{\cdot}50$). However, concatenating the female and blank data from experiments 1b and 2b suggested a significant overall preference for female CHCs over blanks ($z = 2{\cdot}70$, $P = 0{\cdot}007$).

EXPERIMENT 3: IMMUNE CHALLENGE AND THE ATTRACTIVENESS OF MALE VOLATILE PHEROMONES

Females spent a significantly higher amount of time in the arm of the Y-maze leading to the LPS-treated male relative to the control male (Fig. 5; $z = 2{\cdot}51$; $P = 0{\cdot}012$), suggesting that immune-challenged males produce more attractive volatile pheromones. Although live males were used in this experiment as opposed to just their odours, we believe it is

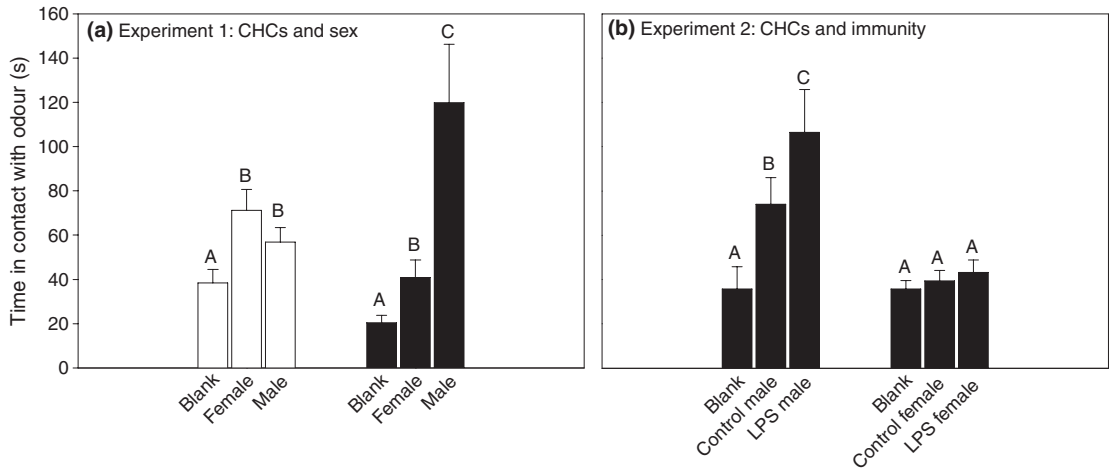

Fig. 4. Female *Tenebrio molitor* can distinguish male and female cuticular hydrocarbons (CHCs) and are more attracted to the CHCs of immune-challenged males. White bars show trials measuring the preferences of males, while black bars show females. Bars sharing a letter are not significantly different (each experiment was analysed separately) and bars show the mean + SE.

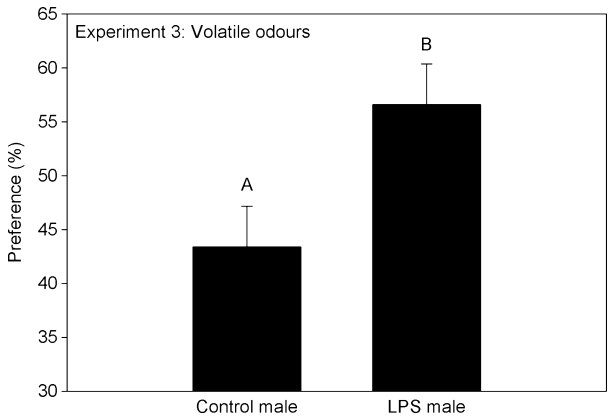

Fig. 5. Females spent more time in the part of the Y-maze closest to the lipopolysaccharide-treated male. Bars show the mean + SE, and the different letters denote a significant difference.

unlikely that non-olfactory differences between the treatment groups were responsible for the observed effect. Females were often far away from males and likely could not perceive their behaviour (Fig. S1). Moreover, immune-challenged males show reduced locomotion (Krams *et al.* 2011), and it is improbable that less mobile males would be more attractive to females.

Discussion

Our study revealed pronounced sexual dimorphism in the cuticular hydrocarbon profile of *T. molitor* and demonstrated that male CHCs and volatile odours become more attractive to females after immune challenge. Although the male and female CHC profiles contain the same compounds, there are many quantitative differences: females produce more long-chain hydrocarbons and methyl-branched alkanes, while males produce a larger total mass of hydrocarbons and had higher relative amounts of two compounds, $C_{25:1}$ and C_{29}. In some respects, immune challenge had a greater effect on the hydrocarbon profile of males, significantly affecting the masses of 3/28 hydrocarbon peaks in males and 0/28 in females. However, females responded to immune challenge by producing more long-chain compounds, while the chain length of male CHCs was unaffected.

Our behavioural experiments confirm that female beetles are able to discriminate between male and female CHCs and are preferentially attracted to male CHCs. Furthermore, some males' extracts were consistently preferred across different females, suggesting that the components of the male CHC profile that are attractive to females differ among males. The CHCs of immune-challenged males were also more attractive to females than those of control males. The attractiveness of female CHCs to other females was unaffected by immune status. These results suggest that males, but not females, altered their CHC profile in a fashion that increased its attractiveness to females. Males simultaneously increased the attractiveness of their volatile odours, such that both their short- and medium-range signals were augmented. We also found no evidence that females identify and avoid the CHCs or volatile odours of immune-challenged individuals, as would be expected if females use olfactory cues to minimize contact with potentially infectious conspecifics.

Together, these results suggest that male CHCs function as a signal used by females to locate and select mates, likely in conjunction with volatile signals, and that sexual selection has shaped the male CHC profile. The higher mass of CHCs produced by males may increase the probability of detection by females (either by direct detection or by deposition of CHCs on the substrate; Bos, Grinsted & Holman 2011), and shorter hydrocarbons are more volatile and may therefore be easier to perceive over a short distance (Brandstaetter, Endler & Kleineidam 2008). The male pattern of CHC production is the apparent opposite of some ant social parasites, which produce small amounts of CHCs with a high average chain

length (i.e. low volatility) to remain chemically inconspicuous (e.g. Lambardi *et al*. 2007).

Our results provide proximate insight into previous data from *T. molitor*, showing that the odours of immune-challenged males are more attractive to females (Sadd *et al*. 2006; Kivleniece *et al*. 2010; Krams *et al*. 2011). Sadd *et al*. adsorbed male odours on filter paper discs, such that CHCs and/or volatile pheromones may have contributed to the female response. The latter studies presented females with caged males that could be touched through a mesh, such that the effect of immune challenge on attractiveness might have been mediated either by CHCs, volatile pheromones and/or male behaviour. Our results show that both male CHCs and volatile odours become more attractive to females after immune challenge.

The altered CHCs and increased olfactory attractiveness of challenged males may represent terminal investment in sexual signalling. This hypothesis has three key assumptions, which we will now evaluate for *T. molitor*: (i) current investment in sexual signalling is traded against future investment, (ii) sexual signalling can be modulated after the animal's perception of its future life span is revised and (iii) immune challenge causes or predicts reduced future survival.

The first assumption implies that sexual signalling has a cost; if the signal were cost-free, there would be no trade-off between current and future signalling. We suggest that the present study and previous works showing increased attractiveness of immune-challenged males (i.e. Sadd *et al*. 2006; Kivleniece *et al*. 2010; Krams *et al*. 2011) provide indirect evidence that chemical signalling is costly; if the olfactory signal were cost-free, males would signal at their maximum capacity throughout their reproductive life. At present, the costs of chemical signals are largely speculative. CHC synthesis may require non-trivial amounts of limiting resources (Blomquist & Bagnères 2010), involve toxic precursors (Zahavi & Zahavi 1997) or may be inextricably linked (e.g. by shared biochemical networks) to other functions that are themselves costly. Compounds that are specialized for signalling might also be suboptimal for other functions; for example, short-chain hydrocarbons are more volatile and less viscous, and hence may be perceived at greater distances and more easily transferred to the substrate (Bos, Grinsted & Holman 2011), favouring signalling. However, shorter hydrocarbons are disadvantageous for desiccation resistance (Kwan & Rundle 2010), which is likely to be an important selective agent in *T. molitor* (which lives in dry habitats such as grain stores). This putative trade-off suggests that sex-specific selection on signalling and desiccation resistance may explain some of the observed sexual dimorphism in CHC chain length in *T. molitor*. Furthermore, females, but not males, responded to immune challenge by producing longer CHCs, suggesting that the inducible aspects of CHC production may be sexually dimorphic for similar reasons. The shift towards longer CHCs in challenged females might represent a prophylactic against further environmental stress and infection, and this response might be absent in males because they are selected to maintain signalling at the expense of survival. Such sexual dimorphism in constitutive and inducible CHC production would represent an example of Bateman's principle, i.e. that males and females are selected to differentially invest in mating success and longevity (Bateman 1948).

The second assumption of the terminal investment hypothesis has clearer support: rapid (<24 h) modulation of the CHC profile following a stimulus has been observed in *T. molitor* (the present study), honey bees (Richard, Aubert & Grozinger 2008) and three ant species (Oppelt & Heinze 2009; Holman *et al*. 2010b; Bos, Grinsted & Holman 2011). The rapid changes may reflect either differential CHC production or transport to the cuticle, or perhaps altered behaviour such as self-grooming. The genetic and biochemical links between immunity and pheromone production remain to be found, although both traits are affected by juvenile hormone (Rantala, Vainikka & Kortet 2003a).

The third assumption that immune status predicts survival also has good support. Immune activation itself carries significant costs (Moret & Schmid-Hempel 2000; Armitage *et al*. 2003; Moret 2006; Sadd & Siva-Jothy 2006; Kivleniece *et al*. 2010), but is also a reliable indicator of a potentially life-threatening infection. In sum, we believe there is good evidence that the increased olfactory attractiveness of immune-challenged male *T. molitor* represents terminal investment.

Terminal investment in sexual signals has been described as 'dishonest' or 'manipulative' signalling (Sadd *et al*. 2006; Vainikka *et al*. 2007; Kivleniece *et al*. 2010), because it increases the frequency with which females choose unhealthy males, which may be of lower average genetic quality. However, caution must be used in applying this description. First, the available data on *T. molitor* imply that all males engage in terminal signalling. Assuming that the strength of the signal is ultimately limited by the sender's underlying quality, high-quality individuals should have the best signal within both the healthy and sick male classes. Therefore, a positive correlation between male quality and the signal will exist across the population despite the extra residual variation ('noise') introduced by differences in immune status. Even if low-quality, terminal males are capable of producing a maximum strength signal, females may still benefit from the signal on average (see Johnstone & Grafen 1993; Kokko 1997; Rowell, Ellner & Reeve 2006; Lindström *et al*. 2009). Secondly, signalling systems with a high rate of dishonesty are predicted to be unstable over evolutionary time, because receivers are selected to evolve resistance to signals that do not increase their fitness. Dishonest signalling systems can only persist under specific conditions (see Searcy & Nowicki 2005), such as when receivers pay a cost to assess senders that outweighs the benefits of checking for dishonesty (Dawkins & Guilford 1991) or when receivers are constrained from evolving resistance (e.g. because the signal exploits a sensory bias that cannot evolve; Holland & Rice 1998). Female *T. molitor* are highly promiscuous (Drnevich 2003), so the benefits of mate choice may be small relative to the costs, allowing for some dishonesty; however, constraints that preclude females from losing their attraction to male CHCs seem unlikely. Finally, the sons of females mating with terminally investing males

should also possess this signalling strategy, which may mitigate some or all of the putative cost of mating with terminal males (Cordero & Eberhard 2003). Overall, we believe that terminal investment in sexual advertisements is unlikely to represent a true manipulation (in which the signal has a net negative effect on the receiver's inclusive fitness) in any species.

Our results highlight how plasticity in sexual signals may impact the efficacy of sexual selection. Whenever males are exposed to partly stochastic (i.e. independent of the male's genotype) factors that raise or lower the intensity of their signal, the extra noise in the relationship between males' advertised quality and their true quality should lead to more type I and II errors by choosy females, who will more frequently accept a low-quality male or reject a high-quality male than in systems with less noise. Although noisy honest signals should allow females to make better choices than if they ignored the signal (Kokko 1997), the extra mate choice errors could lower female fitness relative to a less noisy signal, meaning that inter-locus sexual conflict may exist over male terminal investment in signals. Additionally, mistakes in female choice will cause relaxed selection on the underlying quality trait and perhaps on the signal, and therefore to a greater amount of genetic variation at mutation-selection balance. Stochastic variation in sexual signals, such as that introduced by terminal investment, may thereby contribute to the resolution of the lek paradox (e.g. Rowe & Houle 1996) and slow the rate of sexually selected evolution.

In summary, the CHCs of *T. molitor* are sexually dimorphic and are affected by immune challenge. Sex-specific selection for signalling and defence may have driven the evolution of sexual dimorphism in both the CHC profile and its response to immune challenge. Females can discriminate between male and female CHCs and are especially attracted to the CHCs of immune-challenged males, while males were equally attracted to male and female CHCs. Male volatile odours also became more attractive after immune challenge, suggesting that males terminally invest in more than one sexual signal.

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Supporting Information

Additional supporting Information may be found in the online version of this article.

Fig. S1. The Y-maze apparatus used in experiment 3.

Table S1. The mean representation of each cuticular hydrocarbon, expressed as either a percentage of the total or as absolute mass in μg, for control and LPS-treated males and females.

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