The role of sexual selection in the evolution of insect cuticular hydrocarbons (CHCs)

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ABSTRACT

A substantial body of work supports the role of sexual selection through female choice in the evolution of some of the most striking traits in biology. Most of this work has focussed on auditory and visual traits, and commonly single traits within each study. However, in many species mate choice acts on multiple characters, including those that are less conspicuous to humans. This thesis contributes to our understanding of sexual selection by investigating mate choice based on a multivariate chemical trait in insects, the cuticular hydrocarbons (CHCs). To broaden our knowledge beyond well studied drosophilid fruit flies, key questions are addressed using the Australian field cricket, *Teleogryllus oceanicus*, and the bull-horned dung beetle, *Onthophagus taurus*. Specifically, I used these species to investigate: 1) whether CHCs are a costly trait; 2) what benefits may be derived from using CHCs in mate assessment; 3) the strength and form of selection imposed by female choice on male CHCs within a multi-modal context; and 4) if CHCs evolve in response to a manipulation of sexual selection.

In my first chapter I investigated opposing natural and sexual selection on the CHC profile of *T. oceanicus*. Along with a sexual display function, CHCs provide protection against desiccation. By measuring the CHCs, attractiveness and desiccation resistance of related individuals, I found evidence that producing an attractive CHC profile comes at a cost of reduced desiccation resistance. The genetic correlations between CHCs and attractiveness also revealed that females using CHCs during mate choice can gain 'sexy sons'.

Work on the social insects has indicated that CHCs act as signals of queen fertility/fecundity, but little is known about the role of CHCs as fecundity signals to males in solitary insects. Previous work on *T. oceanicus* had found that CHCs influence female attractiveness. In Chapter 2 I measured the CHCs and fecundity of related individuals, and found evidence that female CHCs are genetically correlated with fecundity in *T. oceanicus*. I conclude that CHCs can provide an honest signal of fecundity in this species, and that males can derive the indirect benefit of more fecund daughters by using female CHCs in mate assessment.

In Chapter 3 I examined sexual selection from female choice acting on male *O. taurus* CHCs. Rather than using laboratory reared individuals, both male and female beetles used in this study were collected from the field. This ensured that some variation in CHCs and female mate preferences relevant to natural populations was present in the study sample. Using multivariate selection analysis, I found significant nonlinear selection acting on male CHCs, independent of the effect of behavioural courtship.

Having found correlational evidence for the role of sexual selection in the evolution of *O. taurus* CHCs, in my fourth chapter I present experimental evidence of an evolutionary response in the CHC profile to sexual selection. I measured the CHCs of beetles that had evolved in populations where sexual selection was relaxed through enforced monogamy, or persisted by allowing males and females to mate freely. Relaxing sexual selection resulted in an evolutionary response in the male CHC profile. This result provides experimental evidence for the role of sexual selection in the evolution of male *O. taurus* CHCs. Conversely, female CHCs diverged in response to the presence of sexual selection. This shows that male and female CHCs can evolve independently in this species.

The evolution of traits under sexual selection is predicted to result in an exaggeration of the trait, imposing additional resource costs on an organism. Costly secondary sexual traits can evolve condition dependence, where the trait is expressed relative to the pool of resources available to an organism, facilitating mate preferences for high condition individuals. In my final chapter I use experimental manipulation of phenotypic condition to show that *O. taurus* CHCs are condition dependent. Female *O. taurus* benefit by mating with high condition males, and the results from this study indicate that CHCs can act as an honest signal of condition in this species.

In conclusion, this thesis provides evidence for the role of sexual selection in the evolution of CHCs in two species of insect. Furthermore, CHCs are shown to be a costly trait in these species, and potential benefits to mate choice based on CHCs are revealed.

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AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

This thesis contains work that has been published or prepared for publication. Specifically, Chapter 3 has been published in the peer reviewed journal *Behavioral Ecology*. The remaining chapters are all presented in manuscript form for submission to peer reviewed journals.

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I, Leigh W Simmons certify that the student statements regarding their contribution to each of the works listed above are correct Coordinating supervisor signature: [redacted] Date: 20th June 2018

PROLOGUE

"We shall hereafter see that many animals exist, of which neither sex is brilliantly coloured or provided with special ornaments, and yet the members of both sexes or of one alone have probably acquired simple colours, such as white or black, through sexual selection."

"On the banks of the Plata I perceived the air tainted with the odour of the male Cervus campestris, at half a mile to leeward of a herd; and a silk handkerchief, in which I carried home a skin, though often used and washed, retained, when first unfolded, traces of the odour for one year and seven months…The development of these organs is intelligible through sexual selection, if the most odoriferous males are the most successful in winning females,…"

Charles Darwin (1871)

Discussions of sexual selection through mate choice commonly include references to some of the more visually and acoustically extravagant characters found in the natural world. This is unsurprising, as it is the evolution of these characters that remains unexplained by the theory of natural selection. However, Darwin did not restrict his theory of sexual selection to the most exaggerated traits, nor those that only act on the visual/acoustic sensory modalities. As the introductory quotes highlight, Darwin argued that less conspicuous traits, as well as chemical traits, could evolve through sexual selection. Accepting Darwin's arguments has important implications. Specifically, investigations of broad patterns exhibited by secondary sexual traits can suffer from sampling bias if the majority of the research they rely upon focuses on only a subset of secondary sexual traits. In this thesis I aim to contribute to our understanding of sexual selection by focussing on a chemical trait that is less conspicuous to human observers, the cuticular hydrocarbons (CHCs) of insects. Along with representing a trait from a less studied sensory modality, insect CHC profiles have the "advantage" that they are by definition a multivariate trait, and studies of multivariate traits can reveal a different perspective of secondary sexual traits compared to studies that focus on univariate traits. Much of the work on sexual selection and CHCs has focussed on fruit flies from the genus *Drosophila* and to a lesser extent crickets. In the first two chapters I extend upon previous work on one species of cricket, the Australian field cricket *Teleogryllus oceanicus*. Prior studies on this species had found evidence that both male and female CHCs are subject to sexual selection, with some indication of opposing natural and sexual selection on male CHCs. This made *T. oceanicus* an ideal system to further investigate the role of sexual selection in the evolution of insect CHCs.

Taxonomic biases can skew our understanding of sexual selection in much the same way as a focus on particular types of traits. I therefore use the final three chapters to extend our understanding of sexual selection and CHCs taxonomically, by conducting investigations using the bull-headed dung beetle, *Onthophagus taurus*. Throughout the thesis I address key questions in sexual selection/mate choice research, primarily asking whether CHCs are a costly trait, and what (if any) benefits can be derived by males or females in using CHCs for mate choice. To answer these questions I use a combination of quantitative genetics, multivariate selection analyses, experimental evolution and a manipulative phenotypic experiment.

In the first chapter I use a quantitative genetic framework to ask whether producing an attractive CHC profile is opposed by natural selection in male *T. oceanicus*. Specifically, CHCs protect insects against desiccation, and I investigate whether investment in compounds that increase the attractiveness of an individual results in a reduced capability to survive desiccation stress. Testing each compound separately ignores how different blends of CHCs may act together in influencing attractiveness and desiccation resistance. I therefore also use multivariate methods to score each individual's CHC profile for attractiveness/desiccation resistance and test for a trade-off between these different blends.

In Chapter 2 I again use a quantitative genetic framework with *T. oceanicus*, in this case to investigate the benefits that males can receive by using female CHCs during mate choice. Male mate choice is relatively understudied compared to female mate choice, and we know little about the genetic benefits males can derive by being choosy. Interestingly, work on the social insects has revealed that CHCs commonly act as queen pheromones, with this function linked to fertility/fecundity signalling. This raises the hypothesis that CHCs may act as signals of female fecundity to males in insects that share a common ancestor with the social insects. In Chapter 2 I test the hypothesis that female CHCs can honestly signal fecundity, and whether males using the female CHC profile in mate assessment can receive the indirect benefit of more fecund daughters. I do this by examining whether CHCs are genetically correlated with female fecundity in *T. oceanicus*.

Chapter 3 begins the investigations of sexual selection on CHCs in *O. taurus*. In this chapter I use no-choice mating assays coupled with a multivariate selection analysis to test whether female mate choice results in linear and/or non-linear selection on male *O. taurus* CHCs. I include a documented behavioural sexual display in the investigation and explore whether CHCs act alongside this trait during mate choice. The use of field collected beetles in this study ensured that some phenotypic variation relevant to natural populations was captured in the study sample.

Multivariate selection analyses provide correlational evidence for an association between a trait and fitness. Where such an association is found, manipulative experiments are required to provide further evidence for the influence of sexual selection in the evolution of CHCs. In Chapter 4 I examine the CHC profiles of male and female *O. taurus* beetles that were derived from replicate populations evolving under experimentally manipulated sexual selection. Sexual selection was either removed by enforcing monogamy, or allowed to continue by allowing males and females to mate freely. Testing for a response to these two selection regimes provides a test for whether sexual selection documented in Chapter 3 influences the long term evolutionary divergence of CHCs. Moreover, by measuring both male and female CHC profiles, I examine whether the sexes differ in their response to this manipulation.

In Chapter 5 I ask whether *O. taurus* CHC profiles display condition dependence. The concept of condition dependence is important in sexual selection research, as secondary sexual traits that co-vary with "condition" can potentially provide an honest signal of mate quality. However, because chemical traits are generally produced in small amounts relative to other fitness enhancing traits, it is unclear whether the expression of CHCs will depend upon an individual's resource pool. *Onthophagus taurus* provided the ideal model system to test whether CHCs are condition dependent, as previous work had found significant additive genetic variance in a proxy for condition in this species, and that females targeting high condition males in mate choice receive genetic benefits from doing so.

Finally I summarise the findings presented in this thesis in the epilogue, and outline some potential avenues for future research.

CHAPTER 1

Natural and sexual selection on cuticular hydrocarbons: A quantitative genetic analysis

This chapter is presented in manuscript form.

1.1 Abstract

While the reproductive benefits of sexual displays have been widely studied, we have relatively limited evidence of the fitness costs associated with most display traits. Insect cuticular hydrocarbon (CHC) profiles are sexually selected traits that also protect against desiccation. These two functions are thought to oppose each other, with investment in particular compounds believed to increase attractiveness at the expense of compounds that protect against water-loss. We investigated this potential trade-off in a quantitative genetic framework using the Australian field cricket, *Teleogryllus oceanicus*. We found that investment in compounds thought to increase attractiveness (shorter-chained CHCs) was positively genetically correlated with male attractiveness, but was not negatively genetically correlated with desiccation resistance. However, scoring each individual's overall CHC profile for its level of attractiveness and desiccation resistance indicated a negative genetic correlation between these multivariate phenotypes, supporting a genetic trade-off between sexually and naturally selected functions of the CHC profile as a whole. We suggest that the production of an attractive CHC profile may be costly for males, but highlight the need for further work to support this finding experimentally. Genetic covariation between the CHC profile and attractiveness suggests that females can gain attractive sons through female choice.

1.2 Introduction

Increased male mating success through the evolution of elaborate secondary sexual traits is thought to come at a cost of decreased viability (Darwin 1871; Fisher 1930; Andersson 1994). For example, sexual signals can attract not only mates but predators and parasitoids (Zuk and Kolluru 1998). While being more attractive or producing more exaggerated sexual traits can increase mating success, it can also reduce lifespan (Brooks 2000; Robinson et al. 2006). Costs associated with sexual trait expression have important implications for the evolution of secondary sexual traits, providing a constraint to further elaboration (Fisher 1930), a mechanism to link sexual displays to individual quality (Zahavi 1975; Grafen 1990b), and an avenue for the maintenance of genetic variation (Roff 2002; Johnston et al. 2013). Despite the importance of costs to our understanding of the evolution of secondary sexual traits, we still have a limited knowledge of the costs associated with many well studied sexual displays (Kotiaho 2001).

The cuticular hydrocarbons (CHCs) of insects act as a sexual display in a number of species (Chenoweth and Blows 2003; Van Homrigh et al. 2007; Thomas and Simmons 2009b; Curtis et al. 2013; Steiger et al. 2013; Ingleby et al. 2014; Steiger et al. 2015; Lane et al. 2016; Berson and Simmons 2018), but we have only a limited understanding of the viability costs (if any) of producing an attractive CHC profile. Cuticular hydrocarbons are long-chained compounds that have both a signalling function and provide a barrier to water loss across the cuticle (Gibbs and Rajpurohit 2010). Generally a number of different compounds make up the CHC profile and these compounds can vary in both their chain length and the presence or absence of methyl branches and double bonds (Blomquist and Bagnères 2010b). Variation in the structure of CHC compounds is associated with variation in their physical properties, such that increasing chain length increases their melting temperature, which is decreased by double bonds and methyl branches (Gibbs and Pomonis 1995). Variation in the composition of insect CHC profiles, and consequently variation in melting temperatures, are thought to have important implications for both water-proofing and signalling functions. In particular, a CHC profile that displays a greater degree of melting at ecologically relevant temperatures should facilitate the detection of signalling compounds but have reduced water-proofing abilities. In contrast, a more solid CHC profile is likely to hinder the detection of these compounds but reduce water loss across the cuticle (Gibbs 2002; Montooth and Gibbs 2003; Menzel et al. 2017). Using CHCs as a sexual display trait can therefore result in a viability cost, where investment in compounds that increase attractiveness decreases the water-proofing functionality of the CHC layer, and consequently the ability to withstand desiccation.

Despite the potential for the production of an attractive CHC profile to be costly in terms of desiccation resistance, little empirical evidence of such a trade-off exists. Most investigations of the association between CHCs and male mating success have found complex patterns of linear and nonlinear selection (Steiger and Stökl 2014), with the association between the level of attractiveness of a CHC blend and its water-proofing properties unclear. Experimental evolution using the fruit fly, *Drosophila simulans*, found that a principal axis of CHC variation favoured under high temperatures only evolved in the absence of sexual selection (Sharma et al. 2012). However, as the effect of this particular blend of CHCs on the water-proofing properties of *D. simulans* is unknown, and the evolution towards greater investment in total and long-chained CHC production was unimpeded by the operation of sexual selection (Sharma et al. 2012), it is not possible to ascertain whether this evolutionary response reflects a trade-off between CHC attractiveness and desiccation resistance. Although the potential for trade-offs between attractive and protective CHC profiles certainly exists, increased investment in compounds with mid-range effects on the fluidity of the CHC layer could be favoured by both natural and sexual selection (Chung and Carroll 2015). For example, a series of experiments found that one methyl-branched compound had positive effects on both mating success and desiccation resistance in male *D. serrata* (Chung et al. 2014). The general lack of evidence that producing an attractive CHC profile results in a viability cost through decreased desiccation resistance, along with the possibility that natural and sexual selection may favour investment in the same CHCs, necessitates further investigation of this topic.

Testing for negative genetic correlations is a useful approach for addressing the question of whether or not the production of an attractive CHC profile comes at a cost of reduced desiccation resistance (Roff 2002). For example, provided that the necessary genetic variance exists, if investment in a particular compound increases attractiveness at the expense of reduced desiccation resistance, we would expect to see a positive genetic correlation between that compound and attractiveness, and a negative genetic correlation with desiccation resistance. Similarly, if the blend of CHCs that maximizes mating success differs from the blend that maximizes desiccation resistance, then a negative genetic correlation between the production of the different blends can reveal the trade-off between them. There is evidence that investment in longer-chained CHC compounds is positively genetically correlated with desiccation resistance in *D. melanogaster* (Foley and Telonis-Scott 2011), though whether this corresponds to a negative genetic correlation with attractiveness is not known. Similarly, the results from two artificial selection experiments indicate the existence of a genetic correlation between mating success and the blends of CHCs predicted to be attractive in male *D. serrata*,

and these attractive blends of CHCs appear to be opposed by natural selection (Hine et al. 2011; Gosden et al. 2018). However, whether the opposing natural selection arises through desiccation associated viability selection is unknown. Although there is evidence for genetic correlations between CHCs and attractiveness, and between CHCs and desiccation resistance, ascribing desiccation related costs to an attractive CHC profile requires both to be measured in the one study. We sought to investigate this question by testing for genetic correlations between the CHC profile, attractiveness and desiccation resistance using the Australian field cricket, *Teleogryllus oceanicus*.

Cuticular hydrocarbons have been found to play a significant role in influencing male mating success in *T. oceanicus*. Ablation of female chemoreceptors reduces the likelihood they will mount a courting male (Balakrishnan and Pollack 1997), and a combination of linear and nonlinear selection exerted by female mate choice appears to be acting on male CHC profiles (Thomas and Simmons 2009b; Simmons et al. 2013). CHCs are heritable in *T. oceanicus* (Thomas and Simmons 2008a), and although the estimates are low, attractiveness is significantly repeatable (Thomas and Simmons 2009b), indicating that this fitness component may also be heritable. Among isolated populations of *T. oceanicus* introduced to the Hawaiian Islands there have been independent evolutionary origins of males unable to produce song, favoured by selection from the acoustically orienting parasitoid fly *Ormia ochracea* (Zuk et al. 2006; Tinghitella 2008; Pascoal et al. 2014). These 'flatwing' males are no longer able to attract females using song, but they have an increased investment in short-chained CHC compounds, potentially as a method for increasing attractiveness in the absence of song (Simmons et al. 2014). The same study found that males derived from islands with higher precipitation also produced a greater proportion of short-chain compounds, suggesting a role for both sexual and natural selection in shaping variation in CHC profiles (Simmons et al. 2014). Collectively, these findings raise the hypothesis that greater investment in shorter-chained compounds increases attractiveness, but that this comes at a cost of reduced desiccation resistance in *T. oceanicus*.

We used a quantitative genetic framework (Falconer and Mackay 1996; Lynch and Walsh 1998) to test the hypothesis that greater investment in shorter-chained CHCs will be positively genetically correlated with attractiveness, but that these positive genetic correlations will be mirrored by negative genetic correlations with desiccation resistance. The reverse relationship is expected to occur with increasing CHC chain-length. Though intuitive, this hypothesis relies on a relatively simple relationship between CHC chain length and the fluidity/permeability of the CHC profile, and ignores how particular blends of CHCs may act

together in influencing attractiveness and desiccation resistance. We therefore also use multivariate methods to generate univariate scores of attractiveness / desiccation resistance for each individual's CHC profile. If producing a more attractive CHC profile comes with a viability cost of reduced desiccation resistance, we would expect to see a negative genetic correlation between these scores.

1.3 Methods

Breeding design

Quantitative genetics uses the similarity among relatives to partition the additive genetic variance / covariance (V_A / COV_A) from other sources of phenotypic variation, and subsequently derive estimates of the genetic correlations between traits (calculated as $COV_{Aij} / \sqrt{(V_{Ai} \times V_{Aj})}$, where the subscripts i and j represent two different traits) (Falconer and Mackay 1996; Lynch and Walsh 1998). For example, a widely used breeding design in laboratory settings is the paternal half-sib design, where each of multiple males (Sires) is mated to two or more females (Dams) and the proportion of variance explained by Sires (that is, the degree of similarity among half-siblings) is taken to represent ¼ of the population's V_A / COV_A (Falconer and Mackay 1996; Lynch and Walsh 1998). A more commonly used approach in natural populations is the animal model that estimates V_A / COV_A using varying degrees of relatedness among individuals represented in a pedigree (Kruuk 2004; Wilson et al. 2010; Charmantier et al. 2014). We combined these two approaches by employing a paternal half-sib design over two generations in which female offspring from F1 were used as Dams in the second generation.

For the first generation, fifteen male *T. oceanicus* crickets were collected near Carnarvon, Western Australia, and brought to the laboratory where each was mated to three virgin females from a laboratory stock population derived from the same location. Each female was then housed separately and allowed to oviposit in moist cotton wool. At least two replicate groups of offspring from each Dam were housed in 15 litre plastic containers, given egg cartons for shelter and free access to food (cat chow) and water. Fresh water and additional food if required were given weekly. To reduce common environment effects, at five to seven weeks post-emergence all nymphs from the same mother were amalgamated and randomly assigned to separate containers of 30-40 individuals. Crickets were removed from these containers at the penultimate instar and placed individually in plastic containers (7 x 7 x 5 cm) with free access to food and water. Upon adult eclosion the container was cleaned and each cricket was given a

restricted diet of 190 ± 10 mg cat chow. Variation in resource acquisition (van Noordwijk and de Jong 1986; Houle 1991) and the availability of surplus resources (Roff 2002) can both mask trade-offs between life history traits. For example, a previous study using *T. oceanicus* revealed a trade-off between immunity and sperm viability when crickets were fed a restricted diet, but not when they were fed ad libitum (Simmons 2012). Crickets were therefore provided a restricted diet to increase the likelihood that trade-offs would be revealed. A preliminary study had found that the average total cat chow consumed by an individual male cricket over 12 days was 356 mg (N = 21, SE = 24 mg). The diet we provided therefore represented 53% of the average consumption over this time period. Crickets were kept in a controlled temperature room on a 12:12 h light:dark cycle at 26°C for 12±2 days before being assayed and/or freeze killed for later measurement.

Female offspring from the above families were allowed to remain in their original containers and given free access to food and water until sexual maturity. Groups of three of these female offspring were then selected across different half-sibling families and mated to one male offspring derived from each of 19 field-inseminated females that had been collected at the same location and time as the males described above (one male was mated to two females that shared a Sire but had different Dams). We then housed each mated female separately and repeated the protocol described for the first generation. Over both generations this breeding design resulted in a total of 34 Sire and 85 Dam families, with some Sires having offspring with less than three Dams due to either Dam death or infertility (mean Dams per Sire = 2.5, minimum = 1, maximum = 3). The Sire-Dam combinations produced both full siblings and half siblings, and the use of female offspring from the first generation as Dams for a second generation provided additional pedigree links including maternal cousins, and uncle-nephew relations.

Attractiveness and desiccation resistance

To measure attractiveness, at 12±2 days post-eclosion, male offspring were placed in a clean container with a single stock female in a no-choice mating trial. Following commencement of the male courtship song, pairs were observed for five minutes and the time taken (courtship time) for a female to mount the male and receive a spermatophore was recorded. Those males that were not mounted within two minutes were assigned a status of unmated. Two minutes was selected because a preliminary survival analysis revealed that 50 % of males were successfully mounted by the female two minutes after the commencement of courtship (males not mated within the five minutes of observation were assigned a census time

of five minutes for the preliminary survival analysis). We will refer to this binary measure (mated versus unmated) of attractiveness as "Attractiveness$_1$" throughout the text. Attractiveness$_1$ captured data for all males, including those that were not mated within the five minutes of observation. However, this measure obscures the continuous variation in attractiveness of all males that mated within the five minute observation period. We therefore used the duration of courtship necessary to elicit a mount for those males that were mounted within the five minutes of observation as a second attractiveness measure to capture this variation. As increased courtship time represents relative unattractiveness, to aid in the interpretation of results we reverse scored all correlations involving courtship time so that a positive correlation represents a positive association with attractiveness. We will refer to this measure of attractiveness as "Attractiveness$_2$" throughout the text. Females used in the mating assay were between seven and nine days old. These females had been kept in female only groups until the day before the mating trial, at which time they were housed individually with a single stock male for eight hours to ensure prior mating experience, as this has been shown to increase the level of female choosiness in another species of cricket (Bateman et al. 2001). All observations took place at 26°C under red light to minimize disturbance of the crickets. Following the mate choice trial, male crickets were returned to their original container to be used in a desiccation assay the following day.

To measure desiccation resistance, each cricket was placed in a new container that held 30 g of desiccant (silica gel, SiO_2), separated from the cricket by a plastic barrier, and then immediately placed in an incubator set at 30°C and left for 18 hours. After this time crickets were checked every hour for a further 18 hours and their time of death recorded. The majority (88.64%) of crickets died between 19 and 36 hours. However, some crickets were found dead at the first check (6.58%) or still alive after 36 hours (4.78%). To provide conservative estimates of desiccation resistance for these crickets we assigned them a time to death of 18 and 37 hours respectively. Crickets were frozen after death or at the end of the trial if they were still alive. Throughout the text we will use "Desiccation-resistance" to refer to the time to death under desiccation. Both assays (attractiveness and desiccation resistance) were conducted blind to an individual's pedigree links.

Cuticular hydrocarbons

At 12 days of age, crickets that had not undergone a mating or desiccation trial were frozen and placed in a clean glass vial that was plugged with cotton wool and stored at -20°C. To extract the CHCs, crickets were immersed for five minutes in 5 ml of hexane containing

0.02 g L^{-1} of *n*-dotriacontane as an internal standard. One µl of the sample was injected using the splitless mode into a Shimadzu QP2010 gas chromatography – mass spectrometry (GC-MS) machine fitted with a Stabilwax column (30 m x 250 µm x 0.10 µm). The initial column temperature was set at 40°C for one minute and then increased at 20°C per minute until reaching 250°C for 20 minutes. In GC-MS the compounds are visualized as peaks with the area under each peak representing the amount of the compound. Peaks were integrated using Shimadzu GCMSsolution software (Ver 4.41) and matched to compounds previously identified in this species. To control for variation in the amount of solution injected across samples, all peak areas were divided by the area of the internal standard. For consistency with the multiple regression analyses (see below) we restricted our quantitative genetic analyses to the seven compounds of greatest abundance.

Analyses

Prior to conducting the quantitative genetic analyses, we tested for an effect of Generation (essentially representing a Block effect) on each of the traits individually. We used standard regression analyses with permutation testing for significance tests as some traits did not have normally distributed residuals. Generation was found to have a significant effect on Desiccation-resistance and two of the CHC compounds (Table 1.1). For consistency across traits we included Generation as a fixed effect in all further analyses.

We next fit univariate mixed effects 'animal' models in a REML framework incorporating information from the pedigree to estimate the additive genetic variance for each trait (Wilson et al. 2010). To assess whether the estimated additive genetic variance (V_A) for each trait was statistically different from zero we performed log-likelihood ratio tests (LLRT) on twice the difference in log-likelihoods between models that included the pedigree information and those without, assuming a chi-square distribution with one degree of freedom. Heritability (h^2) was calculated as the additive genetic variance divided by the total phenotypic variance (V_A / V_P), with V_P calculated as V_A plus the residual variance (V_R). Following Garcia-Gonzalez et al. (2012) we calculated the coefficients of variation without the 100 multiplier and also present the complementary measure of evolvability, I_A (Houle 1992; Hansen et al. 2011). Approximate standard errors for these parameters were calculated following equations six and nine of Garcia-Gonzalez et al. (2012). We tested for maternal effects using LLRT to compare the above animal model with a model that also included Dam identity as an additional random effect, as the simpler model can potentially return inflated estimates of h^2 (Kruuk and Hadfield 2007). However, for the majority of traits the effect of Dam identity was estimated to

be on the boundary of parameter space, indicative of low associated variance but rendering statistical tests invalid (Table 1.1). We therefore fit a Sire + Dam model to each trait, and calculated h^2 as 4 * Sire Variance / V_P. Eight individuals that did not have half-siblings were excluded from these analyses. The Sire + Dam models provided an estimate of h^2 from which maternal effects are excluded. In all cases h^2 was either greater in the Sire + Dam model or differed by less than 0.05 compared to the animal model (Table 1.1), indicating that the estimates from the animal model were not inflated by non-modelled Dam effects. We therefore excluded Dam ID as a random effect from further analyses.

We tested for a trade-off between the attractiveness and desiccation resistance of *T. oceanicus* CHC profiles in two ways. First we estimated the genetic correlation between each CHC compound with the two measures of attractiveness and with desiccation resistance. If investment in a particular compound increases attractiveness at the expense of decreased desiccation resistance, we would expect to see a positive genetic correlation between the compound and attractiveness, and a negative genetic correlation between the compound and desiccation resistance. To estimate the genetic correlations we fit bivariate animal models for each of the CHC compounds with the measures of attractiveness and desiccation resistance. As the CHCs were measured on different (but related) individuals to those that we measured both attractiveness and desiccation resistance, the residual (within individual) correlation was not estimable so we constrained it to zero. To test the significance of each genetic correlation we ran a separate model that constrained the genetic correlation to zero and used LLRT to compare the two models. A square root transformation of $Attractiveness_2$ improved the normality of the model residuals and was undertaken for inference testing but not for estimating the genetic correlations. Although one of our attractiveness measures ($Attractiveness_1$) is a binary trait, we estimated the genetic correlations that included this trait assuming a Gaussian distribution of the residuals as this method should provide unbiased estimates of the genetic correlations (Olausson and Rönningen 1975; Roff 2001; Walling et al. 2014). The model estimating the genetic correlation between one CHC compound ($4\text{-}MeC_{33}$) with $Attractiveness_1$ had a boundary effect on the residual variance of the CHC compound. We therefore used a regression of sire family means to estimate this particular genetic correlation. Although this method will likely underestimate the magnitude of the genetic correlation, it should provide an unbiased indication of its sign (see Buzatto et al. (2015) for further details and for a comparison with the animal model). Bootstrapping was used for standard error estimation and inference in this instance.

Rather than investment in individual CHC compounds being important for attractiveness / desiccation resistance, the overall blend of compounds may be more pertinent. Accordingly, trade-offs between the attractiveness and desiccation resistant properties of *T. oceanicus* CHC profiles may occur at the level of the overall composition of the CHC profile, rather than at the individual compound level. Therefore, we derived a univariate score in order to predict a given CHC profile's attractiveness / desiccation resistance in multivariate space. A trade-off would be apparent if the predicted attractiveness scores were negatively genetically correlated with the predicted desiccation score. We used linear multiple regression (Lande and Arnold 1983) to model the multivariate association between the CHC profile and attractiveness / desiccation resistance. To do so, we haphazardly selected a subset of individuals for which we had obtained attractiveness and desiccation resistance data (N=252) from across different families, and measured their CHCs following the same procedures outlined above. We performed separate multiple regression analyses using either Attractiveness_1, Attractiveness_2 or Desiccation-resistance as the dependent variable, and the seven CHC compounds produced in greatest abundance along with Generation as explanatory variables. Each of the seven CHC compounds individually comprised > 4% of the total CHC profile and together represented over 80% of total CHC abundance. We only included the seven largest peaks as these captured the majority of the CHC abundance of each individual but avoided statistical issues of multicollinearity among peaks (all variance inflation factors were < 10 in each analysis) and large numbers of explanatory variables relative to our sample size. Peak areas were scaled to a mean of zero and a standard deviation of one prior to the regression, and the attractiveness / desiccation resistance measures were scaled by their mean (Lande and Arnold 1983).

To predict the univariate score of multivariate CHC profile attractiveness / desiccation resistance for the individuals that had not undergone either a mating trial or a desiccation assay, we applied the vector of coefficients returned from the multiple regression analyses to each individual's scaled CHC data (McGuigan et al. 2008; Delcourt et al. 2012; Gershman and Rundle 2016). We will refer to these univariate scores as Attractiveness_1-CHCβ, Attractiveness_2-CHCβ and Desiccation-resistance-CHCβ. The coefficients from the regression with Attractiveness_2 as the measure of attractiveness were reverse scored so that higher scores would now represent greater attractiveness. Testing for negative genetic correlations between Attractiveness_1-CHCβ or Attractiveness_2-CHCβ and Desiccation-resistance-CHCβ allowed us to assess whether producing a more attractive CHC profile trades-off genetically with producing a more desiccant resistant CHC profile. CHCs are known to respond to changes in

environmental and social conditions in several species (Ingleby 2015; Otte et al. 2018) including *T. oceanicus* (Thomas et al. 2011; Thomas and Simmons 2011b; Pascoal et al. 2016). Using individuals that had not been exposed to a female or the desiccating environment allowed us to exclude potential effects of these factors on the CHC profile. The estimation and testing of genetic correlations were conducted as described above, except that the residual correlation was no longer constrained to zero because both traits were estimated from the CHC profile of the same individuals. Genetic correlations between the fitness components ($Attractiveness_1$ or $Attractiveness_2$ and Desiccation-resistance) were tested in the same manner. We also tested for phenotypic associations between attractiveness and desiccation resistance using logistic regression with $Attractiveness_1$ as the response variable and Desiccation-resistance as the explanatory variable. We used Spearman's correlation (ρ) to test for a phenotypic association between $Attractiveness_2$ and Desiccation-resistance. All analyses were performed in R (R Core Team 2017) using *ASReml-R* for fitting the animal models (Butler et al. 2009), *OIsurv* for the survival analysis (Diez 2013), *ggplot2* for plotting (Wickham 2009), and the *boot* package for bootstrapping (Canty and Ripley 2016).

1.4 Results

We measured a total of 1,038 offspring spread across 34 Sire and 85 Dam families. We obtained data for $Attractiveness_1$ from 736 offspring, of which 529 also provided data for $Attractiveness_2$. Of the individuals that had data on attractiveness, 623 also provided data for Desiccation-resistance. The lower number for Desiccation-resistance was a result of space constraints in the desiccating incubator. An additional 46 individuals were exposed to a female in a mate choice assay but we were unable to acquire attractiveness data because they failed to court the female; we therefore only obtained data for Desiccation-resistance from these individuals. This gave a total of 669 crickets that provided data for Desiccation-resistance. Two hundred and fifty six individuals that were not exposed to a female or the desiccating environment had their CHCs assayed. The CHC data from these individuals were used in the quantitative genetic analyses. The multiple regression analyses used CHC data from 252 individuals that had been measured for both attractiveness and desiccation resistance. Of these 252 individuals, 132 (52%) were mounted by the female within two minutes after the commencement of courtship, and 185 (73%) had data on courtship time. The results of the univariate animal models are presented in Table 1.1, which shows significant additive genetic variance (V_A) in all traits.

Table 1.1 Results of tests for the effect of Generation (Gen *P*) as well as the univariate animal models for $Attractiveness_1$ (Att_1), $Attractiveness_2$ (Att_2), Desiccation-resistance (Des) and the seven most abundant cuticular hydrocarbon (CHC) compounds for the field cricket *Teleogryllus oceanicus*. Included are estimates of additive genetic variance (V_A), residual variance (V_R), total phenotypic variance (V_P), coefficients of variation for each variance component (CV), a measure of evolvability (I_A), heritability (h^2) and p-values from log-likelihood ratio tests used for inference for significant additive genetic (V_A *P*) and maternal (Dam *P*) effects. Standard errors (SE) for the quantitative genetic parameters are given in parentheses. The h^2 estimates from a Sire + Dam model are also presented (h^2 Sire). Note that the h^2 estimates for the binary trait $Attractiveness_1$ were calculated on the observed scale, converting these to the liability scale following Dempster and Lerner (1950) results in h^2 estimates of 0.100 from the animal model and 0.170 from the Sire + Dam model. The CHC compounds correspond to those labelled Peaks 5, 9, 12, 14, 17, 21 and 22 in Thomas and Simmons (2008b).

Trait	Mean	V_A	V_R	V_P	CV_A	CV_R	CV_P	I_A	h^2	h^2 Sire	Gen *P*	V_A *P*	Dam *P*
Att_1	0.523	0.016 (0.010)	0.235 (0.015)	0.250 (0.013)	0.239 (0.080)	0.926 (0.03)	0.957 (0.025)	0.057 (0.038)	0.062 (0.041)	0.108 (0.071)	0.724	**0.043**	-
Att_2	1.401	0.167 (0.099)	1.329 (0.114)	1.496 (0.094)	0.292 (0.087)	0.823 (0.035)	0.873 (0.028)	0.085 (0.051)	0.112 (0.065)	0.201 (0.112)	0.310	**0.036**	-
Des	28.214	8.918 (2.998)	26.518 (2.502)	35.435 (2.148)	0.106 (0.018)	0.183 (0.009)	0.211 (0.006)	0.011 (0.004)	0.252 (0.077)	0.226 (0.138)	**0.012**	**<.001**	0.555
4-MeC_{30}	0.485	0.005 (0.002)	0.007 (0.002)	0.012 (0.001)	0.150 (0.029)	0.171 (0.020)	0.227 (0.011)	0.023 (0.009)	0.437 (0.144)	0.458 (0.271)	0.708	**<.001**	-
$C_{31:1}$	0.58	0.018 (0.006)	0.011 (0.004)	0.029 (0.003)	0.232 (0.035)	0.181 (0.031)	0.295 (0.015)	0.054 (0.016)	0.622 (0.148)	0.936 (0.323)	**0.040**	**<.001**	-
$C_{31:2}$	0.997	0.062 (0.021)	0.061 (0.016)	0.123 (0.012)	0.249 (0.042)	0.248 (0.032)	0.352 (0.017)	0.062 (0.021)	0.503 (0.142)	0.636 (0.282)	0.578	**<.001**	-
4-MeC_{33}	0.293	0.001 (0.000)	0.002 (0.000)	0.003 (0.000)	0.111 (0.026)	0.159 (0.015)	0.194 (0.009)	0.012 (0.006)	0.329 (0.138)	0.343 (0.256)	**<.001**	**0.002**	0.800
$C_{33:1}$	0.463	0.004 (0.002)	0.008 (0.002)	0.013 (0.001)	0.142 (0.032)	0.197 (0.019)	0.243 (0.012)	0.020 (0.009)	0.341 (0.137)	0.331 (0.253)	0.972	**0.001**	0.641
$C_{33:2}$	0.821	0.026 (0.009)	0.021 (0.006)	0.047 (0.005)	0.196 (0.032)	0.175 (0.026)	0.263 (0.013)	0.039 (0.013)	0.557 (0.149)	0.533 (0.287)	0.162	**<.001**	-
$C_{33:2}$	2.029	0.095 (0.035)	0.114 (0.027)	0.209 (0.021)	0.152 (0.028)	0.166 (0.020)	0.225 (0.011)	0.023 (0.009)	0.455 (0.143)	0.494 (0.283)	0.176	**<.001**	-

Table 1.2 presents the results of tests for genetic correlations (r_G) between the CHC compounds of males and the two attractiveness measures and desiccation resistance measured on their male relatives. The typically large standard errors on all of the estimates indicates that we had low statistical power to detect where the estimated correlations were different from zero. Nevertheless, three of the longer-chained CHC compounds were significantly negatively genetically correlated with one of the attractiveness measures. Interestingly, for both attractiveness measures, we found negative genetic correlations with compounds that have a chain-length of 33 carbons, and positive genetic correlations with compounds that have a chain-length of 30 or 31 carbons. This suggests a positive genetic association between attractiveness and investment in shorter-chained CHCs, and a negative genetic association between attractiveness and investment in longer-chained CHCs. To investigate this further we calculated the ratio of investment in compounds with a chain length of 30 and 31 carbons to investment in compounds with a chain length of 33 carbons. Both measures of attractiveness were significantly positively genetically correlated to this ratio (Attractiveness$_1$: r_G = 0.740, SE = 0.338, P = 0.025; Attractiveness$_2$: r_G = 0.704, SE = 0.275, P = 0.016), indicating that genotypes that invest relatively more in short-chained CHCs are more attractive to females.

The estimated genetic correlations between the CHC compounds and desiccation resistance were generally lower than those with attractiveness, and none were statistically significant (Table 1.2). Furthermore, unlike the attractiveness measures there was no clear pattern between the sign of the genetic correlations and the CHC chain length. Accordingly there was no evidence for a genetic correlation between the ratio of investment in the shorter versus longer-chained CHCs and desiccation resistance (r_G = -0.016, SE = 0.216, P = 0.942). However, there was some evidence for a genetic association between desiccation resistance and two alkenes ($C_{31:1}$ and $C_{33:1}$), with the sign of these two genetic correlations being opposite to those estimated for the two attractiveness measures (Table 1.2). Calculating the ratio of investment in $C_{31:1}$ versus $C_{33:1}$ revealed a significant negative genetic correlation between this ratio and desiccation resistance (r_G = -0.441, SE = 0.181, P = 0.029), indicating that investing proportionally more in the shorter-chained of the two alkenes reduced an individual's ability to withstand desiccation. This ratio was positively genetically associated with the two attractiveness measures (Attractiveness$_1$: r_G = 0.510, SE = 0.324, P = 0.117; Attractiveness$_2$: r_G = 0.538, SE = 0.309, P = 0.044), suggesting that the proportional investment in compounds of different chain-length in one class of CHCs might generate trade-offs between the attractiveness and desiccation resistance functions of *T. oceanicus* CHC profiles.

Table 1.2 Results of the bivariate animal models testing for genetic correlations (r_G) between each of the cuticular hydrocarbon (CHC) compounds with the two measures of attractiveness and desiccation resistance. The standard errors (SE) and p-values (*P*) from log-likelihood ratio tests are also presented.

	Attractiveness$_1$			**Attractiveness$_2$**			**Desiccation resistance**		
CHC	**r_G**	**SE**	***P***	**r_G**	**SE**	***P***	**r_G**	**SE**	***P***
4-MeC_{30}	0.224	0.357	0.528	0.407	0.310	0.192	0.215	0.245	0.393
$C_{31:1}$	0.239	0.334	0.466	0.403	0.317	0.086	-0.298	0.210	0.174
$C_{31:2}$	0.303	0.354	0.382	0.379	0.311	0.164	0.076	0.235	0.744
4-MeC_{33}[†]	-0.459	0.181	**0.001**	-0.685	0.374	0.132	0.093	0.275	0.744
$C_{33:1}$	-0.904	0.350	**0.024**	-0.695	0.356	0.097	0.381	0.247	0.156
$C_{33:2}$	-0.268	0.327	0.416	-0.278	0.298	0.520	0.191	0.233	0.426
$C_{33:2}$	-0.701	0.295	**0.033**	-0.424	0.304	0.378	-0.185	0.240	0.450

[†]The genetic correlation between 4-MeC_{33} and Attractiveness$_1$ was estimated using a regression of sire family means with bootstrapping for SE estimation and inference. This method is likely to underestimate the strength of the correlation but was used in this instance due to problems fitting the animal model.

The results of the multiple regression analyses using data from males that had been measured for all traits are presented in Table 1.3. Consistent with the genetic analyses, the phenotypic associations (represented by the β regression coefficients) between the CHC compounds and the two attractiveness measures were generally stronger compared to those with desiccation resistance. Applying these vectors of β regression coefficients to the scaled CHC data of those individuals that had not undergone a mating trial or desiccation assay returned two univariate measures of predicted CHC profile attractiveness ($Attractiveness_1$-CHCβ, $Attractiveness_2$-CHCβ) and a measure of predicted CHC profile desiccation resistance (Desiccation-resistance-CHCβ). Using these scores to test for genetic correlations revealed a negative genetic correlation between the predicted attractiveness of the CHC profile and its predicted ability to withstand desiccation (Desiccation-resistance-CHCβ - $Attractiveness_1$-CHCβ: r_G = -0.623, SE = 0.156, P = 0.003; Desiccation-resistance-CHCβ - $Attractiveness_2$-CHCβ: r_G = -0.671, SE = 0.146, P = 0.001). The sire family means are plotted in Fig. 1.1 to visualize these negative correlations. Figure 1.1 provides evidence for an evolutionarily significant trade-off between producing an attractive blend of CHC compounds versus a desiccation resistant CHC blend. Despite the presence of this trade-off in the CHC profile, there was no evidence to suggest that overall attractiveness trades off directly with desiccation resistance, with the opposite trend indicated by positive phenotypic associations ($Attractiveness_1$ – Desiccation resistance: β = 0.029, χ^2_1 = 4.400, P = 0.036; $Attractiveness_2$ – Desiccation resistance: ρ = 0.101, P = 0.032) and genetic correlations ($Attractiveness_1$ – Desiccation resistance: r_G = 0.381, SE = 0.301, P = 0.228; $Attractiveness_2$ – Desiccation resistance: r_G = 0.590, SE = 0.240, P = 0.081). That is, although attractive individuals trade-off a more desiccation resistant CHC profile for a more attractive CHC profile, they may not pay a cost in overall desiccation resistance.

Table 1.3 Modelling cuticular hydrocarbon attractiveness and desiccation resistance. Coefficients (β) from multiple regression models with relative attractiveness or desiccation resistance as the response variable and the seven highest abundance cuticular hydrocarbon (CHC) compounds as explanatory variables. These coefficients were used to predict the univariate measures of CHC profile attractiveness / desiccation resistance ($Attractiveness_1$-CHCβ, $Attractiveness_2$-CHCβ and Desiccation-resistance-CHCβ). Bootstrapping was used for standard error estimation (SE) and permutation tests were used to assess statistical significance.

	$Attractiveness_1$			**$Attractiveness_2$**			**Desiccation resistance**		
CHC	**β**	**SE**	***P***	**β**	**SE**	***P***	**β**	**SE**	***P***
4-MeC_{30}	-0.093	0.093	0.364	0.016	0.108	0.884	0.010	0.020	0.610
$C_{31:1}$	0.303	0.116	**0.012**	0.303	0.157	**0.010**	0.005	0.023	0.786
$C_{31:2}$	-0.036	0.119	0.796	-0.125	0.117	0.330	-0.018	0.024	0.406
4-MeC_{33}	-0.107	0.112	0.302	-0.191	0.129	0.100	0.000	0.022	0.998
$C_{33:1}$	-0.036	0.122	0.788	0.062	0.143	0.628	0.050	0.023	**0.022**
$C_{33:2}$	0.250	0.107	**0.034**	0.092	0.125	0.422	-0.001	0.023	0.980
$C_{33:2}$	-0.248	0.127	0.094	-0.159	0.172	0.262	-0.014	0.028	0.554

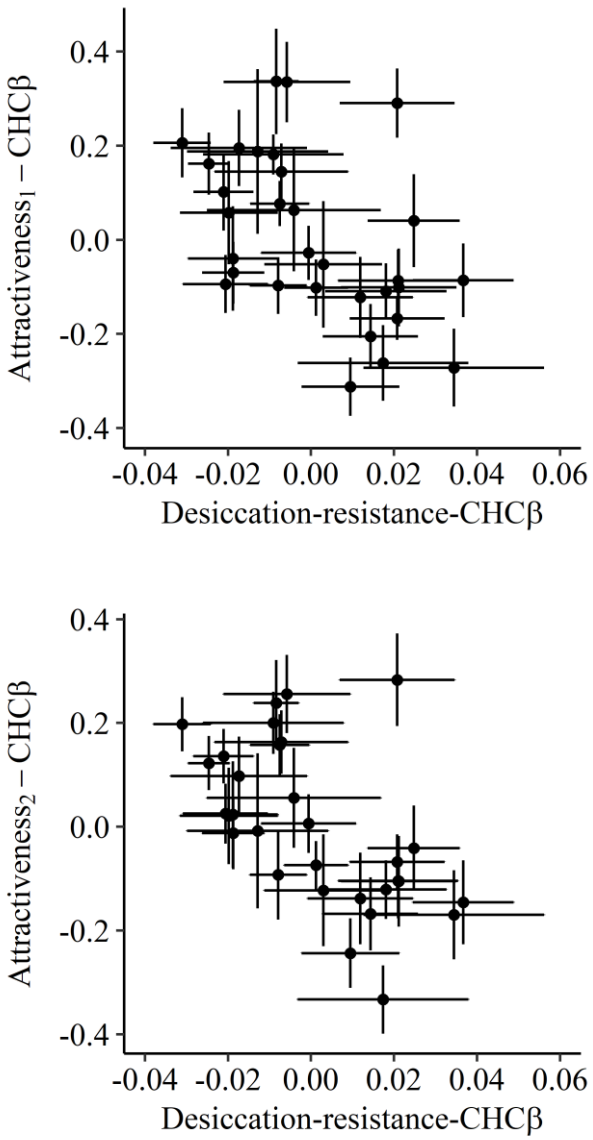

Figure 1.1 Plot of sire family means to visualize the negative genetic correlations between predicted cuticular hydrocarbon (CHC) profile attractiveness ($Attractiveness_1$-CHCβ and $Attractiveness_2$-CHCβ) and predicted CHC profile desiccation resistance (Desiccation-resistance-CHCβ).

1.5 Discussion

We tested whether producing an attractive CHC profile results in a viability cost of increased susceptibility to desiccation for male Australian field crickets, *T. oceanicus*. Consistent with longer-chained CHCs reducing the fluidity of the CHC profile and its detectability by female chemoreceptors, the genetic correlation between investment in longer-chained compounds and attractiveness was negative. Despite this, and contrary to the expectation that increased investment in longer-chained compounds will reduce desiccation risk through a less permeable CHC profile, there was no evidence for a genetic association between overall investment in longer-chained CHCs and desiccation resistance. Some indication of a trade-off at the compound level came from the significant negative genetic correlation between desiccation resistance and investment in the shorter-chained alkene relative to the longer-chained alkene, with this ratio positively genetically correlated with attractiveness. However, only one of the genetic correlations with the two attractiveness measures was significant at the 0.05 level. Stronger evidence for a trade-off was revealed by modelling each individual's multivariate CHC profile. Using these predictive scores revealed that producing the CHC profile that maximized attractiveness was negatively genetically correlated with producing the CHC profile that maximized desiccation resistance.

Despite evidence that producing an attractive CHC profile comes at a cost to its water-proofing properties, we found positive phenotypic associations between both measures of overall attractiveness and desiccation resistance. This may be due to variation in the amount of resources available to individuals within the study sample, with some individuals able to allocate greater resources to both attractiveness and desiccation resistance (van Noordwijk and de Jong 1986; Reznick et al. 2000; Roff 2002). We had attempted to control for variation in resource acquisition by controlling the quantity of food available. However, it is probable that variation in the amount of resources available to each individual remained in the study sample, through for example, variation in the ability to accumulate resources prior to adult eclosion (there was no diet restriction in the pre-adult stages). The CHC profile of crickets is one of multiple traits influencing attractiveness and desiccation resistance, and it is also possible that variation in other unmeasured traits could have contributed to the positive covariation between attractiveness and desiccation resistance. Our results indicate that producing an attractive CHC profile reduces an individual's ability to survive under desiccation, but that this negative effect could be overwhelmed by the positive phenotypic effects of other traits. We suggest that further investigations that include additional life history traits will be useful to gain a more

comprehensive understanding of the association between attractiveness and desiccation resistance.

Water loss across the cuticle has been found to be an important contributor to total water loss in at least some insects, and CHCs provide an important barrier to water loss (Gibbs and Rajpurohit 2010). However, we found only weak phenotypic and genetic associations between desiccation resistance and CHC abundance. There are a number of potential explanations for this result. Although desiccation resistance is a direct viability cost that affects fitness (Kotiaho 2001), CHCs affect this fitness component only indirectly through a reduction in cuticular water loss. Other factors might also affect an individual's ability to withstand desiccation. For example, traits such as the degree of melanisation can influence cuticular water loss (Ramniwas et al. 2012; King and Sinclair 2015), and increased resistance to desiccation in flies artificially selected for desiccation resistance is associated with an increase in carbohydrate as well as water content (Gibbs et al. 1997; Chippindale et al. 1998; Gefen et al. 2006). Variation in the CHC profile may therefore have a large effect on rates of cuticular water loss in *T. oceanicus*, but the effect on overall desiccation resistance could have been obscured by other traits. Other studies have failed to find the predicted association between the rate of cuticular water loss and the physical properties of the CHC profile (Gibbs et al. 1998; Montooth and Gibbs 2003). Gibbs (2002) suggested that these findings may reflect localized variation in CHCs across the insect cuticle, which will be obscured when the body-wide CHC profile is examined. Such regional variation has been found in other insects (Gibbs and Crowe 1991; Wang et al. 2016), and if it occurs in *T. oceanicus*, our measure of the body-wide CHC profile may have prevented the detection of variation relevant to desiccation resistance. Finally, our measure of desiccation resistance (time to death) is likely to underestimated the costs of desiccation imposed from an attractive CHC profile. For example, crickets that produced an attractive CHC profile could have suffered non-lethal physiological costs of water loss in the desiccating environment, such as the ability to produce ejaculates or to transport oxygen or nutrients through the blood stream. All, some or none of the above mentioned factors could explain the weaker association between desiccation resistance and variation in the CHC profile that was initially hypothesized. We suggest that these possibilities provide exciting avenues for future research, particularly in the context of the interaction between natural and sexual selection.

Our finding of a negative genetic correlation between producing a CHC profile predicted to be attractive and one that is predicted to protect against desiccation should be interpreted with some caution. Multiple regression is a statistical technique that allows for the estimation of the effect of a trait on fitness, independent of the effects of the other traits included

in the analysis (Lande and Arnold 1983). An advantage of this form of analysis is the avoidance of assigning an impact of a trait on fitness when this is simply due to its correlation with other traits that have a fitness effect. The disadvantage of multiple regression is that the estimated associations with fitness may be moderated by the effect of traits not included in the analysis (Lande and Arnold 1983; Mitchell-Olds and Shaw 1987). As our predictive models of CHC attractiveness / desiccation resistance were based on multiple regression analyses, it is therefore possible that our finding of a negative genetic correlation between producing a CHC profile that was predicted to be attractive versus one predicted to be desiccation resistant was influenced by the effect of traits not included in the analysis. Nevertheless, results from multiple regression analyses have proven fruitful in CHC research. For example, two artificial selection experiments that selected upon the vector of male CHC attractiveness using *D. serrata* found an evolutionary increase in male attractiveness (Hine et al. 2011; Gosden et al. 2018). Similar studies will be required using *T. oceanicus* to provide further support for our finding of a trade-off between the blend of CHCs that make a male attractive and the blend that make him desiccation resistant. We emphasize that like all correlational studies, the evidence we provide is supportive of a cost for producing an attractive CHC profile, but necessarily demands further experimental work.

The primary role of CHCs has been viewed as one of protecting the insect against desiccation, with signalling functions a subsequent evolutionary innovation (Blomquist and Bagnères 2010b). Even if water-proofing is the primary function of CHCs, this does not explain the complex composition of insect CHC profiles, which under a purely water-proofing scenario could simply comprise of long straight-chained alkanes (Lockey 1988). The results we present support the idea of the signalling function of CHCs being an important driver in shaping the complexity of the *T. oceanicus* CHC profile, with variation in the CHC profile more strongly associated with variation in attractiveness than with desiccation resistance. In particular, we found genetic correlations between male CHCs and attractiveness, indicating that females can use an assessment of the male CHC profile to gain attractive sons, which can have a strong effect on the evolution of sexual display traits (Fisher 1930). Overall, our results suggest that producing an attractive CHC profile trades-off with producing a CHC profile best suited to desiccation resistance. Our understanding of the viability costs of producing an attractive CHC profile is in its infancy, and we believe this topic provides an interesting area for future research.

1.6 Acknowledgements

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CHAPTER 2

Female cuticular hydrocarbons can signal indirect fecundity benefits in insects

This chapter is presented in its manuscript form.

2.1 Abstract

Male mate choice provides an increasingly recognised pathway through which males can increase their fitness. For example, males may increase their number of offspring by targeting more fecund females as mating partners. If fecundity is heritable, males that mate with more fecund females can also receive the indirect benefit of more fecund daughters. In species where female fecundity is not directly assessable, female ornaments may act as signals of fecundity. However, whether female ornaments reliably signal the indirect benefit of more fecund daughters is not well understood. We investigated this question using the field cricket, *Teleogryllus oceanicus*. Previous work had identified the cuticular hydrocarbon (CHC) profile as a female sexual display trait in *T. oceanicus*. To examine whether CHCs can provide a reliable signal of fecundity, we tested whether individual CHC compounds and the major axis of CHC genetic variation (Gmax) are genetically correlated with ovary mass, a proxy for fecundity in this species. We found significant genetic correlations between ovary mass with three CHC compounds and with Gmax. This result indicates that by targeting females as mating partners based on their CHC profile, males can sire more fecund daughters. One genetic correlation with an individual compound was negative in sign, consistent with the expectation of a trade-off between female signalling and fecundity.

2.2 Introduction

Male mate choice has received increasing attention in evolutionary biology, with males thought to commonly target more fecund females as mating partners (Andersson 1994; Amundsen 2000; Bonduriansky 2001; Clutton-Brock 2007; Kraaijeveld et al. 2007; Clutton-Brock 2009; Edward and Chapman 2011). Often, in taxa such as insects, males prefer to mate with larger females where size provides a direct phenotypic indicator of female fecundity (Bonduriansky 2001). Less frequently, male insects may bias their mating effort towards more ornamented females if the female ornament provides a signal of fecundity (LeBas et al. 2003; Cotton et al. 2015; Hopkins et al. 2015). If female fecundity is heritable, and the female ornament is genetically correlated with fecundity, males that prefer females with attractive ornaments can receive the indirect benefit of siring more fecund daughters. Despite the growing interest in male mate choice, we still know little about the reliability of female ornaments as signals of indirect fecundity benefits.

Cuticular hydrocarbons (CHCs) comprise an important composite trait in insects that potentially acts as a signal of female fecundity. For example, in the fruit fly, *Drosophila melanogaster*, there is evidence that CHCs influence female attractiveness, and that individual compounds are genetically correlated with female productivity (Arbuthnott et al. 2017). In *D. serrata*, male choice exerts predominantly stabilising selection on female CHCs (Rundle and Chenoweth 2011), consistent with a theoretical expectation for traits signalling female fecundity (Chenoweth et al. 2006). However, recent work has questioned the influence of CHCs on female attractiveness in this species (Gosden et al. 2018), and whether CHCs act as signals of female fecundity in *D. serrata* is unclear. There is evidence that CHCs act as "queen pheromones" in the social insects, signalling the fertility of reproductive individuals and suppressing the fecundity of other females (Van Oystaeyen et al. 2014; Holman 2018). Moreover, the role of CHCs as queen pheromones appears to be associated with their ability to honestly signal queen fecundity. For example, CHCs are genetically correlated with fecundity in the ant, *Lasius niger*, (Holman et al. 2013). The taxonomically widespread nature of CHCs acting as queen pheromones in the social insects suggests that this role of CHCs may be derived from their role as signals of female fecundity to males in solitary insects (Van Oystaeyen et al. 2014). However, little is known about the potential for CHCs to honestly signal female fecundity in non-social insects. We therefore sought to examine whether CHCs are genetically correlated with female fecundity in a non-social insect that is ancestral to the social-insects. By so doing, our aim is to contribute to the understanding of female ornaments more broadly, and in particular, whether CHCs reliably indicate a female's genetic merit for fecundity.

The Australian field cricket, *Teleogryllus oceanicus*, provides an ideal model species to test for genetic correlations between CHCs and fecundity. Cuticular hydrocarbons are sexually dimorphic in *T. oceanicus*, including compounds that are produced only by females (Thomas and Simmons 2008b). Previous work on *T. oceanicus* indicated that CHCs influence female attractiveness to males (Thomas and Simmons 2010), and that fecundity exhibits significant additive genetic variation (Simmons 2003; Simmons and Garcia-Gonzalez 2007). However, it is not known whether CHCs can provide a reliable signal of a female's genetic merit for fecundity in this species. We therefore used a quantitative genetic framework to test for genetic correlations between female CHCs and fecundity in *T. oceanicus*.

2.3 Methods

Breeding design

We employed a paternal half-sibling design over two generations that used female offspring from the first generation as the Dams in the second generation. Sires were either collected directly from a field population (first generation) or were the offspring of females collected from the same field population (second generation). Dams for the first generation were virgin females from a laboratory stock population. The breeding design provided pedigree links including full and half siblings from the Sire/Dam combinations, as well as maternal cousins and aunt-niece relations from the links across generations. We obtained female offspring from a total of 37 Sire (20 in the first generation and 17 in the second generation) and 96 Dam families (48 in each generation). Some Sires produced offspring with less than three Dams due to either Dam infertility or death (mean Dams per Sire = 2.6, minimum = 1, maximum = 3).

After reaching the penultimate instar, female offspring were kept individually in plastic containers (7 x 7 x 5 cm) and given ad libitum food and water. Following adult eclosion, we provided each cricket with a standardised amount of food, 190 ± 10 mg cat chow. A preliminary study had found that female crickets consume an average of 338 mg (N = 18, SE = 12) over a 12 day period. The amount of food provided represents a restricted diet, being 56 % of the average consumption. Although we were primarily interested in whether genetic correlations exist between fecundity and CHCs, female ornaments are predicted to trade-off with fecundity (Fitzpatrick et al. 1995; LeBas 2006), and previous studies have provided evidence for a trade-off between CHCs and egg production (Schal et al. 1994; Wicker and Jallon 1995; Young et al. 1999; Blows 2002; Holman 2012). If CHCs trade-off with fecundity in *T. oceanicus*, and genetic variance in allocation between CHCs and fecundity exists, we would expect this to

manifest in negative genetic correlations (Roff 2002). However, benign conditions, as well as variation in the amount of resources acquired by individuals, can mask and even reverse the sign of genetic correlations between traits that trade-off against each other (Houle 1991; Roff 2002). We therefore provided a standardised/restricted diet to increase the likelihood of detecting negative genetic correlations, as a test for whether producing CHCs is costly to fecundity. At adult eclosion crickets were haphazardly assigned to being assayed for either fecundity or CHCs. Allocation to CHC measurement was capped at a maximum of five offspring per Dam family due to logistical constraints on the total number of individuals that could have their CHC profiles assayed.

Fecundity

At four days post-eclosion, females that had been allocated to have their fecundity measured were transferred to a clean container and left to mate with a stock male for eight hours, after which they were returned to their original container. These females were frozen at 12 days post-eclosion and stored at -20°C until dissection. Crickets were thawed and their ovaries dissected and weighed using an electronic balance. Previous studies using *T. oceanicus* have found that ovary mass is strongly correlated to the number of eggs present (Simmons 2003) and is significantly associated with the number of eggs laid in a 10 day period (Simmons and Garcia-Gonzalez 2007). We therefore consider ovary mass a reasonable proxy for fecundity in this species.

Cuticular hydrocarbons

Females allocated to have their CHCs measured were frozen at 12 days post-eclosion and placed in a glass vial plugged with cotton wool. The CHCs were extracted by immersing each cricket in 5 mL of hexane that contained 0.02 g L^{-1} of *n*-dotriacontane as an internal standard. We used a Shimadzu QP2010 fitted with a Stabilwax column (30 m x 250 µm x 0.10 µm) for gas chromatography – mass spectrometry (GC-MS) analysis. The initial GC temperature was kept at 40°C for one minute, after which it was increased at a rate of 20°C per minute, before being held at 250°C for 20 minutes. The GC-MS was operated on the splitless mode, and one µl of the sample was injected. We used GCMSsolution software (Ver 4.41) to integrate the peak areas, representing the compound abundances. We divided the peak areas by the internal standard to control for variation in the amount of sample injected in the GC-MS. We restrict our analyses to compounds that comprise at least 4% of the total peak abundance. Ten compounds met this criteria and together accounted for the majority of the CHC profile,

comprising 81% of the total CHC abundance. These compounds were labelled CHC 1 through to CHC 10 and correspond to those labelled Peaks 5, 8, 9, 12, 14, 17, 18, 21, 22 and 24 in Thomas and Simmons (2008b). A small peak reported in Thomas and Simmons (2008b) as Peak 14 did not separate well from CHC 5 in the majority of samples in this study. For consistency across samples we therefore amalgamated this small peak with CHC 5 in those samples in which the two compounds appeared as separate peaks.

Analyses

Prior to quantitative genetic analyses, we tested for an effect of generation on each of the CHC compounds and ovary mass separately, essentially as a test for a block effect. Using least squares regression we found a significant effect of generation on five of the CHC traits and ovary mass (Table 2.1 and Table 2.2). To be consistent across CHC traits, we included generation as a fixed effect in all of the following quantitative genetic analyses.

We used the quantitative genetics "animal model" (Kruuk 2004; Wilson et al. 2010) to estimate the levels of additive genetic variance (V_A) and heritability (h^2) for each of the CHCs and ovary mass. Similar to other forms of quantitative genetic analyses (Falconer and Mackay 1996; Lynch and Walsh 1998), the animal model uses the similarity among relatives to decompose the total variance of a trait into V_A and other non-additive variance components (see Wilson et al. (2010) for a guide on conducting the analyses). However, rather than using information from a narrow set of relative types, such as half- or full-siblings, the animal model uses all of the information available in a pedigree. We tested for significant V_A in each of the traits by fitting univariate animal models for each trait and then using log-likelihood ratio tests (LLRT) to compare these models to those that excluded information from the pedigree. We also tested for significant maternal effects by running models that included Dam identity as a random effect and comparing these using LLRT to the models that only included the pedigree information (Kruuk and Hadfield 2007). We found a significant effect of Dam identity on ovary mass (Table 2.1) but not for any of the CHCs (Table 2.2). Four of the CHCs had Dam effects estimated on the boundary of the parameter space, and we interpret these as indicating Dam variance close to zero (Table 2.2). We therefore present the results of the univariate analyses including Dam for ovary mass, but the simpler model for the CHCs. Heritability was calculated as the additive genetic variance divided by the total phenotypic variance (V_A / V_P). The total phenotypic variance was calculated as the additive genetic variance plus the residual variance ($V_A + V_R$) for the CHC traits, and the additive genetic variance plus the residual variance plus the Dam variance ($V_A + V_R + V_D$) for ovary mass. We calculated the coefficients of variation

without the 100 multiplier, and also present the complementary measure of evolvability, I_A (Houle 1992; Hansen et al. 2011; Garcia-Gonzalez et al. 2012). We calculated approximate standard errors for these parameters using equations six and nine of Garcia-Gonzalez et al. (2012).

A genetic association between ovary mass and the CHC profile as a composite trait could not be estimated by fitting a multivariate animal model that included the ten CHC compounds and ovary mass because of the large number of parameters that would need to be estimated in a single model. An alternative method is to estimate the genetic (co)variance matrix (**G**) of the CHC profile and then use a diagonalization of **G** to estimate its first eigenvector, representing the axis of greatest genetic variation, "Gmax" (Blows 2007; Delcourt et al. 2012). Similar to the use of eigenvectors in a principal component analysis, Gmax can then be applied to the original CHC data to calculate a univariate score for each individual on the major axis of genetic variation. These scores can then be used in tests for a genetic association between the CHC profile as a single composite trait with ovary mass. Preliminary analyses revealed that a multivariate animal model with up to seven CHC compounds could be fit to our data. We therefore fit three multivariate animal models to derive all of the genetic (co)variance estimates required for the complete **G**. We first fit a model that included all of the CHC compounds except CHC 1, CHC 2 and CHC 7. We then fit two more multivariate models that included the three originally excluded compounds, first with CHC 5, CHC 6, CHC 9 and CHC 10; and then with CHC 3, CHC 4 and CHC 8. Combining the parameter estimates from these three models provided us with **G** for all 10 CHC compounds. We used this **G** to calculate Gmax, which we then used to derive the univariate score for each individual on this major axis of genetic variation.

We used bivariate animal models to estimate the genetic correlations (r_G) between ovary mass and each of the CHC compounds separately and Gmax. As the univariate models showed a significant effect of Dam identity on ovary mass, a random effect of Dam identity was included on ovary mass in the bivariate models. By measuring the CHCs and ovary mass on two different sets of related individuals, the residual (within individual) covariance was not estimable and was therefore fixed as zero. The significance of each genetic correlation was tested using LLRT to compare the full model to one where we fixed the genetic correlation to zero. A square root transformation improved the model diagnostics for ovary mass and the majority of the CHC compounds. We therefore square root transformed these traits for inference in both the univariate and bivariate models but used the untransformed data for parameter estimation. Two outliers were removed for inference that involved CHC 1, no other

transformation was required for this compound. No transformation was required for CHC 6 or Gmax. The square root transformation resulted in Dam identity being estimated on the boundary of parameter space for CHC 9. We therefore tested for Dam effects on this compound using the untransformed data. All analyses were performed in R (R Core Team 2017) using *ASReml-R* for the animal model analyses (Butler et al. 2009) and *ggplot2* for plotting (Wickham 2009).

2.4 Results

Across the 37 Sire and 96 Dam families, a total of 329 crickets had their CHCs measured and another 642 had their ovaries weighed. The results of the univariate animal models are presented in Table 2.1 for ovary mass and Table 2.2 for the CHCs. The results in Table 2.1 and Table 2.2 show that all traits displayed significant additive genetic variance (V_A). The genetic (co)variance matrix, **G**, for the CHC profile is included as Supplementary Table S2.1. The first eigenvector of **G** (Gmax) is presented in Table 2.3. This axis explained 83 % of the total variation and represents a contrast between three CHCs with the remaining seven, with the compound of greatest abundance (CHC 9) making the strongest contribution (Table 2.3). This axis therefore captures variation in the compound of greatest abundance, as well as the positive and negative genetic associations between the different CHC compounds (Supplementary Table S2.1). Scoring each individual's CHC profile for Gmax and using these scores in a bivariate animal model with ovary mass revealed a significant positive genetic correlation between the major axis of CHC genetic variation and ovary mass (r_G = 0.463, SE = 0.237, P = 0.046). Table 2.3 presents the results of the bivariate animal models that tested for a genetic correlation between each CHC compound with ovary mass. Two compounds were significantly positively genetically correlated with ovary mass, and one compound was significantly negatively genetically correlated with ovary mass (Table 2.3). Figure 2.1 plots the sire family means to visualize the significant genetic correlations.

Table 2.1 Univariate model results for ovary mass. Results include the test for an effect of generation (Gen *P*), as well as for significant additive genetic variance (VA *P*) and an effect of Dam identity (Dam *P*). A significant effect of Dam identity was detected, the quantitative genetic parameters are therefore taken from the model that included Dam identity. Parameter estimates include additive genetic variance (V_A), Dam variance (V_D), residual variance (V_R), total phenotypic variance (V_P), coefficients of variation for each variance component (CV), a measure of evolvability (I_A), and heritability (h^2). Standard errors are given in parentheses.

Mean	**N**	**V_A**	**V_D**	**V_R**	**V_P**	**CV_A**	**CV_D**	**CV_R**	**CV_P**	**I_A**	**h^2**	**Gen *P***	**V_A *P***	**Dam *P***
61.77	642	158.023 (101.021)	86.945 (48.374)	683.966 (68.501)	928.934 (58.050)	0.204 (0.065)	0.151 (0.042)	0.423 (0.021)	0.493 (0.015)	0.041 (0.026)	0.170 (0.106)	**< 0.001**	**0.005**	**0.028**

Table 2.2 Univariate model results for the CHCs. Results include the test for an effect of generation (Gen *P*), as well as for significant additive genetic variance (VA *P*) and an effect of Dam identity (Dam *P*). No significant effect of Dam identity was detected for any compound, the quantitative genetic parameters are therefore taken from the model that did not include Dam identity. Parameter estimates include additive genetic variance (V_A), residual variance (V_R), total phenotypic variance (V_P), coefficients of variation for each variance component (CV), a measure of evolvability (I_A), and heritability (h^2). Standard errors are given in parentheses. Those traits for which we do not report a p-value for Dam identity had the effect of Dam estimated on the boundary of the parameter space, which we interpret as representing Dam variance close to zero.

CHC	Mean	N	V_A	V_R	V_P	CV_A	CV_R	CV_P	I_A	h^2	Gen *P*	V_A *P*	Dam *P*
1	0.28	329	0.004 (0.002)	0.007 (0.001)	0.012 (0.001)	0.236 (0.044)	0.303 (0.028)	0.384 (0.016)	0.056 (0.021)	0.376 (0.124)	0.171	**< 0.001**	0.875
2	0.20	329	0.016 (0.005)	0.018 (0.004)	0.034 (0.003)	0.635 (0.099)	0.678 (0.070)	0.929 (0.041)	0.404 (0.126)	0.468 (0.123)	0.305	**< 0.001**	-
3	0.19	329	0.016 (0.005)	0.013 (0.003)	0.029 (0.003)	0.654 (0.101)	0.598 (0.078)	0.886 (0.041)	0.428 (0.132)	0.545 (0.136)	**0.006**	**< 0.001**	0.324
4	0.24	329	0.021 (0.008)	0.029 (0.006)	0.050 (0.004)	0.604 (0.109)	0.714 (0.072)	0.935 (0.041)	0.365 (0.132)	0.417 (0.131)	**0.049**	**< 0.001**	0.382
5	0.26	329	0.003 (0.001)	0.004 (0.001)	0.007 (0.001)	0.230 (0.037)	0.236 (0.027)	0.329 (0.015)	0.053 (0.017)	0.489 (0.131)	**< 0.001**	**< 0.001**	-
6	0.14	329	0.004 (0.001)	0.002 (0.001)	0.005 (0.001)	0.424 (0.056)	0.268 (0.057)	0.502 (0.025)	0.180 (0.047)	0.715 (0.135)	0.517	**< 0.001**	-
7	0.12	329	0.006 (0.002)	0.004 (0.001)	0.009 (0.001)	0.644 (0.093)	0.509 (0.080)	0.821 (0.039)	0.415 (0.120)	0.616 (0.137)	**< 0.001**	**< 0.001**	0.897
8	0.27	329	0.019 (0.005)	0.011 (0.004)	0.030 (0.003)	0.514 (0.073)	0.387 (0.065)	0.643 (0.031)	0.264 (0.075)	0.638 (0.139)	0.554	**< 0.001**	0.679
9	0.57	329	0.111 (0.030)	0.050 (0.019)	0.160 (0.016)	0.581 (0.078)	0.389 (0.076)	0.699 (0.034)	0.337 (0.091)	0.690 (0.136)	0.138	**< 0.001**	0.906
10	0.15	329	0.011 (0.003)	0.008 (0.002)	0.019 (0.002)	0.690 (0.104)	0.610 (0.082)	0.922 (0.043)	0.477 (0.144)	0.561 (0.135)	**< 0.001**	**< 0.001**	-

Table 2.3 The first eigenvector (Gmax) of the CHC profile genetic (co)variance matrix, and the genetic correlations (r_G) between each of the CHCs and ovary mass. Standard errors are given in parentheses.

CHC	Gmax	r_G
1	0.088	0.241 (0.300)
2	-0.245	0.182 (0.268)
3	0.262	**0.617 (0.230)***
4	0.308	0.459 (0.282)
5	0.05	-0.252 (0.279)
6	0.126	0.275 (0.236)
7	-0.134	-0.115 (0.274)
8	0.314	0.478 (0.242)
9	0.779	**0.481 (0.226)***
10	-0.166	**-0.605 (0.253)***

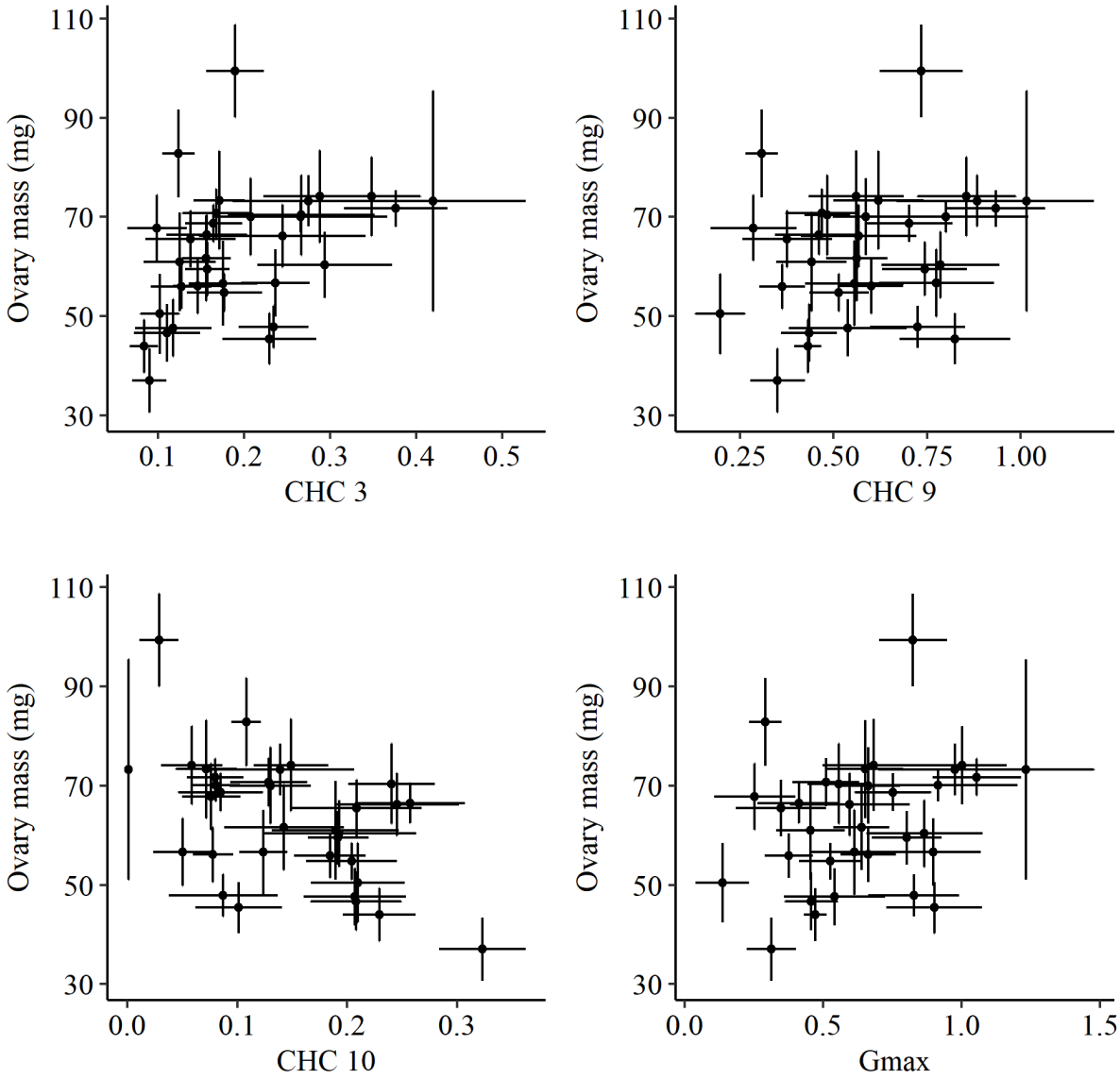

Figure 2.1 Plots of sire family means to visualize the genetic correlations between ovary mass and Gmax, and the three CHCs that displayed significant genetic correlations.

2.5 Discussion

Using the quantitative genetic animal model we have found genetic correlations between both the major axis of CHC genetic variation and individual CHC compounds and a measure of female fecundity in the Australian field cricket, *T. oceanicus*. These genetic correlations show that the female CHC profile can act as an honest signal of female fecundity in this species, enabling males to gain more fecund daughters through their choice of mate. The negative genetic correlation between one CHC compound and fecundity, along with the mix of positive and negative associations between individual CHC compounds and Gmax, indicates that selection may favour females that balance the production of particular CHC compounds with investment in egg production.

Two main hypotheses have been proposed to explain the maintenance of honest signalling. Where signals are costly, and there is variation among females in their ability to bear these costs, it may not pay a female to "cheat" and produce a dishonest signal (Zahavi 1975; Grafen 1990a). Alternatively, some ornaments may be impossible to fake, with their level of expression physiologically constrained to the level of fecundity (Maynard Smith and Harper 2003). Our finding of a negative genetic correlation between one CHC compound (CHC 10) and ovary mass indicates that costs to CHC expression are likely involved in maintaining the reliability of CHCs as fecundity signals. Additionally, the positive genetic correlations between two compounds and ovary mass could be interpreted as indicating a role for constraints in maintaining CHCs as honest signals of fecundity. We caution against viewing this latter interpretation as evidence against a role for costs in maintaining signal honesty for two reasons. First, although we attempted to control for variation in resource acquisition in our study sample by providing a standardised adult diet, it is possible that some variation remained, through genetic variation in food processing efficiency, for example. If this variation was greater than genetic variation in allocation between the CHCs and ovary mass, positive genetic correlations can manifest despite the presence of trade-offs (Houle 1991; Roff 2002). Second, differential costs between high and low quality signallers are predicted to result in the evolution of mechanisms that link individual quality with signal expression (Andersson 1982; Biernaskie et al. 2014). Under these circumstances costs and constraints do not represent alternative hypotheses, rather, costs to low quality individuals of diverting resources from fecundity to signal expression may be the ultimate cause of signal honesty, and physiological mechanisms linking fecundity to signal expression may be the proximate cause (Biernaskie et al. 2014). Positive genetic correlations between CHCs and ovary mass can therefore represent an evolved response to costly signalling, and as such, do not necessarily exclude a role for costs in

maintaining signal reliability. What is clear from our results is that evolutionary changes in CHC expression will result in concomitant changes in fecundity. It is this genetic link that provides evidence for the reliability of CHCs as signals of a female's genetic merit for fecundity, regardless of the precise mechanisms involved.

The evolution of female ornaments is generally considered to be constrained by the costs imposed by ornament expression on fecundity (Fitzpatrick et al. 1995; LeBas 2006). Consistent with this theoretical expectation, we found a negative genetic correlation between fecundity and a CHC compound only expressed in females, CHC 10 (Thomas and Simmons 2008b). Where male mate choice is targeted towards more fecund females, and female ornaments trade-off with fecundity, males may prefer to mate with females that display intermediate levels of ornament expression, representing the optimum balance between investment in signalling and fecundity (Chenoweth et al. 2006). A male preference for intermediate ornament expression will impose stabilizing selection on the ornament, and there is some evidence for stabilizing selection on the major axes of female CHC phenotypic variation in *T. oceanicus* (Thomas and Simmons 2010). We offer an alternative hypothesis that can explain stabilising selection on female CHCs in this species. Female fecundity is negatively genetically correlated with embryo viability in *T. oceanicus* (Simmons and Garcia-Gonzalez 2007), and males will therefore maximize their fitness by mating with females that optimise their investment between quantity and quality of offspring. That is, rather than favouring intermediate ornament expression due to trade-offs between signalling and fecundity, males may prefer intermediate ornament expression if this signals the optimal investment in fecundity versus offspring viability. Furthermore, a multi-component ornament such as a female CHC profile, that includes a combination of positive and negative genetic correlations with fecundity, may increase the precision with which males can assess the number of viable offspring a female is likely to produce. It will be interesting to investigate whether the signs of the genetic correlations we have found between CHCs with ovary mass are reversed for the genetic correlations between CHCs with offspring viability.

In conclusion, we have shown that a proxy for fecundity, ovary mass, is genetically correlated with CHCs in the Australian field cricket, *T. oceanicus*. In combination with previous work that found CHCs influence female attractiveness in this species (Thomas and Simmons 2010), this finding provides evidence for CHCs acting as honest signals of a female's genetic merit for fecundity. Findings from the social insects that CHCs commonly act as queen pheromones and are associated with fecundity has suggested a role for CHCs in fecundity signalling during mate choice in solitary insects (Van Oystaeyen et al. 2014). Our results

support this hypothesis. We suggest that genetic correlations between CHCs and fecundity could be the norm in insects, and that CHCs may be used widely as signals of female fecundity.

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2.7 Supplementary materials

Supplementary Table S2.1 Genetic (co)variance matrix (**G**) used to calculate Gmax. Standard errors are included in parentheses below their respective (co)variance estimate.

	CHC 1	**CHC 2**	**CHC 3**	**CHC 4**	**CHC 5**	**CHC 6**	**CHC 7**	**CHC 8**	**CHC 9**	**CHC 10**
CHC 1	0.004 (0.002)									
CHC 2	-0.001 (0.002)	0.015 (0.005)								
CHC 3	0.006 (0.002)	-0.007 (0.004)	0.015 (0.005)							
CHC 4	0.007 (0.003)	-0.007 (0.004)	0.016 (0.006)	0.020 (0.007)						
CHC 5	0.001 (0.001)	-0.003 (0.002)	0.001 (0.002)	0.001 (0.002)	0.003 (0.001)					
CHC 6	0.001 (0.001)	-0.006 (0.002)	0.004 (0.002)	0.005 (0.002)	0.001 (0.001)	0.003 (0.001)				
CHC 7	0.000 (0.001)	0.007 (0.002)	-0.004 (0.002)	-0.005 (0.003)	0.000 (0.001)	-0.003 (0.001)	0.005 (0.001)			
CHC 8	0.004 (0.002)	-0.012 (0.004)	0.012 (0.004)	0.015 (0.006)	0.002 (0.002)	0.006 (0.002)	-0.006 (0.002)	0.017 (0.005)		
CHC 9	0.010 (0.005)	-0.030 (0.009)	0.030 (0.010)	0.036 (0.013)	0.007 (0.004)	0.016 (0.005)	-0.016 (0.005)	0.038 (0.011)	0.095 (0.027)	
CHC 10	0.000 (0.002)	0.008 (0.003)	-0.006 (0.003)	-0.006 (0.004)	0.001 (0.001)	-0.003 (0.001)	0.006 (0.002)	-0.007 (0.003)	-0.020 (0.008)	0.010 (0.003)

CHAPTER 3

Sexual selection across sensory modalities: female choice of male behavioural and gustatory displays

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3.1 Abstract

The role of cuticular hydrocarbons in sexual displays has received considerable interest over the last two decades. For example, multiple studies have documented significant directional and nonlinear sexual selection acting on the cuticular hydrocarbon profiles of both male and female insects. The majority of these studies have excluded other sensory modalities that may influence attractiveness, and measured selection using laboratory raised individuals. Furthermore, much of this work has been conducted using drosophilid fruit flies and crickets, and investigations using different taxa are necessary to improve our understanding of broader taxonomic trends. Here, we extend our understanding of sexual selection on cuticular hydrocarbons by measuring selection imposed by female mate choice on male bull-horned dung beetles, *Onthophagus taurus*. Both male and female beetles used in our study were collected from the field, ensuring that our estimates of selection incorporated some degree of naturally occurring variation in both cuticular hydrocarbon profiles and female mate preferences. Consistent with previous studies on this species, we found significant directional selection on male courtship displays. We also found significant nonlinear selection on the male cuticular hydrocarbon profile acting independently of the influence of behavioural courtship. Our data are consistent with a role for cuticular hydrocarbons in the mating system of this species and suggest that female *O. taurus* use multiple sensory modalities to assess different aspects of male quality.

3.2 Introduction

Sexual selection through mate choice has resulted in the evolution of some of the most elaborate traits found in biology (Darwin 1871; Andersson 1994). A multitude of studies have documented how an individual's mating success can be influenced by characteristics such as bright coloration (Hill 1991; Brooks and Endler 2001), loud vocalizations (Rebar et al. 2009; Tanner et al. 2017) and conspicuous behavioural displays (Byers et al. 2010; Callander et al. 2012). A recognition that mate choice can be influenced by multiple components within and across sensory modalities (Candolin 2003; Hebets and Papaj 2005; Partan and Marler 2005) has led to a multivariate approach to their analysis (for example: Pryke et al. 2001; LeBas et al. 2003; Bentsen et al. 2006; Gerhardt and Brooks 2009; Cole and Endler 2015; Tanner et al. 2017). Multivariate selection analyses allow for the elucidation of the marginal effect of each measured trait on mating success, and can help avoid potentially false conclusions of selection acting on a given trait that can arise from phenotypic correlations between that trait with other traits that are associated with fitness (Lande and Arnold 1983; Phillips and Arnold 1989; Blows 2007; Chenoweth et al. 2012).

Although the role of chemical traits in mate choice has received relatively less attention (Johansson and Jones 2007; Coleman 2009), in the context of multivariate selection analyses, the cuticular hydrocarbons (CHCs) of insects have received considerable interest (Steiger and Stökl 2014). CHCs form a protective layer on the insect's cuticle, primarily protecting it from desiccation, but also playing a role in close-range/contact sexual communication (Blomquist and Bagnères 2010a). For example, multiple studies have found consistent and relatively strong directional sexual selection acting on the CHC profile of male *Drosophila serrata* (Blows et al. 2004; Hine et al. 2011; Gershman et al. 2014). These, and associated investigations, have contributed greatly towards our understanding of the multivariate nature of mate choice. Notably, by comparing the vector of directional selection gradients with the genetic (co)variance matrix (**G**), a lack of genetic variance in the direction of selection has been revealed, a perspective which contrasts with that gained from examining individual traits in isolation (Blows et al. 2004; Hine et al. 2004; Van Homrigh et al. 2007). However, a weakness of multivariate selection analyses is the potential influence of unmeasured characters (Lande and Arnold 1983; Mitchell-Olds and Shaw 1987). If a trait being examined is phenotypically correlated with an unmeasured character that is itself under selection, the resultant selection gradients may reflect this correlation rather than direct selection on the trait itself (Lande and Arnold 1983). Accounting for all traits influencing attractiveness is therefore an important goal in studies examining the multivariate nature of mate choice. Little attention, however, has been

given to investigating how traits from other signal modalities act alongside CHCs in determining overall attractiveness.

Where both CHCs and nonchemical traits have been incorporated into one study, the evidence to date suggests that the attractiveness of an individual's CHC profile is independent of the attractiveness of trait(s) in other sensory modalities. For example, no association was found between the measures of male attractiveness for calling song and chemical cues (presumed to be CHCs) in the cricket *Gryllus integer* (Leonard and Hedrick 2010). Similarly, no correlation was found between an individual's courtship song attractiveness and CHC attractiveness in the Australian field cricket, *Teleogryllus oceanicus*, (Simmons et al. 2013). Just as a correlated trait can influence estimates of selection gradients, a trait influencing attractiveness independently of the traits of interest should have little impact on the selection gradients when included in the analysis. For example, in an analysis that incorporated two wing shape measures (McGuigan 2009), the estimated linear selection gradients on *D. bunnanda* CHCs were similar to selection gradients found when only CHCs were included (Van Homrigh et al. 2007), suggesting that these two sets of traits influence attractiveness independently. Regardless of whether the attractiveness of an individual's CHC profile is correlated with the attractiveness of traits in other sensory modalities, overall attractiveness is still likely to be influenced by both CHCs and non-CHC traits. For example, Rybak et al. (2002) showed that both CHCs and song are important in male *D. melanogaster* courtship, with the removal/reduction of either modality decreasing the attractiveness of males. Incorporating non-CHC traits into multivariate selection analyses will likely provide a greater understanding of mate choice within the study system, as well as ensuring the estimated selection gradients are not biased by unmeasured characters.

Estimates of selection gradients can also be influenced by the phenotypic (co)variation present in the study sample (Chenoweth et al. 2012). Consequently, our understanding of how mate choice influences the evolution of CHCs in nature will be enhanced by attempts to represent naturally occurring variation within the study sample. We are aware of only two species for which investigations of sexual selection on CHCs have sampled from natural populations. Hine et al. (2004) estimated selection gradients from separate binomial mate choice tests (using lab-reared females) on both laboratory raised and field collected male *D. serrata*. Although the individual gradients differed, including four of the six being of opposite sign, the overall vectors of selection were not found to be significantly different. In the only study that has directly estimated selection on CHCs occurring in a natural population, Steiger et al. (2013) used a natural phenotypic marker of mating success to estimate linear and

nonlinear selection acting on the CHCs of male sagebrush crickets, *Cyphoderris strepitans*. Importantly, along with natural variation in the CHC phenotype, Steiger et al. (2013) also included natural variation in female preference. As laboratory studies generally control the age and mating status of females used to assess male attractiveness, they implicitly assume that the average preference of the laboratory sample of females is representative of that of the larger natural population (and hence an unbiased estimate of selection gradients, see Gershman et al. (2014) for further discussion). Studies that sample from natural populations will therefore not only increase the likelihood of capturing the naturally occurring phenotypic (co)variance, but also the average population preference.

The lack of multivariate selection studies on CHCs that incorporate additional sexual traits provides an imperative for further work on the role of sexual selection in the evolution of CHCs. Additionally, expanding this work beyond drosophilid fruit flies and crickets, as recently done by Lane et al. (2016), will contribute to a taxonomically broader understanding of the evolution of insect CHCs. The dung beetle, *Onthophagus taurus*, has become a model species for studies of sexual selection and provides an ideal candidate for studies of selection on CHCs through female mate choice. Males vigorously drum the dorsal surface of females during courtship, and females prefer males that perform this courtship at a higher rate (Kotiaho et al. 2001; Kotiaho 2002; McCullough and Simmons 2016). Courtship rate is genetically correlated with a measure of male condition (Kotiaho et al. 2001), and females mated to males with a higher courtship rate have offspring of greater viability (Simmons and Holley 2011), suggesting the operation of good genes mate choice in this species (Garcia-Gonzalez and Simmons 2011). However, the influence of male traits from other sensory modalities on male attractiveness has not been determined. Mating occurs in narrow tunnels beneath dung pats, making a role for visual traits unlikely. We therefore hypothesized that olfactory traits, specifically CHCs, might play a role in the mating system of this species. Our aim in this study was to measure the strength and form of selection on *O. taurus* CHCs and, at the same time, assess how CHCs and courtship rate act together in influencing male attractiveness.

3.3 Methods

Beetles were collected from Walpole, Western Australia, and transported to a controlled temperature room (set at 28 °C on a 12:12 h light:dark cycle) where they were maintained in single-sex groups of ~100 individuals in 10-L buckets that contained moist sand topped with cow dung. Mating trials took place two weeks after collection to ensure that all beetles were sexually mature. It is possible that time spent under controlled laboratory conditions altered the

CHC profiles of the subject beetles, and consequently the phenotypic (co)variance of our sample may differ from that found in the field. However, our sample should still capture more of the naturally occurring variation in CHC profiles when compared with the use of laboratory reared individuals, because of variation in age and mating history, for example. We therefore refer to our sample as "field collected" to contrast it to a sample derived from laboratory reared individuals, but highlight the need to interpret the results cautiously when referring to selection occurring in nature. Typically, females collected from the field are already mated, with estimates of the number of previous mates ranging between one and five (mean of 2.8 ± 1.1) (McCullough et al. 2017). We used no-choice trials where a single male and female were introduced into a chamber designed to mimic their natural breeding tunnels (Kotiaho et al. 2001). Pairs were checked every two minutes for one hour and at each check a record was made if the male was displaying courtship activity (drumming the dorsal surface of the female with his forelegs). Courtship rate was calculated as the number of times a male was recorded displaying courtship at these two minute intervals divided by time (60 minutes for unmated males or time elapsed before mating for mated males (Simmons and Holley 2011)). For data analysis, males that were observed courting were assigned either a 1 if mated or 0 if unmated, males that did not court were discarded. Beetles were frozen immediately following mating trials in preparation for CHC extraction and gas chromatography – mass spectrometry (GC-MS) analysis.

To extract CHCs, beetles were immersed in 1 mL of hexane for five minutes, after which the beetle was removed using clean forceps and the hexane allowed to evaporate. Prior to GC-MS, the samples were re-suspended in 0.1 mL of hexane and transferred to a 0.1 mL glass insert within an autosampler vial. We used a Shimadzu QP2010 GC-MS machine fitted with a 20 m x 150 µm x 0.15 µm VF1 column operating on the splitless mode. The initial oven temperature was set at 40°C for one minute, before increasing at 20°C per minute until 200°C where it was held for one minute, and then increased at 10°C per minute before reaching 300°C where it was held for 10 minutes. Peaks were identified from the NIST library by their mass spectra and retention indices. Retention indices were assigned by comparing compounds with a known sample of *n*-alkanes (C_7 – C_{40}). Peaks were integrated using Shimadzu GCMSsolution Version 4.41. As is conventional for CHC analysis (Blows 1998), peak areas were transformed to logcontrasts by taking the log of their relative area divided by the relative area of a randomly chosen peak (in this case *n*-tricosane; we added one before taking the log to account for peak areas of zero). A principal component (PC) analysis was then performed on this set of logcontrasts as a data reduction tool, with only those components with an eigenvalue greater

than one included in the analysis. We consider those peaks with loadings greater than or equal to 70% of the highest value as contributing significantly to a PC (Mardia et al. 1979).

We used the multiple regression method outlined by Lande and Arnold (1983) to estimate linear and nonlinear selection gradients on our retained PCs and courtship rate. This approach first includes only the linear terms in a regression on relative fitness (in our case relative mating success) to estimate the strength of directional selection acting on each trait (β selection gradients). A second regression that includes all correlational and quadratic terms is then fit to estimate nonlinear selection (represented by the γ covariance matrix of correlational and quadratic selection) with nonzero quadratic terms representing curvature in the relationship between the trait and fitness. To allow comparison of selection across traits and studies, we standardized all traits to mean zero and standard deviation one (PCs already have mean zero, so these were only variance standardized). All quadratic coefficients presented in our γ matrix were multiplied by two (Stinchcombe et al. 2008). As nonlinear selection is likely to be underestimated using this technique (Blows and Brooks 2003), we performed a canonical rotation of the γ matrix to estimate multivariate linear and nonlinear selection (Phillips and Arnold 1989). We visualized selection in multivariate space using thin plate splines by applying the canonical axes to the original phenotypic data. Overall significance of selection was tested by fitting a logistic regression (using absolute mating success as the response variable) to the model containing only the linear terms (for directional selection) and to the full second-order model using individual scores on the canonical axes (for nonlinear selection). As relative mating success was non-normally distributed, permutation testing was used for assessing the significance of individual terms, with our procedure for the canonical axes differing from that of Reynolds et al. (2010) in that we kept the canonical rotation constant (Chenoweth et al. 2012). As the detection of significant curvature in the fitness surface does not necessarily represent the presence of an intermediate fitness optima (or minima), we used the methods outlined by Mitchell-Olds and Shaw (1987) to test for significant stabilizing and disruptive selection on our canonical axes. This method involves two tests that constrain fitness to be at its maximum at one or the other extreme of the phenotypic range and tests whether this provides a significantly worse fit compared to the unconstrained model (see Chenoweth et al. (2007) for further details). Again, permutation testing was used to assess the significance of the relevant model term.

To determine how the inclusion of the behavioural trait, courtship rate, influenced the selection gradient estimates for our CHC PCs, we repeated the first- and second-order multiple

regressions, but this time excluding courtship rate. We then compared these linear and nonlinear selection gradients with those on our CHC PCs estimated from the model which included courtship rate. We calculated the angle between our two vectors of selection gradient estimates to compare how the overall estimated direction of selection differed between them (an angle of 0° would indicate perfect alignment, 90° that the overall direction of selection estimates were orthogonal and 180° that selection was estimated to be in the opposite direction). Similarity between our two γ matrices was estimated using the matrix comparison method of Krzanowski (1979), which calculates the similarity of the matrix subspaces that describe the majority of the variation (see Rundle et al. (2008) for an example of how these point estimates can be used for the comparison of multiple γ matrices). In our case we retained the first three eigenvectors of both matrices, with a score of zero indicating that the matrices were completely unaligned, whereas a score of 3 would show perfect alignment. All analyses were performed in R (R Core Team 2017) using the *FactoMineR* package for the PC analysis (Lê et al. 2008), and the *fields* package for thin plate spline visualization (Nychka et al. 2017).

3.4 Results

We analysed hexane washes from a total of 119 male *O. taurus*, 21 (18%) of which were successful in securing a mating. Results from the GC-MS analysis are presented in Table 3.1 and Figure 3.1 shows a typical chromatogram. The vast majority of the CHCs detected were methyl-branched compounds, with alkanes less common but representing some of the larger peaks. Our PC analysis returned seven PCs with eigenvalues greater than one that cumulatively explained 83.13 % of the total variation (Table 3.1). PC1 was weighted positively by all but one compound and most strongly by longer chained compounds. We therefore interpret this axis as describing the relative abundance of heavier compounds. PC2 contrasts straight-chained alkanes with methyl-branched compounds that have a chain-length of 26 carbons or less. PC3 contrasts four methyl-branched compounds and two alkanes with the three longest-chained compounds. PC4 was positively loaded by two 2-methylalkanes, two alkanes and two longer chained 15-methylalkanes. PC5 contrasts two 2-methylalkanes with an 8-methylalkane. PC6 contrasts a 2-methylalkane with a 7-methylalkane and PC7 is weighted by two short-chained alkanes. Though the patterns of variation explained by these PCs are not completely clear, and our description is necessarily a simplification, it is interesting to note that the 2-methylalkanes contribute to the phenotypic variation described by five of the seven PCs.

Table 3.1. Cuticular hydrocarbons of *Onthophagus taurus*, their mean relative amounts, and the results of the principal component analysis. Only PCs with eigenvalues greater than one are shown. Loadings are shown in the body of the table and those in bold have values that are equal to or greater than 70% of the highest loading.

				PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
			Eigenvalue	16.221	5.287	2.787	1.991	1.677	1.566	1.230
			% variance	43.839	14.290	7.533	5.381	4.533	4.232	3.326
Peak	Compound	Mean	SE							
1	*n*-C_{22}	0.10	0.00	0.083	-0.081	0.091	-0.163	-0.089	-0.032	**0.551**
2	*n*-C_{23}	1.22	0.04							
3	11-MeC_{23}	0.86	0.05	0.067	**0.343**	0.025	0.012	-0.302	0.086	-0.010
4	2-MeC_{23}	0.17	0.01	-0.034	**0.249**	-0.046	**0.355**	0.242	-0.158	0.247
5	3-MeC_{23}	0.35	0.02	0.007	**0.338**	0.010	0.063	0.146	0.109	-0.110
6	*n*-C_{24}	0.44	0.01	0.137	0.025	-0.036	0.034	-0.102	0.245	**0.534**
7	2-MeC_{24}	0.19	0.01	0.008	**0.267**	0.067	**0.310**	0.258	-0.100	0.281
8	*n*-C_{25}	5.50	0.13	**0.178**	-0.110	0.029	0.121	-0.144	0.327	0.191
9	11-MeC_{25}	5.53	0.21	0.114	**0.319**	0.070	0.049	-0.106	0.201	-0.067
10	7-MeC_{25}	0.64	0.03	0.057	**0.251**	0.121	-0.078	0.101	**0.428**	-0.221
11	2-MeC_{25}	1.33	0.07	0.091	0.238	**0.300**	-0.071	**-0.320**	-0.181	-0.014
12	3-MeC_{25}	1.86	0.07	0.118	**0.317**	0.196	0.104	-0.008	0.096	-0.029
13	*n*-C_{26}	0.66	0.02	0.139	-0.226	0.166	**0.271**	-0.147	0.166	0.107

14	8-MeC_{26}	1.41	0.05	**0.171**	0.052	0.085	-0.071	**0.457**	0.088	-0.038
15	8,14-diMeC_{26}	0.68	0.03	0.159	0.066	**0.250**	-0.090	0.272	0.021	0.004
16	2-MeC_{26}	0.59	0.02	0.103	-0.013	**0.252**	0.221	0.064	**-0.496**	-0.042
17	*n*-C_{27}	6.23	0.26	0.111	**-0.257**	**0.221**	**0.270**	-0.040	0.168	-0.166
18	13-MeC_{27}	5.62	0.16	**0.190**	0.090	0.176	-0.161	-0.066	0.085	-0.038
19	2-MeC_{27}	2.63	0.11	0.129	0.069	**0.293**	-0.065	**-0.378**	-0.256	0.020
20	3-MeC_{27}	3.10	0.08	**0.190**	-0.102	0.161	-0.007	0.090	-0.157	-0.064
21	*n*-C_{28}	0.62	0.02	0.140	-0.216	**0.222**	0.161	0.125	0.153	-0.062
22	14-MeC_{28}	1.26	0.04	**0.195**	-0.024	0.160	-0.231	0.154	-0.107	0.020
23	*n*-C_{29}	1.99	0.10	0.119	**-0.259**	0.168	0.203	0.051	0.067	-0.075
24	15-MeC_{29}	8.76	0.18	**0.218**	-0.031	0.035	-0.113	-0.057	-0.105	-0.082
25	11,15-diMeC_{29}	1.62	0.04	**0.223**	0.025	-0.049	-0.164	0.130	-0.016	-0.008
26	19,23-diMeC_{29}	3.25	0.08	**0.228**	-0.050	0.018	-0.175	0.058	-0.044	-0.030
27	5,15-diMeC_{29}	2.98	0.08	**0.211**	-0.067	-0.052	-0.183	0.104	0.035	0.161
28	15-MeC_{30}	1.32	0.03	**0.228**	-0.017	-0.083	-0.025	0.098	-0.071	-0.031
29	15-MeC_{31}	12.81	0.25	**0.220**	-0.082	-0.093	0.110	-0.054	0.057	-0.093
30	13,17-diMeC_{31}	1.80	0.05	**0.206**	0.073	-0.191	0.031	-0.012	-0.052	-0.028
31	9,21-diMeC_{31}	3.36	0.12	**0.204**	-0.016	-0.193	-0.173	-0.049	-0.002	-0.015
32	7,17-diMeC_{31}	2.07	0.05	**0.221**	0.015	-0.148	-0.047	0.017	-0.082	-0.036
33	5,17-diMeC_{31}	2.11	0.06	**0.205**	0.016	-0.199	0.037	0.074	-0.063	0.166
34	15-MeC_{32}	0.55	0.02	**0.200**	0.058	-0.154	0.147	-0.032	-0.146	-0.054

35	15-MeC_{33}	3.33	0.10	**0.196**	-0.031	-0.158	**0.269**	-0.146	0.034	-0.116
36	11,21-diMeC_{33}	8.36	0.28	**0.205**	0.018	**-0.244**	-0.077	-0.063	-0.003	-0.068
37	15-MeC_{35}	0.54	0.03	0.133	0.052	**-0.240**	**0.308**	-0.083	-0.076	-0.108
38	11,21-diMeC_{35}	4.17	0.18	**0.183**	0.053	**-0.237**	0.048	-0.070	-0.026	-0.065

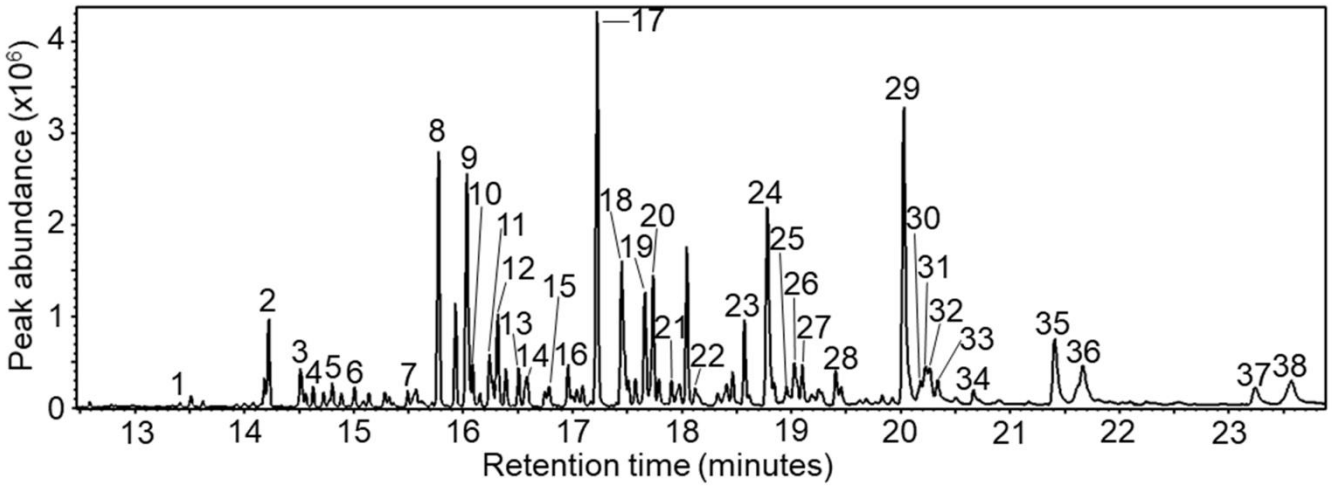

Figure 3.1. Chromatogram of a typical *Onthophagus taurus* CHC profile. The *x*-axis shows the retention time and the *y*-axis peak abundance. Numbers above peaks correspond to those in Table 3.1.

The multivariate selection analyses revealed that courtship rate was subject to directional selection, with nonlinear selection acting on the CHC profile. We detected overall significant linear selection ($\chi^2_8 = 34.938$, $P < 0.001$, $r^2_{(adjusted)} = 0.258$), with this effect driven by courtship rate as no significant linear selection was found for our CHC PCs (Table 3.2). We detected significant negative correlational selection between PC2 and PC5 (Table 3.2) and overall significant nonlinear selection on the canonical axes ($\chi^2_{16} = 83.140$, $P < 0.001$). Significant concave selection was found on axes m_1 and m_2, along with significant convex selection on axes m_7 and m_8 (Table 3.3). Selection acting upon axes m_1 and m_2 was disruptive, with models constraining the highest fitness to be at only the minimum or maximum of the phenotypic variation providing a significantly worse fit ($P < 0.001$ in both cases for m_1, $P = 0.014$ and $P < 0.001$ for m_2). We did not detect significant stabilizing selection on axis m_8 ($P = 0.164$ and $P = 0.422$) nor on axis m_7 ($P = 0.078$ and $P = 0.066$).

Further inspection of the major canonical axes revealed that the disruptive selection was most strongly associated with the CHC profile. Visualizing selection acting on the major canonical axes, m_1 and m_8, revealed a fitness trough at intermediate values of m_1 (as indicated above by the significant disruptive selection) and a peak at decreasing values of m_1 and m_8 (Figure 3.2). An examination of the eigenvectors of axis m_1 revealed that the fitness trough was associated with intermediate values of PC2 and PC5, representing a lack of contrast between short-chained methyl-branched compounds with *n*-alkanes (PC2), as well as a lack of contrast between two 2-methylbranched compounds and 8-methylhexacosane (PC5). Inspecting the eigenvectors for axes m_1 and m_8 together revealed that the fitness peak was associated with high values for PC2 and low values of PC5 (m_1), along with high courtship rate and low values of PC6 (m_8). An inspection of the loadings of the original CHC logcontrasts on these PCs revealed an association between mating success and investment in short-chained methyl-branched compounds (PC2) and 2-methyalkanes (PC5 and PC6).

Table 3.2. Results of the multiple regression analyses including courtship rate (CR). The vector of standardized directional selection gradients (β) and the γ matrix are shown.

	β	PC1	PC2	PC3	PC4	PC5	PC6	PC7	CR
PC1	0.182	0.268	-0.212	0.495	0.050	0.295	0.193	0.039	-0.054
PC2	-0.091		0.341	-0.174	-0.055	**-0.639***	-0.289	-0.104	0.355
PC3	-0.044			-0.316	-0.392	0.197	-0.224	-0.420	0.245
PC4	-0.141				-0.127	-0.393	0.127	0.050	-0.475
PC5	-0.017					-0.003	0.113	-0.038	-0.447
PC6	0.152						-0.646	0.104	0.538
PC7	0.220							0.567	-0.053
CR	**1.137*****								-0.014

* $P < 0.05$, *** $P < .001$

Table 3.3. Results of the canonical analysis derived from the multiple regression including PCs 1-7 and courtship rate (CR). The strength of multivariate linear selection (θ), multivariate nonlinear selection (λ) and the loadings of the original traits on the m_i axes are shown.

m_i	θ	λ	PC1	PC2	PC3	PC4	PC5	PC6	PC7	CR
m_1	-0.183	**1.303****	0.418	-0.611	0.244	-0.088	0.555	0.054	-0.038	-0.270
m_2	0.346	**0.943***	0.161	0.207	0.451	-0.376	-0.003	-0.021	-0.656	0.395
m_3	**-0.918*****	0.515	-0.290	0.005	-0.078	0.314	0.022	-0.314	-0.631	-0.560
m_4	-0.063	0.188	0.742	0.218	0.090	0.525	-0.326	0.044	-0.043	-0.091
m_5	**0.404***	-0.006	-0.164	-0.497	-0.196	0.353	-0.178	0.537	-0.329	0.367
m_6	0.011	-0.516	0.157	0.461	-0.519	-0.024	0.560	0.383	-0.179	-0.009
m_7	0.049	**-0.992***	-0.313	0.239	0.560	0.540	0.418	0.103	0.166	0.161
m_8	**-0.512***	**-1.363***	-0.123	0.141	0.319	-0.245	-0.255	0.671	0.030	-0.537

* $P < 0.05$, ** $P < 0.01$, *** $P < .001$

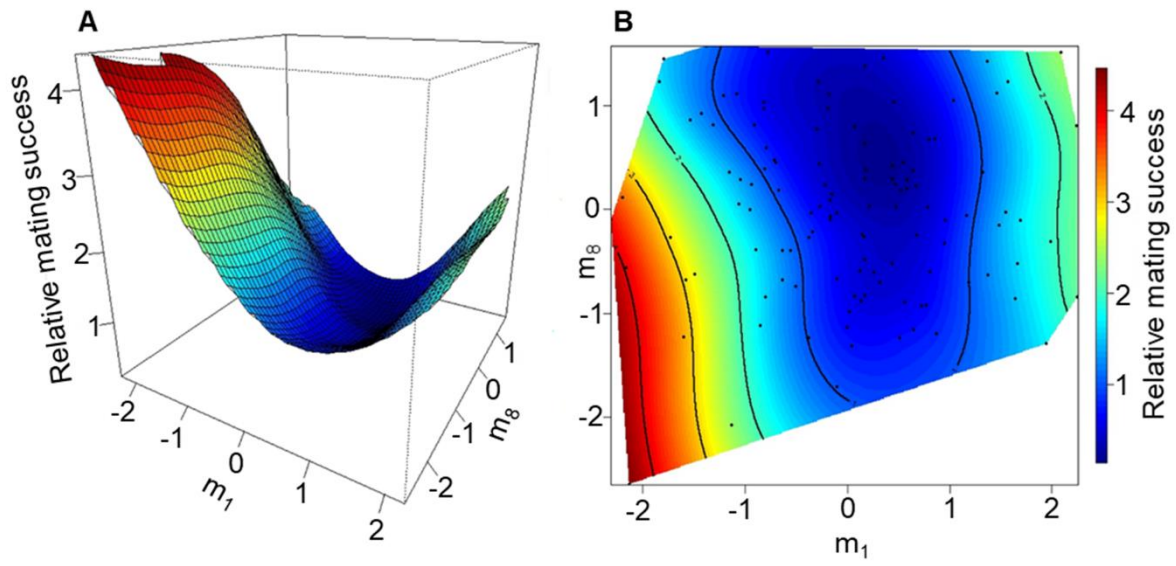

Figure 3.2. Thin plate spline visualization in three dimensions (A) and as a contour plot (B) of selection acting on the major canonical axes, m_1 and m_8. Relative fitness is represented by the vertical axis in A and through colour in both A and B with red representing high fitness and blue low fitness. The points on B are the raw data points.

A comparison of analyses that either included or excluded courtship rate revealed that nonlinear selection on the CHC profile was largely independent of the effect of courtship rate on mating success. Removing courtship rate and repeating the multiple regression analyses resulted in the model of directional selection no longer explaining a significant amount of variation in male mating success ($\chi^2_7 = 9.275$, $P = 0.234$, $r^2_{(adjusted)} = 0.017$). Interestingly, of the small amount of variation that was explained, there was significant directional selection on PC6 (Table 3.4), which out of all the PCs was found to have the strongest correlational selection with courtship rate (Table 3.2), although this was not significant ($P = 0.094$). Comparing the vector of directional selection gradients on the CHC PCs from this analysis with that obtained from our analysis that included courtship rate revealed that the two vectors were oriented 48° from each other and that the individual β estimates were not significantly correlated ($r = 0.689$, $P = 0.087$). The exclusion of courtship rate had little effect on the nonlinear selection estimates, with a comparison of the γ matrices returning a Krzanowski value of 2.513 out of a maximum of 3 (or 84% of the score for complete similarity), and a significant correlation between the individual γ estimates ($r = 0.869$, $P < 0.001$). This was reflected again in finding significant nonlinear selection on the canonical axes derived from the multiple regression that excluded

courtship rate (χ^2_{14} = 59.431, P < 0.001). Although the two sets of canonical axes are not comparable (as they were based on two different sets of traits), it is notable that the CHC PC loadings on the two m_1 axes bear some resemblance (Tables 3.3 and 3.5), and that this axis displays the greatest curvature in our canonical rotation that excluded courtship rate (Table 3.5).

Table 3.4. Results of the multiple regression analyses excluding courtship rate. The vector of standardized directional selection gradients (β) and the γ matrix are shown.

	β	PC1	PC2	PC3	PC4	PC5	PC6	PC7
PC1	-0.029	0.154	-0.251	0.143	0.134	0.431	0.197	0.145
PC2	-0.094		0.148	-0.056	0.163	**-0.690***	-0.396	-0.281
PC3	0.108			-0.281	-0.279	0.081	-0.278	-0.454
PC4	-0.321				0.050	**-0.486***	0.171	0.226
PC5	-0.207					0.131	-0.023	-0.047
PC6	**0.393***						-0.096	0.129
PC7	0.175							0.367

* P < 0.05

Table 3.5. Results of the canonical analysis derived from the multiple regression of PCs 1-7. The strength of multivariate linear selection (θ), multivariate nonlinear selection (λ) and the loadings of the original traits on the m_i axes are shown.

m_i	θ	λ	PC1	PC2	PC3	PC4	PC5	PC6	PC7
m_1	0.106	**1.277****	0.399	-0.601	0.045	-0.226	0.599	0.199	0.167
m_2	0.099	**0.963***	0.102	-0.089	-0.416	0.473	-0.236	0.322	0.652
m_3	-0.141	0.140	0.698	0.153	0.215	0.470	-0.096	0.190	-0.421
m_4	-0.220	-0.046	0.407	0.322	0.140	-0.045	0.115	-0.681	0.481
m_5	0.088	-0.449	0.245	0.553	-0.555	-0.442	0.188	0.300	-0.076
m_6	**-0.505****	-0.494	-0.185	-0.019	-0.479	0.504	0.526	-0.358	-0.278
m_7	-0.027	**-0.920***	0.288	-0.445	-0.471	-0.230	-0.500	-0.371	-0.237

* P < 0.05, ** P < 0.01

3.5 Discussion

Using multivariate selection analyses that incorporated traits from different sensory modalities, we have found significant nonlinear selection on the axes of major CHC phenotypic variation (CHC PCs), and significant directional selection on a behavioural sexual display (courtship rate) in the dung beetle, *O. taurus*. Our finding of significant directional selection on courtship rate is consistent with previous studies on mate choice using this species (Kotiaho et al. 2001; Kotiaho 2002; Simmons and Holley 2011; McCullough and Simmons 2016). What is novel about our results is the finding that CHCs also contribute to mating success in *O. taurus*. Furthermore, the high level of similarity between our two γ matrices (84% of the maximum similarity score) indicates that nonlinear selection on the CHC PCs does not depend on an individual's courtship rate. Our study therefore provides an example of mate choice mediated sexual selection acting on an insect's CHC profile independently of the influence of a well-characterized courtship display. The fact that we have shown this in the laboratory using beetles collected from the field, that would have varied in a variety of life-history traits including age and previous mating history, gives support to the view that sexual selection is likely to be a persistent driver of the evolution of CHCs in natural populations of insects.

Although nonlinear selection on the CHC PCs appeared to act independently of courtship rate, the estimates of directional selection gradients were influenced to some extent by whether courtship rate was incorporated into the analysis. This was most evident in the doubling of the selection gradient (and consequently altering its statistical significance) for PC6, the PC that showed the strongest correlational selection with courtship rate. Similarly, comparing the overall direction of selection acting on the CHC PCs estimated from the two analyses which included or excluded courtship rate resulted in β vector estimates that were angled moderately (48°) away from each other. Although total linear selection on the CHC PCs was not significant, the ability of a non CHC trait to influence the estimate of the overall direction of selection has important implications. For example, such an influence will affect the estimated alignment of selection with genetic variation, potentially altering interpretations of the amount of genetic variation available for selection. The potential for unmeasured characters to influence selection gradients is well known (Lande and Arnold 1983; Mitchell-Olds and Shaw 1987), and we have shown that estimates of selection on CHCs are no exception to this rule. As CHCs are commonly used in studies of sexual selection on multivariate traits (Chenoweth and Blows 2003; Van Homrigh et al. 2007; Thomas and Simmons 2009b, 2010; Curtis et al. 2013; Steiger et al. 2013; Ingleby et al. 2014; Steiger et al. 2015; Lane et al. 2016),

our results indicate that a fuller understanding of mate choice will be gained by incorporating non-CHC traits in future studies. This will of course likely necessitate much larger sample sizes than we have included here, an admittedly challenging logistical proposition.

Here, we were interested in assessing selection imposed on male *O. taurus* CHC profiles through the action of female mate choice. We emphasize that this is highly unlikely to represent the total selection acting on CHCs in this species. The role CHCs play in desiccation resistance (Gibbs and Rajpurohit 2010), along with their production utilizing the shared resource of internal hydrocarbons (Schal et al. 1994; Wicker and Jallon 1995), are both likely to impose strong selection on CHCs. For example, experimental evolution studies have found that altering the environmental temperature (Sharma et al. 2012) and imposing fecundity selection (Blows 2002) lead to an evolutionary response in the CHC profile. The CHCs of some species are also important in male dominance displays (Roux et al. 2002; Kortet and Hedrick 2005; Thomas and Simmons 2009a, 2011b), and Lane et al. (2016) have recently shown that selection on CHCs imposed by female mate choice can differ to that imposed by male-male competition. In investigations of selection via female choice on male CHC profiles, the use of no-choice mating assays is a useful experimental technique that avoids the potential effect of male-male competition. Our study adds *O. taurus* to the limited number of species for which no-choice mating assays have been used to assess selection from mate choice on male CHCs (broad-horned flour beetle, *Gnatocerus cornutus* (Lane et al. 2016); Australian field cricket, *T. oceanicus* (Thomas and Simmons 2009b); decorated cricket, *Gryllodes sigillatus* (Steiger et al. 2015); and *D. simulans* (Ingleby et al. 2014)).

The fact that male *O. taurus* CHCs are subject to selection from female choice is evidence that females of this species use CHCs in their assessment of potential mates. Although our study was not designed to test what benefits such assessment may provide, our results suggest at least one potential hypothesis. Chemical traits have been suggested as good candidates for genetic compatibility based mate choice (Mays and Hill 2004), and there is some evidence that CHCs act in this manner. For example, female cucumber beetles, *Diabrotica undecimpunctata*, prefer males with more dissimilar CHC profiles, and produce offspring with higher immunocompetence when mated to their preferred mates (Ali and Tallamy 2010). Mating is more likely to occur between *T. oceanicus* pairs that have more dissimilar CHC profiles, and genetic distance seems to be correlated with CHC dissimilarity in this species (Thomas and Simmons 2011a). This, and the finding that CHC attractiveness is uncorrelated with a potential signal of good genes (courtship song), suggests that compatibility based mate choice is facilitated through CHCs in these crickets (Simmons et al. 2013). Compatibility and

"good" genes benefits are theoretically uncorrelated (Puurtinen et al. 2009), such that individuals can use one trait to assess good genes benefits, and another trait to assess the genetic compatibility of a potential mate. Such a view of mate choice falls under the "multiple messages" hypothesis which states that multicomponent/multimodal signals evolve to communicate different mate characteristics (Candolin 2003; Hebets and Papaj 2005; Partan and Marler 2005). Our finding of disruptive selection on male *O. taurus* CHCs acting independently of courtship rate is consistent with this scenario. There is strong evidence that courtship rate signals good genes in this species (Kotiaho et al. 2001; Simmons and Holley 2011) and the disruptive selection on the CHC profile we detected is consistent with compatibility based mate choice. Alternatively, variation in courtship rate and the CHC profile in our study sample may be associated with age or other life-history traits that would have varied among individuals collected from the field, and females may be using this variation to select mates based on these characteristics. Although our data are suggestive of a multiple messages/good genes/compatible genes scenario, alternative hypotheses are plausible, and at this point further experimentation is required before firm conclusions can be drawn.

Due to its correlative nature, multiple regression is essentially a hypothesis-generating tool that requires experimental manipulation of traits to test its predictions (Chenoweth et al. 2012). Here, we have provided initial evidence that CHCs play a role in the mating system of *O. taurus*, and suggest hypotheses for future research. Results from multivariate selection studies on CHCs in other species have proven fruitful for providing a basis for further investigation. For example, artificial selection on the linear combination of CHCs found to be subject to sexual selection in *D. serrata* resulted in a correlated increase in male mating success (Hine et al. 2011). Similarly, a positive selection gradient on 2-methylhexacosane suggested an attractiveness function for this compound in *D. serrata* (Chenoweth and Blows 2005), with this role experimentally validated using a perfuming experiment (Chung et al. 2014). These findings indicate that multivariate selection analyses on CHCs can provide useful insights for further experimental work. We suggest that incorporating different sensory modalities will provide further insights into how CHCs influence attractiveness alongside other traits, leading to a fuller understanding of the multivariate nature of mate choice. Conducting these studies across a broad range of taxa will contribute to elucidating general patterns in the relative importance of CHCs in mate choice, as well as consistencies (or otherwise) in the information gained by mate assessment based on CHCs in different taxa. We hope that our findings encourage further research on CHC mediated mate choice across a broader range of species that incorporate traits from other sensory modalities.

3.6 Acknowledgements

We thank Jessica Horn for performing the mating trials and assisting with CHC extraction, Anchal Gupta for integrating the CHC peak areas, and Rowan Lymbery for statistical advice. The quality of the manuscript was improved by comments from two anonymous referees. The authors acknowledge the facilities, and the scientific and technical assistance of the Metabolomics Australia Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments.

CHAPTER 4

Experimental evidence for the role of sexual selection in the evolution of insect cuticular hydrocarbons

This chapter is presented in its manuscript form.

4.1 Abstract

A role for sexual selection in the evolution of insect cuticular hydrocarbons (CHCs) is suggested by observations of selection acting on male CHCs during female mate choice. However, evidence that CHCs evolve in response to sexual selection is generally lacking, and restricted to a single genus of fruit fly. Using the dung beetle *Onthophagus taurus*, we found that the male CHC profile diverged in response to the experimental removal of sexual selection. Conversely, the CHC profile of females diverged in response to the presence of sexual selection but not when it was removed. These results show that sexual selection can be an important mechanism affecting the evolution of insect CHCs, and that male and female CHCs can evolve independently.

4.2 Introduction

Secondary sexual traits are thought to have evolved in response to sexual selection (Darwin 1871; Andersson 1994). The cuticular hydrocarbons (CHCs) of insects were traditionally thought to be naturally selected anti-desiccants, but they can also be subject to sexual selection through female choice (Steiger and Stökl 2014), and a signalling function could explain the complex blends of CHCs commonly produced (Blomquist and Bagnères 2010b). However, evidence that sexual selection leads to the evolutionary divergence of CHCs is currently restricted to a single genus of fruit fly (for example, Chenoweth et al. 2008; Hunt et al. 2012; Sharma et al. 2012). We sought to address this gap in our understanding by examining responses in the CHC profile of the dung beetle, *Onthophagus taurus*, to experimental manipulation of sexual selection.

Male *O. taurus* are subject to sexual selection through female choice based on a male's CHC profile and his courtship behaviour (Kotiaho et al. 2001; Berson and Simmons 2018). Experimental evolution studies of this species, that have manipulated sexual selection through the enforcement of monogamy or maintenance of polygamy, have found divergence in sexual traits such as testes mass and sperm competitiveness (Simmons and Garcia-Gonzalez 2008), and both male and female genital morphology (Simmons and Garcia-Gonzalez 2011). In the present study we asked whether this manipulation of the mating system resulted in an evolutionary divergence of male *O. taurus* CHC profiles. We predicted that removing sexual selection through enforced monogamy would result in an evolutionary divergence in the male CHC profile, but that allowing sexual selection to continue (polygamy) should maintain the CHC profile. Sexual selection on female CHCs is yet to be investigated in this species, but is known from other taxa (Chenoweth and Blows 2003; Thomas and Simmons 2010). Therefore we also looked for responses to the manipulation of sexual selection in the CHC profile of females.

4.3 Methods

Full details of the initiation and maintenance of the experimental populations have been described elsewhere (Simmons and Garcia-Gonzalez 2008) and are summarized in the supplementary materials. Briefly, sexual selection was removed by enforcing monogamy in three laboratory populations of *O. taurus*, while it was maintained in three populations by allowing males and females to mate freely. Five males and five females were randomly selected for CHC analysis from each population after six, 12 and 21 generations of experimental evolution, allowing us to track the evolution of male and female CHC profiles across

generations. CHCs were analysed by gas chromatography – mass spectrometry using a Shimadzu QP2010 with protocols established for this species (Berson and Simmons 2018). As is conventional for CHC analysis (Blows 1998), peak areas were transformed to logcontrasts (using *n*-tricosane as the divisor), and a principal component (PC) analysis performed to reduce the number of explanatory variables. For data analysis, we retained only those PCs that had eigenvalues ≥ 1 and which explained ≥ 10% of the variation. We then conducted separate linear mixed effects models for each retained PC as the response variable, with selection regime, generation and sex as fixed effects, along with all possible interactions, and replicate population as a random effect. We examined how the populations responded to selection over time by testing for a significant selection regime x generation interaction. We also examined whether the CHC profile of females evolved similarly (or otherwise) to males by testing for a significant generation x sex interaction. Model validation was conducted using visual inspection of diagnostic plots. Inspection revealed that models of the first PC (PC1) required a $\log_{10}$-transformation of PC scores (we added 10 to the PC scores before $\log_{10}$-transformation to account for negative values) and we report the results for PC1 using these $\log_{10}$-transformed values. All analyses were performed in R version 3.3.3 (R Core Team 2017), using the *FactoMineR* package for PC analysis (Lê et al. 2008), *lme4* package for mixed model analyses (Bates et al. 2015), the *car* package for testing the significance of model terms using chi-square tests (Fox and Weisberg 2011) and *ggplot2* for data visualization (Wickham 2009).

4.4 Results

We analysed a total of 180 CHC profiles from the six experimental populations. A PC analysis returned two PCs with eigenvalues ≥ 1 that individually explained ≥ 10% of the total variation in CHC profiles (Table 4.1). All two-way interactions were significant for PC1 (Table 4.2). There was an overall evolutionary response to the mating system manipulation (selection regime x generation interaction), with the response of the sexes differing (sex x generation interaction). To see how these significant interactions reflected the response of each sex to the different selection regimes, we tested for an effect of generation on PC1 separately for each sex by selection regime combination. There was a significant effect of generation on PC1 for monogamous population males ($\chi^2_1 = 25.073$, $P < 0.001$) but not polygamous population males ($\chi^2_1 = 0.039$, $P = 0.843$). In contrast, there was a significant effect of generation on PC1 for polygamous population females ($\chi^2_1 = 20.308$, $P < 0.001$) but not monogamous population females ($\chi^2_1 = 2.006$, $P = 0.157$). Plots of the population means at each generation separated

by sex and selection regime are shown in Figure 4.1. The removal of sexual selection through enforced monogamy led to CHC profiles characterized by higher values of PC1 for males, but there was no response to this manipulation in females. In contrast, the presence of sexual selection (polygamous populations) led to CHC profiles characterized by lower values of PC1 for females, but there was no response in males (Figure 4.1). None of the interactions were significant for PC2, although there was a significant effect of generation (Table 4.2).

Table 4.1. Cuticular hydrocarbons of *Onthophagus taurus*, their mean relative amounts, and the results of the principal component (PC) analysis. Loadings are given under the headings PC1 and PC2, those in bold have values that are equal to or greater than 70% of the highest loading (Mardia et al. 1979).

				PC1	**PC2**
			Eigenvalue	13.711	5.294
			% variance	37.058	14.309
Peak	Compound	Mean	SE		
1	*n*-docosane	0.36	0.01	0.138	0.101
2	*n*-tricosane	3.55	0.09		
3	11-methyltricosane	1.06	0.08	0.067	0.046
4	2-methyltricosane	0.42	0.03	-0.049	**0.343**
5	3-methyltricosane	0.44	0.02	0.013	**0.335**
6	*n*-tetracosane	1.05	0.03	0.115	0.029
7	2-methyltetracosane	0.31	0.02	-0.031	**0.335**
8	*n*-pentacosane	11.32	0.25	0.132	-0.208
9	11-methylpentacosane	2.57	0.10	0.114	0.236
10	7-methylpentacosane	0.67	0.03	0.098	0.129
11	2-methylpentacosane	1.08	0.04	0.102	**0.247**
12	3-methylpentacosane	1.50	0.05	0.101	**0.353**
13	*n*-hexacosane	1.17	0.03	**0.172**	**-0.267**
14	8-methylhexacosane	0.58	0.03	0.160	0.071
15	8,14-dimethylhexacosane	0.22	0.01	**0.177**	0.062
16	2-methylhexacosane	0.47	0.02	0.127	0.002
17	*n*-heptacosane	10.74	0.43	0.118	**-0.317**

18	13-methylheptacosane	3.92	0.13	**0.180**	0.057
19	2-methylheptacosane	1.81	0.06	**0.186**	0.022
20	3-methylheptacosane	2.36	0.06	**0.202**	-0.043
21	*n*-octacosane	0.96	0.02	**0.185**	-0.232
22	14-methyloctacosane	0.52	0.02	**0.190**	0.047
23	*n*-nonocosane	3.61	0.14	0.122	-0.170
24	15-methylnonacosane	7.94	0.17	**0.220**	-0.025
25	11,15-dimethylnonacosane	0.53	0.02	**0.211**	-0.012
26	19,23-dimethylnonacosane	1.55	0.05	**0.240**	-0.037
27	5,15-dimethylnonacosane	2.62	0.11	**0.180**	-0.024
28	15-methyltriacontane	1.23	0.03	**0.214**	0.008
29	15-methylhentriacontane	16.82	0.34	**0.201**	-0.064
30	13,17-dimethylhentriacontane	0.64	0.02	**0.222**	0.005
31	9,21-dimethylhentriacontane	1.27	0.05	**0.230**	-0.005
32	7,17-dimethylhentriacontane	1.03	0.03	**0.232**	0.010
33	5,17-Dimethylhentriacontane	1.51	0.06	**0.197**	0.026
34	15-methyldotriacontane	0.82	0.03	**0.180**	0.028
35	15-methyltritriacontane	5.53	0.14	0.165	0.036
36	11,21-dimethyltritriacontane	4.02	0.18	**0.197**	0.108
37	15-methylpentatriacontane	0.86	0.04	0.054	0.167
38	11,21-dimethylpentatriacontane	2.94	0.16	0.154	0.150

Table 4.2. Results of the mixed effects model that included Selection regime (SR), Generation (G), Sex (S) and all possible interactions as fixed effects, and replicate population as a random effect.

	PC1		PC2	
	χ^2_1	*P*	χ^2_1	*P*
Selection Regime (SR)	2.445	0.118	0.993	0.319
Generation (G)	1.813	0.178	16.550	**<0.001**
Sex (S)	5.006	**0.025**	3.189	0.074
SR x G	29.668	**<0.001**	0.097	0.756
SR x S	8.031	**0.005**	0.475	0.491
G x S	16.040	**<0.001**	3.775	0.052
SR x G x S	0.125	0.724	1.735	0.188

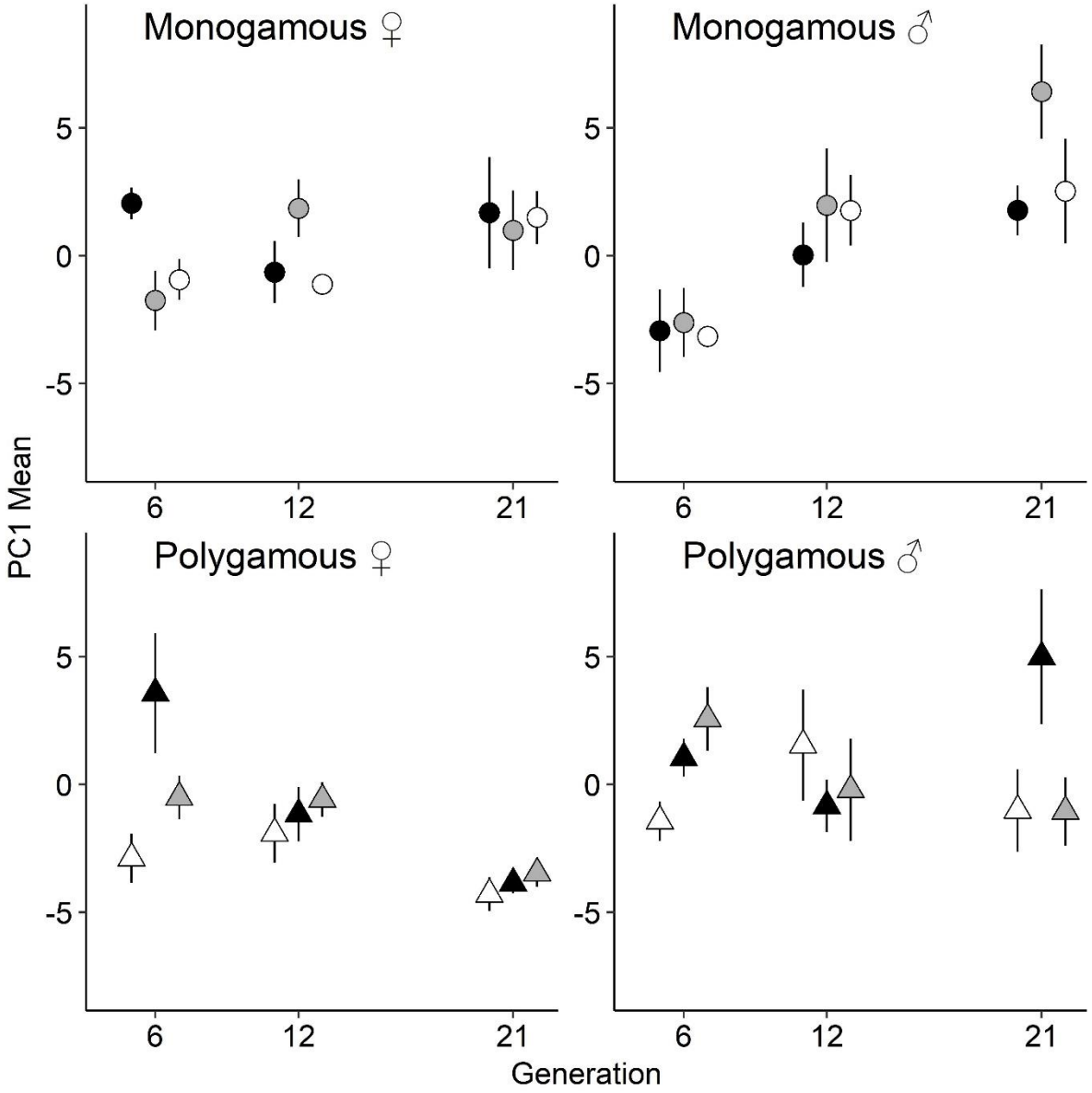

Figure 4.1. Plot of replicate population means at each generation for the major axis of cuticular hydrocarbon phenotypic variation (PC1) separated by sex and selection regime. Error bars represent standard errors. Each population is uniquely shaded within a selection regime.

4.5 Discussion

We found that removing sexual selection from populations of *O. taurus* resulted in an evolutionary divergence in male CHC profiles. In contrast, female CHC profiles diverged when sexual selection was allowed to continue. The response of males to the relaxation of sexual selection was expected from the recent finding that sexual selection acts on male CHCs through female choice (Berson and Simmons 2018). The contrasting response in females suggests independence between the sexes in the evolution of their CHCs. These findings provide important evidence in support of the role of sexual selection in the evolution of insect CHC profiles.

The increase in male PC1 under enforced monogamy suggests that sexual selection in this species may shift the male CHC profile away from its naturally selected optimum. In addition to a sexual display function, CHCs provide a protective barrier against water loss (Gibbs and Rajpurohit 2010), with compounds of increased chain length associated with decreased cuticular permeability (Gibbs and Pomonis 1995). As the loadings on PC1 were weighted towards longer-chained CHCs (Table 4.1), and enforced monogamy resulted in an increase in male PC1 scores, the response of male CHCs may reflect evolution towards CHC profiles of enhanced water-proofing properties following the relaxation of sexual selection. Nevertheless, we suggest caution in this interpretation for two reasons. First, variation in PC1 will reflect both variation in the compounds that load significantly on PC1, as well as variation in the compound used to standardize peaks across the CHC profile. Second, chain-length is just one factor that can affect the water-proofing properties of insect CHCs, and the effect of CHC composition on cuticular water-loss is not always clear (Gibbs and Rajpurohit 2010). With these caveats in mind, consistent with evidence for antagonistic natural and sexual selection on CHCs in fruit flies (Hine et al. 2011; Sharma et al. 2012), our results suggest that natural and sexual selection may act antagonistically on *O. taurus* CHC profiles.

We can offer at least two possible explanations for the evolutionary response seen in female CHCs under polygamy. First, our polygamous treatment may have imposed similar patterns of sexual selection to those acting on males in natural populations, but different patterns on females. Multiple mating is known to increase female reproductive success (Simmons and Holley 2011; McCullough et al. 2018), and sexual selection on females via variation in mating success might act on the female CHC profile. Alternatively, although all females were kept individually and given equal resources when producing offspring, it is possible that the environmental conditions during mating imposed natural selection on females.

For example in the polygamous, but not the monogamous populations, females would have had to compete with other females and males for access to food resources necessary for their future reproduction, and this competition may have selected for changes in female CHC profiles. Regardless of the cause of the evolutionary response in females, our results show that the observed evolution of female CHCs was not simply a correlated response to the evolution of CHCs in males, and their independent evolution may contribute to sexual dimorphism seen in insect CHC profiles (Thomas and Simmons 2008b).

In conclusion, our findings support a role for sexual selection in the evolution of insect CHC profiles. Our results suggest that sexual and natural selection may act antagonistically on *O. taurus* CHC profiles, and that the CHCs of males and females can evolve independently.

4.6 Acknowledgements

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4.7 Supplementary materials

The establishment and maintenance of the experimental populations

Field caught beetles underwent two generations of laboratory rearing before 60 males and 60 females were randomly allocated to each of three replicate monogamous populations and three replicate polygamous populations. All six populations were maintained in isolation from each other and treated identically, except for a manipulation of the mating system. Monogamy was enforced by randomly allocating one male to one female and allowing them to mate for one week. Within each polygamous population, 10 males and 10 females were randomly assigned to one of six containers and left to mate for one week. Following these mating treatments, the males were discarded and the females placed in individual chambers for one week and allowed to produce brood masses (a single brood mass consists of an egg and dung resources for larval development from hatching to adult emergence). For each population, the broods of 50 females were combined. Following offspring emergence, beetles were kept in single-sex cultures for one week of maturation feeding before the above procedures were repeated. Experimental evolution proceeded for 21 generations (approximately 4 years of selection). A random sample of beetles was frozen after maturation feeding each generation to track changes in traits of interest. Full details can be found in Simmons and Garcia-Gonzalez (2008)

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CHAPTER 5

A costly chemical trait: Phenotypic condition dependence of cuticular hydrocarbons in a dung beetle

This chapter is presented in its manuscript form.

5.1 Abstract

Chemical traits are increasingly recognised as important cues used in mate choice. For example, the cuticular hydrocarbons (CHCs) of insects have been shown to influence mating success in a range of taxa. Less is known, however, about how CHCs are expressed in proportion to an individual's condition, and consequently whether CHCs can function as condition dependent signals of quality. We investigated this question using the dung beetle, *Onthophagus taurus*. CHCs are subject to sexual selection in this species through mate choice. A dietary manipulation revealed condition dependence of CHC expression for both sexes: dietary restriction decreased overall CHC production and altered the composition of CHCs. Furthermore, CHC production was associated with a measure of condition in beetles fed a limited diet but not those fed ad libitum. These results implicate a resource cost to CHC production that is likely to result in trade-offs with other fitness components in this species, as these respond similarly to a dietary restriction. The CHC profiles showed sexual dimorphism: males produced more CHCs and the sexes differed in the blend of compounds they produced. There was evidence for a male dimorphism in the CHC profile, in line with the presence of alternative reproductive tactics in this species. However, rather than those males adopting the sneaking tactic mimicking a female CHC profile, they differed more from females than other males. Our results suggest that CHCs are a costly trait in *O. taurus* that has the potential to act as a condition dependent signal of quality.

5.2 Introduction

The opposing effects of natural and sexual selection are predicted to result in an optimum expression of male secondary sexual traits, where the benefits of increased mating success are offset by the costs to viability (Darwin 1871; Fisher 1930; Andersson 1994). If there is variation among males in their ability to bear the costs of a secondary sexual trait, the optimum expression level will also vary, with those males that possess more utilisable resources (in higher condition) better able to afford the costs of further trait exaggeration, and consequently developing a more exaggerated trait (Andersson 1982; Grafen 1990a; Biernaskie et al. 2014). Females that prefer males with the most developed trait will therefore mate with males in high condition, potentially gaining material resources for reproduction or "good genes" for their offspring (Zahavi 1975; Nur and Hasson 1984; Andersson 1986; Grafen 1990b; Rowe and Houle 1996). Condition dependence appears to be a common feature of secondary sexual traits, with examples ranging from behavioural (Kotiaho 2002; Devigili et al. 2013), colour (Kemp 2008; Punzalan et al. 2008), acoustic (Holzer et al. 2003; Hedrick 2005) morphological (Bonduriansky and Rowe 2005; Miller et al. 2016) and chemical (Chemnitz et al. 2015; Jensen et al. 2017) traits.

Although historically chemical traits have received less attention in studies of sexual selection than other forms of signal modalities (Andersson 1994; Johansson and Jones 2007), they are increasingly being recognised as potentially important cues in mate choice (Wyatt 2014). For example, the cuticular hydrocarbons (CHCs) of insects are a class of compounds that have received considerable interest (Steiger and Stökl 2014). CHCs form a complex, multivariate chemical trait secreted onto the cuticle of most insects (Blomquist and Bagnères 2010a). A number of studies have found an association between mating success and CHC expression, particularly in fruit flies from the genus *Drosophila* (Chenoweth and Blows 2003; Van Homrigh et al. 2007; Curtis et al. 2013; Ingleby et al. 2014), to a lesser extent crickets (Thomas and Simmons 2009b, 2010; Steiger et al. 2013; Steiger et al. 2015), and more recently beetles (Lane et al. 2016). There are also indications that CHCs may be costly, for example, there is evidence for a trade-off between egg and CHC production in several taxa (Schal et al. 1994; Wicker and Jallon 1995; Blows 2002; Holman 2012).

Despite the attention CHCs have received in studies of sexual selection, manipulative experiments testing for condition dependence are more limited. CHCs respond to manipulations of diet composition (Liang and Silverman 2000; Otte et al. 2015) including diets of varying quality that presumably alter condition (Delcourt and Rundle 2011; Gosden and Chenoweth 2011; Weddle et al. 2012). The response of CHCs to diets of different

composition/quality can potentially reflect condition dependent responses, and effects that are independent of condition. To isolate changes in CHCs that reflect variation in condition therefore requires a diet restriction or dilution treatment, which directly manipulates the pool of resources available to an individual. This protocol has revealed condition dependence of male CHCs in the fruit fly *Drosophila melanogaster* (Bonduriansky et al. 2015) and the decorated cricket *Gryllodes sigillatus* (Rapkin et al. 2017). It remains an open question as to whether CHCs are condition dependent across a broader range of taxa.

The dung beetle, *Onthophagus taurus*, provides an ideal system to investigate the condition dependence of CHCs. Studies of this species have documented condition dependence of sexually selected traits (Kotiaho 2002; Simmons and Kotiaho 2002; Knell and Simmons 2010), and a measure of condition (relative mass) is both genetically correlated with some of these traits, and displays a high level of genetic variation (Kotiaho et al. 2001; Simmons and Kotiaho 2002). Male *O. taurus* exhibit dimorphic morphologies associated with alternative reproductive tactics; horned major males fight for access to females and offer parental provisions, while hornless minor males sneak copulations with females guarded by horned males (Moczek and Emlen 2000). During mate choice, females target males of high condition based on their courtship rate (Kotiaho et al. 2001) and receive reproductive benefits from doing so (Simmons and Holley 2011). Recently female choice was found to target the male CHC profile in this species (Berson and Simmons 2018), prompting us to ask here whether *O. taurus* CHCs are condition dependent. As *O. taurus* males display alternative mating tactics, we also examine whether the male dimorphism is reflected in variation in the CHC profile.

5.3 Materials and methods

Beetles were collected from a dairy farm located 60 km south-east of Perth, Western Australia. Mixed sex cultures of these beetles were kept in 10 L buckets, ¾ filled with moist sand, and held within a controlled temperature room set at 28°C on a 12 h day-night cycle. Beetles were fed ad libitum cow dung that had been collected from the same location, frozen and then thawed prior to feeding. To generate F1 offspring, male-female pairs were placed in 30 cm high by 9 cm diameter PVC piping ¾ filled with moist sand and topped with cow dung. After seven days the sand was sieved to collect brood balls, each containing a single egg and the dung resources required for larval development. Brood balls were buried in moist sand in individual containers (5 cm x 8 cm x 8 cm). F1 beetles emerged approximately three weeks later. Upon emergence beetles were kept in their individual containers, given fresh moist sand and assigned to either ad libitum food or food limited treatments. These treatments result in ‘high’ and ‘low’ condition

groups respectively, and were based on previous studies examining condition dependence of other sexually selected traits in this species (Kotiaho et al. 2001; Kotiaho 2002; Knell and Simmons 2010). Both ad libitum food and food limited beetles were given 1 teaspoon (20ml) of dung at 2 days and fresh sand at 7 days post-emergence, but only ad libitum food beetles were given a second teaspoon of dung at 7 days. All beetles were frozen at 12 days post-emergence at the same time of day. Beetles were weighed prior to freezing, using digital scales. Beetles were kept at –16°C until CHC extraction. Post CHC extraction, horn length (males only) and pronotum width were measured with a graticule under 100x magnification using a Leica MZ6 dissecting microscope.

CHC Characterisation

To extract CHCs, individual beetles were immersed in 1 mL of hexane for five minutes. The hexane solution was then removed using a Pasteur pipette and placed into a 2 mL autosampler screw top glass vial. The samples were concentrated by evaporating off the hexane using a LABCONCO Centrivap Concentrator and then resuspending in 0.1 mL of hexane, vortexing and placing the solution in a 0.1 mL glass insert within its original vial. Samples were analysed using gas chromatography-mass spectrometry (GC-MS) on an Agilent 7890 GC fitted with an Agilent 5975 MS Detector. The GC was operated using the splitless mode with helium as the carrier gas, fitted with a 20 m x 150 µm x 0.15 µm VF1 column, and programmed with an initial temperature of 140°C held for one minute and then ramped up at 5°C per minute until reaching 300°C for five minutes for a total run time of 38 minutes. Peaks were labelled according to their retention time, and matched to previously identified compounds (Berson and Simmons 2018) by their mass spectra and retention indices. Retention indices were assigned by comparing compounds to a known sample of *n*-alkanes ($C_{20} - C_{30}$). Areas of each peak were found by integrating under the major ion of the compound using Agilent Mass Hunter. To quantitatively describe the major axes of phenotypic variation in the composition of each beetle's CHC profile, we first transformed the individual peak areas to logcontrasts using a $\log_{10}$ transformation of their relative area divided by the relative area of peak 2 (*n*-tricosane; we added one before $\log_{10}$ transformation to allow for instances where peak values are zero) (Neems and Butlin 1995; Blows and Allan 1998). We then performed a principal component (PC) analysis on these logcontrasts, with only those PCs with eigenvalues greater than one and that individually explained ≥ 10 % of the variation retained. As a measure of total CHC production, we calculated the sum of the absolute peak areas (the resulting values were $\log_{10}$ transformed for analyses to ensure normality of model residuals). Although using absolute peak

areas will be subject to error from potential drift in the sensitivity of the GC-MS, we randomised the order of injection across all samples to prevent this error resulting in any systematic bias.

Statistical analysis

To confirm that our diet treatment had an effect on condition, an ANCOVA was performed with mass as the response variable, treatment as the explanatory variable and pronotum width (as a measure of body size) as a covariate. To test if the sexes differed in their treatment response, the analysis was repeated with the addition of sex and its interaction with treatment as factors. We tested for a response to the dietary treatment and whether this response differed between the sexes for overall CHC production and CHC composition using linear models. Included in the models were the PCs and total CHC production as response variables, and treatment (ad libitum food versus food limited), sex and their interaction as the explanatory variables. As relative mass has previously been identified as a biologically relevant estimate of condition in this species (Kotiaho et al. 2001; Simmons and Kotiaho 2002), we conducted a further test for condition-dependent CHC production using separate linear models for each treatment group, with total CHC production as the response variable, and mass and sex as explanatory variables, along with pronotum width as a covariate.

We used the methods of Eberhard and Gutierrez (1991) to determine the pronotum width that represents the switchpoint between the male morphs. Those males that had a pronotum width ≥ the switchpoint were classified as major males, with the remaining classified as minor males. Linear models were again used to determine if the male morphs differed in their CHC profiles, with the PCs and total CHC production as response variables, and male morph (major vs minor), treatment and their interaction as the predictors. To understand how any differences between the male morphs related to the CHC profile of females, we repeated the analyses using major male, minor male and female as independent levels of a sex/morph factor ('status') along with our treatment levels. Tukey post hoc tests were then used to determine which groups differed from each other. All analyses were performed in R version 3.3.3 (R Core Team 2017) using the *FactoMineR* package for PC analysis (Lê et al. 2008), the *car* package for significance tests (Fox and Weisberg 2011) and *ggplot2* for plotting (Wickham 2009).

5.4 Results

There was a significant relationship between body size (pronotum width) and body mass ($F_{1,110}$ = 345.70, P < 0.001; Figure 5.1). Beetles on ad libitum food had a significantly greater relative mass than did food limited beetles ($F_{1,110}$ = 261.48, P < 0.001; Figure 5.1). There was no significant interaction of sex and treatment when included in the model ($F_{1,108}$ = 0.36, P = 0.548) nor of sex when the interaction was removed ($F_{1,109}$ = 0.56, P = 0.457). Access to dung during maturation feeding thereby had a significant effect on the condition of beetles, with ad libitum food beetles having greater relative body mass than food limited beetles.

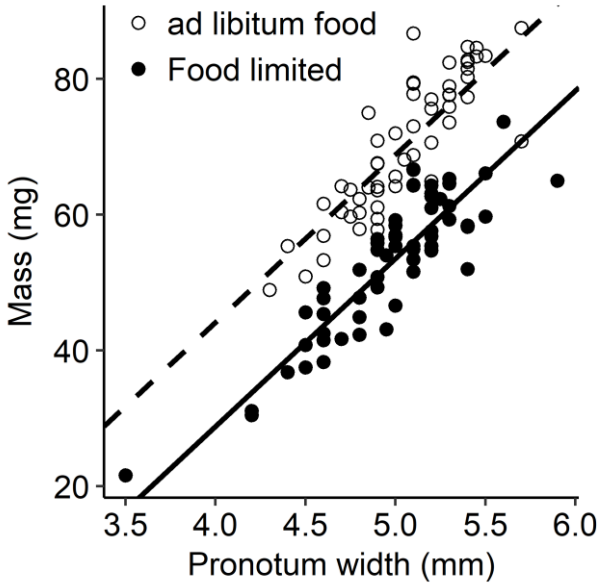

Figure 5.1. Plot of ANCOVA results with mass as the response variable, treatment as the explanatory variable and pronotum width as the covariate. The line of best fit is shown by a dashed line for ad libitum food and a solid line for food limited beetles.

We analysed hexane washes from 20 females in each diet treatment, and 37 food limited and 36 ad libitum food males. We retained two PCs from the principal component analysis that collectively explained 65.1 % of the variation (Table 5.1). Following Mardia et al. (1979) we identified CHC logcontrasts as contributing significantly to a principal component if their loading was equal to or greater than 70% of the highest value. PC1 was loaded significantly by the majority of methyl-branched compounds, particularly those of longer chain length. We thereby interpret this PC as representing relative investment in longer chained methyl-branched compounds. With the exception of *n*-pentacosane, PC2 was positively loaded by all alkanes, and we interpret this PC as representing relative investment in alkanes, although we note that the alkane of greatest abundance (*n*-pentacosane) is not included in this group.

Table 5.1. The best matches for cuticular compounds found in *Onthophagus taurus*, their mean relative amounts and their contributions to the two principal components with eigenvalues greater than one and that individually explain ≥ 10% of the variation. Loadings shown in bold are those whose values are equal to or greater than 70% of the highest loading.

				PC1	**PC2**
			Eigenvalue	18.73	5.36
			% variance	50.62	14.48
Peak	Compound	Mean	SE		
1	*n*-docosane	0.09	0.01	-0.06	0.24
2	*n*-tricosane	3.51	0.16		
3	11-methyltricosane	1.98	0.08	**0.15**	-0.15
4	2-methyltricosane	0.98	0.05	0.10	-0.13
5	3-methyltricosane	1.48	0.07	0.13	-0.24
6	*n*-tetracosane	0.23	0.02	-0.09	**0.32**
7	2-methyltetracosane	0.64	0.02	**0.16**	-0.03
8	*n*-pentacosane	14.22	0.56	0.14	-0.18
9	11-methylpentacosane	7.96	0.28	**0.17**	-0.07
10	7-methylpentacosane	0.90	0.04	0.11	0.01
11	2-methylpentacosane	1.13	0.04	**0.17**	0.06
12	3-methylpentacosane	2.29	0.07	**0.19**	-0.03
13	*n*-hexacosane	0.38	0.03	0.00	**0.40**
14	8-methylhexacosane	0.96	0.05	**0.16**	-0.06
15	8,14-dimethylhexacosane	0.42	0.03	0.11	-0.04
16	2-methylhexacosane	0.49	0.02	**0.17**	0.15
17	*n*-heptacosane	5.57	0.33	0.02	**0.39**

18	13-methylheptacosane	4.85	0.17	**0.18**	-0.01
19	2-methylheptacosane	1.23	0.05	**0.18**	0.10
20	3-methylheptacosane	1.89	0.04	**0.20**	0.11
21	*n*-octacosane	0.23	0.02	0.04	**0.37**
22	14-methyloctacosane	0.65	0.02	**0.20**	0.01
23	*n*-nonocosane	1.36	0.08	0.06	**0.29**
24	15-methylnonacosane	6.51	0.18	**0.20**	0.05
25	11,15-dimethylnonacosane	0.83	0.03	**0.21**	-0.05
26	19,23-dimethylnonacosane	1.68	0.05	**0.21**	0.03
27	5,15-dimethylnonacosane	2.46	0.08	**0.21**	-0.10
28	15-methyltriacontane	1.49	0.05	**0.21**	-0.10
29	15-methylhentriacontane	9.24	0.39	**0.17**	0.20
30	13,17-dimethylhentriacontane	1.35	0.05	**0.20**	-0.03
31	9,21-dimethylhentriacontane	1.37	0.06	**0.20**	0.03
32	7,17-dimethylhentriacontane	1.90	0.06	**0.20**	0.03
33	5,17-Dimethylhentriacontane	3.41	0.12	**0.21**	-0.09
34	15-methyldotriacontane	0.66	0.03	**0.18**	0.03
35	15-methyltritriacontane	2.67	0.14	**0.17**	0.19
36	11,21-dimethyltritriacontane	7.33	0.33	**0.20**	0.05
37	15-methylpentatriacontane	0.88	0.05	**0.17**	0.06
38	11,21-dimethylpentatriacontane	4.79	0.29	**0.19**	0.06

The ANOVA results from the linear models for PC1, PC2 and total CHC production are given in Table 5.2. There were significant diet treatment and sex effects for PC1, PC2 and total CHC production. Beetles with restricted access to food produced a lower total amount of CHCs, and displayed a CHC blend with relatively more longer-chained methyl-branched compounds (increased values of PC1) and less alkanes (lower values of PC2) than those fed ad libitum (Figure 5.2). Relative to females, males produced a greater total amount of CHCs, and displayed a CHC blend with relatively more longer-chained methyl-branched compounds (PC1) and alkanes (PC2) (Figure 5.2). There was no significant interaction between diet treatment and sex for any model, indicating that male and female CHC profiles responded similarly to the diet manipulation. Removing the interaction made little difference to the remaining model terms so the results of the full models are presented.

There was a significant association between relative body mass and the total amount of CHCs produced by food limited ($F_{1,53} = 24.76$, $P < 0.001$) but not ad libitum food ($F_{1,52} = 2.53$, $P = 0.118$) beetles. Body size (measured as pronotum width) had a significant effect on the amount of CHCs produced by food limited ($F_{1,53} = 16.04$, $P < 0.001$) but not ad libitum food ($F_{1,52} = 0.08$, $P = 0.784$) beetles. The total amount of CHCs produced is therefore associated with a measure of condition in this species, with the association only revealed under harsh conditions. The effect of sex on CHC production was significant in beetles supplied ad libitum food ($F_{1,52} = 7.46$, $P = 0.009$) but not-significant for those supplied limited food ($F_{1,53} = 3.75$, $P = 0.058$).

Table 5.2. Results of linear models with PC1, PC2 or Total CHCs ($\log_{10}$ transformed) as response variables, and sex, treatment and their interaction as explanatory variables. Removing the non-significant interactions had little effect on the remaining model terms so the results of the full models are presented.

	Treatment		Sex		Treatment x Sex	
	$F_{1,109}$	P	$F_{1,109}$	P	$F_{1,109}$	P
PC1	7.25	**0.008**	6.57	**0.012**	0.01	0.928
PC2	84.25	**<0.001**	8.08	**0.005**	1.67	0.199
Total CHCs	65.33	**<0.001**	7.51	**0.007**	1.27	0.263

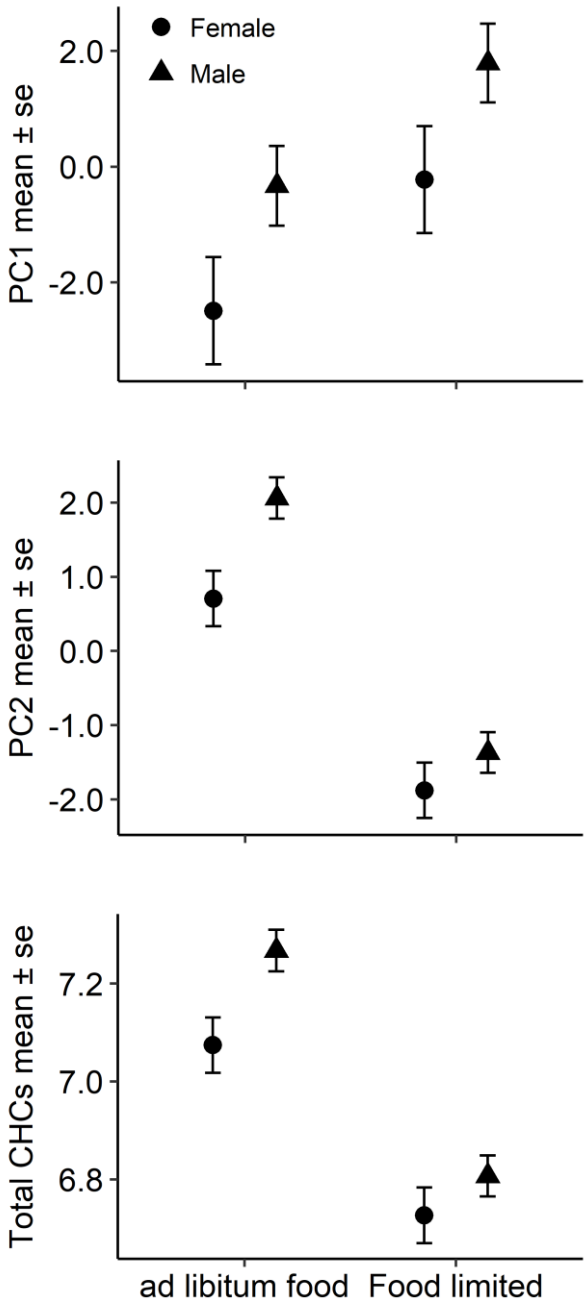

Figure 5.2. Least squares means from the models with total CHC production (Total CHCs, $\log_{10}$ transformed), PC1 or PC2 as the response variables and sex, treatment and their interaction as the explanatory variables. Males are represented by triangles and females by circles.

A pronotum width of 5.08mm was found to represent the body size switchpoint between major and minor males. This resulted in 19 food limited and 17 ad libitum food minors; and 18 food limited and 19 ad libitum food majors. Table 5.3 presents the results of the models testing for differences between the morphs in their CHC profile. Two data points appeared as potentially influential outliers for tests of differences between the male morphs for PC1 and were removed for this analysis. There was no significant interaction between diet treatment and morph for any trait, indicating that the CHC profiles of majors and minors responded similarly to the diet treatment. Removing the non-significant interaction term had little effect on the remaining model terms so the results of the full models are presented. There was a significant effect of morph on PC2 but no significant effect of morph on the total amount of CHCs produced nor PC1. Including the potential outliers for PC1 resulted in statistical significance of the morph effect ($F_{1,69} = 5.13$, $P = 0.027$). Repeating the analyses using both male morphs and females as independent levels of a sex/morph factor ('status') revealed no significant interaction term for any trait (all $P > 0.3$), so the interaction was removed before conducting the post-hoc tests. There was a significant effect of 'status' for all three traits (PC1: $F_{2,107} = 4.17$, $P = 0.018$; PC2: $F_{2,109} = 7.00$, $P = 0.001$; Total CHCs: $F_{2,109} = 3.71$, $P = 0.028$; Figure 5.3) and Tukey post-hoc tests revealed that this effect was driven by differences between minor males and females for PC1 (adjusted $P = 0.013$); minor males with both females (adjusted $P = 0.001$) and major males (adjusted $P = 0.050$) for PC2; and major males and females (adjusted $P = 0.044$) for total CHC production (the difference between females and minor males had an adjusted $P = 0.069$). In conjunction with visual inspection of Figures 5.2 and 5.3, these results indicate that the CHC blend produced by minor males contributed to the sexual dimorphism in the composition of the CHC profile.

Table 5.3. Results of linear models with PC1, PC2 or Total CHCs (log_{10} transformed) as response variables, and male morph, treatment and their interaction as explanatory variables. Removing the non-significant interactions had little effect on the remaining model terms so the results of the full models are presented.

	Treatment		Morph		Treatment x Morph	
	$F_{1,69}$	P	$F_{1,69}$	P	$F_{1,69}$	P
PC1*	5.46	**0.023**	3.05	0.085	1.18	0.282
PC2	73.30	**<0.001**	5.23	**0.025**	0.33	0.566
Total CHCs	51.00	**<0.001**	0.02	0.891	0.24	0.625

***** The results for PC1 are from the model that removed two outliers, the degrees of freedom for the *F* statistics from this model are 1,67.

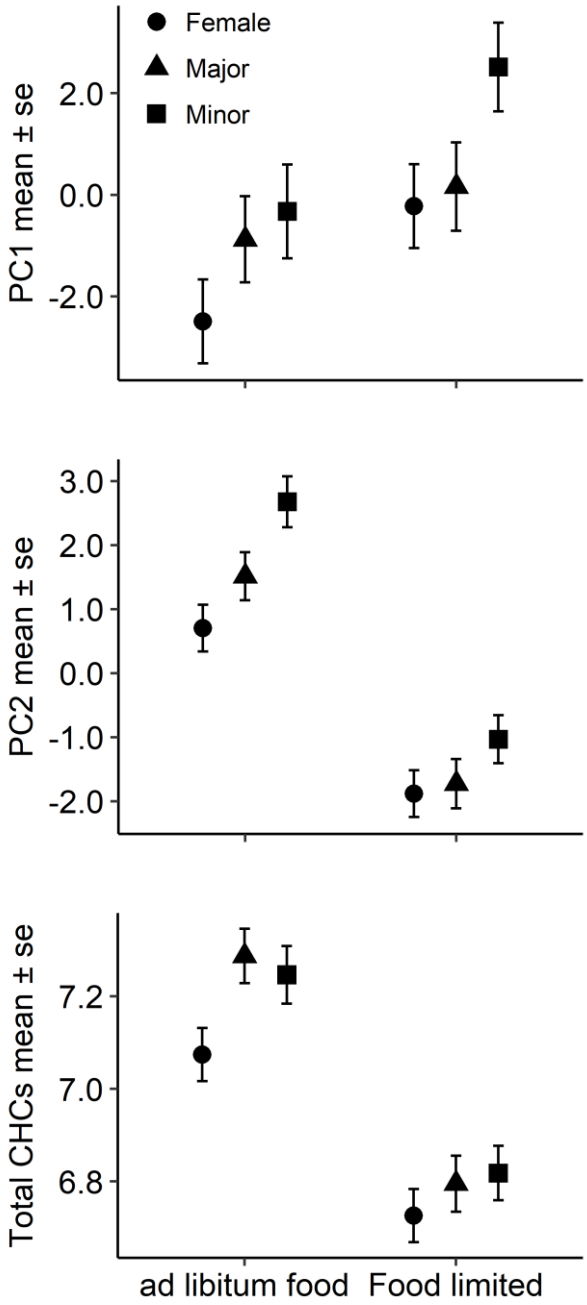

Figure 5.3. Least squares means from the models with total CHC production (Total CHCs, $\log_{10}$ transformed), PC1 or PC2 as the response variables and status (major male, minor male or female), treatment and their interaction as the explanatory variables. Females are represented by circles, major males by triangles and minor males by squares.

5.5 Discussion

Using a dietary manipulation, we have found condition dependent expression of CHCs in male and female *Onthophagus taurus* dung beetles. Beetles fed a restricted diet reduced their investment in CHC production, and produced a different blend of CHCs compared to beetles fed ad libitum. We also found sexually dimorphic CHC expression, with males investing more heavily in CHC production than females, and the sexes differing in their CHC composition. Interestingly, the sexual dimorphism in CHC composition, but not overall CHC production, appeared to be driven to some extent by the CHC profiles of minor males. Our results provide evidence that CHCs are a costly condition dependent trait that has the potential to signal mate quality in *O. taurus*.

Our finding of a reduction in the overall abundance of CHCs under limiting dietary conditions implicates a resource cost of CHC production. A similar response to reduced nutritional availability has been found for several fitness enhancing traits in *O. taurus*, including strength (Knell and Simmons 2010) and courtship rate (Kotiaho et al. 2001). The resources required for CHC production are likely taken from the same pool of resources as those required for these fitness traits, as all respond to dietary limitation in the same way as CHCs, with the potential for trade-offs between them. Furthermore, we found a significant relationship between an estimator of condition (relative mass) and overall CHC production in beetles that were fed a restricted diet but not in beetles that were fed ad libitum. This is expected where the difference in the marginal cost of increased trait expression between low and high quality individuals is increased under harsh conditions (Cotton et al. 2004), and where benign conditions allow all individuals to maximally express a trait. Taken together our results indicate that CHCs are a costly trait in *O. taurus*, and that the costs of increased expression are greater for low quality individuals, particularly in environments with limited food resources.

We have previously found a pattern of disruptive sexual selection on male *O. taurus* CHC profiles, and suggested that females may be using the composition of CHC compounds for compatibility based mate choice (Berson and Simmons 2018). This may appear to contradict our claims of a potential role of CHCs in signalling mate quality. However, our previous investigation used beetles that had been supplied ad libitum food, which is likely to have obscured any correlation between CHC expression and individual quality (as was the case for ad libitum food beetles in the current study). Female *O. taurus* are known to benefit from targeting males of high condition as mating partners based on their courtship rate (Kotiaho et al. 2001; Simmons and Holley 2011). It is possible that females use CHCs in tandem with courtship rate in their assessment of male condition, as well as using CHCs to assess mate

compatibility. Mate choice for compatibility and quality are not mutually exclusive (Colegrave et al. 2002) and both can function within a single trait (Roberts and Gosling 2003). If CHCs are used as indicators of both compatibility and quality we would expect directional and disruptive selection to be acting on them. Our finding that the association between condition and CHC expression is only revealed under limited dietary conditions suggests that future tests for directional selection on *O. taurus* CHCs will benefit from the use of individuals fed a limited diet.

Consistent with the action of directional sexual selection on male CHCs, males produced a greater abundance of compounds relative to females. Under some circumstances this increased CHC expression in males is predicted to result in an increase in their sensitivity to changes in condition (Cotton et al. 2004), a result not found here (there was no significant sex x treatment interaction). However, increased expression of a trait does not necessarily mean it will become more condition dependent, as an increase in sensitivity to condition depends on an increase in the differential cost of further trait expression between low and high quality signallers, rather than the overall cost of trait production (Johnstone et al. 2009). It is also possible that the costs of increased CHC production are greater for females, as could result from a trade-off between CHC and egg production (Schal et al. 1994; Wicker and Jallon 1995; Blows 2002; Holman 2012). This raises the possibility that the sexual dimorphism observed in CHC production is not a result of an exaggerated male CHC profile, but reduced expression in females due to their higher costs of CHC production. It is possible that both male and female CHC profiles act as costly condition dependent signals of quality, but female CHC profiles have a reduced expression due to a trade-off with egg production. Alternatively, both exaggerated male expression and reduced female expression may be the cause of the observed sexual dimorphism, as has been found for plumage brightness among birds (Dale et al. 2015).

We found some evidence for a difference in the CHC blend of minor and major males. Female mimicry of the CHC profile by males displaying an alternative mating tactic has been reported for the tropical ant, *Cardiocondyla obscurior* (Cremer et al. 2002). As *O. taurus* major males are able to distinguish minor males from females and respond to their presence (Hunt and Simmons 2002), female mimicry in the CHC profile of minor males was not expected, nor was it found. Rather, minor males produced a blend of CHCs that differed more to that of females than did the blend of compounds produced by major males (Figure 5.3). It is possible that the variation in CHC profiles between minor males and major males / females is related to variation in behaviour and associated desiccation risk. Along with influencing mating success, CHCs also provide a barrier to water loss in insects (Chung and Carroll 2015). Unlike major

males and females, minor males do not spend time in the dung pat assisting in the provisioning of brood balls (Hunt and Simmons 1998) but rather spend more time excavating side tunnels to enter the breeding chambers of provisioning females (Emlen 1997; Moczek and Emlen 2000). It is possible that these behavioural differences result in minor males spending more time exposed to desiccation pressures. One potential explanation for the CHC dimorphism then, is that selection may have favoured minor males that produce a CHC profile of improved water-proofing properties to suit their alternative mating tactic. How the blend of compounds produced by minor males is related to the water-proofing properties of their CHC profile is currently unknown, and further work is required to reveal if any such associations exist.

In conclusion, we have shown that *O. taurus* CHC expression is condition dependent, and that the CHC profiles of both sexes respond similarly to a restricted diet. Further work is required to understand how the plasticity of CHC expression affects mate choice in this species, particularly if/how CHCs are used as indicators of mate quality. We suggest that investigations of the role of CHCs in mate choice will benefit from using individuals fed a restricted diet, as this may reveal an association with condition otherwise obscured by benign laboratory conditions. Our study adds to the growing body of literature examining the role of chemical traits in mate choice, and suggests that CHCs have the potential to act as costly signals of mate quality.

5.6 Acknowledgements

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EPILOGUE

Understanding the contribution of sexual selection to phenotypic diversity requires the study of the full range of sexual traits, both within species and among a broad taxonomic range of organisms. Compared to visual and acoustic traits, chemical traits have been relatively neglected in sexual selection research. Throughout this thesis I have provided evidence that sexual selection is a significant factor affecting the evolution of an important chemical trait in insects, cuticular hydrocarbons (CHCs). I have shown that CHCs bear some of the hallmarks of sexually selected traits – subject to opposing natural selection, displaying condition dependence, and signalling potential benefits to mating partners. In particular, I have deepened our understanding of the role of CHCs in the mating system of the Australian field cricket, *Teleogryllus oceanicus*, and revealed a previously unknown role for CHCs in the mating system of the dung beetle, *Onthophagus taurus*. Here I briefly summarise the results from each of the thesis chapters, highlight some of the limitations of this work, and provide some direction for future research.

Sexual selection is thought to result in a shift of traits away from their naturally selected optima, such that the positive effect of secondary sexual trait expression on mating success is offset by negative effects on other fitness components. In Chapter 1 I presented evidence for opposing natural and sexual selection on male *T. oceanicus* CHCs. Using multiple regression and quantitative genetics, I found that the blend of CHCs predicted to maximise attractiveness was negatively genetically correlated with the CHC blend predicted to maximise desiccation resistance. Multiple regression presented a useful technique for scoring each individual's CHC profile for its level of attractiveness and desiccation resistance. Nevertheless, these scores can be moderated by the effect of traits not included in the analyses, an issue I explored in greater detail in Chapter 3. A follow-up experiment that uses artificial selection on these scores could be used to test for a correlated response in the corresponding fitness trait. A correlated response would provide further evidence for the influence of CHCs on these two fitness components, and evidence that these scores accurately predict the attractiveness/desiccation resistance of male *T. oceanicus* CHCs.

The weaker than expected association between the CHC profile and desiccation resistance presented in Chapter 1 warrants further investigation. It should be highlighted that the large standard errors on the estimates of genetic correlations in this chapter indicate that I had low power to detect statistically significant genetic correlations between the CHC compounds and desiccation resistance. My use of a paternal half-sib breeding design with links across two generations allowed me to use the quantitative genetic "animal model" for estimating the quantitative genetic parameters. The animal model provides a powerful

approach in quantitative genetics, and future work that incorporates larger sample sizes, a greater number of generations and varying links within the pedigree will assist in detecting the significance, or otherwise, of the weaker genetic correlations revealed in my study. More generally, in Chapter 1 I discuss how the association between the composition of the CHC profile and water-proofing in insects is less clear than what is sometimes assumed. I only reiterate here my belief that investigating the interaction of natural and sexual selection on the evolution of insect CHCs will prove a fruitful area of future research.

Male mate choice has not received the same level of attention as female mate choice, particularly the indirect benefits that males can receive by targeting particular female phenotypes. In Chapter 2 I found evidence for genetic correlations between a proxy for female fecundity in *T. oceanicus*, ovary mass, and both the major axis of CHC genetic variation, and some of the individual compounds that make up the CHC profile. This provided support for the hypothesis that males can receive the indirect benefit of siring more fecund daughters by targeting their mating effort based on female CHCs. Measuring ovary mass and the CHCs on different (but related) individuals removed the potential for residual (within-individual) covariance to obscure any genetic correlations. However, as a consequence this prevented tests for phenotypic associations between CHCs and ovary mass. I was therefore unable to test directly whether males can receive direct benefits by targeting more fecund females based on their CHC profile. Logistical constraints did not allow for measuring other relevant traits in this study, particularly female attractiveness and offspring viability. Testing for a genetic correlation between female attractiveness, total offspring production and the CHC profile, will further elucidate the genetic benefits males can receive by using CHCs for mate assessment in *T. oceanicus*. The potential for CHCs to be a widespread signal of female fecundity in solitary insects also deserves more attention. Expanding the work presented in Chapter 2 to a broader taxonomic range would facilitate comparative analysis. This will be necessary to elucidate whether the queen pheromone role played by CHCs in the social insects is derived from a general signalling of female fecundity to males across the non-social insects.

In Chapter 3 I found that female mate choice imposed significant non-linear selection on male *O. taurus* CHCs. Two main points arise from this study that deserve further attention. First, I was able to show that including a behavioural sexual display trait in the multivariate selection analyses had some influence on the estimated linear selection acting on the CHC profile. The usefulness of CHCs in sexual selection studies is based in part on their multivariate nature. However, CHCs undoubtedly act alongside other traits to influence attractiveness, and the results in Chapter 3 show that an accurate assessment of the strength and form of selection

acting on CHCs and other sexual display traits will require studies that are more inclusive of non-CHC traits. Second, estimates of the strength and form of selection are influenced by the phenotypic (co)variation present in the study sample. I attempted to incorporate naturally occurring variation in male CHCs and female preferences by using beetles collected from the field. Ideally, a combination of selection estimates from both field collected and laboratory raised beetles, the latter at various standardisations for age/mating status/nutritional condition, could be used to gain a greater understanding of selection imposed by mate choice on *O. taurus* CHCs. Measuring sexual selection directly in natural populations of *O. taurus* is difficult due to the tunnelling lifestyle of this species. However, in general, any further work that can provide estimates of selection in the wild will greatly contribute to our understanding of the role of sexual selection in the evolution of insect CHCs.

The information that can be gleaned from multivariate selection analyses sometimes obscures the fact that these are primarily a tool for generating hypotheses. In Chapter 4 I provided experimental evidence that removing sexual selection results in an evolutionary divergence of the male CHC profile. Less explicable in this study was that the continued action of sexual selection resulted in an evolutionary divergence of female CHCs. It is possible that competition among females for mating opportunities was increased in these laboratory populations above the level found in the ancestral population, imposing novel sexual selection on females. Nothing is currently known about the potential for male mate choice to impose selection on female CHCs in *O. taurus*, and this species is an ideal candidate to broaden our understanding of the role of CHCs in fecundity signalling.

Finally, in Chapter 5 I found evidence that CHCs are condition dependent in both male and female *O. taurus*. Interestingly, I also found evidence for a male dimorphism in the CHC profile. Male *O. taurus* display alternative reproductive tactics, with major males fighting over access to females, and minor males sneaking copulations. However, rather than the male dimorphism in CHCs reflecting these different reproductive tactics, with minor males mimicking the female CHC profile to facilitate their sneaking behaviour, minor males differed more from females than did major males. I suggest in the manuscript that the difference between male morphs may be related to differences in their exposure to desiccation risk. By capitalising on the variation between the male morphs, testing this hypothesis will assist in examining the association between CHCs and desiccation resistance more broadly.

In conclusion, I have explored the role of CHCs in the mating systems of two insect species, and by so doing, examined the contribution of sexual selection to the evolution of insect CHC profiles more generally. As happens in science, the answers I have provided

suggest more questions to be asked and mandate more detailed investigations be undertaken. Along with the suggestions already outlined, future work that experimentally manipulates the CHC profiles of *T. oceanicus* and *O. taurus*, and tests how these manipulations affect attractiveness, will help to further understand the contributions of CHCs to mating success in these species.

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