THE ROLE OF CUTICULAR HYDROCARBONS IN DETERMINING MALE REPRODUCTIVE SUCCESS

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature).....

ABSTRACT

Cuticular hydrocarbons (CHCs) are found on the outer cuticle of all terrestrial arthropods. Although their primary function is in desiccation prevention, these compounds have also been shown to play a variety of roles in insect chemical communication, from species and sex recognition to providing cues of dominance and attractiveness. However, despite growing evidence of their versatility as cues, our knowledge of how CHCs are used in mating interactions is limited to *Drosophila* and field crickets. In this thesis I investigate the roles CHCs play in interactions at each stage of the mating process in the broad-horned flour beetle *Gnatocerus cornutus*. I assess the relative importance of CHCs in influencing male reproductive success and examine the complex interplay between different episodes of selection and the mechanisms of sexual selection acting on males. I use a combination of behavioural assays, experimental manipulations and gas chromatography. First, I identify the role of CHCs as cues of sperm competition risk and intensity, demonstrating how the presence of male-derived CHCs on the cuticles of virgin females elicits males to adjust their pre- and post-copulatory investment (chapter 2), by providing information on the state of their competitive environment. I then go on to look at the stability of CHCs as cues of sperm competition over time, finding that they are highly sensitive to environmental degradation (chapter 3) and do not persist in the habitat substrate of this species. Next, I investigate how male CHCs determine fighting and mating success. By estimating and comparing the strength and form of sexual selection imposed by male-male competition and female mate choice, I show that male CHCs are subject to strong antagonistic sexual selection (chapter 4). By experimentally manipulating male CHC profile, I then attempt to verify the selection gradients estimated for female choice

(chapter 5). However, my experimental manipulation fails to verify the importance of male CHCs for female mate choice. Finally, I explore the role of same-sex sexual behaviour (SSB) in determining male reproductive success (chapter 6). I find evidence to suggest that SSB may in fact be a form of aggression in its own right, and demonstrate that SSB and fighting may provide equivalent means for males to overcome female choice and secure a mating advantage. My results indicate that CHCs play key roles as chemical cues throughout the mating process and significantly impact male reproductive success. My thesis reveals the intricate nature of the relationships between mechanisms of sexual selection, alongside highlighting the need to consider both the social and physical environment when investigating the importance of chemical cues. I discuss the implications of these results for the evolution of male CHCs and how my findings can be used to further our knowledge of this field.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

AUTHOR'S DECLARATION

During the research contributing to this thesis Sarah Marie Lane (SML) was supported by a studentship from the Natural Environment Research Council (NERC). All of the experiments presented in this thesis were designed, carried out and written by SML with input and supervision from Clarissa House (CH) and Tom Tregenza (TT), however others contributed to each chapter as discussed below.

Chapter Two

Jon Blount co-supervised the undertaking of this experiment in conjunction with CH. John Hunt (JH) provided access to gas chromatography machinery and equipment while Chris Mitchell (CM) provided training and assistance with running samples. Joanna Solino carried out the data collection for experiment 1 and Kensuke Okada provided dissection assistance via email. A version of this chapter was published in *Behavioural Ecology* **26**: 1021-1029.

Chapter Four

JH provided access and advice with gas chromatography machinery and equipment. CM provided assistance with running samples. Andrew Dickinson collected data from fighting trials and assisted in preparation of samples for chemical analysis. A version of this chapter has been submitted to *Journal of Evolutionary Biology*.

Chapter Six

Alice Haughan and Daniel Evans collected data for experiment 2 of this chapter. A version of this chapter has been accepted for publication in *Animal Behaviour*.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Sexual selection and male reproductive success

Male reproductive success is determined by four main forms of sexual selection, male-male competition, female mate choice, sperm competition and cryptic female choice. Males face each of these mechanisms of sexual selection at different stages during the mating process and each mechanism affects a different aspect of male reproductive success. First and foremost, in order to gain access to receptive females, males often have to engage in agonistic contests with their competitors. Although winning fights may enable males to gain access to females and increase their mating opportunities, it does not necessarily secure them a mate. In most mating systems, females invest a lot more in reproduction than males, and as a result, females tend to be choosy when it comes to deciding who to mate with. Therefore once a male has gained access to a receptive female, there may be a process of assessment before he is accepted or rejected for a mating.

It was traditionally assumed that traits associated with increased fighting success should also be tightly correlated with better mating success, and thus that females should prefer dominant males (Berglund *et al.* 1996; Qvarnström and Forsgren 1998). However, a large body of evidence now demonstrates that while fighting success and mating success can be correlated with one another, this assumption does not always hold true (reviewed in Qvarnström and Forsgren 1998 & Wong and Candolin 2005). In some instances, the same male traits are involved in both processes but the form and/or direction of sexual selection exerted in each case is opposing. For example, in the cockroach *Nauphoeta cinerea*, male pheromone composition is tightly linked to both male dominance status and male mating

success, but females exhibit a preference for the pheromone composition of subordinate males (Moore and Moore 1999), due to fitness costs associated with mating with dominant males (Moore *et al.* 2003). In other cases, female choice acts on traits that have little or no link to fighting success at all, for example in the broad-horned flour beetle *Gnatocerus cornutus* male attractiveness and competitiveness are neither phenotypically nor genetically correlated, with females choosing mates based on their investment in courtship (Okada *et al.* 2014). However, even in these instances where females preferentially mate with subordinates or choose traits unconnected with fighting success, dominant males can still secure a mating advantage over their competitors through force or coercion during male-male competition, even when copulations themselves cannot be forced. Dominant/aggressive males have been seen to override female choice in *N. cinerea* (Moore *et al.* 1995; Moore and Moore 1999; Moore *et al.* 2001) and *G. cornutus* (Harano *et al.* 2010; Yamane *et al.* 2010; Okada *et al.* 2014) along with bitterlings (Reichard *et al.* 2005; Casalini *et al.* 2009), brown trout (Petersson *et al.* 1999) and water striders (Sih *et al.* 2002) (See Wong and Candolin 2005 for a review). Such sexual conflict between male-male competition and female mate choice highlights the complex interplay between these mechanisms of sexual selection and the need to consider both when investigating male reproductive success.

Both male-male competition and female choice can continue to exert selection on males after copulation has occurred. Male-male competition continues in the form of sperm competition and female choice continues as cryptic female choice (I only cover sperm competition in this introduction but see Arnqvist 2014 for a recent review of cryptic female choice). These post-copulatory mechanisms play a major role in generating variation in male

reproductive success when females mate polyandrously (Snook 2005). Sperm competition occurs when the ejaculates of two or more males compete to fertilize a female's ova (Parker 1970), selecting on ejaculate traits that increase a male's competitive fertilisation success (Parker 1998; Wedell *et al.* 2002). According to sperm competition theory, males should adjust their ejaculate expenditure in relation to the risk (the probability of competing with another male's ejaculate – Parker 1970) and intensity (the number of competing ejaculates – Engqvist and Reinhold 2006) of sperm competition. As levels of sperm competition risk and intensity are rarely (if ever) constant between matings, males must be capable of assessing and responding to rapidly changing levels of sperm competition. Consequently sperm competition not only exerts selection on ejaculate traits but on male behavioural plasticity too. Ejaculate expenditure is just one of a suite of plastic traits that can be adjusted in order to maximise male reproductive success under competitive scenarios, other examples including courtship effort, copulation length and mate guarding duration (Bretman *et al.* 2011a). In order to adjust these plastic traits accurately, males must gather information on the risk and intensity of sperm competition from cues in their socio-sexual environment.

Signals and cues provide individuals with an abundance of information. Information is critical at each step throughout the mating process, from the cues used by males to indicate and detect dominance, to the traits of prospective mates assessed by receptive females (i.e. who to mate with) and males (i.e. how much to invest) alike. In this thesis, I focus on the use and importance of cues throughout the mating process. Cues are defined as any feature of the environment (biotic and abiotic), that can be used by an individual to guide their future action, but importantly – unlike a signal – has not evolved for this purpose

(Maynard Smith and Harper 2003, pg 3). Individuals can gain information from a plethora of different cues in their socio-sexual environment, i.e. visual, tactile, acoustic and chemical cues. In this thesis, I aim to investigate the importance of chemical cues, specifically cuticular hydrocarbons (CHCs), in determining male reproductive success. I examine the multifunctional roles played by CHCs during male-male and female-male interactions at different stages of the mating process. In this chapter, I review the current literature surrounding the role of CHCs in sexual communication and outline the aims of the chapters that follow.

1.2 Cuticular hydrocarbons (CHCs)

Cuticular hydrocarbons (CHCs), compounds derived from fatty acids, are found on the outer cuticle of all insects. Individuals produce 30 to 100 hydrocarbons (Ferveur 2005) of differing carbon chain length which together comprise their own distinct CHC profile. The number and type of hydrocarbons expressed by individuals varies from species to species. Furthermore CHC profiles are generally sexually dimorphic, differing between males and females within species either quantitatively (the amount, and relative amounts of CHCs expressed - e.g. The Australian field cricket *Teleogryllus oceanicus* –Thomas and Simmons 2008b) or qualitatively (the type of CHCs expressed - e.g. *Drosophila melanogaster* – Grillet *et al.* 2006) (See Thomas and Simmons 2008b for a comprehensive species list). CHCs play a vital role in desiccation prevention by providing a waterproof barrier (Hadley 1981; Ferveur 2005). Individual CHC profiles can be quantified using gas chromatography (GC), which separates the different hydrocarbons contributing to the overall profile. This technique produces a chromatogram (see fig 1.1), from which the quantity of each compound can be

measured. The chemical composition of individual hydrocarbons can be determined via the addition of Mass Spectrometry (GC-MS), an analytical technique that produces spectra of the masses of the molecules comprising a single compound (in this case a single hydrocarbon molecule), these spectra can then be used to determine the elemental composition of a compound and thus its identity (Wyatt 2003).

Although desiccation prevention is thought to be their primary function, CHCs also play a key role as chemical cues in insect communication (reviewed in - Howard 1993; Ferveur 2005; Blomquist and Bagnères 2010). CHCs can be transferred between individuals at short-range or via contact and are perceived via olfaction (Ferveur 2005). The majority of CHC research centres around the role of CHCs as recognition cues, enabling individuals to identify nestmates, conspecifics and potential mates (see Singer 1998 for a review).This emphasis on CHCs as recognition cues led to the assumption that there is very little intra-species variability in CHCs as recognition cues are selected for stability (Ingleby 2015). However, more recently researchers have begun to investigate the use of CHCs in social interactions and sexual communication, revealing not only that CHCs are highly variable between individuals within a species (in fact CHCs are slowly being recognised as a highly plastic trait – see Ingleby 2015 for a review) but also that CHCs may play a key role in a multitude of social interactions including mating interactions which together determine male reproductive success. These interactions include male-male interactions such as agonistic contests and male-female interactions such as mate assessment.

1.3 CHCs and male-female interactions

1.3.1 Mate choice (female assessment)

The vast majority of mate choice studies have focussed primarily on more conspicuous male sexual traits such as visual, audio and tactile cues. However, studies of chemical traits have increased in recent years and it is now clear that the use of chemical cues in mate choice is widespread (reviewed by Johansson and Jones 2007). Evidence for female mate choice on the basis of male cuticular hydrocarbon profiles however is currently limited to *Drosophila* (Blows 2002; Hine *et al.* 2002; Howard *et al.* 2003; Chenoweth and Blows 2005; Ingleby *et al.* 2014) and crickets (Ivy *et al.* 2005; Kortet and Hedrick 2005; Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2013; Weddle *et al.* 2013; Steiger *et al.* 2015).

Furthermore, only a handful of studies have attempted to quantify the strength and form of sexual selection exerted by female choice on male CHCs, all finding significant evidence of sexual selection. In *Drosophila serrata*, females have been found to exert purely directional selection on male CHC composition (Howard *et al.* 2003; Chenoweth and Blows 2005) while more complicated combinations of linear and nonlinear selection have been displayed in *D. simulans* (directional, quadratic and correlational – Ingleby *et al.* 2014) and *T. oceanicus* (directional and quadratic – Thomas and Simmons 2009c; Simmons *et al.* 2013). Females select not only upon the overall expression of CHCs (i.e the CHC profile as a whole), but also on the expression and relative quantities of specific CHCs, the complexity of this selection reflecting the intricate composition of these multivariate traits.

Although these studies provide good evidence of the importance of CHCs for mating success, they are limited, not only by the fact that they focus on just two groups of insects (*Drosophila* and crickets) but as brought to attention in a recent review (Steiger and Stökl

2014) none of the estimated selection gradients have been verified using experimental manipulation. Multivariate selection analysis is a powerful statistical tool capable of accounting for correlations between measured traits. However, this analysis cannot account for correlations between measured and unmeasured traits, and as it is impossible to measure every trait, this causes a problem. This analysis cannot distinguish between the effects of direct and indirect selection and thus while the selection estimates generated may accurately portray direct selection on the measured traits, they are equally likely to reflect selection on some unmeasured but correlated trait. By using experimental manipulation, it is possible to isolate the effects of specific traits and thus verify previously estimated selection gradients. This is a key step in realising the true influence of CHCs on female mate choice and consequently on male mating success. In chapters 4 and 5, I use a combination of observational and experimental approaches to investigate sexual selection imposed on male CHCs by female mate choice.

1.3.2 Assessing sperm competition risk and female mating status (male assessment)

As well as offering females valuable information about males, CHCs can also inform males about the risk of sperm competition associated with specific females. Males can gain general information on the presence of rivals from several different kinds of cues, for instance *D. melanogaster* males have been shown to rely on a combination of audio, chemical and tactile cues in order to detect rivals (Bretman *et al.* 2011b). However, as mentioned earlier, the risk of sperm competition associated with females is unlikely to be constant across matings and thus males must detect information specific to the females they interact with. Chemical cues can offer males two-fold information regarding both the

local presence of rivals and the mating status of specific females. Chemical cues can be either deliberately deposited by males in order to communicate territory (e.g. scent marking in meadow voles – delBarco-Trillo and Ferkin 2004) or inadvertently left behind on females after physical interactions such as courtship and copulation (Siva-Jothy and Stutt 2002; Andersson *et al.* 2004; Wedell 2005). Residual male CHCs left behind on female cuticles have been shown to have a significant effect on male mating investment and behaviour. For example, male Australian field crickets *T. oceanicus* were found to adjust their ejaculate allocation (measured as sperm viability) in response to the presence of male-derived CHCs on the cuticles of virgin females (Thomas and Simmons 2009a). Males not only adjusted their ejaculate expenditure in response to the presence of these cues but also in relation to the number of individual males contributing to the rival CHCs present on the female (Thomas and Simmons 2009a). Thus male crickets appear to be able to gain information on both sperm competition risk and intensity from the presence of rival male CHCs alone.

One thing that remains unclear however, is what information males are actually acquiring from these rival male cues. The presence of male-derived CHCs may indicate that a female has already mated by mimicking the CHC profile of mated females, as has been suggested in *D. melanogaster*, where experimentally perfuming virgin females with the CHCs of mated females elicited males to mate for longer (Scott 1986; Friberg 2006). Changes to the female chemical profile after mating can be caused by the transfer of male compounds, but also by physiological changes, triggered by copulation, within the female herself (Scott and Jackson 1990; Foster 1993; Karube and Kobayashi 1999). Thus the addition of male-derived CHCs to virgin females may not necessarily mimic the CHC profile of a mated female. Alternatively, the presence of male-derived CHCs may simply make

females “smell” like other males and thus provide more general information concerning the local competitive environment. In chapter 2, I experimentally perfume the CHC profiles of virgin females with rival male CHCs and measure male how males adjust their pre- and post-copulatory investment in response. I then use gas chromatography to examine how our manipulation altered female CHC profile, allowing us to ascertain whether the presence of these male-derived CHCs provide males with specific information regarding female mating status or more general information as to the presence of rivals.

Female insects are able to store sperm for weeks (e.g. Indian meal moth *Plodia interpunctella* – Cook and Gage 1995; *Drosophila melanogaster* – Ala-Honkola *et al.* 2014), months and in extreme cases decades (Hymenopteran species - Hölldobler and Wilson 1990; Tschinkel 1987; den Boer *et al.* 2009) after mating. As a result the heightened risk of sperm competition associated with a mated female persists for far longer than the initial period after mating, meaning it would be beneficial for males to be able to continue to detect and respond to cues of sperm competition for a similar length of time. As chemical cues such as CHCs can persist long after the signaller has gone (from mere seconds [Hölldobler and Wilson 1990] to years [Bordereau and Pasteels 2011]), they may provide males with the means to do so. However, studies investigating the use of chemical cues in the assessment of sperm competition risk have thus far only looked at their short term use, i.e. five minutes to three hours after the female has mated or been chemically manipulated (DelBarco-Trillo and Ferkin 2004; Friberg 2006; Carazo *et al.* 2007; Thomas and Simmons 2009). Thus whether or not these cues continue to provide males with information over time is still to be investigated. In chapter 3, I examine whether rival male CHCs left behind

on the female cuticle continue to provide males with information on sperm competition risk over extended time periods.

1.4 CHCs and male-male interactions

1.4.1 Dominance and fighting success

In species across a diverse range of taxa from cockroaches (Moore *et al.* 1997) to crayfish (Schneider *et al.* 2001) to lizards (Martín *et al.*2007), males possess chemical "badges" of dominance status. Chemical signals of dominance usually reflect dominance status but have also been shown to determine it. For example in a study of *N. cinerea*, male cockroaches that were perfumed with either a dominant or subordinate pheromone composition were not only treated as per their artificial status by other individuals, but they themselves behaved accordingly (Moore *et al.* 1997). Male *N. cinerea* pheromones consist of three highly volatile components which together determine dominance status. Although most research has focussed on these pheromones, dominance status in *N. cinerea* has also been linked to CHC expression. Analysis of CHC extracts from established dominant and subordinate males has revealed quantitative differences in CHC expression between the two social statuses (Roux *et al.* 2002). However, the relative importance of CHCs in signalling dominance (especially in comparison to the well-studied volatile pheromones) has yet to be investigated in this species.

CHC expression has also been linked to dominance in field crickets. Evidence from a study on *Gryllus integer* suggests that the CHC profiles of dominant and subordinate males may differ and moreover that females may prefer the CHCs of subordinate males (Kortet

and Hedrick 2005). However, in this study females were not presented with CHC extracts but rather filter paper that had been walked on by a dominant or subordinate male for 24 hours and thus the link between CHC profile, dominance status and female choice remains arguably speculative in this species. In *T. oceanicus* on the other hand extraction and analysis of male CHCs has demonstrated a dramatic difference in CHC expression between dominant and subordinate males. Subordinate males invest significantly more in attractive CHCs known to increase male mating success than dominant males (Thomas and Simmons 2009b). Because subordinate males exhibit lower ejaculate quality than dominant males, it appears that by investing more in attractive CHCs, subordinates may be increasing their reproductive success via an alternate route, by increasing their chances of obtaining successful matings (Thomas and Simmons 2009b). In a further study, this differential investment in CHC expression was shown to be a plastic trait, with males modifying CHC expression to match changes in their own dominance status (i.e. going from dominant to subordinate after losing a fight) (Thomas and Simmons 2011b). This suggests that males are capable of modifying CHC expression in order to maximise their reproductive success through an alternative mating strategy.

As discussed earlier, dominance and fighting success do not necessarily equate to high mating success. As such the form and direction of sexual selection imposed by male-male competition and female mate choice on male CHCs may not be the same. However, despite evidence of a clear link between male dominance, fighting success and CHC expression, to date no one has investigated the strength and form of sexual selection exerted by male-male competition on male CHCs. In chapter 4, I estimate and formally

compare the strength and form of sexual selection exerted by male-male competition and female choice on male CHCs.

1.4.2 Same-sex sexual behaviour (male-male)

A largely neglected area of study is that of same-sex sexual behaviour (SSB) which describes sexual interactions that occur between individuals of the same sex. SSB occurs in various forms from courtship to mounting to copulation and is widespread across taxa (see Bailey and Zuk 2009 for a review), but particularly common in insects (see supplementary material in Sharf and Martin 2013 for a list of over 100 insect species in which SSB has been observed). Many hypotheses have been proposed to explain the occurrence of SSB, both adaptive and non-adaptive (reviewed in Bailey and Zuk 2009). However, the occurrence of SSB in insects is largely regarded as the result of mistaken identity, the idea that individuals are unable to discriminate clearly between males and females, and thus accidentally exhibit SSB whilst trying to find a mate. But, as demonstrated in the literature cited here, a large proportion of insects are known to possess excellent discriminatory abilities on the basis of chemical cues, specifically CHCs (reviewed in Antony and Jallon 1982; Svensson 1996; Singer 1998; Johansson and Jones 2007). CHCs are largely sexually dimorphic within species, and female hydrocarbons have been found to elicit male mating behaviour even in species lacking sexually dimorphic CHC profiles (Cobb and Jallon 1990; Nemoto *et al.* 1994). Furthermore, individuals of many insect species can detect a vast array of subtle differences in CHC profile, for example *T. oceanicus* individuals are capable of identifying the mating status (Thomas and Simmons 2009a) and even genetic similarity (Thomas and Simmons 2011a) of a potential mate all on the basis of CHCs.

One adaptive hypothesis for the occurrence of SSB is that it acts to intensify or diminish intrasexual aggression and that by carrying out SSB males may be able to increase their mating success relative to that of their competitors (Bailey and Zuk 2009). However evidence for this hypothesis in insects is currently only anecdotal (Preston-Mafham 2006) and it remains unclear who benefits from SSB, whether it is the males carrying out SSB or the males receiving SSB (i.e. being courted by other males). Male parasitoid wasps *Pysttalia concolor* and *D. melanogaster* who receive courtship from other males when young have been shown to subsequently exhibit higher courtship rates and lower copulation latency with females when older, but this behaviour did not result in heightened mating success for these males (Benelli and Canale 2012; Dukas 2010; McRobert and Tompkins 1988). Thus the potential role of SSB as a component of male-male competition and its influence on male reproductive success remains unknown. In chapter 6, I investigate the role of SSB in mediating aggression and examine its effect on the subsequent mating success of males who carry out SSB and the males who receive SSB.

1.5 Study system

The broad-horned flour beetle *Gnatocerus cornutus* is a cosmopolitan stored-product pest, populating food processing facilities and flour mills where they feed on a variety of flours, grains and seeds. Relatively little is known about their habitat in the wild, but there is some evidence to suggest that various flour beetle species lived beneath the bark of trees feedings on fungus and dead plant matter (Linsley 1944). Their invaded environment however is now largely considered to be their natural habitat and is easily replicated under laboratory conditions. Both male-male competition and female mate choice play key roles in

the mating system of *G. cornutus*. Males possess enlarged mandibles which are used as weapons during male-male contests. Males fight for access to females and territories, and success in these fights is determined by mandible size (larger mandibles, higher fighting success – Okada *et al.* 2006). Males who win fights restrict the access of loser males to females and attain a significant mating advantage under competitive scenarios as a result (Harano *et al.* 2010; Okada and Miyatake 2010; Yamane *et al.* 2010; Okada *et al.* 2014). Males who lose fights consequently disperse to new territories and have been found to exhibit a loser effect which lasts for four days after a loss. For these four days males actively avoid engaging in further contests and instead allocate more resources to sperm production (Okada *et al.* 2010; Yamane *et al.* 2010). This response indicates that males are capable of responding to sperm competition risk in this species, but whether males can perceive the risk and intensity of sperm competition in the physical absence of competitors remains unknown.

Although winning fights grants winner males a mating advantage in competitive scenarios, female choice is not linked to fighting success or mandible size but rather to courtship effort (Okada *et al.* 2014). This suggests that male-male competition and female mate choice are not aligned in this species, and moreover that male-male competition can override female choice. Courtship is an essential step in the mating process of *G. cornutus* as males cannot force copulation and females never initiate mating. A male mounts a female and then commences to court her by continuously drumming his tibia along the back of her elytra, a female can then accept a mating by extending her ovipositor or reject it by dislodging the male from her back. Mating only lasts for a few seconds, but courtship can continue for up to and over 10 minutes (SML personal observation), providing ample

opportunity for the transfer of CHCs between the sexes. The majority of research on *G. cornutus* focusses on male fighting behaviour, but males also exhibit high levels of same-sex sexual behaviour (SML personal observation) in which males mount and court one another, mirroring heterosexual courtship behaviour. Despite its prevalence, the occurrence of SSB has yet to be investigated in this species.

1.6 Thesis aims and outline

Using the broad-horned flour beetle *Gnatocerus cornutus*, this thesis aims to clarify the role(s) of cuticular hydrocarbons in informing decisions and behaviours throughout the mating process which ultimately determine male reproductive success. By looking at the role of CHCs at different stages of the mating process, I aim to build a more complete picture of the importance of CHCs in determining male reproductive success in an effort to further our understanding of the multiple roles played by CHCs in sexual communication. Specifically, I begin in chapter 2 by investigating the role of male-derived CHCs left behind on the cuticles of females in informing male perception of sperm competition risk and intensity, measuring how males adjust both their pre- and post-copulatory investment in response to the presence of these cues. Then using gas chromatography I examine how male-derived CHCs change female chemical profile in order to determine what information they provide i.e. do they make females smell mated and thus present information on female mating status, or do they simply make females smell like other males. In chapter 3, I go on to investigate how stable these cues of sperm competition are over time, measuring how long the presence of male-derived CHCs continue to elicit a behavioural response to heightened sperm competition risk. In chapter 4**,** I measure and compare the strength and

form of sexual selection exerted on male CHCs via male-male competition and female mate choice using a multivariate selection analysis. I then attempt to verify the selection gradients estimated in chapter 4 by conducting an experimental manipulation in chapter 5, perfuming random males with the CHC extracts of attractive and unattractive males as identified by their positions on the fitness surface created in chapter 4. Finally, in chapter 6**,** I investigate how same-sex sexual behaviour influences male reproductive success, focussing on its potential role as an extension of male-male competition.

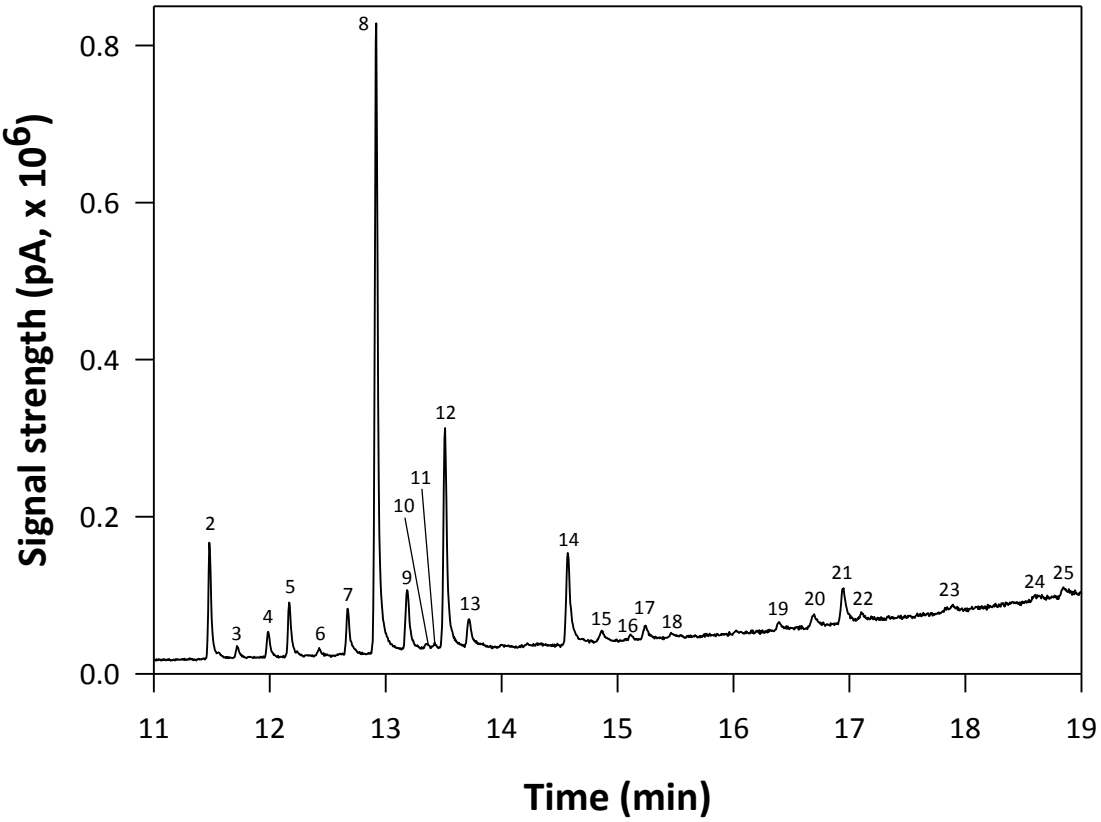

Figure 1.1 A typical example of a chromatogram obtained from the solvent extracted CHCs of a male broad-horned flour beetle *Gnatocerus cornutus*. The *x*-axis shows retention time, indicating the size of the compounds (smaller, more volatile compounds burn off quicker than larger more stable compounds) whilst the *y*-axis shows the signal strength (abundance) of each of these compounds measured in picoamperes.

The following data chapters were created in collaboration with those mentioned in the 'Author's Declaration' section of this thesis. I use the term "we" throughout the data chapters as per publication standard practice and for consistency, it is not intended to suggest that any part of this thesis is not my own work.

CHAPTER 2: RIVAL MALE CHEMICAL CUES EVOKE CHANGES IN MALE PRE- AND POST-COPULATORY INVESTMENT IN A FLOUR BEETLE

2.1 ABSTRACT

Males can gather information on the risk and intensity of sperm competition from their social environment. Recent studies have implicated chemosensory cues, for instance cuticular hydrocarbons (CHCs) in insects, as a key source of this information. Here, using the broad-horned flour beetle (*Gnatocerus cornutus*) we investigated the importance of contact-derived rival male CHCs in informing male perception of sperm competition risk and intensity. We experimentally perfumed virgin females with male CHCs via direct intersexual contact and measured male pre- and post-copulatory investment in response to this manipulation. Using chemical analysis we verified that this treatment engendered changes to perfumed female CHC profiles, but did not make perfumed females 'smell' mated. Despite this, males responded to these chemical changes. Males increased courtship effort under low levels of perceived competition (from 1-3 rivals), but significantly decreased courtship effort as perceived competition rose (from 3-5 rivals). Furthermore our measurement of ejaculate investment showed that males allocated significantly more sperm to perfumed females than to control females. Together, these results suggest that changes in female chemical profile elicited by contact with rival males do not provide males with information on female mating status, but rather inform males of the presence of rivals within the population and thus provide a means for males to indirectly assess the risk of sperm competition.

2.2 INTRODUCTION

Sperm competition occurs when sperm from two or more males compete within the female genital tract to fertilize a female's ova (Parker 1970). There is good evidence that the relative number of sperm represented within the female is an important determinate of success in sperm competition (Wedell *et al.* 2002; Kelly and Jennions 2011; Parker and Pizzari 2010). However, males cannot always produce limitless supplies of sperm as sperm production can be costly (Wedell *et al.* 2002). Furthermore, the energetic costs associated with sperm production (Olsson *et al.* 1997) are expected to trade off with other aspects of reproduction such as obtaining a mate or investing in future reproductive events (Liljedal *et al.* 1999; Fitzpatrick *et al.* 2012; Parker *et al.* 2013). Consequently, males should adjust their ejaculate investment according to the benefit accrued from a mating and the risk (the probability that sperm from different ejaculates will compete - Parker 1970) and intensity (the number of competing ejaculates - Engqvist and Reinhold 2006) of sperm competition. Under this scenario, males should maximize ejaculate expenditure when under sperm competition risk but actually decrease ejaculate expenditure as the intensity of sperm competition increases beyond one competing ejaculate and the benefits of investing more diminish (Engqvist and Reinhold 2006).

A male's ability to respond to changes in sperm competition risk and intensity is entirely dependent on his ability to gather information to assess this risk and intensity accurately (Parker *et al.* 1997). Males can acquire such information from a variety of cues in their socio-sexual environment (e.g. visual - presence of rival males during mating *Drosophila pseudoobscura* [Price *et al.* 2012], Mediterranean fruit flies *Ceratitis capitata* [Gage 1991]; acoustic - male song in crickets *Teleogryllus oceanicus* [Gray and Simmons

2013]; Tactile - *Drosophila melanogaster* [Bretman *et al.* 2011b]) and recent empirical evidence illustrates that males rely on these cues, often in combination (Bretman *et al.* 2011b; Thomas 2011). However, not all cues provide an equal breadth of information. Visual, audio and tactile cues for instance can reliably indicate the local presence of competitors but denote nothing about a female's mating status. Chemosensory cues on the other hand offer males a two-fold insight into the risk and intensity of sperm competition (e.g. Carazo *et al.* 2004; Garbaczewska *et al.* 2013; Sirot *et al.* 2011). Males of many species use olfactory cues in the form of scent marking to communicate their presence to rival males (e.g. Meadow voles, delBarco-Trillo and Ferkin 2004). Simultaneously, courtship and copulation can elicit changes in a female's chemical profile, changes that can be triggered by the transfer of male-derived chemicals (Andersson *et al.* 2004; Siva-Jothy and Stutt 2002; Wedell 2005) or through physiological mechanisms within the female herself (Scott and Jackson 1990; Foster 1993; Karube and Kobayashi 1999). Therefore unlike the aforementioned cues, chemical cues facilitate both the detection of competitors and the assessment of female mating status.

In insects, cuticular hydrocarbons (CHCs), semiochemicals transferred directly from male to female via contact, have been shown to elicit behavioural responses to sperm competition risk. In the fruit fly, *Drosophila melanogaster,* experimentally perfuming virgin females with the CHCs of mated females induced males to mate for longer (Scott 1986; Friberg 2006). Furthermore male Australian field crickets (*Teleogryllus oceanicus*) have been shown to distinguish between the individual profiles of rival males left behind on the female cuticle in order to detect both the risk and intensity of sperm competition (Thomas and Simmons 2009a). These studies implicate the importance of CHCs as cues of female mating

status and therefore the risk and intensity of sperm competition. However, research to investigate how CHCs may select on male reproductive traits is limited to species in the genus *Drosophila* and *Teleogryllus*. Therefore further studies across a wider number of taxa are required to determine whether male responsiveness to contact-derived CHCs is a general phenomenon that will drive the evolution of male sexual traits.

Females of the broad-horned flour beetle *Gnatocerus cornutus* exhibit moderate levels of polyandry and repeated mating in populations maintained at an equal sex ratio (*CMH* unpublished data). Highly aggressive males limit the access of loser males to females through male-male competition, repeated mating with the same female and extended periods of post-copulatory mate guarding (*CMH* unpublished data). Previous studies have shown that males who lose fights become less aggressive and increase their investment in ejaculates for four days after a fight (Okada *et al.* 2010), a response to relative social competitiveness which has also been shown in birds (Pizzari *et al.* 2007).This response indicates that males of this species respond to sperm competition risk, but it is unknown whether males can perceive the risk and intensity of sperm competition in the physical absence of local competitors. *G. cornutus* exhibit a highly tactile form of courtship, in which the male mounts the female and stimulates her, drumming his tibia along her abdomen until she allows him to mate with her. Such tactile courtship can last for over 10 minutes (SML *personal observation*), which may provide an opportunity for the exchange of CHCs. Thus, there is the potential for contact-derived semiochemicals to elicit changes in the female chemical profile and provide information on the risk and intensity of sperm competition in *G. cornutus.*

Here we manipulated the chemical profile of virgin females by facilitating contact between males and females to investigate whether contact-derived male CHCs retained on female cuticles influence pre-copulatory (courtship effort) and post-copulatory (ejaculate expenditure) male investment. First we tested the hypotheses that males can assess sperm competition risk and intensity from chemical cues of female mating status and invest most in courtship when the risk of sperm competition is high and least with increased intensity of sperm competition. Next, we tested the hypothesis that males respond to the risk of sperm competition perceived via these chemical cues by allocating more sperm to an ejaculate. Finally, using Gas Chromatography-Mass Spectrometry (GC-MS) we tested whether perfuming virgin females with the CHCs of males changed their chemical profiles such that these females were more chemically similar to mated females and thus whether rival male CHCs provide a cue of female mating status.

2.3 MATERIAL AND METHODS

Stock populations and rearing protocols

G. cornutus are a stored product pest that feed on a variety of grains, flours, yeasts and dry animal products (Linsley 1944; Zakladoni and Ratanova 1987) so replicating their natural environment is easy. Beetles used in this study were taken from stock populations of *G. cornutus* derived from the Japanese National Food Research Institute (NFRI), at which beetle cultures have been maintained for over 50 years (see Okada *et al.* 2006 for details of origin and culture conditions). In our laboratory in the UK, we replicated these culture conditions closely. In brief, mixed sex populations have been maintained since 2012 in pots (Thermoscientific Nalgene 500mL, 120mm OD) containing 50 individuals. These stock

populations are reared on wholemeal flour enriched with 5% yeast and incubated at 27°c with 60% humidity on a 14L: 10D lighting cycle (Okada *et al.* 2006). Every 3 – 4 weeks, a random selection of final instar larvae are randomly removed from each stock pot (n = 18) and placed into six 24-well plates as pupation is inhibited at moderate to high larval density (Tsuda and Yoshida 1985). At eclosion, 25 male and 25 female adults are randomly selected to form the parents of the next generation.

Preliminary investigations

During our preliminary investigations, we conducted 2 hour observations of small, equal sex-ratio populations (No. of populations= 59; n= 4♀ and ♂4 per population; Total N = 236♀ and 236♂) of uniquely marked males and females that were held in close proximity (mating/fighting arenas). We recorded the number of mates acquired by females and males and the number of male-male agonistic contests. Average female mating success (i.e. mating with different males) was 1.01 with a variance of 1.68 compared to males whose average mating success was 1.21 with a variance of 2.51 (CMH unpublished data, calculated according to Shuster and Wade 2003). During this time period, males repeatedly mated with the same female up to 8 times (mean = 2.73), which is likely to dilute or displace rival males sperm. Models of sperm competition integrate the patterns of male sperm precedence and the probability that a female will re-mate with another male (Engqvist and Reinhold 2006). However, in this system a female will engage in polyandry as well as repeated mating with the same male which should influence the numerical representation of rival sperm in the female sperm storage organs. Thus, it is unclear when male *G. cornutus* should perceive a risk of sperm competition after a female has mated one or more times.

Experimental animals

We collected final instar larvae from lab stocks daily and placed them into 24-well plates until eclosion. The day after eclosion, we transferred adults into single sex 24-well plates to prevent interactions between conspecifics. The lids of the male-only 24-well plates were secured with masking tape to prevent tactile and visual contact between males which have previously been shown to influence investment in ejaculates (Okada *et al.* 2010). All adults were provided with ad libitum wholemeal flour and maintained as described above.

Experiment 1: Pre-copulatory investment

To determine the potential for males to detect cues about the risk and intensity of sperm competition from CHCs transferred from males to females via contact, we perfumed 17 day old virgin females by vortexing them either alone (control), or with 1, 3 or 5 virgin males. Females were placed into Eppendorf tubes (1.5mL) containing the males and vortexed for 30 seconds on a low setting, facilitating contact and CHC transfer between the sexes whilst preventing courtship and copulation. The males used during vortexing were discarded immediately after and were not used in subsequent mating trials. Thirty minutes after vortexing, we paired the vortexed females with random virgin males of the same age and recorded the number of times the males courted with them during a 40 minute observation period. These observations continued for the whole 40 minutes, even if mating occurred, as a male will continue to court the same female even after he has successfully mated with her (CMH personal observation).

Statistical analyses

To analyse the effect of perfuming treatment on male courtship effort, we conducted a generalised linear model. Because courtship effort was not normally distributed and highly overdispersed, we used a quasi-Poisson error family in our model which allowed us to account for this overdispersion. To further investigate the effect of treatment, we conducted multiple post-hoc comparisons between the four treatments (control, one male, three males, five males) using a Tukey's HSD test.

Experiment 2: Post-copulatory investment and re-mating rate

(a) Perfuming and mating trials

To investigate the effect of the presence of male-derived CHCs on ejaculate investment, we assigned virgin females to one of three treatments - control, sham or perfumed – six days after eclosion (as above). On day 17, perfumed females were individually placed into Eppendorf tubes (1.5mL) containing 3 random virgin males of the same age. These beetles were then vortexed for 30 seconds, 30 minutes before mating (as described above) and separated immediately afterwards. Once again males used as a source of chemical cues were not used for the mating trials. Sham females were used to investigate the effects of vortexing per se on mating behaviour and were vortexed alone for 30 seconds.

Previous studies have shown that virgin males produce significantly larger ejaculates than mated males (Svärd and Wiklund 1986; also see Torres-Vila and Jennions 2005 for a review) and so to eradicate first-mating effects on ejaculate size and content, all males in our study were singly mated to a random non-focal virgin female 20 minutes prior to their focal mating (after which time, males were receptive to re-mating – SML *personal*

observation). Females used in this first mating were frozen and discarded. For their focal mating we paired males with a female from one of the three treatments outlined above. To control for any potential effects of female quality on ejaculate allocation, female age was standardised across all matings and all females were randomly allocated to males. We held pairs in a mating arena and observed them for 45 minutes. Focal pairs who failed to mate were discarded from the experiment. We continued to conduct mating trials until we had obtained 40 successful focal matings for each of our treatments. Unsuccessful matings were recorded and later analysed to examine the effect of treatment on re-mating rate. If copulation occurred, females from this second mating were removed and kept individually at 27°C for 4 hours. This allowed adequate time for the sperm to travel up the reproductive tract to the spermatheca (SML *personal observation*), before the experimental females were frozen at -20°C. If a pair failed to mate within 45 minutes they were removed from the trial and discarded from the experiment. Twice mated males were frozen for subsequent body measurement, whereas males who failed to re-mate were discarded from the experiment. We captured digital images of the dorsal view of the males' bodies using a Leica M125 microscope with mounted camera (Leica DFC295, Leica microsystems Ltd. CH-9435 Heerbrugg) that conveyed images to a PC. We measured the width of the pronotum (to the nearest 0.01mm) as an index of body size (Okada *et al.* 2006) using Image J (version 1.46r). We measured each pronotum twice to calculate the repeatability of this measure based on the variance components derived from an analysis of variance (Lessells and Boag 1987), showing high repeatability ($F_{24,25} = 120.33$, $r = 0.992 \pm 0.0034$, $P < 0.001$).

(b) Measuring ejaculate investment

Twenty four hours after being frozen, females were removed from the freezer for dissection. We placed each female directly onto a fresh microscope slide, abdomen facing upwards. Using two pairs of fine watchmaker's forceps, we gently squeezed the female's abdomen and carefully grasped and pulled out the reproductive tract. Removing all other tissue from the slide, we carefully separated the spermatheca from the surrounding reproductive tissue. We added 10µl of deionised water to the centre of the slide (away from the dissection area to avoid contamination of the sample), crushed the spermatheca between the forceps and placed it directly into the droplet. We stirred the sample to prevent the sperm clumping and drew a circle around the drop to aid identification of the area under high magnification. After leaving the sample to air-dry fully, we recorded total sperm count using an Olympus BX61 microscope (Olympus Corporation, Tokyo, Japan) under phase contrast at 20x magnification. *G. cornutus* produce relatively small ejaculates of <2000 sperm making full counts possible. We thus performed sperm counts manually and the repeatability of counts of the same ejaculate was measured as described above, showing high repeatability ($F_{6,7}$ = 652.464, r = 0.997±0.0012, P < 0.001). All sperm counts were performed blind by the same person throughout.

Statistical analyses

To analyse the effect of treatment on re-mating rate, we conducted a GLM with a binomial error family, giving individual males a binary score of either 1 or 0 to represent their success

or failure to re-mate, respectively. Body size data and re-mating data for sham females was not available for this analysis and thus was not included. All statistical analyses were carried out using R (version 2.12.0).

To analyse the effect of treatment and vortexing per se on the total number of sperm transferred, we first removed all females to which no sperm had been transferred, classing these as unsuccessful matings. We then conducted a generalised linear model on the remaining data from all three treatment groups. As sperm number was not normally distributed and highly over-dispersed we used a quasi-Poisson error family which allowed us to account for this over-dispersion in our model. Next we conducted a separate analysis to control for the potentially confounding effect of body size on sperm number, including pronotum width as a covariate and examined the interactions between body size and treatment. We were unable to include our sham group in this analysis as we did not have body size data for this group of females.

Experiment 3: GC-MS analysis of experimental male and female CHC extracts

To investigate the effects of our experimental perfuming treatment on female CHC profile, we analysed the CHC profiles of an additional subset of control and perfumed virgin females (generated as above, i.e. perfumed with 3 males but not used in the behavioural assays) along with a set of mated females, virgin males and mated males using Gas Chromatography-Mass Spectrometry (GC-MS). All beetles used for GC-MS analysis were stored at -20°c for two months before the commencement of CHC extraction. Firstly, we randomised samples prior to the CHC extraction process to avoid any bias caused by column

degradation during GC-MS. Next, we extracted cuticular hydrocarbons from individuals by full-body immersion in 50µl of HPLC-grade hexane with 10ppm pentadecane as an internal standard. Individual beetles were left to soak for 5 minutes and during the last minute each sample was vortexed to maximise CHC extraction. After 5 minutes we removed the beetle from the vial using metal forceps which we cleaned in pure hexane between each sample to avoid contamination. 2µl of the extracted CHC sample was injected into a GC-MS (Agilent 7890A Gas Chromatograph coupled with an Agilent 5975B Mass Spectrometer and an Agilent CTC PAL Autosampler chilled to 5 °C, Agilent Technologies, Cheshire, UK) fitted with a DB1-MS column (30 m x 0.25 mm ID x 0.25um film thickness) using helium as the carrier gas. The inlet and MS transfer line were set at 250 and 300 °C, respectively, and the injection was run in the pulsed splitless mode. The GC oven temperature profile started at 100°C for 1 minute, ramping at 20°C/min to 250°C, then 5°C/minute to 320°C. GC extraction methods were uniquely designed to optimise chemical separation for *G. cornutus* on the instrument in use and thus the methods described here were uniquely designed for this study. Peaks were quantified using MSD Chemstation software (Agilent Technologies, version E.02.00.493), using ion 57 as the target ion to quantify the abundance of each CHC compound. Methyl-branched alkanes were identified by their mass spectra (Nelson *et al.* 1972), and the identities of the peaks were confirmed using retention indices (Francis and Veland 1981) which were calculated by running a straight-chain alkane standard that contained all alkanes from C_7 to C_{40}. The positions of double bonds in unsaturated hydrocarbons were determined by interpreting the mass spectra of the dimethyl disulphide derivatives (DMDS). In brief, treating unsaturated hydrocarbons with dimethyl disulphide removes C=C bonds, creating a weak point in the molecule which is cleaved to produce two characteristic fragments. The size of these fragments can then be used to determine the

position of the double bond and thus identify the compound (see Nelson *et al.* 1972; Buser *et al.* 1983 for more details).

Statistical analyses

GC-MS analysis identified 24 individual CHC peaks, producing quantitative data on all 24 compounds. To calculate the concentration of each compound, the area of each peak was divided by the area of the internal standard peak (peak 1) and the resulting data was log10 transformed. This allowed us to look at the variation between individual beetle's CHC profiles as variation in this species is quantitative not qualitative (i.e. all individuals possess the 24 identified CHC compounds but in varying amounts).We then used discriminate function analyses (DFA) in order to obtain a reduced number of functions which capture and describe the between-group variation in CHC profiles. We conducted two separate DFA analyses in order to test two separate predictions 1.) CHC profiles of perfumed females were chemically similar to those of mated females and 2.) CHC profiles of perfumed females were more chemically similar to virgin males (with whom they were perfumed) than mated males. All data analysis was conducted using SPSS (version 20).

2.4 RESULTS

Experiment 1: Pre-copulatory investment

Our analyses showed that contact-derived male CHCs retained on female cuticles had a significant effect on courtship effort ($F_{3,115}$ = 3.096, P=0.03). Multiple post-hoc comparisons

revealed that males courted females who had been vortexed with 5 males significantly less than females who had been vortexed with 3 males (P=0.025; see fig 2.1). Despite an increase in courtship effort between the control, 1 male and 3 male perfuming treatments, the difference was non-significant between these groups (fig 2.1). Nonetheless, males were, on average, most responsive to females who had been vortexed with three males and consequently we used this perfuming treatment in our second experiment.

Experiment 2: Post-copulatory investment and re-mating rate

Treatment had a significant effect on re-mating rate (χ^2= 5.48, P=0.019). 42% of males failed to re-mate in the perfumed group compared with 22% in the control group. This result suggests that either males less readily mated with perfumed females or perfumed females less readily allowed males to mate with them, but as we did not measure courtship effort in this second experiment we are unable to determine which.

The number of sperm transferred to females differed significantly across the treatments ($F_{2,92}$= 5.86, P= 0.004). Post-hoc analyses showed that males transferred significantly more sperm to perfumed females than to sham and control females ($F_{1,93}$= 11.64, P= 0.00096) (see Figure 2.2). There was no significant difference in the number of sperm transferred to control females and sham females ($F_{1,93}$= 0.14, P= 0.71). In our control and perfumed females, there was no significant interaction between body size (measured here as pronotum width) and treatment ($F_{1,67}$= 2.62, P= 0.11). However, body size had a significant effect on the number of sperm transferred ($F_{1,68}$= 5.05, P= 0.03), with larger males transferring more sperm.

Experiment 3: GC-MS analyses of experimental male and female CHC extracts

Male and female hydrocarbon profiles were comprised of a mixture of straight-chained alkanes, mono- and di-methyl alkanes, and alkenes ranging from 25 to 33 hydrocarbons in length (see table 2.1 for more details). Our first DFA examined the variation in the CHC profiles of our three groups of females - control females, perfumed females and mated females – and produced two functions that together explained 100% of the between-group variation in CHCs. Estimates based on generalised cross-validation values showed that the predictive model correctly classified groups with 70.6% success.

Function 1 explained 98.9% of the variance in CHCs (canonical r^2= 0.98), discriminating mated females from both control and perfumed females (see figure 2.3a and table 2.2). Examination of the factor loadings for each of the 24 CHC peaks indicated that this discrimination was due to the amount of pentacosane, 11-methylpentacosane and 11-methylhexacosane (peaks 2, 3 and 6 respectively). Loading factors of 0.25 or higher were interpreted as significant (Tabachnick and Fidell 1989). Function 2 described a further 1.1% of the variance in CHCs (canonical r^2= 0.36), distinguishing control females from perfumed and mated females. Examination of the factor loadings showed that function 2 was positively loaded to nonacosane, 3-methylnonacosane and 3-methylhentriacontane (peaks 14, 17 and 22 respectively) whilst also being negatively loaded to 5-hexacosane and 13-methylnonacosane (peaks 7 and 15 respectively). This analysis indicates that perfumed females separate slightly from our control group but overall, the CHC profiles of mated females are very different from those of the control and perfumed females. Thus, whilst our

perfuming treatment altered the CHC profile of perfumed females, it did not make them more chemically similar to mated females than control females.

Our second DFA examined the variation in CHC profiles between perfumed females, virgin males and mated males. This DFA produced two functions that together described 100% of the between group variation in CHCs. Estimates based on generalised cross-validation values show that the predictive model correctly classified groups with 97.3% success. Function 1 explained 88.4% of the variance in CHCs (canonical r^2=0.98), discriminating both virgin groups (perfumed females and virgin males) from mated males (see figure 2.3b and table 2.2). This separation was predominantly due to pentacosane (peak 2) to which function 2 was positively loaded. Function 2 explained 11.6% of the variance in CHCs and separated perfumed females from both virgin and mated males (canonical r^2= 0.86). Examination of the factor loadings showed that this discrimination was due to amounts of 5-hexacosane (peak 7) which was negatively loaded to function 2.

2.5 DISCUSSION

Our findings indicate that male *G. cornutus* are able to detect the local risk and intensity of sperm competition from chemical cues transferred between males and females during contact, as well as through physical interactions with rival males as has been shown previously (Okada *et al.* 2010). We found that we were able to experimentally alter the CHC profile of virgin females through direct intersexual contact which mimicked the tactile courtship of this species and altered the relative abundance of several hydrocarbons. In accordance with our predictions we found that males initially increased courtship effort when under risk of competition but decreased their investment significantly as the number

of rivals rose above 3, suggesting that males are sensitive to cues of both sperm competition risk and intensity. During post-copulatory investment, males responded to perfuming by significantly increasing their ejaculate expenditure when mating with perfumed females in comparison to control females, even though the chemical profile of perfumed females was not more chemically similar to mated females. An increase in ejaculate expenditure should increase a male's probability of achieving fertilisation (Parker 1990; Parker *et al.* 1997) however, more work is needed to demonstrate that this increase in sperm number in *G. cornutus* is adaptive. Furthermore it is important to note that our measure of ejaculate investment in this study (sperm counts from the spermatheca) as in other studies (Okada *et al.* 2010; Yamane *et al.* 2010) is only a proxy of ejaculate investment. Sperm utilisation and storage can also be affected by female-driven factors (i.e. *Tribolium casteneum;* Edvardsson and Arnqvist 2000 and field crickets; Bretman *et al.* 2009) and whilst we are not aware of any such factors in *G. cornutus*, it is possible that our measure of ejaculate expenditure is reflective not just of patterns of male sperm allocation but female sperm utilisation also.

Contrary to our initial prediction, comparison of the CHC profiles of females perfumed with 3 rival males, control females and mated females revealed that perfuming did not make females 'smell' mated and therefore it is clear that our experimental males were not responding to cues about females mating status. Instead, these results raise an interesting possibility that male-derived CHCs retained on the female cuticle may provide information about the presence and density of rival males within the population and thus offer males a way to indirectly assess sperm competition risk and intensity. Specifically, our data suggests that males adjust their pre- and post-copulatory reproductive investment (i.e. courtship effort and ejaculate investment) in response to the risk and intensity of sperm

competition that is detected from either the overall concentration of CHCs or the number of males' CHCs present - we cannot say which with certainty. These results mirror evidence from Weir *et al.*'s 2011 meta-analysis in which an increase in OSR (operational sex ratio) bias lead to a decrease in male courtship rate and aggression but an increase in copulation duration and mate guarding, further supporting the idea that males may be able to assess rival density using these chemical cues. Our results are also similar to previous studies that have directly illustrated male use of chemical cues to assess female mating status (Carazo *et al.* 2004; Friberg 2006) and sperm competition risk (Carazo *et al.* 2007; Friberg 2006; Thomas and Simmons 2009a; Garbaczewska *et al.* 2013;). However, to our knowledge, this is the first study to explicitly show that the presence of rival male chemical cues present on the cuticle of virgin females can elicit a behavioural response in males even though these cues do not make virgin females 'smell' mated.

A general prediction from sperm competition models is that males are expected to allocate more sperm when mating with a virgin (Engqvist and Reinhold 2006) or in the presence of a single competitor, which is well supported empirically (Kelly and Jennions 2011). Our results do not conform exactly to these sperm competition models as by virtue of our experimental design, males responded to the chemical cues of three rival males not one as these models simulate. However, the results of experiment 1 indicate that male *G. cornutus* do not perceive a risk of sperm competition in the presence of a single competitor. In this species, it is possible that males lack the sensory apparatus to detect the chemical signature of a single rival or perhaps the tendency of males to repeatedly mate with the same female is sufficient to dilute or displace the sperm of a single rival male and therefore a single competing ejaculate does not constitute a 'risk'. Male field crickets have been

shown to be able to detect the exact number of different male CHC profiles present on a female and to adjust their ejaculate in response (Thomas and Simmons 2009a).Here, the results of experiment 1 suggest that *G. cornutus* males are similarly sensitive to either the overall concentration of CHCs or the number of distinct male profiles present, but further investigation is needed. If males of this species are indeed able to distinguish between individual male profiles, this should select on sensory organs that detect unique CHCs and plasticity in ejaculate expenditure, especially if males are able to gain information about the age and quality of rivals from their CHCs alone.

Despite their potential importance, the role of chemical cues in shaping male perception of sperm competition risk is unknown, with the notable exceptions of studies in *Drosophila* (Friberg 2006) and a field cricket (Thomas and Simmons 2009a). The aforementioned studies implicate (Friberg 2006) or have shown (Thomas and Simmons 2009a) the importance of CHCs as a key source of socio-sexual information for male sperm competition assessment, consistent with our findings. More generally there is growing evidence that CHCs transferred via contact are a key source of socio-sexual information for both sexes. Empirical studies of *Nauphoeta cinerea* (Harris and Moore 2005), *Drosophila melanogaster* (Scott 1986; Scott *et al.* 1988) and *Gryllodes sigillatus* (Weddle *et al.* 2013) indicate that males and females use contact-derived CHCs transferred during socio-sexual interactions to inform their mating choices. For example, female N. cinerea preferentially mate with males who bear the epicuticular rubbing of a single female over those who bear the rubbings of multiple females (Harris and Moore 2005), appearing to use this information to avoid mating with sperm-depleted males. Behavioural assays in *D. melanogaster* show that sex-specific CHCs transferred during mating in this species, act as antiaphrodisiacs

when present on the reciprocal sex. These antiaphrodisiacs confer potential fitness benefits by reducing the chances of mating with an already mated female or a sperm depleted male (Scott 1986; Scott *et al*. 1988). Finally, female *G. sigillatus* actively avoid mating with males that bear their own CHC profile, facilitating the avoidance of mating again with a previous mate (Weddle *et al.* 2013).

The detection of a rival male's CHCs prior to mating is likely to have important consequences for the evolution of traits used during sperm competition. Whenever the environment provided by one individual influences the phenotype of another and variation in this environment reflects (at least in part) genetic differences between individuals, then indirect genetic effects (IGEs) will exist and the environment will be heritable (Wolf *et al.* 1998). In theory, IGEs can have a number of widespread implications for the evolution of phenotypic traits, including biasing the rate and direction of evolutionary change, generating evolutionary time lags in the response to selection and enabling phenotypic traits to evolve in the complete absence (or reduced levels) of additive genetic variance (Wolf *et al.* 1998). Moreover, IGEs may also play a central role in the maintenance of genetic variance in traits subject to strong selection (Miller and Moore 2007). It is possible that the CHCs transferred to females by rival males during mating may represent an IGE and hold important implications for the evolution of ejaculate characteristics in *G. cornutus*. Although we currently do not know the genetic basis of male CHCs in *G. cornutus*, CHCs are known to be heritable in a variety of other terrestrial arthropods (e.g. Hine *et al*. 2004; Thomas and Simmons 2008a; Ingleby *et al.* 2013; Weddle *et al.* 2013) including beetles (Yezerski *et al.* 2004) and our current study shows that males are able to adjust the number of sperm in their ejaculates in response to the CHCs transferred at mating by rival males. What we do

not know, however, is whether this male response varies with the genotype of rival males. Work on the field cricket (*Teleogryllus oceanicus*) suggests that IGEs between competing males engaging sperm competition is indeed possible and can have important consequences for male reproductive success (Garcia-Gonzalez and Simmons 2007). More work is needed, however, to demonstrate the existence of IGEs in *G. cornutus* and this is the focus of our current research.

To adjust their ejaculate expenditure in response to the risk and intensity of sperm competition, males must gather information to accurately assess these states (Parker *et al.* 1997; Parker *et al.* 2013). Our research demonstrates that males are able to indirectly assess sperm competition risk and intensity from rival male CHCs derived from contact and retained on the female cuticle. We show that these chemical cues do not provide males with information about female mating status, but rather appear to equip males with information on the presence and perhaps density of rivals within their mating environment, and this information alone elicits an increase in reproductive investment.

Table 2.1 Chemical characterization of male CHCs in *Gnatocerus cornutus*. KRI: Kovats Retention Index for each chemical compound, DMDS: diagnostic ions used for compound identification after derivation with dimethyl disulphide.

Peak	KRI	Compound	Diagnostic ions
2	2495	C_{25}	352
3	2531	11-MeC_{25}	168, 227, 351
4	2568	3-MeC_{25}	337, 57, 351
5	2594	C_{26}	366
6	2629	11-MeC_{26}	168, 238, 365
7	2661	5-C_{26}-ene	DMDS: 458, 117, 341
8	2693	C_{27}	380
9	2728	11-MeC_{27}	168, 252
10	2748	Unknown	
11	2759	11,15-diMeC_{27}	267, 168, 197, 239
12	2769	3-MeC_{27}	365, 57
13	2794	C_{28}	394
14	2894	C_{29}	408
15	2927	13-MeC_{29}	252, 196
16	2956	11,15-diMeC_{29}	295, 168, 224, 239
17	2969	3-MeC_{29}	57, 393
18	2993	C_{30}	422
19	3093	C_{31}	436
20	3126	15-MeC_{31}	224, 252
21	3152	3,19, 3,17-diMeC_{31}	196, 224, 267, 295, 435
22	3169	3-MeC_{31}	57, 421
23	3250	4,12-diMeC_{31}	435, 71, 309, 197
24	3325	11-MeC_{33}	169, 337, 225, 281
25	3349	15,17-diMeC_{33}	295, 225, 253, 267

Table 2.2. Unstandardized canonical discriminant functions evaluated at group centroids, which represent the averages. Positive values are highlighted to show which treatments are distinguished between at each function.

Discriminant Analysis 1		
Treatment	**Function**	
	1	**2**
Control females	3.983	**-0.881**
Perfumed females	4.391	0.858
Mated females	**-11.551**	0.031
Discriminant Analysis 2		
Treatment	**Function**	
	1	**2**
Perfumed females	-4.207	**2.886**
Virgin males	-4.478	-2.906
Mated males	**10.392**	-0.064

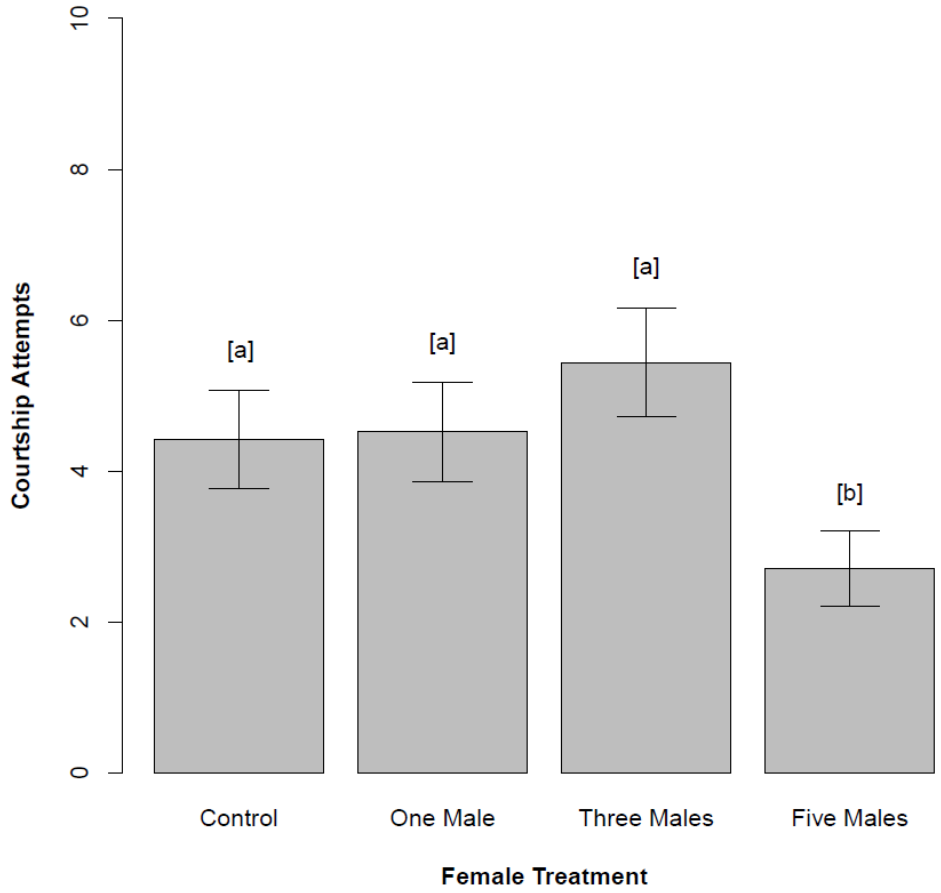

Figure 2.1. Mean (±SE) number of courtship attempts by males to females of each treatment group in experiment 1. Different letters indicate a significant difference, males courted significantly less with females perfumed with 5 males compared to females perfumed with 3 males (*P* =0.025).

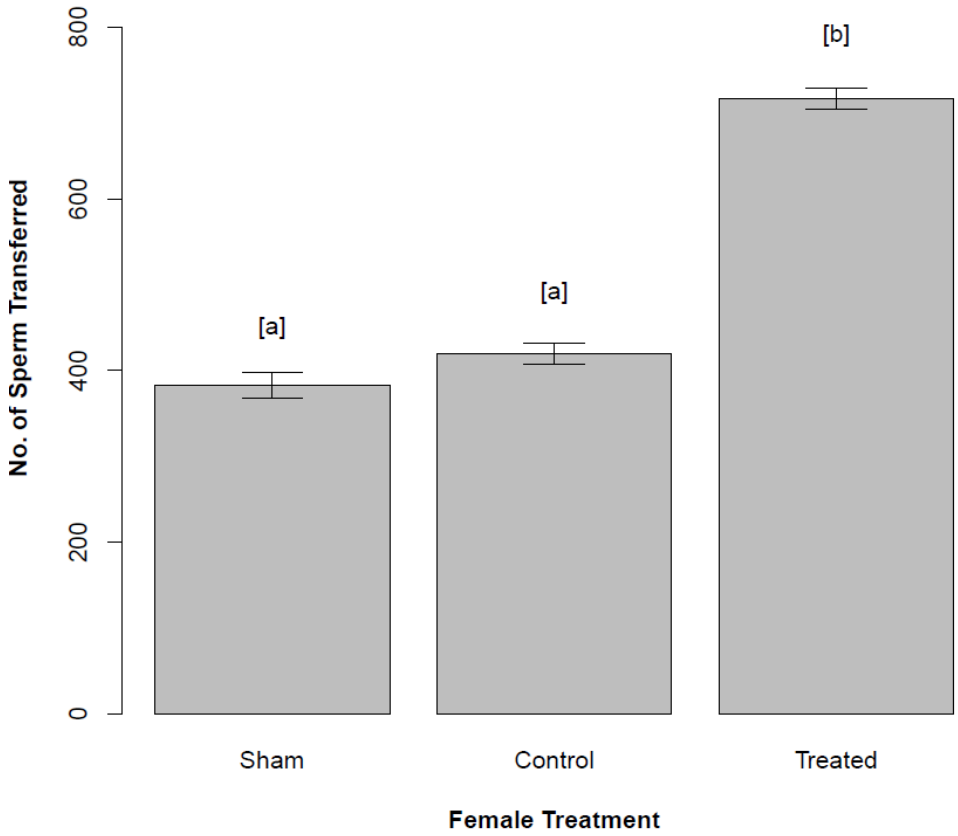

Figure 2.2. Mean (±SE) number of sperm transferred by males to females of each treatment group in experiment 2. Different letters indicate a significant difference, males transferred significantly more sperm to females in the perfumed treatment *(P* <0.001).

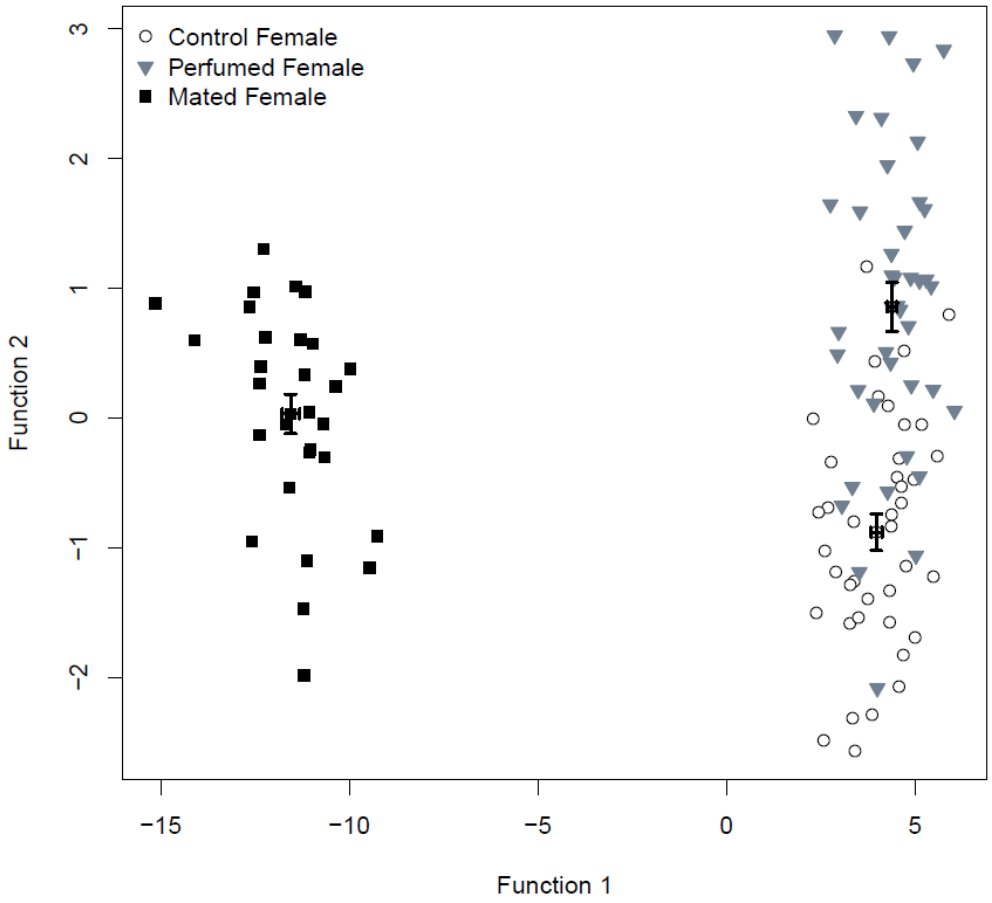

Figure 2.3a. Combined-groups plot showing functions 1 and 2 derived from the discriminant function analysis of control, perfumed and mated females. Function 1 explains 98.9% of between-group variance, separating mated females and both groups of virgin females. Function 2 explains 1.1% of the variance, discriminating control females from perfumed and mated females. Centroids represent the averages and standard errors of each treatment.

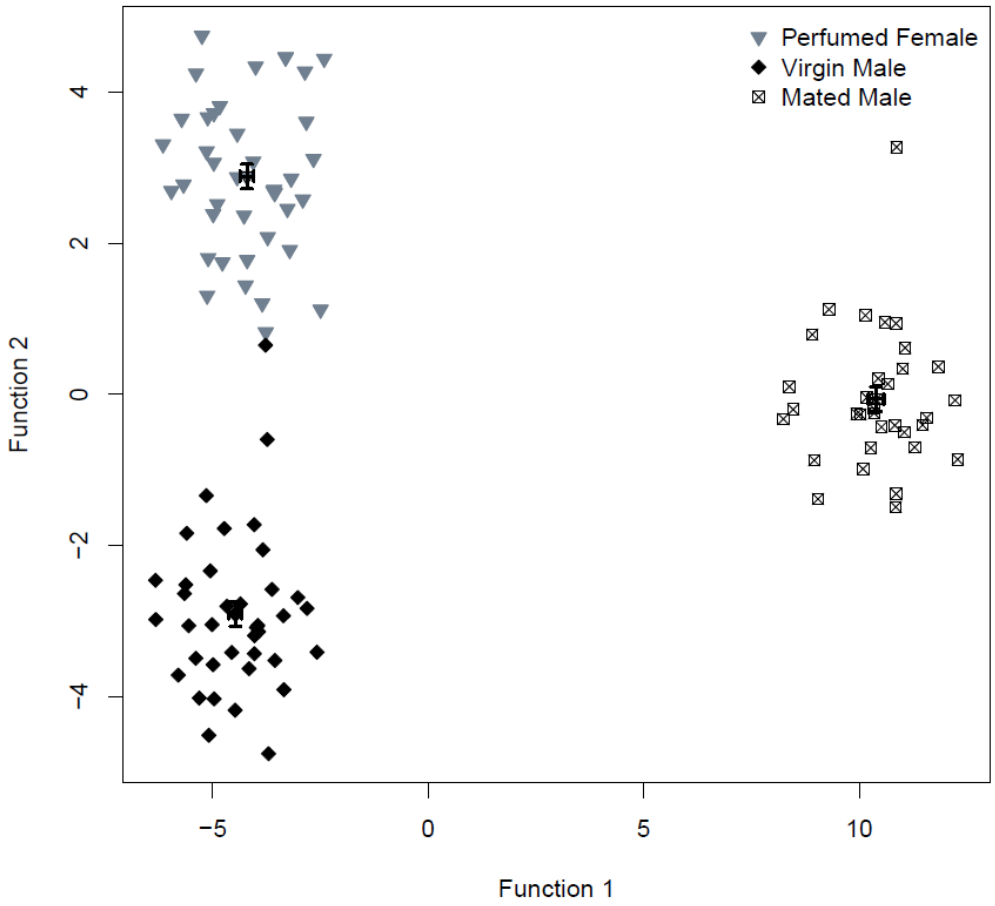

Figure 2.3b. Combined-groups plot showing functions 1 and 2 derived from the discriminant function analysis of perfumed females, virgin males and mated males. Function 1 explains 88.4% of between-group variance, separating perfumed females and virgin males from mated males. Function 2 explains 11.6% of the variance, discriminating perfumed females from both groups of males. Centroids represent the averages and standard errors of each treatment.

CHAPTER 3: RAPID DEGRADATION OF CHEMICAL CUES OF SPERM COMPETITION

3.1 ABSTRACT

Females of many species, including most insects, can store and utilise sperm for long periods after mating. In such species, the sperm competition risk associated with mated females persists long after their initial mating and hence males can benefit from being able to detect and respond to cues of mating status for an equally long period. Males may be able to do so by utilising chemical cues deposited on females by other males, as chemical cues can persist long after the signaller has gone. Previous studies have shown that chemical cues can inform males about sperm competition risk and intensity, but their signal 'strength' has only been investigated shortly after perfuming. Here we investigate the stability of chemical cues as indicators of sperm competition risk over time in the broad-horned flour beetle *Gnatocerus cornutus.* In chapter 2 we found that male-derived cuticular hydrocarbons (CHCs) elicit a significant increase in ejaculate investment in *G. cornutus.* Here we perfume females with male-derived CHCs at four different time points before mating. In our current study CHCs failed to elicit a behavioural response at any of the four different time intervals after perfuming, including the same time point at which a significant effect was seen in chapter 2. The methodology of the two studies was identical, except that in the current study females were returned to flour in between perfuming and mating. Our findings thus suggest that environmental conditions may limit the capacity of males to respond to cues of sperm competition risk.

3.2 INTRODUCTION

Sperm competition theory predicts that males should adjust their ejaculate investment depending upon female mating status (Parker *et al.* 1997), and evidence from a growing number of empirical studies supports this prediction (see Wedell *et al.* 2002 for review). In order to adjust their ejaculate expenditure, males must gather and respond to information about female mating status and sperm competition risk from their environment. Behavioural responses in males to visual, audio and more recently chemical cues of sperm competition risk have been shown both in isolation (Thomas and Simmons 2009; Price *et al.* 2012; Gray and Simmons 2013) and in combination (Bretman *et al.* 2011b). Chemical signals and cues differ from those of other sensory modalities in that they can persist long after the signaller has gone (Wyatt 2014). Their duration ranges from the alarm pheromones of ants that last mere seconds (Hölldobler and Wilson 1990), to termite trails that remain detectable for years (Bordereau and Pasteels 2011). However, studies investigating the use of chemical cues in the assessment of female mating status have thus far only looked at their short term use, i.e. five minutes to three hours after the female has mated or been chemically manipulated (DelBarco-Trillo and Ferkin 2004; Friberg 2006; Carazo *et al.* 2007; Thomas and Simmons 2009). Female insects can store and utilise sperm for significantly longer periods of time, for example females of the indian meal moth *Plodia interpunctella* (Cook and Gage 1995), *Drosophila melanogaster* (Ala-Honkola *et al.* 2014) and the wool-carder bee *Athidium manicatum* (Lampert *et al.* 2014) have been shown to store sperm for at least one week after mating, whilst more extreme examples of sperm storage come from the queens of many eusocial Hymenopteran species who mate only once at the beginning of their reproductive lifetimes and store sperm for years, even decades in some instances

(Hölldobler and Wilson 1990; Tschinkel 1987; den Boer *et al.* 2009). Therefore it may be advantageous for males to be able to detect and respond to cues of sperm competition risk and mating status for far longer than the initial period after a female has mated.

Cuticular hydrocarbons (CHCs) – compounds found on the outer cuticle of all insects – have been shown to play an important role as both signals and cues in chemical communication among insects. Social insects for instance, have been found to use CHCs for colony and nestmate recognition (Thomas *et al.* 1999; Liang and Silverman 2000; Wagner *et al.* 2000), suppressing worker reproduction (Endler *et al.* 2004) and even to find their way home (Bordereau and Pasteels 2011). Hydrocarbons are predominantly transferred via contact and changes to female CHC profile elicited by courtship and mating have recently been demonstrated to significantly shape male perception of sperm competition risk. For example, male *Drosophila melanogaster* presented with virgin females perfumed with the CHCs of mated females, significantly increased both copulation length and mate guarding duration (Friberg 2006). Furthermore, perfuming virgin females with the CHCs of rival males induced male field crickets and flour beetles to significantly increase the number of sperm transferred during mating (Thomas and Simmons 2009; Lane *et al.* 2015) and male field crickets even tailor the size of their ejaculate to the number of competitors perceived (Thomas and Simmons 2009).

Due to their unreactive nature, hydrocarbons are thought to be very stable (Martin *et al.* 2009), but few studies have investigated this in detail. Of the few studies that have, one examined the long-term stability of CHCs in museum specimens (Martin *et al.* 2009), while the main focus of the other studies has been the stability of synthetic hydrocarbons applied experimentally (Ginzel and Hanks 2002; Witjes and Eltz 2009). These studies

quantified stability as the amount of hydrocarbons remaining over time on either the individual or the substrate as detected by gas chromatography, however, their results are inconclusive, with evidence of CHC stability over periods from anywhere between 24 hours (Witjes and Eltz 2009), 2 months (Ginzel and Hanks 2002) and 20 years (Martin *et al.* 2009). Whilst measurements of quantity can give us an idea of the chemical stability of CHCs, they provide little indication of the qualitative value of these compounds to elicit behavioural responses. Thus, this measurement alone reveals nothing of the temporal dynamics of cuticular hydrocarbons as chemical cues.

In chapter 2 we demonstrated the ability of male broad-horned flour beetles *Gnatocerus cornutus* to detect and respond to rival male-derived CHCs retained on the cuticles of virgin females as cues of sperm competition risk. Males allocated significantly more sperm to virgin females whose CHC profiles had been manipulated with the CHCs of three rival males in comparison to control virgin females (Lane *et al.* 2015). Here, we investigate whether these cues continue to provide a stable signal of sperm competition risk over time. Like most insects, females of this species can store sperm for at least 6 days (SML personal observation) and thus it may be advantageous for males to be able to detect these cues for an extended length of time. We manipulated female chemical profile at 4 different time points – 7 days, 72 hours, 24 hours and 30 minutes before mating – and then measured male ejaculate expenditure.

3.3 MATERIALS AND METHODS

(a) Stock populations and rearing protocols

Beetles used in this study were taken from stock populations of *G. cornutus* derived from the Japanese National Food Research Institute (NFRI), at which beetle cultures have been maintained for over 50 years (see Okada *et al.* 2006 for details of origin). In our laboratory mixed sex populations have been maintained for 2 years in pots (Thermoscientific Nalgene 500mL, 120mm OD) containing 50 individuals. These stock populations are reared on wholemeal flour enriched with 5% yeast and incubated at 27°c with 60% humidity on a 14L: 10D lighting cycle (Okada *et al.* 2006). Every 3 – 4 weeks, final instar larvae are randomly removed from each stock pot (n = 18) as pupation is inhibited at moderate to high larval density (Tsuda and Yoshida 1985) and mixed at random with larvae from all other pots to maintain gene flow between the populations. At eclosion, 25 male and 25 female adults are randomly selected to form the parents of the next generation.

(b) Experimental animals

Final instar larvae were collected from lab stocks daily and placed into five 24-well plates until eclosion. The day after eclosion, adults were transferred to single sex 24-well plates to prevent interactions between conspecifics and provided with *ad libitum* wholemeal flour. The lids of the male-only 24- well plates were secured with masking tape to prevent male-male interactions, which have been previously shown to influence investment in ejaculates (Okada *et al.* 2010).

To determine the stability of CHCs transferred from males to females via contact, virgin females were allocated to five different treatments. Females were perfumed by vortexing with three males at different time points, either; 7 days (7 day virgins), 72 hours (72 hour virgins), 24 hours (24 hour virgins), or 30 minutes (30 minute virgins) prior to mating (n=40 per treatment). Vortexing involved placing a single virgin female into an eppendorf (1.5mL) with 3 virgin males of the same age and vortexing on a low setting for 30 seconds (Lane *et al.* 2015). Males used during vortexing were immediately discarded and not used in mating trials. Additionally, we set up a control treatment group referred to as control virgins (n=40). Control virgin females were vortexed alone 30 minutes before mating to control for any potential effects of vortexing *per se.* Following vortexing, all experimentally perfumed females were returned back to flour prior to mating.

(c) Mating trials and measuring ejaculates

Mating trials were undertaken on day 17 after eclosion. To eradicate potential first-mating effects on ejaculate size and content (Svärd and Wiklund 1986; see Torres-Vila and Jennions 2005 for a review), all males were first paired and mated to a random non-focal virgin female 20 minutes before their focal mating. Females used in this first mating were frozen and discarded. For their focal mating, males were paired with a female from one of the five treatments outlined above and observed for 45 minutes. If copulation occurred, females were removed immediately afterwards and kept individually at 27°c for 4 hours. This allowed adequate time for the sperm to travel up the reproductive tract to the spermatheca (SML, personal observation), after which females were frozen at -20°c. If a pair failed to mate within the 45 minutes, they were removed from the trial and discarded from the

experiment. Twice mated males were frozen for subsequent body measurements. Digital images of the dorsal view of males' bodies were taken using a Leica M125 microscope with mounted camera (Leica DFC295, Leica Microsystems Ltd. CH-9435 Heerbrugg) using standardised settings. Male pronotum width was measured to the nearest 0.01mm as an index of body size (Okada *et al.* 2006) using imaging software ImageJ (version 1.46r). The repeatability of this measure was calculated based on the variance components derived from an analysis of variance (Lessells and Boag 1987), demonstrating a high repeatability ($F_{24,25}$ = 120.33, r = 0.992± 0.0034. P < 0.001).

Twenty four hours after being frozen, females were removed from the freezer for dissection. Each female was placed onto a fresh microscope slide, abdomen facing upwards. Using fine forceps, the female's the abdomen was gently squeezed revealing her reproductive tract, which was gently grasped and pulled out. The spermatheca was carefully removed from the surrounding reproductive tissue and all other tissue was removed from the slide. A drop of 10µl of deionised water was added to the centre of the slide, the spermatheca was crushed between the forceps and placed directly into the droplet. The sample was stirred to prevent sperm clumping and left to air-dry fully. Total sperm count was recorded using an Olympus BX61 microscope (Olympus Corporation, Tokyo, Japan) under phase contrast at 20x magnification. As *G. cornutus* produce relatively small ejaculates of <2000, full counts of sperm stored in the spermatheca were possible. Sperm counts were performed manually. The repeatability of counts of the same ejaculate was calculated using the variance components derived from an analysis of variance (Lessells and Boag 1987), showing high repeatability ($F_{6,7}$ = 652.464, r = 0.997±0.0012, P < 0.001).

(d) Statistical analyses

Before analysing our data we removed all females to which no sperm had been transferred, classifying these as failed copulations (final sample sizes once zeroes removed – control = 28; 30 minutes = 30; 24 hours = 31; 72 hours = 23; 7 days = 23). We then analysed the number of sperm transferred to females from one of the five treatments using a general linear model (GLM). As our response variable was non-normally distributed and highly overdispersed, we incorporated a quasi-Poisson error family into our model which accounts for such overdispersion. Male pronotum width was included in the model to control for the potentially confounding effects of male body size on ejaculate size. All statistical analyses were carried out in R (version 3.1.0 - R Core Team 2014).

3.4 RESULTS

Our analysis revealed no significant difference in the number of sperm transferred across all five treatments ($F_{4,130}$= 0.21, P= 0.93), indicating that rival male-derived CHCs failed to elicit a behavioural response. Thus it appears that rival male-derived CHCs on the cuticle of virgin females are highly unstable and are lost rapidly (see fig. 3.1). Furthermore there was no significant interaction between pronotum width and treatment ($F_{4,125}$= 0.83, P= 0.51) and no significant effect of pronotum width (our proxy of body size) on sperm number ($F_{1,129}$= 2.29, P= 0.13).

3.5 DISCUSSION

Our results indicate that the presence of male-derived cuticular hydrocarbons on the cuticles of virgin females failed to elicit a behavioural response at any of the time points measured after perfuming. We found no significant difference in ejaculate allocation across the four time points, including 30 minutes, the same time point at which we found the same cues to elicit a highly significant increase in ejaculate expenditure in chapter 2 (Lane *et al.* 2015). While the two studies were not run simultaneously, they were both carried out using beetles from the same outbred stock populations in the same controlled laboratory environment. The studies differ in just one methodological aspect, whether or not the females were placed back into flour in between being perfumed and mated. In chapter 2, the behavioural response of males was measured only once – 30 minutes after perfuming – and as such females were not returned to flour but rather kept in clean plasticware before being paired for mating. In our current study however, due to the elongated time intervals (24 hours, 72 hours and 7 days), it was necessary to return females to flour to avoid starvation effects on behaviour. To standardise across treatments we returned all females, including those in the 30 minute treatment to flour in between perfuming and mating. Furthermore we have used the same experimental perfuming technique in multiple other studies without flour and found it to elicit significant behavioural responses (SML and CMH unpublished data). Thus while we cannot exclude the possibility that the differences observed between studies are the result of temporal variation, our results strongly suggest that flour significantly accelerated the degradation of these chemical cues, rendering them undetectable within half an hour of application.

Flour beetles are a stored product pest worldwide, and as this invaded environment is now generally considered to be their natural environment (Sokoloff 1978; Wade 1990), natural and lab conditions are very similar. Consequently if rival male CHCs do not provide any information to males when in flour, it raises the question as to why males respond to these cues outside of their habitat substrate. Relatively little is known about the habitat of flour beetles before they populated flour mills and food processing facilities, but there is some evidence to suggest that many species lived as scavengers underneath the bark of trees feeding on fungus and dead plant materials (Linsley 1944). Thus the ability to detect and respond to contact-derived chemical cues may be vestigial in this species, leftover from their previous habitat in which such chemical cues may have remained detectable. Evidence of vestigial responses to chemical cues has been suggested by studies of some snake and lizard species, in which naïve individuals were found to respond to the cues of predators who have not shared their habitat for generations (Burghardt 1968; Van Damme *et al.* 1995; Van Damme and Castillo 1996).

To date only a handful of studies have considered the temporal dynamics of chemical cues from a behavioural perspective as we have done here, measuring behavioural responses over time in order to estimate signal duration. These few studies focus on different kinds of chemical cues in different environments - predator and alarm cues in aquatic environments (Chivers *et al.* 2013; Ferrari *et al.* 2007; Peacor 2006) and recruitment cues left on soil (vanOudenhove *et al*. 2012) – but they all find that chemical cue degradation is highly dependent on environmental conditions, and moreover that the degradation rate of cues seen under laboratory set ups did not reflect that found under natural conditions. For example, the recruitment trails of mass-recruiting ants *Tapinoma*

nigerrimum show high sensitivity to temperature fluctuations when left on soil, vanishing completely as temperature rises (van Oudenhove *et al.* 2012). Alarm cues of the wood frog *Lithobates sylvatica* degrade at a significantly faster rate under natural aquatic conditions (i.e. ephemeral ponds) compared to observations in laboratory set ups. Similarly, the behavioural response of the bullfrog *Lithobates catesbeianus* to predator cues is limited by water type (Peacor 2006; Ferrari *et al.* 2007) and the alarm cues of the coral reef fish *Pomacentrus ambonensis* evoke a strong antipredator response initially but degrade within 30 minutes under natural aquatic conditions (Chivers *et al.* 2013).

Environmental conditions can have profound effects on the expression of chemical compounds and in doing so may significantly change the message conveyed by chemical signals (Gershman *et al.* 2014). For instance, the CHC profiles of workers within a colony of the harvester ant *Pogonomyrmex barabatus* are modified by the amount of environmental exposure associated with specific tasks, meaning that individuals within the colony may be able to recognise workers for the specific task they perform based solely on their CHC profiles (Wagner *et al.* 2000). Similarly, Liang and Silverman (2000) showed that the stability of CHCs used for colony recognition in the Argentine ant *Linepithema humile* is highly reliant on environmental stability. Changing the diets of a subset of ants within the colony lead to alterations in CHC expression and furthermore to the development of intra-colony aggression between ants reared on different diets (Liang and Silverman 2000). Environmental changes to signal expression and transmission can also have complex effects on sexual selection. For example, the presence of environmental noise has been shown to significantly alter the shape of female preference function for male calling song in the grasshopper *Chorthippus biguttulus* (Reichert and Ronacher 2014). Thus seemingly short-

term changes to signal composition and transmission can translate into long-term shifts in evolutionary trajectories (Endler 1992).

Despite these potentially far-reaching effects, to date very few studies have investigated the stability of CHCs. Those that have, quantified the stability of CHCs based on the concentration of compounds found on museum specimens (Martin *et al.* 2009) or on individuals (Ginzel and Hanks 2002) or substrates (Witjes and Eltz 2009) following the application of synthetic hydrocarbons but not on how long these cues continued to elicit a behavioural response, thus revealing little to us about the temporal dynamics and biological utility of CHCs as chemical cues. To our knowledge ours is the first study to specifically investigate the stability of CHCs as chemical cues, and moreover as chemical cues of sperm competition risk. While we cannot conclude with absolute certainty that the lack of behavioural response seen here was the direct result of the presence of flour, this potential highlights the necessity of considering environmental conditions when investigating the use and importance of chemical cues. If we are to understand the actual ecological relevance of cues, we must consider them in the context of the environment in which they are transmitted and received.

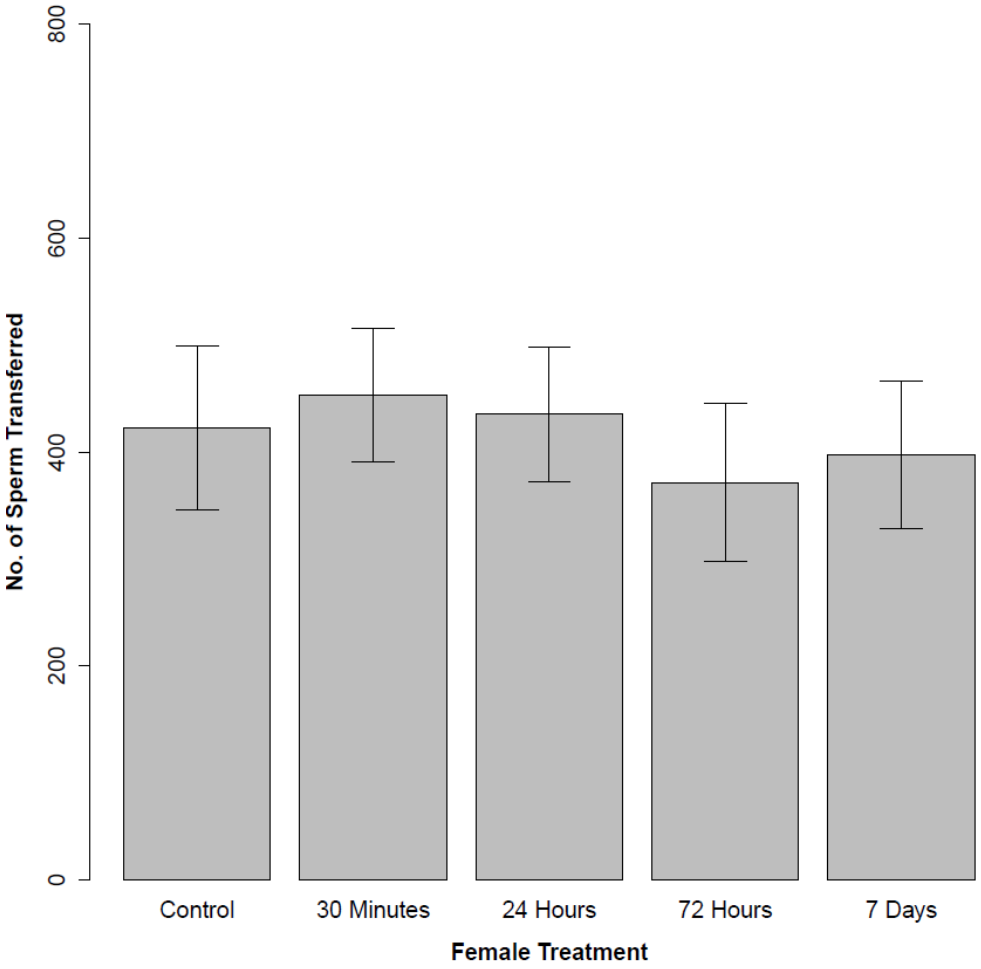

Figure 3.1. Mean ± SE number of sperm transferred by males to control virgin females (*n* = 28) and virgin females that had been perfumed with three males and returned to flour for either 7 days (*n* = 23), 72 hours (*n* = 23), 24 hours (*n* = 31) or 30 minutes (*n* = 30) prior to mating. The number of sperm transferred did not differ significantly across treatments (*P* = 0.78).

CHAPTER 4: SEXUAL SELECTION ON MALE CUTICULAR HYDROCARBONS VIA MALE-MALE COMPETITION AND FEMALE CHOICE

4.1 ABSTRACT

Traditional views of sexual selection assumed that male-male competition and female mate choice work in harmony, selecting upon the same traits in the same direction. However, we now know that this is not always the case and that these two mechanisms often impose conflicting selection on male sexual traits. Cuticular hydrocarbons (CHCs) have been shown to be linked to both social dominance and male attractiveness in several insect species. However, whilst several studies have estimated the strength and form of sexual selection imposed on male CHCs by female mate choice, none have established whether these chemical traits are also subject to sexual selection via male-male competition. Here using a multivariate selection analysis, we estimate and compare sexual selection exerted by male-male composition and female mate choice on male CHC composition in the broad-horned flour beetle *Gnatocerus cornutus*. We show that male-male competition exerts strong linear selection on both overall CHC abundance and body size in males, while female mate choice demonstrates a complex blend of linear and non-linear selection, targeting not just the overall amount of CHCs expressed but the relative abundance of specific hydrocarbons as well. We discuss the potential implications of this antagonistic selection with regards to male reproductive success.

4.2 INTRODUCTION

The widespread elaboration of male sexual traits results from two mechanisms of sexual selection first proposed by Darwin – male-male competition and female mate choice. A long-held assumption of sexual selection was that these two mechanisms should be reinforcing, (i.e. impose the same form and direction of selection on the same suite of male traits), resulting in a scenario whereby females always preferred dominant males (Cox and LeBoeuf 1977; Berglund *et al.* 1996; Wiley and Poston 1996). However, a large body of evidence now demonstrates that the selection pressures of male-male competition and female mate choice are often conflicting (reviewed in Qvarnström and Forsgren 1998 & Wong and Candolin 2005).

Nevertheless even when mechanisms of sexual selection are antagonistic and females do not exert a preference for dominant males, it remains possible for dominant males to gain a mating advantage over their competitors through force or coercion, increasing their own mating opportunities and ultimately overriding female mate choice (Qvarnström and Forsgren 1998; Wong and Candolin 2005). Evidence of conflict between male-male competition and female mate choice has been observed in flour beetles (Harano *et al.* 2010; Yamane *et al.* 2010; Okada *et al.* 2014), cockroaches (Moore and Moore 1999), bitterlings (Reichard *et al.* 2005; Casalini *et al.* 2009), brown trout (Petersson *et al*. 1999) and water striders (Sih *et al.* 2002). The consequences of mating with dominant, non-preferred males can be severe, for example female cockroaches *Nauphoeta cinerea* mated to non-preferred males had a reduced lifespan and produced fewer offspring (Moore *et al.* 2001; Moore *et al.* 2003). This effect is linked to dominant male's pheromone composition – a sexually selected trait in this species (Moore *et al.* 2003).

Although chemical cues have received relatively little attention in comparison with more conspicuous male sexual traits (e.g. visual and audio traits), recent years have seen a surge in studies investigating the role of chemical traits in determining male mating success (Wyatt 2014). Chemical cues have now been shown to signal a multitude of male characteristics including dominance status (e.g. cockroaches – Moore *et al.* 1997; South *et al.* 2011; field crickets – Thomas and Simmons 2009b), condition (e.g. meadow voles – Ferkin *et al.* 1997; Hobbs and Ferkin 2011), infection status (reviewed in Penn and Potts 1998) and even genetic compatibility (e.g. field crickets – Thomas and Simmons 2011a; Capodeanu-Nägler *et al.* 2014). Cuticular hydrocarbons (CHCs), semiochemicals found on the cuticles of most terrestrial arthropods have been shown to be particularly important in providing cues of a males socio-sexual environment and signals of male quality [see Ingleby 2015 for a review] (as well as playing a key role in species and mate recognition [see Howard and Blomquist 2005 & Johansson and Jones 2007 for a review]) in insects.

CHCs are known to convey information about male competitive ability and attractiveness. For example, male CHC profiles often determine the outcome of both male-male competition (e.g. cockroaches - Roux *et al.* 2002; field crickets - Kortet and Hedrick 2005; Thomas and Simmons 2009b; 2011b) and female mate choice (e.g. field crickets - Kortet and Hedrick 2005; Ivy *et al.* 2005; Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2013; 2015; *Drosophila* – Blows 2002; Howard *et al.* 2003; Chenoweth and Blows 2005; Ingleby *et al.* 2014). However to date only a handful of studies, limited to *Drosophila* and field crickets, have investigated the strength and form of sexual selection imposed on CHCs (reviewed in Steiger and Stökl 2014). Futhermore, these few studies only look at the strength and form of sexual selection imposed by female mate choice not by

male-male competition. As discussed above, sexual selection during one episode of selection may not be reflective of selection occurring at another. Therefore, it cannot be assumed that selection on CHCs during male-male competition is reinforced during selection on CHCs from female mate choice (Hunt *et al.* 2009).

Here we examine the form and strength of sexual selection imposed by male-male competition and female mate choice on male CHCs in the broad-horned flour beetle *Gnatocerus cornutus*. Male-male competition is an important component of the mating system of *G. cornutus.* Males possess enlarged mandibles, a trait absent in females, which they use to fight over territories and mates. Mandible size is a major determinant of fight outcome and although females do not prefer males with large mandibles or males that win fights, evidence suggests that aggressive males are still able to secure a mating advantage under competitive scenarios (Harano *et al.* 2010; Yamane *et al.* 2010; Okada *et al.* 2014). Cuticular hydrocarbon profiles are sexually dimorphic in *G. cornutus*, consisting of 24 compounds which vary in concentration between the sexes (Lane *et al.* 2015). Males of this species have previously been shown to be able to detect slight changes in female CHC profiles in order to respond to the risk and intensity of sperm competition (Lane *et al.* 2015), indicating that CHCs are an important source of information in this species.

We begin by using a multivariate approach to estimate the strength of linear and non-linear sexual selection on male CHCs during both male-male competition and female mate choice. We then test whether the strength and form of sexual selection on male CHCs changes during these two episodes of selection.

4.3 MATERIALS AND METHODS

(a) Stock populations and rearing protocols

Beetles used in this study were taken from stock populations of *G. cornutus* derived from the Japanese National Food Research Institute (NFRI), at which beetle cultures have been maintained for over 50 years (see Okada *et al.* 2006 for details of origin and culture conditions). In our laboratory mixed sex populations have been maintained since 2012 in pots (Thermoscientific Nalgene 500mL, 120mm OD) containing 100 individuals (50 ♀ and 50 ♂). These stock populations are reared on wholemeal wheat flour enriched with 5% yeast and incubated at 27°C and 60% humidity on a 14L: 10D lighting cycle (Okada *et al.* 2006). Every 3 – 4 weeks, final instar larvae are randomly removed from each stock pot (n = 18) and placed into six 24-well plates as pupation is inhibited at moderate to high larval density (Tsuda and Yoshida 1985). At eclosion, 50 male and 50 female adults are randomly selected to form the parents of the next generation of each pot.

For the purposes of this study, final instar larvae were collected from lab stocks daily and placed into 24-well plates until eclosion. After eclosion, adults were separated by sex, transferred into fresh 24-well plates and provided with ad libitum wholemeal wheat flour. Animals remained in these well-plates until the day of their trial which occurred from 7-15 days after eclosion, a range which ensured sexual maturity.

(b) Fighting success trials

Twenty four hours before fighting trials, males were randomly allocated as either the focal or non-focal male and those allocated as non-focal males were marked with tippex on their

elytra and returned to individual cells to allow the tippex to dry. Focal males would later be run through the GC-FID and thus could not be marked with tippex – tippex marking has no effect on the outcome of male-male competition (*CMH personal observation*) and returned to individual cells to allow the tippex to dry. The next day, focal and non-focal males were randomly paired in fighting arenas and observed for 20 minutes. All acts of aggression were recorded along with the winner of each bout. Aggressive encounters were classified as: - (1.) Males repeatedly placed their mandibles beneath an opponent's body and attempted to lift and flip them onto their back. (2.) Males shoved and bit their opponent with their mandibles (Okada *et al.* 2006). Losers were determined as those who were successfully flipped onto their back or whom retreated from the encounter under the pursuit of the victor. At the end of the 20 minutes, focal individuals were frozen at -20°c for subsequent chemical analysis. For each focal individual, the number of wins and losses were tallied to reveal whether they won or lost the majority of aggressive encounters. Continuous fighting data was not comparable across focal males as the number of fights that occurred within the total 20 minute period differed between pairs. Therefore, focal male fighting success was calculated for each individual as the proportion of fights won (i.e. number of fights won/total number of fights entered).

(c) Mating success trials

Males and females were paired randomly in mating arenas and observed for 20 minutes, during which time we noted all courtship attempts and successful copulations. Males have a stereotypical courtship display which commences when a male mounts a female and drums her abdomen with his tibia. This may be followed by a brief copulation that lasts only 3-4

seconds. Males who obtained a mating were given a fitness score of 1 (n =353), whilst those who exhibited courtship behaviour but failed to obtain a mating were given a fitness score of 0 (n = 147). All males were removed from the mating arena at the completion of the trial and were frozen at -20°C for CHC extraction.

(d) CHC extraction and analysis

Samples were randomised before the extraction process to eliminate any possible effects of column degradation over time. Cuticular hydrocarbons were then extracted from individuals via full-body immersion in 50µl of HPLC-grade hexane with 10ppm pentadecane as an internal standard. Individual beetles were left to soak for 5 minutes, during the final minute of which they were vortexed to maximise CHC extraction. After the 5 minutes, beetles were removed from the extraction vials using metal forceps which were cleaned in pure hexane between samples to avoid contamination. Individual beetles were then placed back into their corresponding eppendorfs to be re-frozen for later body measurements.

2µl of the extracted CHC sample was injected into a GC-FID (Agilent 7890) fitted with two injectors, and two DB-1 columns (of 30m x 0.25mm with an internal diameter of x 0.25µm film thickness) using helium as a carrier gas. The inlets were set at 250°C, and the injection was run in the pulsed splitless mode. The GC oven temperature profile started at 100°C for 1 minute, ramping at 20°C/min to 250°C, then finally 5°C/min to 320°C. The FID detector heaters were set at 300°C, the H_2 flow was 22ml/min, and the air flow was 200ml/min. Nitrogen was used to make up the column flow to 30ml/min.

(e) Morphological measurements

We captured images of the dorsal view of the focal males' bodies using a Leica M125 microscope with mounted camera (Leica DFC295, Leica microsystems Ltd. CH-9435 Heerbrugg) which conveyed images to a PC. We then measured the width of the pronotum (to the nearest 0.01mm) as an index of body size (Okada *et al.* 2006) using ImageJ (version 1.46r). We measured a subset of these pronota twice to calculate the repeatability of this measure [using the R code (Wolak *et al.* 2012)] and find that the repeatability is high ($F_{28,29}$ = 93.37, r = 0.98, CIs: 0.99,0.96 P<0.001).

Statistical analyses

(i) Principal components analysis

GC-FID analysis identified 24 individual CHC peaks. After dividing all peaks by the internal standard (peak 1), the resulting data were $\log_{10}$ transformed. We pooled the data from both of our datasets (i.e. male-male competition and female mate choice) and ran a principal components analysis (PCA) in order to obtain a reduced number of eigenvectors which capture and describe the variation in CHC profiles. All data analysis was carried out using SPSS (version 20).

(ii) Measuring sexual selection

(a) Multivariate selection analysis

Individuals in our analysis are independent as males were used once only (i.e. for either mating or fighting trials). We quantified the strength and form of sexual selection acting on male CHCs across the two episodes of selection using a standard multivariate selection analysis as described by Lande and Arnold (1983). We first calculated individual relative fitness by dividing individual absolute fitness scores by the mean fitness score for each population within each selection episode. As our response variables were measured in different units, it was necessary to standardise them for statistical comparison. Therefore we standardised pronotum width (PW) to have a mean of zero and a standard deviation of one using a Z-transformation. Our CHC data was already standardized by the principal components analysis conducted on our full dataset (see section above).We fitted linear regression models for each episode of selection that included our measure of body size and the PCs that described the composition of the CHCs as the predictor variables and relative fitness as the response variable to obtain estimates of standardized linear selection gradients during male-male competition ($\boldsymbol{\beta}_{mc}$) and mating success ($\boldsymbol{\beta}_{ms}$). We then fitted a quadratic regression model that incorporated all linear, quadratic and correlational terms to estimate the matrix of nonlinear selection gradients during male-male competition ($\boldsymbol{\gamma}_{mc}$) and mating success ($\boldsymbol{\gamma}_{ms}$). As regression models tend to underestimate quadratic selection by a factor of 0.5, the resulting quadratic selection gradients were doubled as recommended by Stinchcombe *et al*. 2008. As our measures of fitness (fighting and mating success) did not conform to a normal distribution, we assessed the significance of our linear and nonlinear selection gradients for each data set using a re-sampling procedure where fitness scores were randomly shuffled across individuals in the dataset to obtain a null distribution for each gradient where there is no relationship between trait and fitness (Mitchell-Olds and Shaw 1987). Probabilities are the number of times (out of 9,999 permutations) in which the

gradient pseudo-estimate was equal to or less that the original estimated gradient. We conducted separate randomization analyses for the multiple regression models for directional selection (i.e. model containing only linear terms) and for the full quadratic model (i.e. model containing linear, quadratic and correlational terms).

If the size and significance of the γ-coefficients are interpreted individually, it is possible to underestimate the strength of nonlinear selection (Phillips and Arnold 1989; Blows and Brooks 2003). Consequently, we used the car package of R (See appendix for the R script) to determine the extent of nonlinear sexual selection by conducting a canonical analysis as suggested by Reynolds *et al.* (2010) to locate the major axes of fitness surfaces described by $\boldsymbol{\gamma_{mc}}$ and $\boldsymbol{\gamma_{ms}}$ (Phillips and Arnold 1989). Permutation tests of the eigenvalues were conducted by randomly permutating the fitness variable 1000 times for each simulated data set to estimate the numbers of times the observed F statistic exceeded the F statistic from the permuted datasets (Reynolds *et al.* 2010). This tests therefore, whether the test statistic of the eigenvectors extracted from the canonical analysis of $\boldsymbol{\gamma_{mc}}$ and $\boldsymbol{\gamma_{ms}}$ is larger than expected by pure random error (Reynolds *et al.* 2010).

(b) Visualizing the fitness surface

To visualise the fitness surfaces from the canonical rotation of $\boldsymbol{\gamma_{mc}}$ and $\boldsymbol{\gamma_{ms}}$ we used thin plate splines (Green and Silverman 1994). We used the Tps function in the fields package of R (version 2.13.0; available via http://www.r-project.org) to fit spline surfaces using the value of the smoothing parameter ($\boldsymbol{\lambda}$) that minimized the generalized cross-validation (GCV) score. We then plotted surfaces in R using both the perspective and contour map views.

Finally, we used a sequential model building approach as outlined in Appendix A of Chenoweth and Blows (2005) to examine whether the form and strength of sexual selection acting on male CHCs differed significantly between the two episodes of selection. In short this sequential approach tests the difference in the sign and magnitude of the linear, quadratic and correlational selection gradients across the different episodes of selection by comparing the change in variance explained by a regression model that fits a single relationship through the two selection episodes being compared (model 1) to a regression model that fits a separate relationship for each episode of selection (model 2). If model 2 explains significantly more variance than model 1, as determined by a partial *F* test, this demonstrates that the selection gradients differ across selection episodes. We began by running a reduced regression model which included selection episode as a dummy variable (coded as ms or fs) and contained only the standardised linear terms:

$$S = \beta_0 + \alpha_0 Episode + \sum_{i=0}^{n} \beta_i \, \mathrm{C}_i + \varepsilon, \qquad (1)$$

Where *S* was the binomial fighting/mating success measure, C_i refers to the log-contrast concentration of the *i*th principal component (PCs representing CHCs), *n* represented the number of PCs in the model and ε is the unexplained error. From (1), the unexplained (i.e. residual) sum of squares for this reduced model (SS_r) was compared to the

same quantity (SS_c) from a second complete model that included all of the terms in (1) with the addition of the terms $\alpha_i C_i$Epsiode, which represents the linear interaction of the dummy variable, selection episode, and the *i*th PC:

$$S = \beta_0 + \alpha_0 Episode + \sum_{i=0}^{n} \beta_i \, C_i + \sum_{i=0}^{n} \alpha_i \, C_i \, Episode + \varepsilon,$$

(2)

A partial *F*-test (Bowerman and O'Connell 1990) was used to compare SS_r and SS_c from (1) and (2) respectively, to test whether linear sexual selection on male CHCs differed between the selection episodes:

$$F_{a,b} = \frac{(SS_r - SS_c)/a}{SS_c/b}$$

(3)

where *a* is the number of terms that differ between the reduced and complete model and *b* is the error degrees of freedom for SS_c.

To test whether the quadratic gradients of selection acting on male CHCs differed between selection episodes, the SS_r from the reduced model:

$$S = \beta_0 + \alpha_0 Episode + \sum_{i=0}^{n} \beta_i \, C_i + \sum_{i=0}^{n} \alpha_i \, C_i \, Episode + \sum_{i=0}^{n} \beta_i \, C_i^2 + \varepsilon,$$

(4)

was compared to the SS_c of the complete model:

$$S = \beta_0 + \alpha_0 Episode + \sum_{i=0}^{n} \beta_i C_i + \sum_{i=0}^{n} \alpha_i C_i Episode + \sum_{i=0}^{n} \beta_i C_i^2 + \sum_{i=0}^{n} \beta_i C_i^2 \; Episode + \varepsilon, \quad (5)$$

using (3).

Finally to test whether correlational selection gradients different significantly between selection episodes, the SS_r from the reduced model:

$$S = \beta_0 + \alpha_0 Episode + \sum_{i=0}^{n} \beta_i C_i + \sum_{i=0}^{n} \alpha_i C_i Episode + \sum_{i=0}^{n} \beta_i C_i^2 + \sum_{i=0}^{n} \beta_i C_i^2 \; Episode + \sum_{i=0}^{n} \sum_{j \geq 1}^{n} \beta_{ij} C_i C_j + \varepsilon, \quad (6)$$

was compared to the SS_c of the complete model,

$$S = \beta_0 + \alpha_0 Episode + \sum_{i=0}^{n} \beta_i C_i + \sum_{i=0}^{n} \alpha_i C_i Episode + \sum_{i=0}^{n} \beta_i C_i^2 + \sum_{i=0}^{n} \beta_i C_i^2 \; Episode + \sum_{i=0}^{n} \sum_{j \geq 1}^{n} \beta_{ij} C_i C_j + \sum_{i=0}^{n} \sum_{j \geq 1}^{n} \alpha_{ij} C_i C_j Episode + \varepsilon,$$

(7)

In summary, the comparison of model (1) versus (2), (4) versus (5), and (6) versus (7) provides a test for the overall significance of the interaction between selection episode and the linear, quadratic and correlational selection acting on male CHCS, respectively. Therefore significant differences in these model comparisons (as detected by a partial *F*-test) demonstrate that the linear, quadratic and/or correlational selection gradients imposed by the selection episodes differ, respectively. We also inspected the interaction of individual principal components with the selection episodes from the full model (7) to determine which of the PCs were responsible for the significance of the overall partial *F*-test.

4.4 RESULTS

(a) Principal components analysis

GC-MS analysis identified 24 individual CHC peaks. Individual profiles consisted of a mixture of straight-chained alkanes, mono- and di-methyl alkanes, and alkenes ranging in length from 25 to 33 hydrocarbons (see table A1 in appendix for more details). Principal components analysis resulted in 3 PCs that collectively explained 80.6% of the total variation in male CHC expression (see table 4.1) and CHCs with factor loadings >0.25 were considered to have contributed significantly to that PC (Tabachnick and Fidell 1989). PC1 explained 60.4% of the variance and was heavily positively loaded to all CHC peaks except for peak 16 to which it was negatively loaded, thus we interpreted PC1 as representing overall investment in CHC production. PC2 explained another 14.3% of the variance and was

significantly loaded to 16 out of 24 CHCs, some positively and some negatively. PC3 explains a further 5.8% of the variance and was only significantly loaded to 5 CHCS. PC3 was positively loaded to peaks 1, 3, 11 and 16, and negatively loaded to peak 17. Due to the complex nature of these loadings, PC2 and PC3 were interpreted as representing specific blends of CHCs that are present in larger or smaller amounts. For example, PC3 was positively loaded to peaks 1,3,11 and 16 and negatively loaded to peak 17 so represents a trade-off between four different peaks and a single peak.

(b) Male fighting success

Our selection analysis revealed significant negative linear selection acting on PC3 and positive linear selection on pronotum width but no significant nonlinear gradients of selection (table 4.2). Canonical analysis of the **γ** matrix revealed significant directional selection on two positive eigenvectors ($\mathbf{m_1}$ & $\mathbf{m_3}$) and two negative eigenvectors ($\mathbf{m_2}$& $\mathbf{m_4}$) (table 4.3). Visualization of the fitness surface against the axes of strongest linear selection ($\mathbf{m_2}$ & $\mathbf{m_3}$) revealed a region of highest fighting success at low positive values of $\mathbf{m_2}$ (representing high amounts of PC2 and low amounts of PC3) and extreme positive values of $\mathbf{m_3}$ (overall CHC abundance as represented by PC1) (figure 4.1A and B).

(c) Male mating success

Standardised linear, quadratic and correlational selection gradients are presented in table 4.2. There was significant sexual selection favouring lower values of PC1 and PC3 (negative **β**) and higher values of pronotum width (positive **β**). There was also stabilising (negative **γ)** selection on both PC1 and PC3 (see table 4.2). Canonical analysis resulted in three eigenvectors ($\mathbf{m_2}$, $\mathbf{m_3}$ & $\mathbf{m_4}$) with significant linear sexual selection (table 4.3). There was also

significant stabilizing selection acting on vectors $\mathbf{m_3}$ and $\mathbf{m_4}$. Visualization of the fitness surface and the contour-view of the same fitness surface against the major axes of nonlinear selection ($\mathbf{m_3}$ & $\mathbf{m_4}$) revealed a region of highest mating success at intermediate to negative values of $\mathbf{m_4}$ and positive values of $\mathbf{m_3}$ which represents low to intermediate levels of PC1 (overall abundance of CHCs) and PC3 (specific blend of 5 CHCs). The area of lowest mating success is situated at extreme negative values of m3 and high values of $\mathbf{m_4}$, representing males with high levels of PC1 and PC3 (see table 4.3 and figure 4.1C and D). Finally, significant negative directional selection was found to be acting on $\mathbf{m_4}$ which was heavily weighted to PC1.

(d) Comparison of sexual selection across male-male competition and female mate choice

Gradients of linear sexual selection differed significantly between the two episodes of selection ($F_{4,990} = 6.40$, $P < 0.0001$) due to the episodes exerting opposing gradients of linear selection on PC1 ($P = 0.003$). Quadratic selection also differed significantly between the two selection episodes ($F_{4,982} = 3.16$, $P = 0.014$) as a result of quadratic selection on PC3 ($P = 0.029$) imposed by mating success and the absence of any significant quadratic selection at all under fighting success. Correlational selection on the other hand did not differ significantly between the two episodes of sexual selection ($F_{6,970} = 0.68$, $P = 0.668$).

4.5 DISCUSSION

Male CHCs are known to be associated with social dominance and fight outcomes (Roux *et al.* 2002; Kortet and Hedrick 2005; Thomas and Simmons 2009b; 2011a) but to date, to our knowledge, no one has investigated whether this association results in sexual selection on male CHCs via male-male competition. Here, by measuring sexual selection exerted by male-

male competition and female mate choice we show that male CHCs in *G.* cornutus are subject to strong sexual selection via both mechanisms and furthermore that the strength and form of selection exerted on male CHCs differs significantly between the two.

Male-male competition exerted strong linear sexual selection on male CHC composition and body size. Overall CHC abundance (PC1) and body size were subject to positive linear selection, indicating that large males with a high overall amount of CHCs had the greatest fighting success. Males who were least successful in fights had high levels of specific CHC blends (PC3), suggesting that fighting success is maximised by increased investment in overall CHC abundance and decreased investment in relative amounts of specific CHCs. Female choice on the other hand imposed linear and stabilising non-linear sexual selection on both the overall abundance of CHCs and on specific CHC blends. For instance, mating success was highest for males with a low to intermediate total amount of CHCs and low to intermediate levels of the specific CHC blend represented by PC3. Although sexual selection on male CHCs was statistically significant in two different contexts, we cannot rule out the possibility that male CHCs are correlated with another trait that influences fighting and/or mating success and thus are under indirect rather than direct selection. Nonetheless, the patterns that we found are consistent with previous studies in crickets (Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2013; 2015) and *Drosophila* (Ingleby *et al.* 2014) which have shown similarly complex patterns of linear and nonlinear sexual selection acting on male CHCs as a result of female mate choice. More generally, complex sexual selection on multivariate signalling traits via female choice appears to be a common phenomenon. For example females have been shown to impose similarly complex patterns of selection on acoustic signals such as male courtship song in crickets (Bensten *et al.* 2006; Simmons *et al.* 2013) and visual signals such as the intricate

coloration of male guppies (Brooks *et al.* 2003). Here, it appears that complex selection on male CHCs may reflect the complexity of the information that CHCs convey. Furthermore, our findings highlight that although overall trait expression is important (e.g. total CHC abundance), females are selecting upon multiple components within these composite traits.

Correlational selection did not differ between the two episodes of selection (being absent in both), however gradients of both linear and quadratic selection acting on male CHCs did differ significantly between the selection episodes. The significant difference in quadratic selection is not surprising given that the strongest selection exerted by mating success was non-linear in form while no significant gradients of non-linear selection were found for fighting success. Specifically, fighting success was more strongly linked to an increased investment in overall CHC profile rather than to specific CHC blends. Mating success on the other hand was tightly linked to both a lower investment in overall CHC expression and a lower relative abundance of specific CHC blends. In some species of *Drosophila* and cricket (Ingleby *et al.* 2014; Steiger *et al.* 2015), overall CHC abundance has been found to correlate with male body size, potentially explaining why males with increased fighting success were both larger and possessed higher overall CHC abundance. Intrasexual selection commonly exerts positive directional selection on male sexual traits (Jones *et al.* 2012). However, we did not find evidence of correlational selection in this study. The form of selection imposed by female mate choice is more varied across the literature, with some studies showing female choice to target the relative abundance of specific CHCs as opposed to their overall abundance (Howard *et al.* 2003; Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2015) while other studies show as we have here that sexual selection targets both specific CHC blends as well as the total abundance of CHCs (Steiger *et al.* 2013; Ingleby *et al.* 2014).

Our findings indicate that sexual selection by male-male competition and female choice target different components of male CHC profile and as a result, male CHCs linked with fighting success and mating success are not the same. This suggests that males who win fights may not also able to achieve high mating success under female choice. A similar conclusion was reached in a recent study investigating female preference for morphological traits known to be associated with fighting success in this species (Okada *et al.* 2014). The authors found that females chose mates not on the basis of fighting traits but rather on male courtship rate, and consequently mating success was not correlated with fighting success. Thus, like the aforementioned study, our results suggest that winning a fight does not equip males with any benefit when it comes to mating. However, antagonistic selection during male-male competition and female choice may not always restrict the mating success of dominant or aggressive males, namely if they are able to override this conflict. For instance in *G. cornutus*, males who win fights gain priority access to females and achieve a mating advantage under competitive scenarios (Harano *et al.* 2010; Yamane *et al.* 2010; Okada *et al.* 2014), circumventing female choice. Thus although our study provides evidence that male-male competition and female choice are antagonistic, the consequences for males under competitive scenarios are more difficult to predict.

An overwhelming number of studies of sexual selection on male CHCs have focused on the influence of female mate choice for male CHC profiles. Here, we provide the first evidence, to our knowledge, that male CHC composition can have a profound influence on the outcome of male-male competition and that sexual selection on male CHCs during fighting is opposed by sexual selection on CHCs during mating. However, although multivariate selection analyses are a powerful tool for estimating selection on multiple traits, this approach cannot separate the effects of direct and indirect selection (Wade and

Kalisz 1990; Schluter and Nychka 1994; Krakauer *et al.* 2010). To date no one has attempted to verify observed sexual selection patterns on CHCs with experimental manipulation (Steiger and Stökl 2014) and thus more work is needed to show that these chemical traits are indeed important determinants of mating and fighting success and this is an area of research that we aim to address.

Table 4.1 Results of principal components analysis for male CHCs. Compounds with a loading factor >0.25 were classified as biologically significant and are shown in bold (Tabachnick and Fidell 1989). CHCs are listed in ascending order of chain length.

	PC1	**PC2**	**PC3**
Eigenvalue	14.501	3.442	1.394
% variance	60.423	14.341	5.808
Loadings			
1	**0.541**	-0.062	**0.732**
2	**0.797**	**0.396**	-0.096
3	**0.758**	**0.385**	**0.408**
4	**0.882**	**-0.261**	0.230
5	**0.825**	**0.439**	-0.091
6	**0.458**	0.246	-0.176
7	**0.739**	**-0.387**	0.240
8	**0.869**	**0.358**	0.149
9	**0.792**	0.056	-0.249
10	**0.654**	**0.355**	-0.172
11	**0.911**	-0.019	**0.257**
12	**0.862**	**-0.378**	-0.006
13	**0.738**	**-0.594**	0.079
14	**0.890**	0.201	0.019
15	**0.772**	**-0.568**	-0.030
16	**-0.711**	**0.537**	**0.323**
17	**0.723**	0.246	**-0.302**
18	**0.669**	**-0.661**	-0.153
19	**0.893**	**0.288**	-0.067
20	**0.771**	**0.389**	-0.221
21	**0.675**	**-0.630**	-0.186
22	**0.828**	0.013	-0.102
23	**0.880**	0.216	0.137
24	**0.827**	**0.284**	-0.101

Table 4.2 The vector of standardised directional selection gradients (***β***), and the matrix of quadratic and correlational selection gradients (**γ**) for male CHC expression (i.e. PCs) and body size (PW) with respect to fighting and mating success in *G. cornutus*. Significant values (*P* <0.05 after randomisation tests) are shown in bold.

		γ			
	β	PC1	PC2	PC3	PW
Fighting success					
PC1	0.044	-0.002			
PC2	0.029	0.047	0.018		
PC3	***-0.149	0.044	0.016	0.016	
Pronotum width (PW)	***0.162	0.004	-0.041	-0.036	0.106
Mating success					
PC1	***-0.152	**-0.138			
PC2	-0.002	0.062	-0.082		
PC3	***-0.151	-0.034	0.007	**-0.112	
Pronotum width (PW)	*0.063	0.037	0.002	-0.029	-0.018

Table 4.3 The **M** matrix of eigenvectors from the canonical analysis of **γ** for male CHC (i.e. PCs) and body size. θ_i is the strength of directional selection and λ_i is the strength of non-linear along each of the eigenvectors m1-m4 across the two episodes of sexual selection. Significant values (*P* <0.05) are indicated in bold.

	M				Selection	
	PC1	PC2	PC3	PW	***ϑi***	***λi***
Fighting Success						
m1	-0.549	-0.592	-0.551	0.210	***0.074**	0.914
m2	0.020	-0.688	0.726	0.019	***-0.124**	0.001
m3	0.806	-0.308	-0.325	0.386	***0.137**	-0.036
m4	0.219	-0.285	-0.253	-0.898	**-0.107**	-0.131
Mating Success						
m1	-0.384	-0.254	0.291	-0.840	-0.038	0.011
m2	0.401	0.821	0.065	-0.410	***-0.098**	-0.053
m3	0.210	-0.203	-0.893	-0.343	**0.082**	***-0.115**
m4	0.806	-0.473	0.341	-0.110	***-0.179**	*-0.194

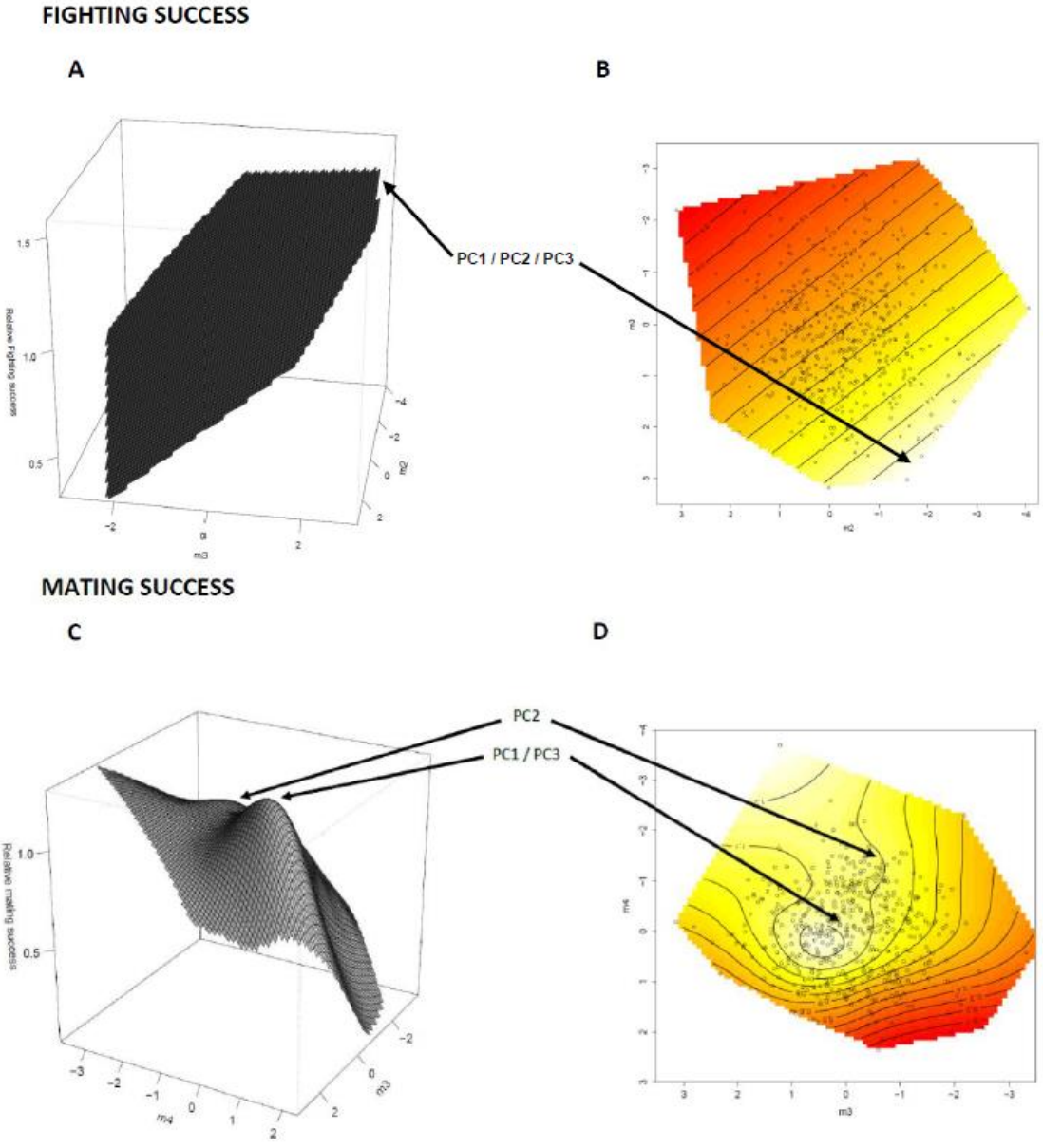
FIGHTING SUCCESS
A
B
PC1 / PC2 / PC3
MATING SUCCESS
C
D
PC2
PC1 / PC3

Fig. 4.1 Thin-plate spline visualization of the major canonical axes for fighting success (A and B) and mating success (C and D). The three-dimensional surfaces on the left (A and C) show a perspective-view while the contour plots on the right (B and D) show the same surfaces from above. The highest peaks are labelled with the traits that contribute most strongly to these regions of high/low fitness when the coefficients of both eigenvectors are interpreted together. Points on the contour plots represent actual males and white/very pale yellow areas indicate areas of highest/high fitness whereas red areas indicate areas of lower fitness.

CHAPTER 5: SEXUAL SELECTION ON MALE CUTICULAR HYDROCARBONS: EXPERIMENTAL MANIPULATION OF MALE HYDROCARBON PROFILE

5.1 ABSTRACT

Selection is rarely univariate, and as traits targeted by selection are often phenotypically and genetically correlated with other traits, it can be difficult to identify the true targets of selection from observational analyses alone. Selection gradients estimated from our multivariate selection analysis in chapter 4 indicate that male CHCs are subject to sexual selection via female mate choice in *Gnatocerus cornutus*. Here we attempt to verify these selection gradients using experimental manipulation. We identify attractive and unattractive CHC profiles based on their position in the fitness surface generated in chapter 4, and then use these attractive and unattractive CHC extracts to experimentally perfume random males before measuring their mating success. We found no significant difference in the mating success of males manipulated with attractive and unattractive CHC extracts. We discuss the possible explanations for this non-significant result, and its implications.

5.2 INTRODUCTION

For decades after Darwin first postulated sexual selection, the true complexity of this process remained unappreciated. The focus of sexual selection was thought to be univariate and the two main mechanisms of sexual selection – male-male competition and female

mate choice – were presumed to work together in harmony, selecting for the same traits in the same direction (Berglund *et al.* 1996; Qvarnström and Forsgren 1998). However, we now know that the process of sexual selection is not so clear cut. Today, evolutionary biologists widely recognise that sexual selection is rarely, if ever univariate, for example a large body of evidence now shows that females often rely on multiple cues to decide with whom to mate (see Candolin 2003 for a review). Furthermore, these multivariate targets of selection are often phenotypically and genetically correlated with each other (Lande and Arnold 1983; Grafen 1988; Wade and Kalisz 1990; Krakauer *et al.* 2011). Standard multivariate selection analyses designed by Lande and Arnold (1983) measures selection on correlated traits, allowing researchers to separate the effects of direct and indirect selection between multiple measured traits. However, one cannot measure every trait, and consequently multivariate analyses are unable to account for correlations between measured and unmeasured traits. Conducting this type of analysis alone cannot tell us if a particular trait is actually a target of selection, or whether this trait is merely being pulled along via indirect selection on some unmeasured but correlated trait (Krakauer *et al.* 2011). To confirm selection gradients, multivariate selection analyses need to be paired with experimental manipulation (Grafen 1988; Wade and Kalisz 1990; Krakauer *et al.* 2011). Experimental manipulation allows researchers to isolate the effects of a single trait and therefore verify whether the trait in question is indeed a target of selection (Grafen 1988; Wade and Kalisz 1990; Krakauer *et al.* 2011).

Cuticular hydrocarbons (CHCs), semiochemicals found on the outer cuticle of all insects have been shown to play a multitude of roles in insect communication. Male CHCs are thought to have significant effects on male mating success, having been shown to be

linked to both male competitive ability (e.g. cockroaches – Roux *et al.* 2002; Field crickets – Kortet and Hedrick 2005; Thomas and Simmons 2009b; 2011a) and attractiveness (e.g. crickets – Kortet and Hedrick 2005; Ivy *et al.* 2005; Thomas and Simmons 2009c; 2011b; Steiger *et al.* 2013;2015; *Drosophila* – Blows 2002; Howard *et al.* 2003; Chenoweth and Blows 2005; Ingleby *et al.* 2014). Although many studies have demonstrated an association between male CHC composition and mating success under female choice, only a handful of studies have estimated the strength and form of sexual selection imposed on male CHCs by female choice (reviewed in Steiger and Stökl 2014). The results of these multivariate selection analyses all indicate that male CHC composition is subject to strong and often complex sexual selection via female choice (Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2013; 2015; Ingleby *et al.* 2014). However, to date no study has attempted to verify these selection gradients using experimental manipulation, and therefore the true importance of male CHCs in determining male mating success remains unknown.

In chapter 4 we used a multivariate selection analysis to estimate the strength and form of sexual selection imposed on male CHCs by male-male competition and female mate choice in the broad-horned flour beetle *Gnatocerus cornutus*. Here, we attempt to verify the selection gradients obtained for female choice, by experimentally manipulating male CHC profile with extracts from the individual CHC profiles of 'attractive' and 'unattractive' males identified in the fitness surface generated in chapter 4. Similar perfuming techniques have been successfully employed to investigate the importance of CHCs for sexual isolation in leaf beetles – perfuming females with conspecific and heterospecific CHCs (Petersson *et al.*

2007) - and male detection of sperm competition risk in field crickets – perfuming females with rival male CHCs (Thomas and Simmons 2009a).

5.3 MATERIALS AND METHODS

Stock populations and rearing protocols

Beetles used in this study were taken from stock populations of *G. cornutus* derived from the Japanese National Food Research Institute (NFRI), where beetle cultures have been maintained for over 50 years (see Okada *et al.* 2006 for details of origin and culture conditions). In our laboratory, mixed sex populations have been maintained since 2012 in pots (Thermoscientific Nalgene 500mL, 120mm OD) containing 100 individuals (50 ♀ and 50 ♂). These stock populations are reared on wholemeal wheat flour enriched with 5% yeast and incubated at 27°C and 60% humidity on a 14L: 10D lighting cycle (Okada *et al.* 2006). Every 3 – 4 weeks, final instar larvae are randomly removed from each stock pot (n = 18) and placed into six 24-well plates as pupation is inhibited at moderate to high larval density (Tsuda and Yoshida 1985). At eclosion, 50 male and 50 female adults are randomly selected to form the parents of the next generation of each pot.

The CHC profiles of randomly selected males were manipulated by perfuming individuals with single CHC extracts of extreme 'attractive' or 'unattractive' males. We identified these extreme males based on their position in the fitness surface created in chapter 4. Identifying 'unattractive' males as those positioned in the area of lowest fitness (negative m3 and m2 scores) and 'attractive' males as those located in the area of highest fitness (i.e. positive m3 and m2 scores) (see fig. 5.1a).

Next, focal males were randomly allocated to either the 'attractive' or 'unattractive' treatment on day 11 post-eclosion and perfumed in the following way. Males were vortexed alone in a 1.5mL Eppendorf on a low setting for 30 seconds (Lane *et al.* 2015) to remove a proportion of the focal males' own CHCs after which they were removed and left to recover for 5 minutes. After this time males were placed individually into autosampler vials containing the residual CHC extracts of either an 'attractive' or 'unattractive' male and vortexed on a low setting for a further 30 seconds. Males were then removed from these vials and placed into individual cells to recover for 30 minutes. This vortexing technique has previously been used to perfume individuals in a number of studies (Thomas and Simmons 2009a; Weddle *et al.* 2013; Capodeanu-Nägler *et al.* 2014; Lane *et al.* 2015), in which it has successfully elicited behavioural responses. Each CHC extract was used once only during the experiment. This approach provided a total of 69 'attractive' extracts and 55 'unattractive' extracts.

After 30 minutes, each male was paired with a random virgin female of the same age and observed for 20 minutes, during which time we recorded any courtship attempts and copulations that occurred. If copulation occurred, pairs were separated immediately afterwards. At the end of the 20 minute trial, males were assigned a binary fitness score of 1 or 0 (successful or unsuccessful respectively) in relation to their mating success. Males were then placed into individual Eppendorf tubes and frozen at -20 °C for subsequent body measurements. Courtship is essential in this species as males cannot force females to mate and females never initiate mating. As a result, males who failed to court were removed from the experiment entirely (Resulting sample sizes – 'attractive' n= 45, 'unattractive' n = 42).

To control for the potential effects of vortexing per se on male mating behaviour (measured here as propensity to court), two additional control treatments were included, a vortexed control group and a non-vortexed control group. The vortexed control males (n=20) were reared and vortexed in exactly the same way as our manipulated males, only the vials used were clean, leaving their CHC profiles unchanged. Non-vortexed control males (n=20) were reared as above but were not vortexed at all. All treatments were subject to the same mating conditions.

Male body size was measured by capturing images of the dorsal view of focal males' bodies using a Leica M125 microscope with mounted camera (Leica DFC295, Leica microsystems Ltd. CH-9435 Heerbrugg) which conveyed images to a PC. We then measured the width of the pronotum (to the nearest 0.01mm) as an index of body size (Okada *et al.* 2006) using ImageJ (version 1.46r). We measured a subset of these pronota twice to calculate the repeatability of this measure [using the R code (Wolak *et al.* 2012)] and found that the repeatability was high ($F_{28,29}$ = 93.37, r = 0.98, CIs: 0.99,0.96 P<0.001).

Statistical analyses

To analyse the effects of our experimental perfuming treatment on male mating success we performed a generalized linear model fitted with a binomial error structure, including pronotum width as a covariate. We then used the same type of model to examine whether vortexing *per se* affected male courtship propensity.

5.4 RESULTS

After our study was complete, we discovered that the selection analysis from which we had originally identified and extracted our "extreme" males (as described above) was in fact incorrect (the correct analysis is presented in chapter 4). Re-running the selection analysis altered the loadings of the eigenvectors and the major axes of sexual selection. Thus this new analysis changed which males now represented the most extreme CHC profiles. We identified the positions of our original "extreme" males on the new fitness surface and although the majority of these males still resided in the areas of highest and lowest fitness, some did not (see fig. 5.1b). To attempt to correct our comparison in the context of this new fitness surface, we removed the least extreme males (those that no longer fell within the areas of highest and lowest fitness on the new surface) until we had 30 males remaining in each treatment (N = 60) (see fig. 5.1c). We then analysed these 60 males.

There was no significant difference between the mating success of 'attractive' and 'unattractive' males ($\chi^2_{1,59}$ = 0.11, P = 0.73) (see fig 5.2). In accordance with a previous study we also found no significant effect of body size on mating success ($\chi^2_{1,58}$ = 1.41, P = 0.23). Vortexing *per se* had no significant effect on male courtship behaviour (courtship propensity) ($\chi^2_{1,46}$ = 0.003, P = 0.96).

5.5 DISCUSSION

Despite the well-established view that selection gradients estimated from multivariate analyses need to be confirmed using experimental manipulation (Wade and Kalisz 1990; Krakauer *et al.* 2010), ours is the first study, to our knowledge, to attempt such verification

for sexual selection on CHCs. We found no significant difference in the mating success of males who had been perfumed with the CHCs of 'attractive' and 'unattractive' males. Unfortunately, we cannot know whether this lack of significance was simply due to our manipulation failing to establish a biologically significant difference between our treatment groups, or whether CHC profile alone is not an important determinant of male mating success.

The error found in our previous analysis from which attractive and unattractive males were identified will have weakened the effect of our treatments, but as our extreme males still mapped onto the areas of highest and lowest fitness on the fitness surface, this is unlikely to explain our convincingly non-significant result. A more likely explanation is that our experimental manipulation did not work. It is possible that the concentration of a single individual's CHC extract was not enough to significantly alter the manipulated male's overall CHC profile, which will include the individual's own profile. Previous experiments have applied CHC extracts from single individuals in a similar manner and successfully yielded behavioural responses (e.g. Petersson *et al.* 2007; Thomas and Simmons 2009a). Another technique used by many studies is to pool individual CHC extracts together to perfume individuals (e.g. Thomas and Simmons 2009a; Weddle *et al.* 2013; Capodeanu-Nägler *et al.* 2014; Lane *et al.* 2015), however this was not possible in our study as extracts were limited by the number of 'attractive' and 'unattractive' males identified in the fitness surface generated in chapter 4.

An alternate explanation for the lack of behavioural response is that male CHC profiles in isolation are not important determinants of male mating success but rather become important in the context of other male traits. Mounting evidence demonstrates that females often base their mate choice upon multiple cues (see Candolin 2003 for a

review). There are several hypotheses for why females rely on multiple cues, for example multiple cues may provide information on different aspects of male quality (the multiple messages hypothesis - Møller and Pomianowski 1993) or alternatively may act as back-up signals, reinforcing the same information (Johnstone 1996). The use of multiple cues can decrease the value or importance of individual cues in several different ways. Firstly multiple cues can be additive, such that the strength of female preference is proportional to the number of cues available for assessment. Alternatively, cues can interact with one another, in which case the importance of a particular cue can be completely reliant on the expression of another cue (Candolin 2003). For example female side-blotched lizards exhibit a preference for barred dorsal patterning, but only if males are also yellow throated (Lancaster *et al.* 2009). In many species, females assess cues sequentially, requiring that the first cue exceed a certain threshold before the next cue is even considered (e.g. European bitterling – Candolin and Reynolds 2001; Satin bowerbirds – Coleman *et al.* 2004; Bower-building cichlid fish – Young *et al.* 2010). It is thus possible that the reason our manipulation failed to elicit a behavioural response is because CHCs alone are not determinants of male mating success, but rather are evaluated by females in the context of other traits. It is equally possible that male CHCs are not important for male mating success at all and that the selection gradients estimated in our selection analysis (chapter 4) reflect indirect selection on male CHCs as a result of direct selection on some unmeasured trait. However, we cannot know for sure why our manipulation did not elicit a behavioural response and thus can only speculate as to the true importance of CHCs in determining male mating success.

Our study highlights the difficulties with finding a suitable method for experimentally manipulating complex multivariate traits, especially chemical traits. CHC profiles are particularly complex as they consist of so many compounds (i.e. 24 compounds in *G. cornutus*). Less complex chemical traits can be manipulated using synthetic compounds (where available), for instance male pheromones in the cockroach *Nauphoeta cinerea* are made up of 3 components, all of which have been synthetically manufactured and can be easily manipulated as a result (e.g. Moore *et al.* 1997; Moore and Moore 1999). Identifying a suitable method for successfully manipulating CHC profiles will be a key challenge in verifying the importance of these chemical traits in determining male fitness across species.

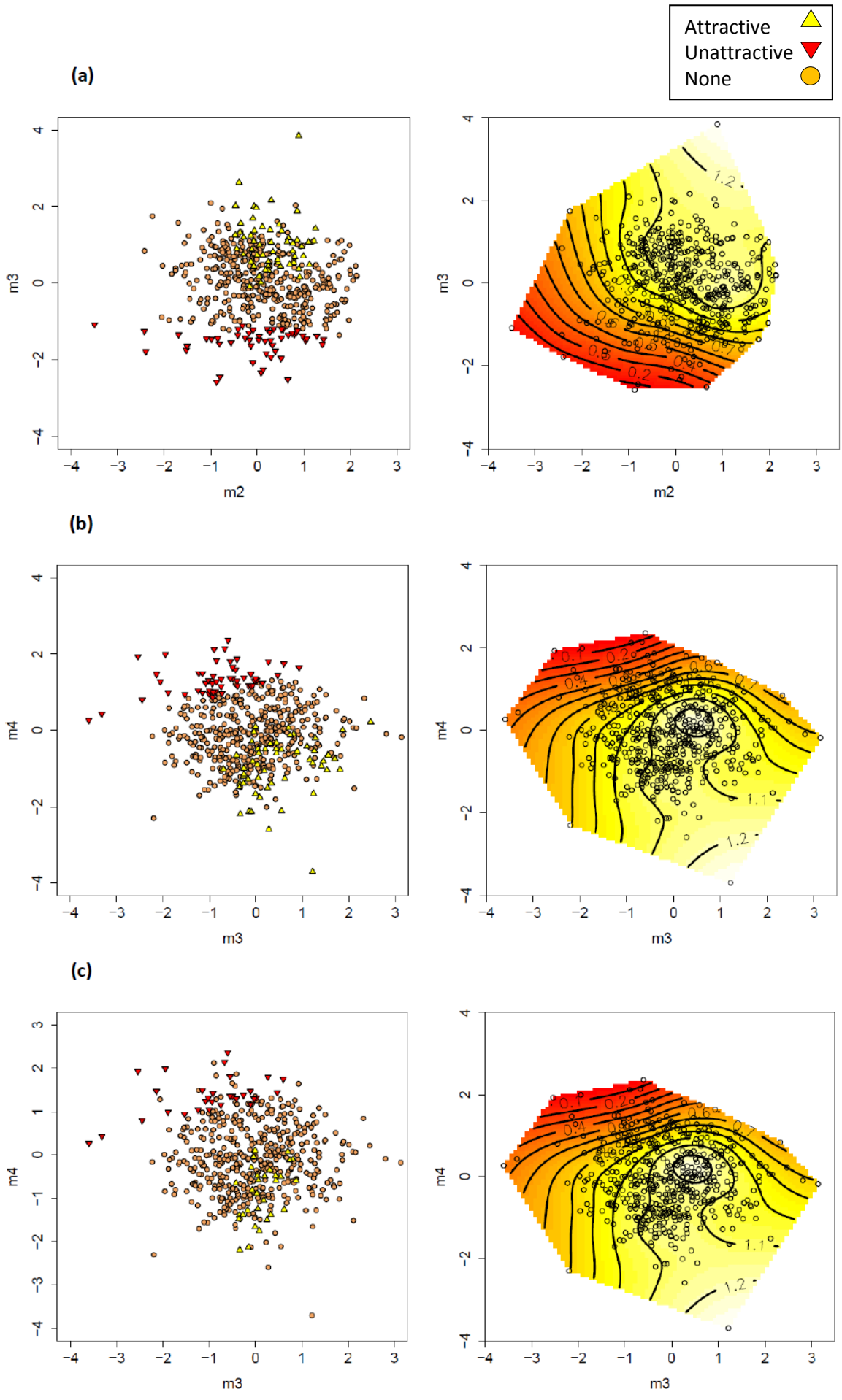
Attractive
Unattractive
None
(a)
m3
m2
(b)
m4
m3
(c)
m4
m3

Figure 5.1 (a) Original fitness surface generated in chapter 4 showing positions of 'attractive' and 'unattractive' males taken for manipulation. The contour plot on the right shows the same surface. **(b)** Corrected fitness surface showing positions of 'attractive' and 'unattractive' males originally identified and used for the experimental manipulation along with contour plot of new surface. **(c)** Corrected fitness surface showing the positions of the final 30 'attractive' and 'unattractive' males retained after least extreme males removed.

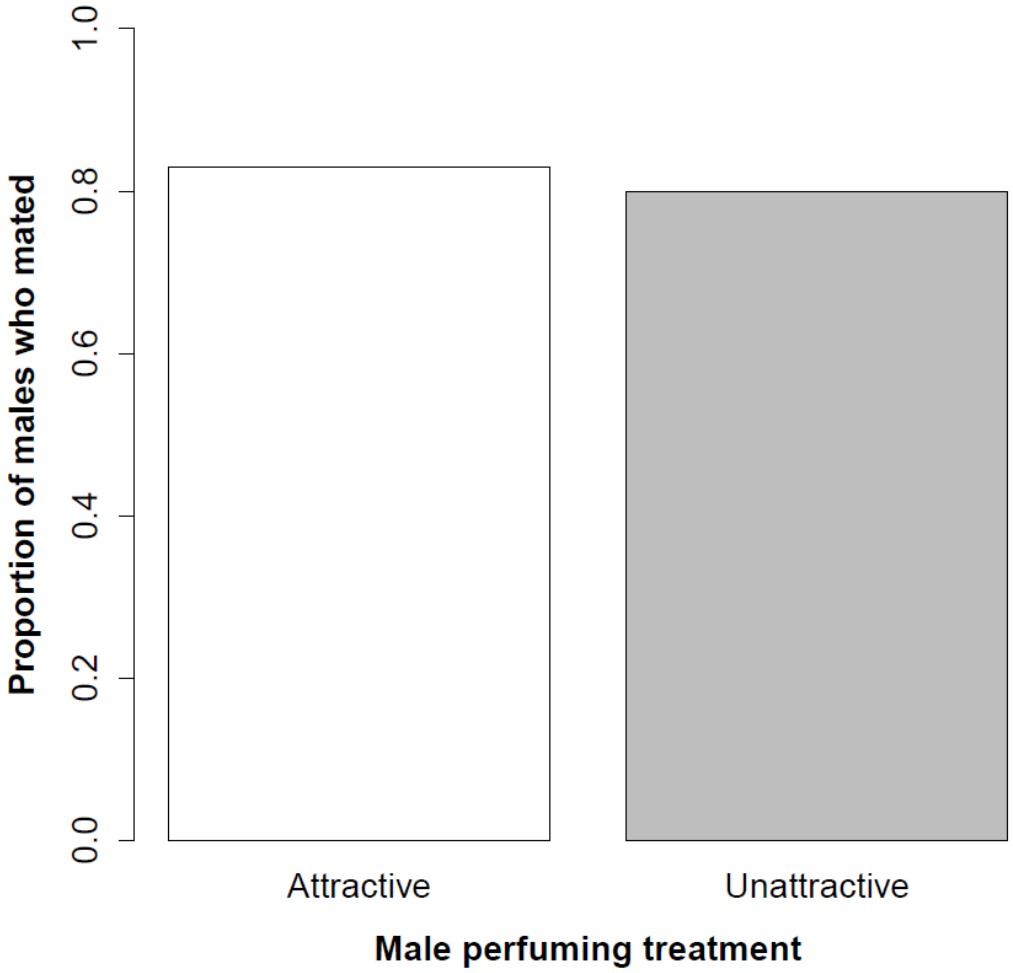

Figure 5.2 The proportion of males who successfully mated. There was no significant difference in male mating success between treatments ($\chi^2_{1,59} = 0.11$, $P = 0.73$).

CHAPTER 6: SAME-SEX SEXUAL BEHAVIOUR AS A DOMINANCE DISPLAY

6.1 ABSTRACT

Same-sex sexual behaviour (SSB) is widespread across taxa. One adaptive hypothesis to explain the occurrence and maintenance of SSB, is that it acts to intensify or diminish aggression by providing males with a means to reinforce or resolve dominance. However, evidence for this hypothesis is very limited across taxa and the possibility that same-sex sexual behaviour acts as an extension of intra-sexual competition remains contentious. We investigated the role of SSB in intensifying or diminishing aggression in the broad-horned flour beetle *Gnatocerus cornutus*. We tested the hypothesis that SSB is an extension of male-male competition by observing how the occurrence of SSB and the stability of SSB courtship roles (i.e. whether males switched between mounting and being mounted) influenced levels of aggression within pairs. We found that typically, males rapidly establish fixed SSB roles and moreover that the occurrence of SSB and the stability of SSB roles had a highly significant effect on levels of aggression observed within pairs. Pairs in which one male consistently mounted the other showed significantly lower levels of aggression than in pairs in which neither male exhibited SSB or in which males continuously switched SSB roles and attempted to mount each other. Furthermore, males who were consistently on the receiving end of SSB demonstrated lower propensity to court females and had a lower mating success than active males. This pattern was analogous to that found in loser males as a result of fighting. Males who lost fights also courted less and had lower mating success

than males who won fights. Our findings provide the first empirical support for the hypothesis that same-sex sexual behaviour is an extension of male-male competition. Furthermore, our results suggest that SSB may act as a display, allowing males to resolve dominance hierarchies without escalating into an injurious fight.

6.2 INTRODUCTION

Same-sex sexual behaviour (SSB) is a widespread phenomenon seen across a huge variety of taxa (see Bailey & Zuk, 2009 for a review). SSB ranges from courtship to mounting to even long-term pairing in some species (e.g. Laysan albatross [Young, Zaun, & VanderWerf, 2008]). There are many different hypotheses for the existence and maintenance of same-sex sexual behaviour, both adaptive and non-adaptive (reviewed in Bailey & Zuk, 2009). Examples of adaptive hypotheses include social bonding (e.g. Bottlenose dolphins [Mann, 2006]; Japanese macaques [Vasey, Chapais, & Gauthier,1998]), practice for future mating (*Drosophila spp.* [McRobert & Tompkins, 1988]) and even increasing attractiveness to potential mates (Atlantic mollies [Bierbach, Jung, Hornung, Streit & Plath,2013]). Mistaken identity, in which individuals fail to distinguish between the sexes and thus to recognise potential mates, constitutes the major non-adaptive hypothesis for the occurrence of SSB and has been invoked to explain the majority of SSB cases observed in insects (SSB occurs in over 100 species of insects – see supplementary material of Sharf & Martin, 2013 for details).

One adaptive hypothesis for the occurrence of SSB is that it provides a way for males to reinforce or resolve dominance hierarchies (Bailey & Zuk, 2009). Furthermore, by carrying

out SSB, dominant individuals may increase their reproductive success relative to that of their competitors (Bailey & Zuk, 2009). However, while these hypotheses have been investigated in a range of species, evidence for SSB as an extension of intra-sexual competition remains contentious. When testing for a link between dominance, aggression and SSB in female Japanese macaques, Vasey et al., (1998) found that SSB was not carried out more often by dominant females, nor were levels of aggression affected by the occurrence of SSB. In the male American Bison, same-sex sexual behaviour is commonplace and although there is some link between SSB and dominance, SSB appears to be more clearly linked to age rather than social rank and it remains unclear whether this behaviour is an attempt to vie for dominance or simply an act of play between immature bulls (McHugh, 1958; Reinhardt, 1985; reviewed in Vervaecke & Roden, 2006). In insects the evidence is similarly lacking. Many studies have investigated the dominance/aggression hypothesis but as in the Japanese macaques, found no evidence to support it (e.g. the flour beetle *Tribolium castaneum* Levan, Fedina & Lewis, 2009 and the parasitoid wasp *Pysttalia concolor* Benelli & Canale, 2012). Although some insect studies have linked SSB to a reduction in aggression (Peschke, 1985, 1987; Ruther & Steiner, 2008; Steiner, Stiedle & Ruther 2005), the evidence is indirect. These studies show that when males deliberately mimic a female's chemical profile they are treated as females (i.e. courted and not fought with) but they do not directly show that SSB causes a reduction in aggression. Although this pattern is interesting, it seems more likely that SSB is driven by mistaken identity as opposed SSB to establish dominance. Finally, other studies have found anecdotal evidence to support a direct link between same-sex behaviour and decreased aggression but this evidence has yet to be backed up empirically (Iguchi, 1996; Preston-Mafham, 2006).

Although the occurrence of SSB is undisputed, it remains unclear who benefits from SSB, the male performing SSB (referred to hereafter as the active male) or the male receiving SSB (referred to hereafter as the passive male) (*sensu* Sharf & Martin, 2013). In the dung fly *H. livens* males are thought to mount other males in order to deny them mating opportunities, increasing their own mating success by eliminating competition (Preston-Mafham, 2006), however this hypothesis has again yet to be empirically tested. In contrast, many studies of SSB have found that male-male courtship has a positive effect on the subsequent mating behaviour of the males who have received SSB. For example *Drosophila melanogaster* and *P. concolor* males who received courtship from other males while still young subsequently exhibited significantly higher levels of courtship with females as well as shorter copulation latency, in comparison with control males who had never received SSB (McRobert & Tompkins, 1988; Dukas, 2010; Benelli & Canale, 2012). However this behaviour did not translate into increased mating success for passive males. Thus whether or not SSB serves to increase or decrease aggression by reinforcing dominance and who might benefit from its occurrence remains unclear.

Males of the broad-horned flour beetle *Gnatocerus cornutus* are armed with enlarged mandibles which they use to push, bite and flip each other over during fights. Males fight to guard both territories and mates and males who lose fights disperse to new territories, where they actively avoid engaging in further contests for four days after the fight, investing instead in increased sperm production (Okada , Yamane & Miyatake, 2010; Yamane, Okada, Nakayama & Miyatake, 2010). Female mate choice in *G. cornutus* is not based on traits associated with fighting ability (i.e. mandible size) but rather on male courtship effort, traits which are neither phenotypically nor genetically correlated (Okada,

Katsuki, Sharma, House & Hosken, 2014). However, as more aggressive (winner) males are better able to secure access to females, they attain a significant mating advantage under competitive scenarios (Harano, Okada, Nakayama, Miyatake & Hosken, 2010; Yamane et al., 2010). Alongside this fighting behaviour, males also exhibit SSB which is clearly distinguishable from aggression and is characterised by a male mounting another male and drumming his tibia along the other male's elytra, closely mimicking male-female courtship behaviour. Fighting has been extensively studied in *G. cornutus* (Okada, Miyanoshita & Miyatake, 2006; Okada, Yamane & Miyatake, 2010; Okada et al., 2014; Okada & Miyatake, 2009, 2010; Yamane et al., 2010; Demuth, Naidu & Mydlarz, 2012), but the role of SSB is yet to be examined. Furthermore, cuticular hydrocarbons are highly sexually dimorphic in this species (Lane et al., 2015), which suggests that mistaken identity is less likely to be driving SSB in *G. cornutus*.

Here, we investigate whether same-sex sexual behaviour is an extension of male-male competition in *G. cornutus* by testing three main hypotheses. Firstly we investigate whether SSB is the result of mistaken identity: If males are unable to identify mates we would expect that males would direct similar levels of courtship behaviour towards females and other males; we also expect a positive intra-male correlation between levels of same-sex and heterosexual courtship, reflecting the activity levels of individual males. Secondly, we investigate whether SSB diminishes aggression by providing a non-injurious way for males to establish dominance. If this is the case, we predict that levels of aggression will be significantly reduced in male-male pairs where a single male consistently mounts the other (i.e. SSB roles are fixed), as we expect SSB role stability to reflect whether males have been able to resolve dominance using SSB alone. Finally, we investigate whether experiencing SSB

(i.e. being the passive male within a pair) has negative consequences on subsequent male mating success. If SSB is an extension of male-male competition, we expect the consequences of SSB and male-male fighting to be similar. Thus, we compare the effects of these two interaction types on the subsequent mating success of passive and loser males, respectively.

6.3 MATERIALS AND METHODS

Stock populations and rearing protocols

G. cornutus are a stored product pest that feed on a variety of grains, flours, yeasts and dry animal products (Linsley, 1944; Zakladnoi & Ratanova, 1987). Beetles used in this study were taken from stock populations of *G. cornutus* derived from the Japanese National Food Research Institute (NFRI), (see Okada et al., 2006 for details) and reared in our laboratory in the UK following the protocol outlined in Lane et al. (2015). For this experiment, 120 final instar larvae were collected from stock pots daily and monitored daily for eclosion. On eclosion, adults were moved into individual wells in a 24-well plate (one larva per well), provided *ad libitum* wholemeal wheat flour and maintained at 27°c with 60% humidity on a 14L:10D lighting cycle (Okada et al., 2006; Lane et al., 2015).

Experiment 1: Same-sex behaviour, aggression and heterosexual mating behaviour

Behavioural trials took place 11-15 days after eclosion. On the morning of the trials we randomly assigned males of the same age to the categories ‘focal’ or ‘non-focal’ and marked the tip of their elytra accordingly with either a green or pink gel pen (Pentel Hybrid Gel Grip

DX Metallic), the colour of the focal male was alternated between trials to control for any potentially confounding effects of marking. After marking we returned males to individual petri dishes with *ad libitum* flour until the afternoon. To observe male-male behaviour, focal and non-focal males were paired in arenas and observed for 20 min. We recorded the number of courtship attempts observed within the 20 min and noted whether they were made by the focal or non-focal male. We also recorded the number of aggressive acts that occurred between the males. At the end of the 20 min we removed the non-focal males and allowed our focal males to rest for 5 min before introducing a single female (of the same age) to each of them. We then observed these opposite-sex pairs for a further 20 min recording the number of courtship attempts (courtship effort) along with copulation latency if a successful mating occurred. A male will continue to court with the same female even after he has mated with her and thus we recorded courtship effort throughout the whole trial regardless of whether or not a pair had mated. All individuals used in trials were frozen in Eppendorf tubes at -20°c for subsequent measurements (N = 622 [311 pairs]).

In order to assess the potential effect of male body size we captured digital images of the dorsal view of focal and non-focal males' bodies (N= 622) (see Lane et al., 2015 for details on protocol used). We then measured the width of the pronotum (to the nearest 0.01mm) as an index of body size (Okada et al., 2006) using Image J (version 1.46r). We measured a subset of pronota twice to calculate the repeatability of this measure based on the variance components derived from an analysis of variance (Lessells & Boag, 1987), showing high repeatability ($F_{24,25}$ = 120.33, r = 0.992±0.0034, P < 0.001).

Statistical analyses

We tested for an effect of sex on courtship effort (no. of courtship attempts) using a generalised linear model (referred to hereafter as a GLM) fitted with a quasi-poisson error family to compensate for overdispersion of our count data (Crawley, 2005). To test for a relationship between same-sex courtship effort and heterosexual courtship effort we calculated the repeatability of these behaviours within focal males using the R code of Wolak et al. (2012).

We tested for an effect of SSB on male-male aggression in two ways. First, we split our aggression data into two different variables (1.) a binary measure of whether or not aggression occurred (coded as 0 or 1) and (2.) a count of the number of aggressive acts that were observed in pairs in which aggression did occur. This separation allowed us to investigate the effects of SSB on both the occurrence of aggression and on the amount of aggression. We then conducted two separate GLMs to analyse the effect of same-sex courtship on these two aggression variables (throughout our analyses, GLM models were fitted with either binomial [for binary variables – e.g. occurrence of aggression] or quasi-poisson [for count variables – e.g. no. of aggressive acts] error structures).

We analysed the effect of courtship role stability (i.e. no SSB at all [0 active males], SSB roles were fixed [1 active male], males switched between roles [2 active males]) on the occurrence and amount of aggression using two separate GLMs fitted with binomial and quasi-poisson error families, respectively.

Finally, we determined focal male SSB status as being either always active, always passive, both (male switched between active and passive roles) or neither (no SSB was

observed within the pair). We then analysed the effect of focal male SSB status on his subsequent heterosexual mating behaviour measured as - (1.) courtship propensity (0 = did not court or 1 = did court), (2.) courtship effort (no. of courtship attempts) and mating success (0 = unsuccessful or 1 = successful) – again using a series of GLMs including focal male body size as a covariate. We then conducted further post-hoc comparisons to identify significant differences between the four SSB statuses using a Tukey's HSD test.

All statistical analyses were carried out in R (version 3.1.2).

Experiment 2: Male fighting success and subsequent mating behaviour

To examine the effect of fighting success on subsequent male mating behaviour, groups of 4 males were randomly chosen and placed together in an arena. Males were then observed until a fight occurred from which a clear winner could be identified. The winner was considered to be the male who initiated and won most fights. At this stage the winner was removed from the arena and placed into a separate dish. The remaining 3 males were observed further until a clear overall loser could be identified. The loser was considered the male who was attacked and flipped over most and/or fled the other males most. This loser male was removed from the arena and placed into a separate dish.

After a 5 min rest period, individual winners and losers were each paired with a single female and observed for 20 min as described above. Binary measures of courtship propensity and mating success were recorded. After the trial, males were frozen for subsequent body measurement as described above.

Statistical analyses

Generalised linear models (GLMs) fitted with a binomial error family were used to analyse the effect of fighting status (winner or loser) on courtship propensity (0 = did not court or 1 = did court) and mating success (0 = unsuccessful or 1 = successful). Body size was included as a covariate to control for any potential effects on mating behaviour or mating success.

6.4 RESULTS

Experiment 1: male-male courtship, aggression and mating behaviour

82% of all male pairs (*N* = 311) exhibited SSB, and of these pairs, 27% also exhibited aggression. In 33% of all male pairs, aggression, but not SSB was observed.

The repeatability of same-sex courtship effort and heterosexual courtship effort within individual males was weak but significant ($F_{310,311}$ = 1.45, *r* = 0.18, CIs: 0.29, 0.08, *P* <0.001), suggesting that an element of same-sex courtship was driven by the overall activity levels of males. Males who exhibited higher levels of same-sex courtship also elicited higher levels of heterosexual courtship. However, our analyses also revealed that sex had a significant effect on male courtship effort. Males courted significantly more with females than with other males ($F_{1,620}$ = 13.903, *P* <0.001).

Out of all male pairs, 71% showed fixed SSB roles throughout the 20 min observation period, indicating that males establish stable dominant and subordinate roles. Furthermore, SSB role stability had a significant effect on both the occurrence of aggression ($\chi^2_{2,308}$ = 13.9,

$P < 0.0001$) and amount of aggression exhibited within pairs ($F_{2,308} = 8.32$, $P < 0.0001$). The occurrence (Tukey's HSD: $P < 0.001$ – figure 6.1a) and amount (Tukey's HSD: $P < 0.001$) of aggression observed was significantly higher in pairs in which both males exhibited SSB and SSB roles were therefore unstable (i.e. males switched between active and passive roles [no. active males = 2]) compared to pairs in which only one male exhibited SSB (i.e. SSB roles were fixed [1 active male]). Additionally, the amount of aggression exhibited by pairs in which only one male exhibited SSB was significantly lower than that seen in pairs in which neither male exhibited SSB (0 active males) (Tukey's HSD: $P = 0.02$ - figure 6.1b).

Subsequent focal male mating behaviour was significantly affected by SSB status (i.e. active, passive, both or neither). Focal male SSB status significantly affected subsequent heterosexual courtship propensity ($\chi^2 = 34.57$, $P < 0.001$) (figure 6.2a). Multiple post-hoc comparisons revealed that passive males were significantly less likely to court females than active males or males who had experienced no same-sex courtship at all ($P < 0.001$). There was no significant interaction between focal male SSB status and body size ($\chi^2 = 1.95$, $P = 0.58$), nor any significant effect of body size on courtship propensity ($\chi^2 = 0.007$, $P = 0.93$). Among males that courted a female, courtship effort ($F_{1,166} = 2.57$, $P = 0.056$) and mating success ($\chi^2 = 0.44$, $P = 0.93$) did not differ significantly between males of different statuses. However, in this species males must court to mate, as females will never initiate mating and when we included those males who didn't court into our analysis, we found that the mating success of passive male was significantly lower than active males and males who had no experience of same-sex courtship (SSB status = neither) ($\chi^2 = 16.38$, $P < 0.001$) (figure 6.2b). There was no significant difference in mating success between passive males and those who had switched between active and passive roles throughout the observation period (SSB

status = both). Nor was there a difference in the mating success of males who switched between roles, active males and males who were not involved in SSB at all (SSB status = neither). Thus the effect of SSB appears to be most detrimental for passive males who are less likely to court a female following SSB. Mating success was not significantly affected by body size ($\chi^2 = 0.06$, $P = 0.802$).

Experiment 2: Male fighting success and subsequent mating behaviour

Male fighting success had a significant effect on courtship propensity ($\chi^2 = 14.7$, $P = 0.0001$), losers were significantly less likely to court females than winners (figure 6.3a). We were unable to look at the effect of body size on courtship propensity as we did not have this data for males who failed to court. However of males who did court, we found no significant interaction between body size and status ($\chi^2 = 0.07$, $P = 0.933$) and no significant effect of body size on mating success ($\chi^2 = 0.195$, $P = 0.907$). Fighting success had a significant effect on overall mating success ($\chi2 = 8.1$, $P = 0.004$), the mating success of loser males being significantly less than that of winners (figure 6.3b).

6.5 DISCUSSION

Our findings indicate that male *G. cornutus* rapidly established fixed active and passive SSB roles in more than 70% of pairs. The stability of these roles significantly impacted both (a) whether or not aggression occurred within a pair and (b) how much aggression occurred. When both males in a pair displayed SSB (i.e. switched between being active and passive), aggression was not only more likely to occur, but occurred at a significantly higher rate in

comparison to pairs in which only one male exhibited SSB. Furthermore, aggression was significantly lower in pairs in which only one male held the active SSB role compared to pairs in which neither male displayed SSB. Together these results support our prediction that same-sex sexual behaviour is used by males to establish dominance. If one male displays SSB and is not challenged by the other (i.e. the other male does not attempt SSB in response), dominance is resolved and aggression is unlikely to occur. However, if both males attempt to mount one another (i.e. are both vying for dominance), and they are thus unable to resolve dominance using displays of SSB, it is then that they escalate to a physical contest. The use of non-injurious displays as a means to settle contests without escalation into injurious fighting is the cornerstone of the classic Hawk-Dove contest model first proposed by John Maynard Smith and Geoff Parker (1974). In short this model predicts that escalation into injurious contests should be avoided as long as the costs incurred by these fights outweigh the potential benefits (Maynard Smith & Parker, 1974). Displays are commonplace among a variety of taxa, for instance mantis shrimp possess one of the deadliest weapons in the animal kingdom, but recent research has shown that rather than use this weapon to its full potential during fights, mantis shrimp engage in non-injurious sparring to settle conflict before resorting to escalation (Green & Patek, 2015). Similarly, red deer use roaring contests and walking displays to assess their opponent and avoid the potential costs of escalating to a fight (Clutton-Brock & Albon, 1979). Our results indicate that SSB may be equivalent to such displays. As well as giving males a chance to weigh up the costs and benefits of fighting, SSB may allow males to avoid aggression by playing one of two strategies depending on their phenotype. If a male is of a more aggressive phenotype he may choose to take the role of the active partner, displaying his dominance via SSB. If a male is in some way inferior, it may pay for him to “allow” a more dominant male to mount

him in an effort to reduce the chances of becoming engaged in a contest. Similar behaviour has been suggested in the rove beetle *Aleochara curtula* in which immature, starved and multiply mated males mimic female CHC profiles in an apparent effort to avoid fights. This mimicry has been shown to significantly reduce the amount of aggression directed towards a male although increasing the amount of SSB to which he is subjected (Peschke, 1985, 1987).

Our results further show that active males subsequently courted females more and achieved higher mating success than passive males (who consistently received SSB). This pattern was similar to the relationship with male-male fights: Males who won fights had higher mating success than loser males, who were less likely to court and mate with females. A key question is whether losing a fight, or being the passive partner in a same-sex courtship interaction directly results in the subsequent decrease in mating success, or whether these roles reflect a generally inferior phenotype. Poor quality males may be more likely to be mounted by other males (or allow males to mount them as discussed above), suffer more defeats in fights, and be less likely to attempt courtship with females. However, if overall quality and inactivity was the main factor underlying the observed difference in courtship behaviour we would expect males who were not involved in SSB at all (i.e. didn't carry out or receive SSB) to exhibit similarly low levels of heterosexual courtship, but in fact, we see the opposite. Males who were not involved in SSB at all were just as likely to court females as males who had actively carried out SSB (active males). Therefore, although a correlation with general inactivity cannot be ruled out entirely, it seems more likely that there is a negative relationship between losing fights or taking the passive role in SSB and subsequent mating behaviour. One way of disentangling these two possibilities would be to

manipulate the condition of males (e.g. via dietary manipulation – House et al., 2015) and observe how condition affects whether a male is active or passive.

To date, studies of same-sex sexual behaviour across taxa have found limited evidence to support the hypothesis that SSB acts to mediate intrasexual aggression (Reviewed in Vervaecke & Roden, 2006 and Bailey & Zuk, 2009). Our results indicate that SSB is equivalent to ritualised fighting displays, acting as a non-injurious way of resolving dominance in *G. cornutus* without escalation to injurious fighting. Only when dominance cannot be resolved by SSB, do males escalate to physical conflicts.

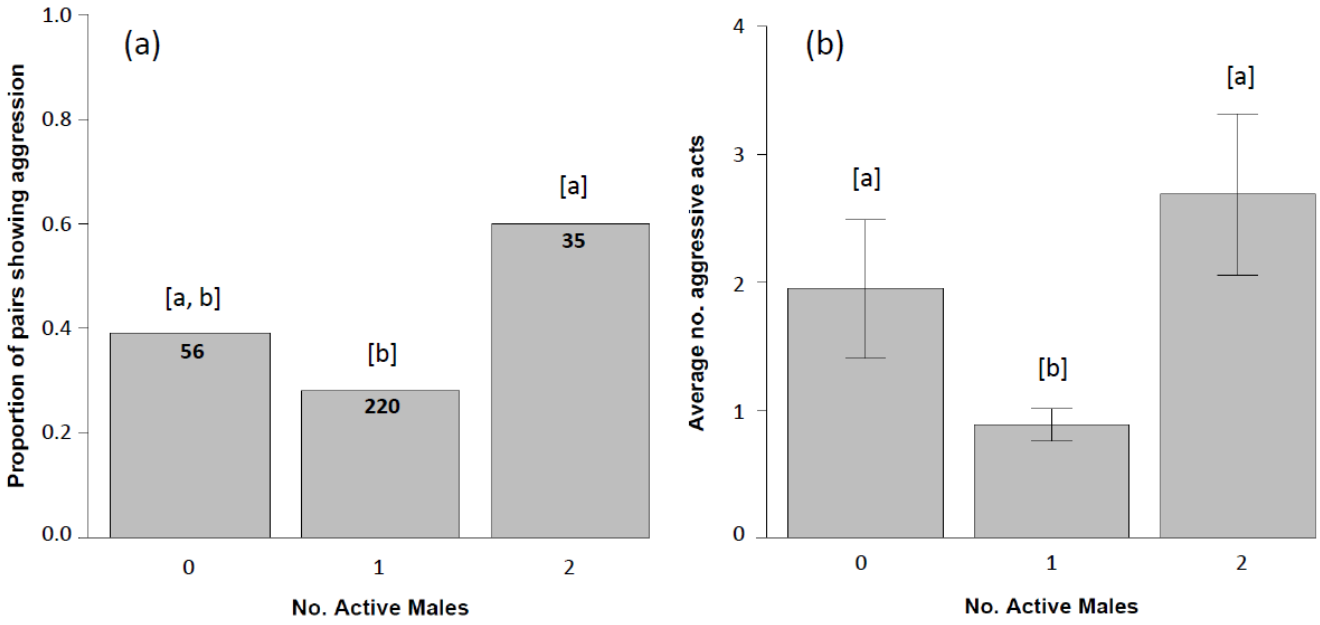

Figure 6.1 Effect of courtship role stability on aggression (a) The proportion of male-male pairs showing aggression and (b) the average number of aggressive acts that occurred ± standard error in relation to the number of active (mounting) males within pairs. Sample sizes are shown within bars and letters indicate significant differences at $P<0.05$ (Tukey's HSD).

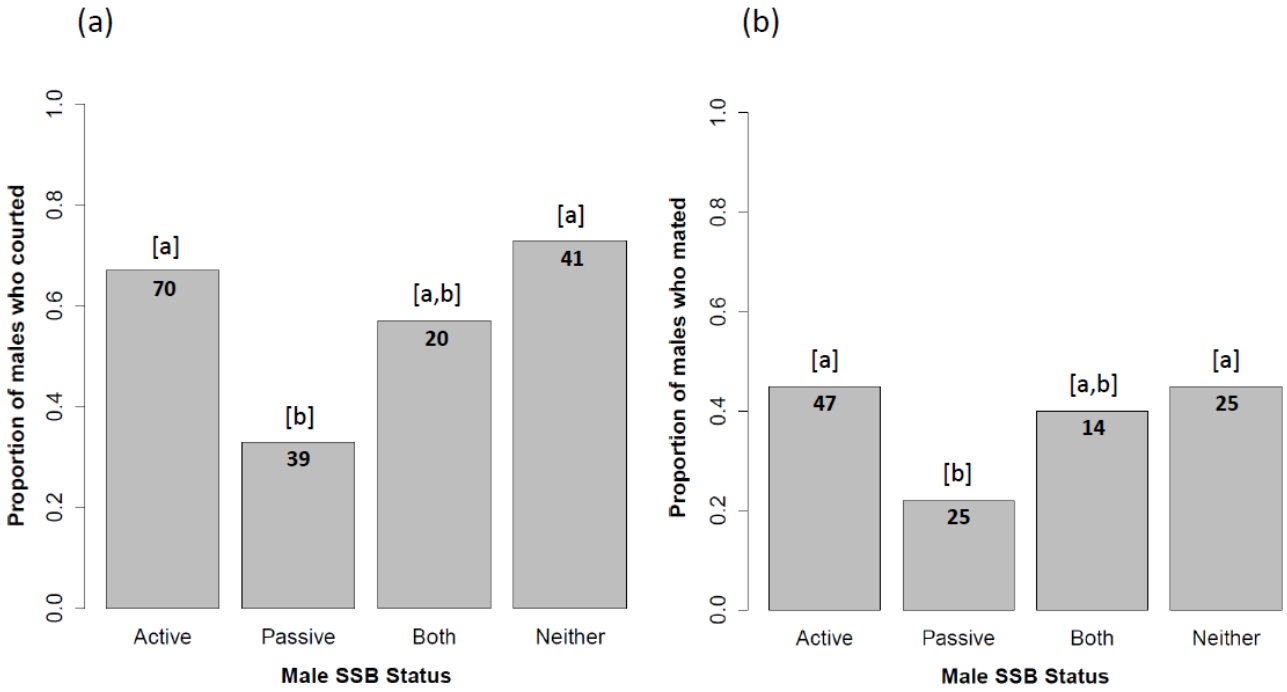

Figure 6.2 SSB status and heterosexual mating behaviour (a) The proportion of focal males who subsequently courted females differs according to their SSB status (χ^2 = 34.57, *P* <0.001). Active = consistently the courting/mounting male; Passive = consistently the male receiving courtship; Both = Males switched between active and passive roles; Neither = Males not involved in any SSB. Sample sizes are shown within bars and letters indicate significant differences at *P* <0.05 (Tukey's HSD).

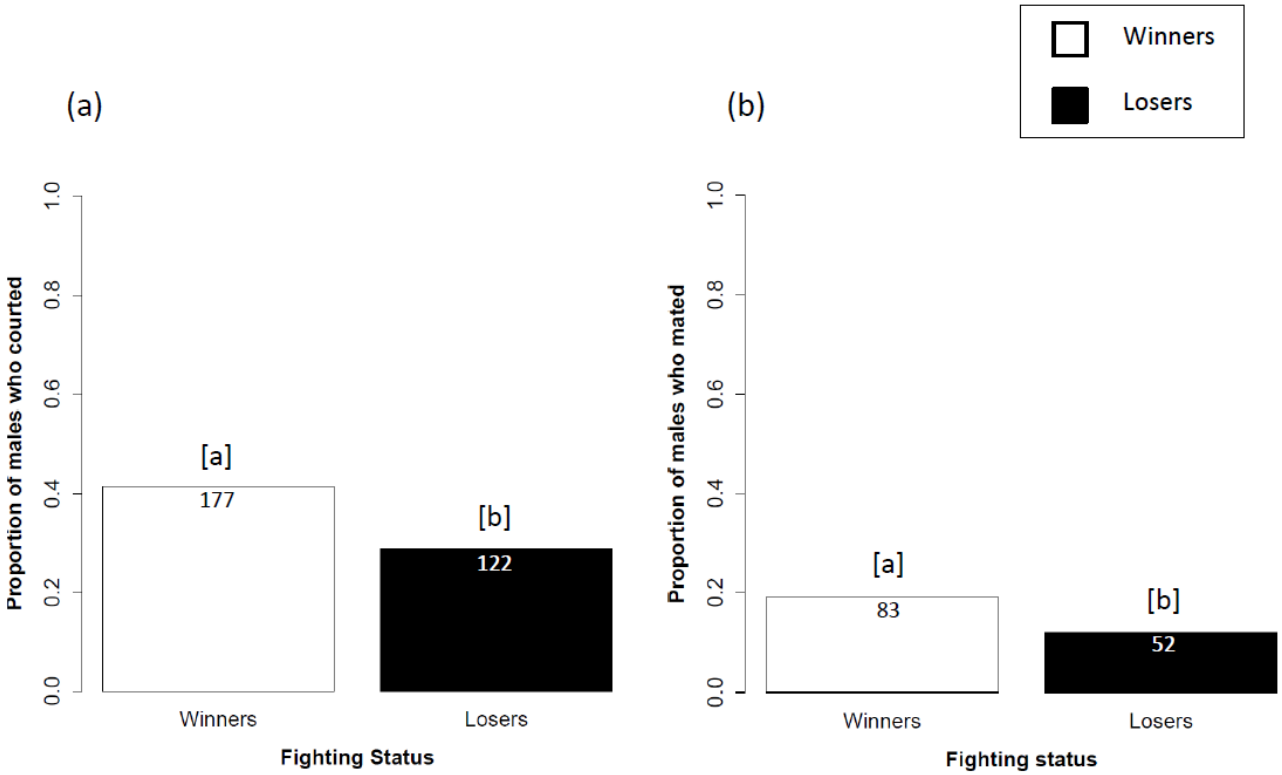

Figure 6.3 Fighting success and subsequent mating success (a) Proportion of males who subsequently courted females after a fight. (b) The proportion of males who mated (including those who failed to court) in relation to their fighting success. Sample sizes are shown within bars and letters indicate significant differences at P <0.05 (Tukey's HSD).

CHAPTER 7: General Discussion

Interest in insect cuticular hydrocarbons has grown considerably over the past thirty years. However, despite accumulating evidence of their potential importance in sexual communication and mating, research into the roles of CHCs during mating interactions and their impact on male reproductive success has been limited to *Drosophila* and field crickets (see Steiger and Stökl 2014 for a review). This thesis aimed to expand our understanding of this field by investigating the role of CHCs in sexual communication in broad-horned flour beetles. Overall my findings indicate that CHCs play important roles at each stage of mating, providing information to both males and females. In this final chapter I will discuss how the research in my thesis contributes to this field, highlighting both the knowledge gained through my work as well as the methodological limitations realised. Finally I will discuss avenues for future research.

7.1 CHCs as cues of sperm competition

Virgin females perfumed with the residual CHCs of males (Thomas and Simmons 2009a) have been shown to elicit significant adjustments in male mating investment in the field cricket *T. oceanicus*. However the precise information about the risk of sperm competition that these cues provide for males remains unclear. For example, do residual male CHCs on females provide general information about the competitive environment or specific information regarding the mating status of their current mating partner? In chapter 2 I found that as in *T. oceanicus*, the presence of male-derived CHCs elicited a response in male post-copulatory investment in *G. cornutus*. Males also adjusted their pre-copulatory

investment in response to these cues, in a manner which indicated that males (like those of *T.* oceanicus – Thomas and Simmons 2009a) were sensitive not only to the presence of these cues but also to the number or concentration of male CHCs (I cannot distinguish between these alternatives) that contributed to them. Further to these findings, I investigated how the addition of these male CHCs altered the chemical profile of perfumed females. I found that the addition of male CHCs did not make the profiles of perfumed females more similar to those of mated females. Thus male-derived CHCs do not appear to provide information about female mating status, but rather general information about their competitive environment. Whether this information about the risk and intensity of sperm competition is perceived by males as being specific to their current mating partner and is thus 'discarded' after their current mating, or is held onto and used as a general rule of thumb for future mating investment remains to be explored.

The findings of chapter 2 led me to consider the stability of CHCs once they have been applied to a female's cuticle and therefore whether they provide males with information about the risk and intensity of sperm competition beyond a single intersexual interaction. This consideration is arguably very important as females of most insect species can store sperm for an inordinate length of time (e.g. Tschinkel 1987; Hölldobler and Wilson 1990; Cook and Gage 1995; den Boer *et al.* 2009; Ala-Honkola *et al.* 2014; Lampert *et al.* 2014) and thus the sperm competition risk associated with mated females continues long after their initial copulation. However, I was unable to uncover anything about the stability of these cues as they failed to elicit a behavioural response from males at any time point when the females had been kept in flour after perfuming. Although as I recognised in the discussion of chapter 3, there may be some unknown underlying cause to the lack of

behavioural response seen, it seems more likely that these cues simply do not persist in flour. If the environment was indeed the causative factor, it begs the question as to why males respond to residual male CHCs at all when these very cues are rendered undetectable in their habitat substrate. *Drosophila melanogaster* males have been shown to rely on a combination of interchangeable cues in order to detect rivals (Bretman *et al.* 2011b). It is possible then that male-derived CHCs are just one in a suite of cues available to and utilised by *G. cornutus* males in order to assess competition. Furthermore the detection and thus relative importance of, these chemical cues may then be dependent on the environment in which a male resides. For example in the three-spined stickleback *Gasterosteus aculeatus*, females generally assess males on the basis of visual cues, however in more turbid conditions visual cues become redundant and females switch their focus to olfactory cues instead (Heuschele *et al.* 2009). Despite evidence of environmental effects on the expression of CHCs (Ingleby *et al.* 2013; 2014), surprisingly little work has been done to examine the effects of the environment on their efficacy as cues. My work here suggests that the natural environment is vitally important for the perception of chemical cues and this is an important consideration for laboratory studies that often test hypotheses under contrived conditions.

7.2 CHCs and male mating success – The combined effects of male-male competition and female choice

CHCs are known to be associated with male competitive ability (Roux *et al.* 2002; Kortet and Hedrick 2005; Thomas and Simmons 2009b; 2011b) and male attractiveness (Blows 2002; Howard *et al.* 2003; Chenoweth and Blows 2005; Ivy *et al.* 2005; Kortet and Hedrick 2005;

Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2013; 2015; Ingleby *et al.* 2014). However, studies investigating sexual selection acting on male CHCs have so far focussed solely on selection imposed by female choice (reviewed in Steiger and Stökl 2014). In chapter 4 I estimated and compared the strength and form of sexual selection imposed by both of these mechanisms. I presented the first evidence of sexual selection exerted by male-male competition on both the overall and relative abundance of male CHCs. I found that the form of selection imposed by male-male competition and female choice was significantly different. Male-male competition imposed only linear selection, while female choice exerted a much more complex combination of linear and non-linear selection. Such complex patterns of selection are commonly associated with female choice on multivariate male traits (e.g. acoustic – Bensten *et al.* 2006; Simmons *et al.* 2013, visual – Blows *et al.* 2003, chemical - Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2013; 2015; Ingleby *et al.* 2014). The significant difference in selection imposed by male-male competition and female choice suggests that males cannot be both good fighters and lovers on the basis of their CHCs. These results resonate with those of a previous study which demonstrated that females do not preferentially mate with males who win fights in *G.* cornutus, and thus that males who win fights do not necessarily secure a mate via female choice (Okada *et al.* 2014). However, in chapter 6 we found evidence to suggest that males who win fights may be able to overcome female choice by deterring their rivals from even attempting courtship with a female. These results also suggested that conducting same-sex behaviour may provide an alternative (potentially less costly than fighting) means to the same end. Together these findings suggest that winner males who possess less attractive CHC profiles (and potentially less attractive courtship behaviour – Okada *et al.* 2014) may not lose out on mating opportunities after all if they are able to circumvent female choice.

Such possibilities highlight the limitations of selection analyses that quantify selection under a restrictive set of conditions.

In an effort to further decipher what selection analyses indeed really do tell us, in chapter 5 I attempted to verify the selection gradients estimated in chapter 4 by experimentally manipulating male CHC profile. Despite the need to verify correlational selection estimates with experimental manipulation in order to ascertain the true targets of selection (Wade and Kalisz 1990; Schluter and Nychka 1994; Krakauer *et al.* 2010), combined correlational and experimental selection studies are still relatively rare. Although a number of studies have manipulated CHCs to follow other lines of enquiry (e.g. CHCs as cues of sperm competition – Thomas and Simmons 2009a; Self-referencing – Capodeanu-Nägler *et al.* 2014; Weddle *et al.* 2013), to date no one has attempted to verify estimates of sexual selection on CHCs using experimental manipulation (Steiger and Stökl 2014). Despite using perfuming techniques similar to those that have previously proved successful (Petersson *et al.* 2007; Thomas and Simmons 2009a), I was unable to confirm that CHCs are indeed an important component of male attractiveness. One possible reason for the lack of response to my manipulation is that it simply didn't work. Another potential reason however is that CHCs are just one component of attractiveness. Females often use multiple cues to assess males (Candolin 2003) and we know from a previous study that courtship effort contributes to male attractiveness in *G. cornutus* (Okada *et al.* 2914). Different components of overall attractiveness may provide different information about a male and thus may be utilised at different stages of male-female mating interactions (i.e. sequential cue use – See Candolin 2003 for a review). For example in *T. oceanicus* CHCs have been suggested to signal genetic compatibility while courtship song (another sexually selected trait) is thought to provide

information about male condition (Simmons *et al.* 2013). If a male 'smells' attractive, a female may be more likely to allow him to mount and court her, however if he does not then also exhibit attractive courtship behaviour, the female may decide not to mate with him after all. In other words, perhaps CHCs are only important for female mate choice in the context of other sexually selected traits.

However as discussed in chapter 5, it is extremely difficult to interpret these results as we cannot disentangle the possibility that my method of manipulation simply did not work from the potential biological significance of the results. This dilemma highlights the need to find a suitable and perhaps more sophisticated way of manipulating CHC profiles. One clear possibility is to use experimental evolution or inbred lines. Inbred lines have been used successfully in other CHC studies to investigate the ability of females to self-reference in order to avoid mating with related males (Capodeanu-Nägler *et al.* 2014) and previous mating partners (Weddle *et al.* 2013). Experimental evolution lines on the other hand could provide a means of long term manipulation, whereby lines are selected for 'attractive' and 'unattractive' CHC profiles over multiple generations. Male CHC extracts from 'attractive' and 'unattractive' lines could then be pooled together to perfume random males with a higher concentration of CHCs, potentially providing a more powerful methodology than the individual extracts I used in chapter 5.

7.3 Future directions

In chapter 2 I investigated male behavioural plasticity in response to the presence of CHCs, however there is increasing evidence to show that CHC expression itself is a highly plastic

trait (reviewed in Ingleby 2015). Just as CHCs were once thought to demonstrate little to no intra-species variability, CHC expression has been thought to be fixed and in many ways is still viewed this way. But evidence shows that CHC expression can be subject to rapid short-term changes and this is likely to have an immense impact on what we think we know about CHCs. At perhaps the simplest level, mating alters CHC expression (Everaerts *et al.* 2010; Weddle *et al.* 2013). This means that the post-mating CHC profile is unlikely to reflect that assessed by the selecting female (or male) before copulation. However, as most commonplace CHC extraction methods require that individuals are killed, all studies of sexual selection on CHCs (including chapter 4 of this thesis) are thus far based upon measures of CHC expression after mating has occurred – you cannot kill an individual first and then observe its mating behaviour later (Thomas and Simmons 2009c; Simmons *et al.* 2013; Steiger *et al.* 2013; 2015; Ingleby *et al.* 2014). This casts doubt on what we can realistically learn from these analyses. They clearly provide evidence of selection, but selection on what exactly? How similar/different are the pre- and post-mating hydrocarbon profiles of the same individual? This is a very key point that needs to be assessed if we are to go forward with confidence in this field. Non-lethal techniques are available (for instance solid phase microextraction [SPME] which facilitates multiple sampling of the same individual over time), and although they are time consuming and decisively more expensive than the traditionally used hexane extractions, they have already been shown to be fruitful tools for research into CHC plasticity. For example, using SPME to take samples from the same individuals before and after a fight, Melissa Thomas and Leigh Simmons (2011b) demonstrated that *T. oceanicus* males modify CHC expression when their social status changes as a result of fighting outcome. The CHC profiles of dominant males who lost fights

and became subordinate were found to have altered CHC profiles that were more similar to that of subordinate males.

With plasticity comes the question of what is under selection, the trait itself or the ability to plastically alter the expression of said trait? *Drosophila serrata* males have been shown to alter their CHC expression immediately after seeing or briefly touching a female (Petfield *et al.* 2005). Furthermore, the nature of this modification depends on the condition and CHC expression of the female with whom they interact – an example of an indirect genetic effect (IGE). However, whether or not there is variation between males in the ability to carry out this plastic response is unknown. If there is, just as sperm competition selects on the ability of males to detect and respond to cues of sperm competition risk, the ability of males to plastically alter their CHC profile in response to specific females in itself is likely to fall under sexual selection.

7.4 Concluding remarks

Cuticular hydrocarbons play a diverse range of roles in insect communication yet despite a growing interest in these chemical cues, our knowledge of their role in sexual communication and moreover in determining male reproductive success remains limited. The aim of this thesis was to expand our understanding of the roles CHCs play during intra-, inter- and post-copulatory sexual selection and how together these episodes of selection influence male reproductive success. I have shown that CHCs do indeed play important functions at each episode of selection, providing information to both males and females. I have demonstrated that CHCs can provide males with information on their competitive

environment, but that the persistence of these cues overtime is likely to be highly dependent on the physical environment. I have presented the first evidence that males CHCs are subject to sexual selection via male-male competition and that same-sex sexual behaviour may provide males with a means of circumventing female choice. However, my work has also brought to light, the many gaps that still require filling before we are to fully understand the true importance of CHCs for male reproductive success. Incorporating the incredible plasticity of CHCs into future research will likely provide key insights into the true importance of these traits in mating interactions and shed a new light on previous CHC research.

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APPENDIX

R code for canonical analysis from Reynolds *et al.* 2010

```
#############################################################################
# R Script for Permutation Based Hypothesis Tests of the Eigenvalues from a
# Canonical Analysis of the Gamma Matrix of Quadratic and Correlational
# Selection Gradients
# Questions and bug reports to npajewski at ms.soph.uab.edu
# Updated: 9/04/2009
#############################################################################
#                        PROGRAM NOTES                                      #
#############################################################################
# 1. R available from http://www.r-project.org/
# 2. This program uses the "car" package for R, therefore this package needs to
# be installed prior to using the script. To install, type the following at the
# command prompt upon opening R.
install.packages("car", dependencies=TRUE)
# and then select an appropriate mirror for download (say USA(MI)).
# 3. Users simply need edit the file paths and parameter settings within the
# user input section. The entire code can then simply be copied
# (CNTL-A then CTNL-C) and pasted into the R window
#############################################################################
#                  DESCRIPTION OF INPUT FILE FORMATS                        #
#############################################################################
# The script expects 2 files + an optional covariates file as input. The default
# is to have these files in comma delimited format (.csv), although appropriate
# changes for tab delimited data are documented below.
# 1. Fitness components file. Each fitness component is a column, rows denote
# samples. This file can have multiple components.
# 2. Traits file. Rows index individuals (samples), columns index traits.
# 3. Covariates file. Same format as traits file, although inclusion of
# covariates is optional
# Note that each file should contain a header row containing names for the
# appropriate fitness component, trait, or covariate.
#############################################################################
#                     BEGIN USER INPUT SECTION                              #
#         (Note the direction of slashes in specifying file paths)          #
#############################################################################
dset_path<-"fs_traits_prop.csv" # Path to traits file; e.g.,"F:/05trts.csv"
pset_path<-"fs_relfit_prop.csv" # Path to fitness components file; e.g.,"F:/05fit.csv"
cov_flag<-0 # 0=No covariates, 1=Include covariates
cov_path<-"...." # Path to covariates file; e.g.,"F:/05cov.csv"
trait_col<-1 # column to use in fitness components file
std_traits<-0 # Standardize traits? 1=Yes, 0=No
std_fitness<-0 # Convert fitness component to relative fitness, 1=Yes, 0=No
num_perm<-10000 # Number of permutations
piter<-100 # Print out progress from permutation testing every X permutations
######################### Reading in Datafiles ###################################
```

```
# Code is expecting comma delimited data, for tab delimited change to sep=" "
dset_input<-read.table(dset_path, header=TRUE, sep=",")
pset_input<-read.table(pset_path, header=TRUE, sep=",")
if(cov_flag==1){
 cset_input<-read.table(cov_path, header=TRUE, sep=",")
}
#########################################################################################
#                       END USER INPUT SECTION                          #
#########################################################################################
library(car)
cat("Cleaning out observations with missing fitness, trait, or covariate measurements...\n")
if(cov_flag==0){ # No covariates
 tset_input<-cbind(dset_input, pset_input[,trait_col])
 tset<-na.omit(tset_input)
 num_traits<-ncol(dset_input)
 fit<-tset[,num_traits+1]
 end_idx<-num_traits+1
 traits<-tset[,-end_idx]
 num_obs<-nrow(traits)
}else{ # Include covariates
 tset_input<-cbind(dset_input, cset_input, pset_input[,trait_col])
 tset<-na.omit(tset_input)
 srt_idx<-ncol(dset_input)+1
 end_idx<-ncol(dset_input)+ncol(cset_input)
 traits<-tset[,1:ncol(dset_input)]
 cset<-tset[,srt_idx:end_idx]
 fit<-tset[,end_idx+1]
 num_traits<-ncol(traits)
 num_obs<-nrow(traits)
 num_cov<-ncol(cset)
}
#################################
# Standardize traits and fitness
#################################
if(std_fitness==1){
 cat("Converting to relative fitness....\n")
 relfit<-fit/mean(fit)
}else{
 relfit<-fit
}
relfit<-as.data.frame(relfit)
names(relfit)<-c("relfit")
if(std_traits==0){
 strait<-traits
}else{
 cat("Standardizing traits....\n")
 strait<-matrix(rep(0.0, num_obs*num_traits), nrow=num_obs)
 for(j in 1:num_traits){
  for(i in 1:num_obs){
   strait[i,j]<-(traits[i,j]-mean(traits[,j]))/sd(traits[,j])
  }
```

```
  }
}
strait<-as.data.frame(strait)
names(strait)<-names(traits)
# Create cross-product and quadratic terms for response surface model
cat("Creating cross-product and quadratic model terms....\n")
for(i in 1:num_traits){
  for(j in i:num_traits){
    newterm<-strait[,i]*strait[,j]
    temp_names<-names(strait)
    strait<-cbind(strait,newterm)
    names(strait)<-c(temp_names,paste(names(strait)[i],names(strait)[j],sep="_"))
  }
}
#################################
# Build formula object
#################################
cat("Fitting response surface model....\n")
if(cov_flag==1){
  cat("Including covariates in model....\n")
  jstrait<-strait
  strait<-cbind(strait,cset)
}
main_effects = paste(names(strait),collapse="+")
form1<-as.formula(paste(names(relfit),"~",main_effects,sep=""))
dset2<-as.data.frame(cbind(relfit,strait))
rsmod<-lm(form1,dset2)
cat("Constructing gamma matrix and calculating canonical coefficients....\n")
gamma<-matrix(rep(0.0, num_traits*num_traits), nrow=num_traits)
num_terms<-ncol(strait)
index<-num_traits+2
for(i in 1:num_traits){
  for(j in i:num_traits){
    if(i==j){
      gamma[i,j]<-2.0*rsmod$coefficients[index]
      gamma[j,i]<-gamma[i,j]
      index<-index+1
    }else{
      gamma[i,j]<-rsmod$coefficients[index]
      gamma[j,i]<-gamma[i,j]
      index<-index+1
    }
  }
}
m<-eigen(gamma)$vectors
obs_m<-m
can_coef<-eigen(gamma)$values
y<-as.matrix(strait[,(1:num_traits)])%*%m
for(i in 1:num_traits){
  for(j in i:num_traits){
    newterm<-y[,i]*y[,j]
```

```
   y<-cbind(y,newterm)
  }
}
temp_names<-c("Z_1")
numc<-ncol(y)
for(i in 2:numc){
  temp_names<-c(temp_names,paste("Z",i,sep="_"))
}
Z<-as.data.frame(y)
names(Z)<-temp_names
if(cov_flag==1){
  Z<-cbind(Z,cset)
}
main_effects = paste(names(Z),collapse="+")
form1<-as.formula(paste(names(relfit),"~",main_effects,sep=""))
dset2<-as.data.frame(cbind(relfit,Z))
rsmod2<-lm(form1,dset2)
BAsum<-Anova(rsmod2, type="III")
# Pick off p-values from double regression
idx<-num_traits+2
BApval<-matrix(rep(0.0, num_traits), ncol=num_traits)
BAtstat<-matrix(rep(0.0, num_traits), ncol=num_traits)
for(i in 1:num_traits){
  for(j in i:num_traits){
    if(i==j){
      BApval[1,i]<-BAsum$"Pr(>F)"[idx]
      BAtstat[1,i]<-BAsum$"F value"[idx]
    }
    idx<-idx+1
  }
}
cat("Performing permutation test of canonical coefficients....May take awhile..be patient!\n")
main_effects = paste(names(strait),collapse="+")
form1<-as.formula(paste("permfit~",main_effects,sep=""))
temp_names<-c("Z_1")
numc<-ncol(y)
for(i in 2:numc){
  temp_names<-c(temp_names,paste("Z",i,sep="_"))
}
for (c in 1:num_perm){
  if((c%%piter)==0){
    cat("Iteration ",c," out of ",num_perm,"\n")
  }
  # Need to permute covariates along with fitness measure
  if(cov_flag==1){
    permset<-as.data.frame(cbind(relfit,cset))
    permset2<-permset[order(sample(permset[,1])),]
    permfit<-permset2[,1]
    end_pt<-ncol(permset2)
    cset_perm<-permset2[,2:end_pt]
    strait<-as.data.frame(cbind(jstrait,cset_perm))
```

```
  main_effects = paste(names(strait),collapse="+")
  form1<-as.formula(paste("permfit~",main_effects,sep=""))
}else{
  permfit<-sample(relfit[,1])
}

# Fit RSM model to permuted dataset
dset2<-as.data.frame(cbind(permfit,strait))
rsmodp<-lm(form1,dset2)
gamma<-matrix(rep(0.0, num_traits*num_traits), nrow=num_traits)
num_terms<-ncol(strait)
index<-num_traits+2
for(i in 1:num_traits){
  for(j in i:num_traits){
    if(i==j){
      gamma[i,j]<-2.0*rsmodp$coefficients[index]
      gamma[j,i]<-gamma[i,j]
      index<-index+1
    }else{
      gamma[i,j]<-rsmodp$coefficients[index]
      gamma[j,i]<-gamma[i,j]
      index<-index+1
    }
  }
}
# Compute test statistics from double regression method
m<-eigen(gamma)$vectors
y<-as.matrix(strait[,(1:num_traits)])%*%m
for(i in 1:num_traits){
  for(j in i:num_traits){
    newterm<-y[,i]*y[,j]
    y<-cbind(y,newterm)
  }
}
Z<-as.data.frame(y)
names(Z)<-temp_names
if(cov_flag==1){
  Z<-cbind(Z,cset_perm)
}
main_eff = paste(names(Z),collapse="+")
form2<-as.formula(paste("permfit~",main_eff,sep=""))
dset2<-as.data.frame(cbind(permfit,Z))
rsmod2<-lm(form2,dset2)
BAsum<-Anova(rsmod2, type="III")
# Pick off p-values from double regression
idx<-num_traits+2
p_tstat<-matrix(rep(0.0, num_traits), ncol=num_traits)
for(i in 1:num_traits){
  for(j in i:num_traits){
    if(i==j){
      p_tstat[1,i]<-BAsum$"F value"[idx]
```

```
    }
    idx<-idx+1
   }
  }

  if(c==1){
   stat_track<-p_tstat
  }else{
   stat_track<-rbind(stat_track, p_tstat)
  }
}
cat("Finished with permutation testing....\n")
exceed<-matrix(rep(0.0,num_perm*num_traits), nrow=num_perm)
for(c in 1:num_perm){
 for(j in 1:num_traits){
  if(stat_track[c,j]>BAtstat[j]){
   exceed[c,j]<-1.0
  }
 }
}
pvalues<-matrix(rep(0.0, num_traits), ncol=num_traits)
for(c in 1:num_traits){
 pvalues[1,c]<-mean(exceed[,c])
}
results<-rbind(can_coef, pvalues)
rownames(results)<-c("Eigenvalues","Permutation p-values")
print(results)
cat("Eigenvectors of Gamma...\n")
eigvec<-as.data.frame(obs_m)
rownames(eigvec)<-names(traits)
print(eigvec)
```

Table A1. Chemical characterization of male CHCs in *Gnatocerus cornutus*. KRI: Kovats Retention Index for each chemical compound, DMDS: diagnostic ions used for compound identification after derivation with dimethyl disulphide.

Peak	KRI	Compound	Diagnostic ions
2	2495	C_{25}	352
3	2531	11-MeC_{25}	168, 227, 351
4	2568	3-MeC_{25}	337, 57, 351
5	2594	C_{26}	366
6	2629	11-MeC_{26}	168, 238, 365
7	2661	5-C_{26}-ene	DMDS: 458, 117, 341
8	2693	C_{27}	380
9	2728	11-MeC_{27}	168, 252
10	2748	Unknown	
11	2759	11,15-diMeC_{27}	267, 168, 197, 239
12	2769	3-MeC_{27}	365, 57
13	2794	C_{28}	394
14	2894	C_{29}	408
15	2927	13-MeC_{29}	252, 196
16	2956	11,15-diMeC_{29}	295, 168, 224, 239
17	2969	3-MeC_{29}	57, 393
18	2993	C_{30}	422
19	3093	C_{31}	436
20	3126	15-MeC_{31}	224, 252
21	3152	3,19, 3,17-diMeC_{31}	196, 224, 267, 295, 435
22	3169	3-MeC_{31}	57, 421
23	3250	4,12-diMeC_{31}	435, 71, 309, 197
24	3325	11-MeC_{33}	169, 337, 225, 281
25	3349	15,17-diMeC_{33}	295, 225, 253, 267