Sexual selection and reproductive isolation in field crickets

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.

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Acknowledgments

First of all my thanks go to my supervisors Tom Tregenza & Rolando Rodríguez-Muñoz for interpreting my incoherent babbling during meetings, reading endless drafts of chapters, as well as providing support throughout the PhD. To Xavier Harrison for teaching me most of what I know about molecular genetics, and for taking on much more than he bargained for, as well as Amanda Bretman and Michelle Hares for fielding endless questions. To Yannick Pauchet who patiently helped me get to grips with transcriptomics. To Iain Stott - my walking-talking guide to statistics, and to Fiona Ingleby for introducing me to the world of PCA. To Thor Veen for his constant enthusiasm, and for enduring my company in a confined space while driving around the middle of no-where in rural Spain. To Laurence Albert who braved sea-sickness, mossie bites and sunburn to help collect crickets. To Jeff Stoltz for helping look after crickets, and to Rochishnu Dutta for the tedious task of watching them do very little. To Tanya Pennell and Dave Ellis for collecting preliminary data (and for pioneering a release programme at Tremough). To Joe Faulks, Corrina Lowri and Chris Mitchell for acting as lab sat-navs. To Helen Leggett for casting her eye over the entire thesis. Finally, to all those (there are many) who listened to endless moaning, and shared bottles of wine and laughs through the good and the bad.

Abstract

Barriers to interbreeding limit gene flow between sister taxa, leading to reproductive isolation and the maintenance of distinct species. These barriers come in many forms, and can act at different stages in the reproductive process. Pre-copulatory barriers may be due to individuals discriminating against heterospecifics in mate choice decisions. These decisions may be informed through a range of sensory modalities. If a female is mated and inseminated, then there may be multiple postmating-prezygotic barriers that affect the success of heterospecific sperm in attaining fertilisations. Post-zygotic barriers can be very early acting, resulting in embryonic fatality, or may be later acting, affecting the fitness of hybrid offspring. In this thesis I investigate potential reproductive barriers between the interbreeding field crickets *Gryllus bimaculatus* and *G. campestris*.

I find that females of both species show only weak preference for conspecific calling song, and may even respond phonotactically to songs typical of heterospecific males. Female *G. bimaculatus* are repeatable in their preferences and strength of response. *G. bimaculatus* females presented with synthetic songs prefer those with longer inter-pulse intervals, whereas *G. campestris* show no discrimination between these songs.

Upon meeting, *G. campestris* females strongly discriminate against heterospecific males, behaving aggressively towards them. This is likely driven by females responding to close range species recognition cues, including chemoreception. The species differ in their cuticular hydrocarbon profiles, and females that are no longer able to use their antennae to receive chemosensory information reduced their aggressive behaviour towards heterospecific males.

G. bimaculatus females will mate with heterospecific males, though less readily than to conspecifics. When sequentially mated to both conspecific and heterospecific males, these females will preferentially take up and store sperm from the conspecific male, and sperm from conspecific males is more likely to sire offspring than would be predicted from the proportion of sperm in storage.

Eggs from inter-species mating pairs are less likely to begin embryogenesis, and are more likely to suffer developmental arrest during the early stages of embryogenesis. However hybrid embryos that survive to later stages of development have hatching success similar to that of pure-bred embryos.

After mating, phonotaxis of *G. bimaculatus* females towards male songs follows a pattern of suppression and subsequent recovery, likely triggered through detection of seminal proteins

transferred in the male ejaculate, or detection of mechanical filling of the spermatheca. This pattern of suppression and recovery of phonotaxis does not differ between females mated to conspecific or heterospecific males. Females that lay few or no eggs do not experience a refractory period.

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Author's Declaration

During the research contributing to this thesis I was supported by a studentship from the European Social Fund. All of the chapters presented in this thesis were written by me, with comments and editing by Tom Tregenza and Rolando Rodríguez-Muñoz. Cricket collections in 2010 were carried out with the help of Thor Veen, and in spring 2011 with the help of Laurence Albert. Cricket collections in summer 2011 and spring 2012 were carried out by Rolando Rodríguez-Muñoz. Further contributions to sections are detailed below.

Chapter 2 - Song recordings and extraction of data from these was carried out by Thor Veen, who also created two of the synthetic songs used. I created all other synthetic songs, and performed all other behavioural trials and analyses.

Chapter 3 - Cuticular hydrocarbon samples were processed through the GCMS by Christopher Mitchell.

Chapter 4 - I carried out the molecular analyses in collaboration with Xavier Harrison, who took part in all aspects of this work. Amanda Bretman provided technical advice. Thor Veen performed some of the mating trials, though I performed the majority. A version of this chapter has been accepted for publication in *Molecular Ecology.* We are grateful for the comments of three anonymous reviewers.

> Tyler F, Harrison X, Bretman A, Veen T, Rodríguez-Muñoz R & Tregenza T (In Press) Multiple post-mating barriers to hybridisation in field crickets. *Molecular Ecology*.

Chapter 5 - A version of this chapter is currently in review with *BMC Evolutionary Biology*.

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Appendix B - cDNA preparation & sequencing was carried out by Yannick Pauchet, who also taught me the skills required to identify candidate genes for behaviour

Appendix C – Cuticular hydrocarbon samples were collected by Rolando Rodríguez-Muñoz and Tom Tregenza. Processing of samples was carried out by Patrizia d'Ettorre & group.

Chapter 1 General Introduction

1.1 Speciation

Speciation is the evolutionary process through which new species arise. Populations within an ancestral species diverge until members of each group no longer recognise the other as mates, or reproductive attempts between them are unsuccessful. These behavioural or physiological barriers prevent gene flow in areas of sympatry, allowing the divergent groups to be defined as reproductively isolated species, as defined by the Biological Species Concept (Mayr, 1942). There are a range of biogeographical modes through which speciation can act, the importance of which are debated in the literature. Likely the most commonly acting is allopatric speciation, whereby an ancestral species is divided geographically and populations diverge in isolation (Jordan, 1905). Divergence might come about through random accumulation of new mutations in each population, or alleles being lost through genetic drift (Nei *et al.*, 1983). This may be particularly true of small populations, in which changes such as these are more likely to spread to fixation. Additionally, if new populations are established by a small number of individuals, non-random sampling of the gene pool of the source population can create genetic differences between the parent and offspring populations, called founder effects (Mayr, 1954). Isolated populations will also experience different environments and so be subjected to different selection pressures. Adaptation to the local environment through natural selection may drive divergence in traits and eventually speciation (Schluter, 2000). Differences in sexual selection pressures experienced across locations may cause sexual traits, and the preferences for them, to diverge, ultimately leading to speciation (Panhuis *et al.*, 2001). If species later come into secondary contact, barriers to interbreeding may be strengthened through the process of reinforcement. If there are costs associated with the production of hybrid offspring, then there will be selection against interbreeding, driving reinforcement of traits used in mate choice that reduce the production of hybrid offspring (Servedio & Noor, 2003).

1.2 Reproductive barriers

For species to remain distinct units whilst in contact with closely related sister taxa, there need to be barriers to gene flow, which Mayr (1963) described as reproductive barriers. More divergent species tend to have greater behavioural and physiological differences, and associated with these, stronger barriers to interbreeding. These barriers come in many forms, and act at different stages in the reproductive process. Traditionally these were categorised as those acting before, or after, zygote

formation (Dobzhansky, 1937). For many years, the study of pre-zygotic barriers focussed on those occurring prior to mating, with little consideration of anything later acting due to the difficulties of studying internally acting mechanisms. In recent decades there has been growing interest in cryptic processes (Parker, 1970; Thornhill, 1983; Eberhard, 1996) and technical advances in our ability to study them. The significance of these mechanisms in reproductive isolation is widely recognised, and barriers are now more often categorised as those acting prior to copulation, after copulation but before zygote formation ('postmating-prezygotic'), or after zygote formation (Coyne & Orr, 2004; Howard *et al.*, 2009).

Pre-copulatory barriers may be due to individuals no longer recognising heterospecifics as potential mates, or discriminating against them in mate choice relative to conspecifics. A number of cues can be used to inform these mate choice decisions, through a range of sensory modalities, for example auditory (Ryan & Rand, 1993), visual (Wiernasz & Kingsolver, 1992), or chemosensory cues (Coyne & Charlesworth, 1997). Alternatively, pre-copulatory barriers may be due to behavioural or morphological incompatibilities between individuals that attempt to mate, preventing successful copulation and insemination (Coyne, 1993; Eberhard, 2001).

If insemination is successful, there may be postmating-prezygotic barriers that affect the success of an inter-species mating. These may be non-competitive, for example inviability of heterospecific sperm in the female reproductive tract (Gregory & Howard, 1994), or incompatibilities between gametes (Shaw *et al.*, 1994). Alternatively they may be competitive, whereby the success of heterospecific sperm is dependent upon the presence of ejaculates from other males (Parker, 1970). In a number of polyandrous interbreeding species, the relatively greater success of conspecific males in siring offspring has been reported, a phenomenon known as conspecific sperm precedence (Howard, 1999).

Post-zygotic barriers can be very early acting, resulting in embryonic fatality (Baird & Yen, 2000), or may be later acting, affecting the fitness of hybrid offspring. Hybrids are often sterile, directly preventing introgression of genes (Dobzhansky, 1936), or may be less fit than pure-bred offspring leading to natural or sexual selection against them (Hatfield & Schluter, 1999). Their intermediate phenotypes may make them poorly suited to the environments to which the parental species are adapted, or their intermediate sexual traits may be unattractive to members of either parental species.

Rather than any one barrier causing reproductive isolation between species, there are likely multiple barriers that cumulatively contribute to the prevention of gene flow. Among species pairs

these barriers are likely to range in strength and in number, with some species being isolated by few barriers of strong effect, or others being isolated by many barriers of weak effect (Veen *et al.*, 2001; Lemmon, 2009; Leonard & Hedrick, 2009).

1.3 Study species

The sister species *Gryllus bimaculatus* (Fig 1.1, 1.2) and *G. campestris* (Fig 1.3, 1.4) comprise the European clade of field crickets (Huang *et al.*, 2000). In Europe *G. bimaculatus* are distributed along the Mediterranean coast (Gorochov & Llorente, 2001), occupying relatively arid conditions. They can be found sheltering under rocks or logs, or in grass, and are predominantly nocturnal. They are capable of flight, and are multivoltine, producing many generations through the year. *G. campestris* occupy less arid regions (Gorochov & Llorente, 2001). They require meadow grass lands to live, where they create burrows in which to shelter. They are territorial and flightless, which limits dispersal. They are diurnal and univoltine, mating in the spring-summer to produce nymphs that diapause through winter before emerging as adults in the following spring. Among other locations (Popov & Shuvalov, 1977; Gangwere & Llorente, 1992), the distributions of these species overlap through central / southern Spain (Fig 1.5) (Pardo *et al.*, 1993; Gorochov & Llorente, 2001). The study populations used in this thesis originated from allopatric locations in Spain, the *G. bimaculatus* near Valencia, Mediterranean coast, and the *G. campestris* near Oviedo, Cantabrian coast.

Though their geographic ranges overlap, it is not known whether individuals of these species encounter each other, or if they interbreed in the wild, since they occupy different habitats. They will interbreed in the laboratory, though only unidirectionally. While female *G. bimaculatus* will mate with male *G. campestris* (though less readily than to conspecific males), female *G. campestris* will only rarely interbreed despite attempts by male *G. bimaculatus* to court these females (Veen *et al.*, 2011; Veen *et al.*, 2013). The extent to which they will interbreed is uncertain. While modern studies have never recorded a *G. campestris* female interbreeding, earlier studies have, though there is very limited information about the conditions in which these matings occurred (Cousin, 1933; von Hörmann-Heck, 1957). Females of both species are polyandrous, mating with multiple males over their lifetime (Bretman & Tregenza, 2005; Rodríguez-Muñoz *et al.*, 2011).

Although the hatching success of hybrid eggs is less than that of pure-species eggs, those offspring that do hatch are both viable and fertile. The mean lifespan of hybrids is longer than that of the parental species, and they are often larger in size. Female *G. bimaculatus* respond

phonotactically to hybrid calling song and will mate with hybrid males to produce viable backcross offspring (Veen *et al.*, 2011; Veen *et al.*, 2013).

Figure 1.1. *Gryllus bimaculatus* male

Figure 1.2. *Gryllus bimaculatus* female

Figure 1.3. *Gryllus campestris* male

Photographs: Thor Veen

Figure 1.4. *Gryllus campestris* female

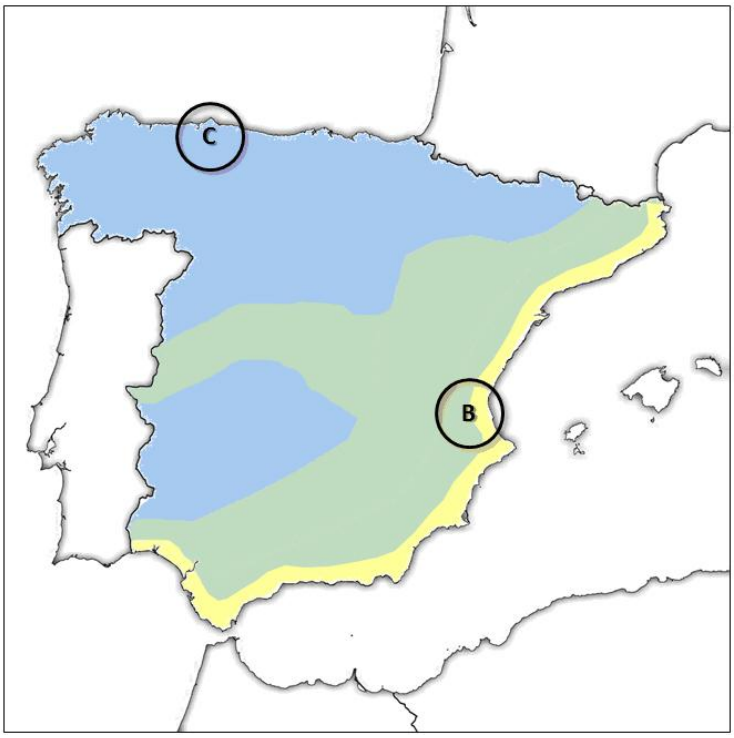


Figure 1.5. Estimated distributions of *G. campestris* (blue) and *G. bimaculatus* (yellow) across mainland Spain. Their distributions overlap through the centre / south of the country (green). (The south-west section in which *G. bimaculatus* are not represented is likely to reflect a lack of sampling effort rather than absence of this species). Study populations of *G. campestris* (**C**) and *G. bimaculatus* (**B**) were collected from allopatric locations.

1.4 The field cricket mating system

The aim of this thesis is to explore reproductive isolation between *G. bimaculatus* and *G. campestris*, investigating potential barriers to gene flow between them. These barriers may occur at a range of stages through the reproductive process, acting prior to and after mating, both before and after zygote formation.

Males advertise themselves by producing long-range calling song. Females use these songs to orientate themselves towards the males through a process of phonotaxis (Thorson *et al.*, 1982). Traits of these songs differ between individuals (Popov & Shuvalov, 1977), and females likely use these differences in mate choice decisions. Species differences in song may be used in species recognition, though evidence for this, particularly in *G. campestris*, is not strong (Veen *et al.*, 2013). In Chapter 2, I build upon these previous studies, aiming to obtain a reliable estimate of *G. campestris* preference, and to investigate female response to particular song traits.

Upon meeting, there are a range of sensory cues that may be used to asses potential mates. Males produce a courtship song that differs from their long-range calling song (Alexander, 1961), which females may use to discriminate between males. Visual cues may play a role, particularly in *G. campestris*, which are diurnal. However, potentially the most important close-range cues are received through the antennae. Individuals often touch antennae and investigate each other's body surfaces, receiving both tactile and chemosensory information (Balakrishnan & Pollack, 1997). Like other insects, crickets' bodies are covered in cuticular hydrocarbons, or CHCs. Individuals vary in the expression of these molecules, and these differences can be used in mate choice (Hardy & Shaw, 1983; Tregenza & Wedell, 1997; Thomas & Simmons, 2011a). In Chapter 3, I compare CHC profiles of *G. campestris* and *G. bimaculatus*, and investigate whether or not females use species differences in chemical composition to recognise heterospecific males.

If the female chooses to mate, she mounts the male, and the male attaches an external spermatophore to her. The ejaculate is subsequently transferred to the reproductive tract, a process that takes around 1 hour to complete (Simmons, 1986). The process of sperm uptake and storage is at least partly controlled by the female, creating the opportunity for cryptic female choice. *G. bimaculatus* females can discriminate against related males in the uptake and storage of sperm (Bretman *et al.*, 2009), and this mechanism might likewise be used to discriminate against heterospecific males. Since these females are polyandrous, and in the laboratory will mate to different males in quick succession, ejaculates are likely to face sperm competition in the female reproductive tract. In Chapter 4, I use a competitive microsatellite technique (Wooninck *et al.*, 2000; Bussière *et al.*, 2010) to determine the relative success of competing conspecific and heterospecific males in representation in the spermathecae of *G. bimaculatus* females, and relate this to subsequent success in siring offspring.

Between sperm storage and egg hatching there are a cascade of mechanisms that potentially affect the reproductive success of inter-species pairings. Prior to zygote formation there may be incompatibilities between sperm and the reproductive tract of the female or between the sperm and egg (Gregory & Howard, 1994; Shaw *et al.*, 1994). After zygote formation, hybrid embryogenesis may fail at any one of a number of developmental stages (Baird & Yen, 2000; Sellier *et al.*, 2005; Álvarez & Garcia-Vazquez, 2011). In Chapter 5, I assess whether eggs laid from inter-species pairings are less likely to begin development, and secondly assess whether those eggs that do begin to develop subsequently fail prior to hatching.

A male can improve his reproductive fitness by limiting the competition faced from other males, and hence it is in that male's interests to prevent a female from re-mating. Immediately after

mating the male exhibits guarding behaviours (Simmons, 1991b) which prevent the female from removing the spermatophore before its entire contents have been transferred, and also to prevent the female from searching out other males (Alcock, 1994). The influence on female mate search behaviour may be extended beyond the initial guarding phase. As well as sperm, many other compounds are transferred to the female within the ejaculate, some of which may have manipulative effects on female behaviour (Loher *et al.*, 1993; Green & Tregenza, 2009). In Chapter 6, I aim to confirm that female phonotaxis is suppressed post-mating, and to investigate whether the extent of this suppression depends upon whether the ejaculate is from a conspecific or heterospecific male.

Chapter 2 The use of long-range calling song for species recognition in field crickets

2.1 Abstract

Traits of male calling songs differ between individuals, and females likely use these differences in mate choice decisions, including identifying conspecifics. We firstly presented the females of *Gryllus bimaculatus* and *G. campestris* with the same synthetic songs as used in a previous study, to confirm whether or not females prefer conspecific song, and whether response to song is similar across different methodologies. Since preferences are only likely to be evolutionarily important if individuals are repeatable in their choices, we then repeatedly trialled *G. bimaculatus* females to get a measure of their consistency in choice of songs, and in the strength of their phonotactic responses. Finally we investigated the importance of inter-pulse interval (IPI) in species recognition and estimated the shape of female preference function for this trait. Unlike previous research, we found that neither *G. bimaculatus* nor *G. campestris* females show preferences for conspecific songs. In *G. bimaculatus* this was not due to females erratically choosing song, rather they were repeatable in their preferences, and also repeatable in their latency to respond phonotactically. Contradictory to our first study, in the second we found a preference for heterospecific song. Finally, we demonstrated that the females of these species respond differently to songs varying in IPI. While overall, *G. campestris* females moved faster towards songs, they showed no change in response to differing IPIs, whereas *G. bimaculatus* showed increasingly strong preferences as IPI increased.

2.2 Introduction

Males of many species produce advertisement calls to attract potential mates. As well as providing information about the location of the male, songs differ between individuals and can be used to inform mate choice decisions. Females may be able to use song characteristics to assess the quality of males, or to identify conspecifics (Hoy *et al.*, 1977; Baker, 1991; Ryan & Rand, 1993). Species recognition may be particularly important when closely related species are found in the same place. In these cases interspecific matings may still take place, often bearing costs in terms of offspring viability or fertility (Dobzhansky, 1937). Individuals can avoid these costs by determining the species identity of potential partners prior to mating (Mayr, 1963; Mayr, 1969). Preferences are only likely to be evolutionarily important if individuals consistently exhibit the same choices. Hence determining repeatability is the first step to understanding the role of mate preference in reproductive isolation.

The more similar the songs produced by closely related species, the more finely tuned a female's recognition of song traits needs to be to avoid interbreeding. The nature of the male trait will determine the form of female preference. If traits of an individual's song change during a single bout of calling (for example due to fatigue), then females will consistently prefer extreme values associated with high quality. This will exert directional selection on these traits. However if male traits are static, and change very little over a breeding season, then selection should be stabilising. In these cases, values of traits that are nearest to the mean value of conspecifics should be preferred, and extreme values should be less attractive, creating a unimodal preference function (Gerhardt, 1991). This pattern has been observed in a handful of species, predominantly anurans and orthopterans (Gerhardt, 1991; Ritchie, 1996; Castellano & Giacoma, 1998; Wollerman, 1998; Shaw & Herlihy, 2000).

The field crickets *Gryllus bimaculatus* and *G. campestris* are found across Europe, their ranges overlapping through southern Spain (Pardo *et al.*, 1993; Gorochov & Llorente, 2001). In the laboratory *G. bimaculatus* females will mate with *G. campestris* males, producing hybrid offspring that are both viable and fertile. *G. campestris* females however, are hostile towards heterospecific males and will rarely accept courtship attempts (Cousin, 1933; von Hörmann-Heck, 1957; Veen *et al.*, 2011). Like most crickets, males of *G. bimaculatus* and *G. campestris* use a long range-calling song to attract females (Alexander, 1961). This song is produced through stridulations of their wings that create pulses of sound. Rather than producing a continuous trill of pulses like some species, these crickets produce groups of 3 or 4 pulses to form distinct chirps interspersed by silence. There is variation within and among individuals, but also between species in the structure of songs. The number of pulses produced per chirp, the spacing between pulses, and the spacing between chirps all vary to different degrees (Popov & Shuvalov, 1977; Veen *et al.*, 2013). While chirp rate in *G. bimaculatus* is highly variable and can vary according to the motivational state of the individual, the number of pulses per chirp and the inter-pulse interval (IPI) tend to be consistent (Popov & Shuvalov, 1977) at a given temperature (Doherty, 1985). Traits such as these with low intra-male variation might be more reliable cues in species recognition than others. Some song traits are more important than others in eliciting a phonotactic response from females, and while there are species differences in responses to some traits, IPI is important in both *G. bimaculatus* and *G. campestris* phonotaxis (Popov & Shuvalov, 1977; Thorson *et al.*, 1982).

The fact that there are species differences in song traits, and that female response varies with these traits, suggests that calling song is likely used in species recognition, and there is evidence that this is the case. Veen *et al.* (2013) created synthetic songs that reflected the mean trait values

of each of the species, and played them to females in no-choice and two-choice trials. *G. bimaculatus* females did not show preference for song in no-choice trials, however when both song types were simultaneously presented, they preferred conspecific song. *G. campestris*, on the other hand, showed little or no preference for song regardless of how the songs were presented.

Song preferences were measured using a track-ball system, whereby each female was suspended over a polystyrene sphere that moved in response to the female's normal walking movements (Veen *et al.*, 2013). Sensors detected and measured the movement of the sphere, allowing turning effort and forward motion of the female to be quantified. There are a number of benefits to using a track-ball system to measure female phonotaxis, however a major drawback is that *G. campestris* behave very erratically when placed on the sphere, making the collection and interpretation of data difficult. Here, we replicate aspects of the study by Veen *et al.* (2013), but instead use an arena in which females can walk freely towards a sound source, a method that *G. campestris* respond more favourably to.

We firstly present the females of both species with the same synthetic songs as used by Veen *et al.* (2013) to confirm whether or not female response to song is similar across these different methodologies, aiming to get a less erratic measure of *G. campestris* response to conspecific and heterospecific songs. With the expectation that song preferences are heritable, we then repeatedly trialled *G. bimaculatus* females, predicting that individuals will be consistent in their choice of songs, and in the strength of their phonotactic responses. Finally we investigated the importance of IPI in species recognition, expecting to find differences between the species in the mean IPI produced by males, and unimodal preference functions in the females of these species, with the mean IPI values of conspecific males being most preferred.

2.3 Methods

2.3.1 Song analysis and construction

For the two-choice trials we used the same synthetic songs as were used by Veen *et al.* (2013). These two songs had been designed to be typical of either *G. campestris* or *G. bimaculatus*. Number of pulses, chirp length and inter-chirp interval were manipulated to match population means, but not IPI.

For the IPI trials, songs were created based upon the recordings of 42 male *G. campestris* and 30 *G. bimaculatus.* The calling songs of males were recorded at 28 ^{o}C +/- 1 ^{o}C, and temporal parameters (Figure 2.1) were measured using JAVA software, a custom program that calculates

distances between peaks from .wav file waveforms. Means of parameters were calculated for individual males, taken from samples of song 21 chirps long. A mean IPI was calculated for each species, and an overall mean for the species combined (weighted to account for the larger sample size for *G. campestris*). Difference in IPI between the species was confirmed with a t-test. Mean inter-chirp interval was likewise calculated (Table 2.1). Frequency histograms of IPI measured from song recordings are shown in Figure 2.2.

Using Audacity software (http://audacity.sourceforge.net) synthetic song was constructed by choosing a single pulse at random from a sample of songs, which was then repeated to make a 4-pulse chirp. Chirps were spaced to match the mean inter-chirp interval taken from the combined species. Each song was then made to differ in the spacing of these pulses, with IPI increasing in increments of 1 ms. The range of IPIs covered ~1 standard deviation around the combined species mean. This resulted in 7 synthetic songs with an IPI of 6, 7, 8, 9, 10, 11 or 12 ms. Manipulating the IPI had the knock on effects of altering overall chirp length, as well as duty cycle for each song (Table 2.2), however these parameters have little effect on female response to song (Popov & Shuvalov, 1977).

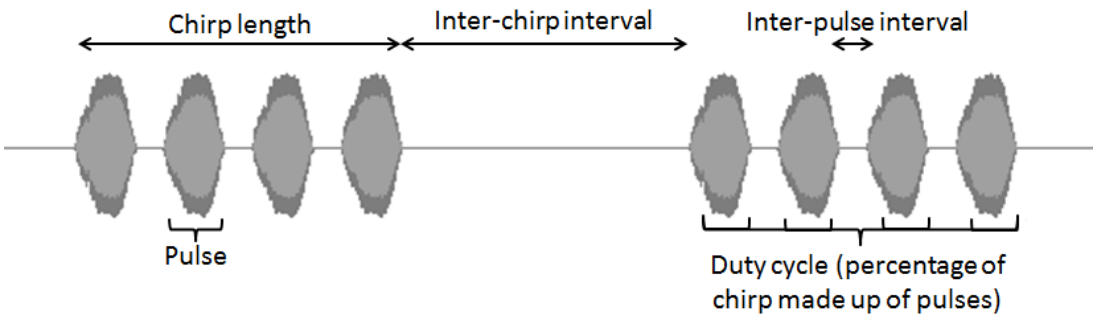


Figure 2.1. Example parameters of calling song

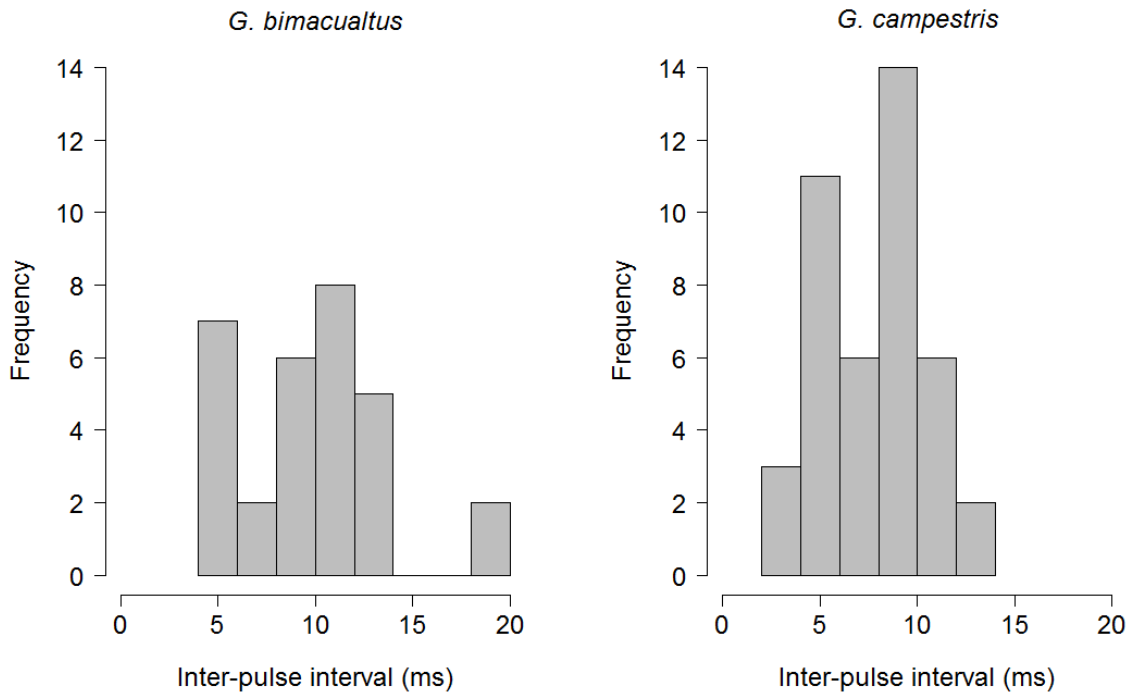


Figure 2.2. Inter-pulse intervals measured from the songs of *G. bimaculatus* (left) and *G. campestris* (right) songs.

Table 2.1. Parameters calculated from song recordings, used to inform the construction of synthetic 'IPI' songs

Parameter	**Length (ms)**
Mean IPI, *G. bimaculatus*	9.7
Mean IPI, *G. campestris*	7.5
Mean IPI, species combined	8.6 (± 3.3)
Mean inter-chirp interval, species combined	185

IPI significantly differs between *G. bimaculatus* and *G. campestris* (t = -2.81, df = 47.4, P = 0.007).

Table 2.2. Parameters of synthetic songs. *'G bimaculatus'* and '*G. campestris'* songs were taken from Veen *et al.* (2013), whereas 'IPI' songs were newly created for this study

Song	Pulses per chirp	Inter-pulse interval (ms)	Chirp length (ms)	Inter-chirp interval (ms)	Chirp duty cycle (%)
'*G. bimaculatus*' (Veen *et al.*, 2013)	3	18	95	245	62.7
'*G. campestris*' (Veen *et al.*, 2013)	4	13	120	250	68.6
'IPI 6'	4	6	92	185	78.3
'IPI 7'	4	7	95	185	75.8
'IPI 8'	4	8	98	185	73.5
'IPI 9'	4	9	101	185	71.3
'IPI 10'	4	10	104	185	69.2
'IPI 11'	4	11	107	185	67.3
'IPI 12'	4	12	110	185	65.5

2.3.2 Study population

G. bimaculatus females were collected from Valencia, southern Spain in 2005 and reared in the lab where the generation time was approximately 2 months, until trials were carried out in 2010 and 2011. Crickets were housed at 28 ^{o}C under a 16:8 light:dark cycle, with food and water provided *ad libitum*. Last instar nymphs were isolated to ensure virginity upon becoming adult. *G. campestris* were collected as last instar nymphs or adults from Gijon, northern Spain in 2010 (two-choice trials) and 2011 (IPI trials). These wild caught individuals were kept in the laboratory for at least 7 days prior to use in trials. All individuals had matured for a minimum of 7 days after adult emergence before being used in experimental trials. All individuals were isolated and housed in small plastic boxes.

2.3.3 Phonotactic trials

Prior to phonotactic trials, food, water and debris were removed from the boxes in which the females were housed, the lids loosely replaced, and the boxes inverted to leave the females standing on the lids. These were then kept in a polystyrene container and left for at least 15 minutes to allow the females to settle. Trials were conducted at 28 ^{o}C +/- 1 ^{o}C under red light within a 126 x 67 x 29

cm wooden arena, constructed with two speakers in adjacent corners angled towards the centre of the arena at 70 degrees. A semi-circle with a radius of 5 cm was marked out in front of each speaker. Each female was transferred in turn to the arena while still within her upturned box, and the body of the box removed to leave the female in the centre of the arena. Song was then played to the female at ~65 Db (re 20 μPa) from the speakers for a maximum of 5 min.

To assess response to conspecific and heterospecific song, both *G. bimaculatus* and *G. campestris* females were used. Conspecific and heterospecific songs were simultaneously played, having been randomly assigned to the left and right speakers for each female. Response to the song was recorded as choice of speaker. The trial was terminated when the female's entire body was within one of the marked semi-circles, or after 5 min.

To assess repeatability of response to the songs only *G. bimaculatus* female were used. Again the songs were simultaneously played, and response was recorded as choice of speaker but also latency to reach the speaker. If the female did not reach a speaker within the 5 min trial their latency was recorded as 5 min. We aimed to trial each female on 12 consecutive days, each day the side from which the two songs were played alternated, carrying out a minimum of 4 trials. Only females trialled more than 3 times were included in the dataset.

For the IPI trials both *G. bimaculatus* and *G. campestris* females were used. These were no-choice trials, in which song was only played from the left or right speaker during each trial. The females were trialled with each of the 7 songs on consecutive days. The order of song presentation was random, as was use of the left or right speaker. Response was measured as latency to reach the speaker playing song. If a female did not reach a speaker during any of her trials, data for this female were removed from the dataset.

2.3.4 Analyses

All analyses were performed using R 2.14.1 software (R Development Core Team, 2011). Choice of conspecific or heterospecific song by the two species of females was analysed using a Chi-squared test. Repeatability of song choice and repeatability of latency in *G. bimaculatus* females were calculated following Nakagawa & Schielzeth (2010) (rpt: R rptR library (Schielzeth & Nakagawa, 2011)). To determine which of the songs each female chose more often, we used a sign test, tested against an expected equal response to the songs. To estimate female response to song depending on IPI, a generalised linear mixed model (GLMM) was fitted (lmer: R lme4 library (Bates *et al.*, 2011)) with latency to reach the speaker as the response variable. Song (coded as 6 - 12) and female

species were entered as explanatory variables, as well as the interaction between these variables. Female ID, a unique number for each individual, was entered as a random effect to control for repeated measures from individuals. Model selection was carried out through sequential removal of explanatory variables and their interactions, until only significant terms remained in the model, their significance determined through likelihood ratio tests (Crawley, 2007).

2.4 Results

2.4.1 Responses to conspecific and heterospecific song

In the initial two choice tests, in which each female was tested once, 58 *G. bimaculatus* and 94 *G. campestris* females were successfully tested. Neither *G. bimaculatus* nor *G. campestris* females preferentially chose the speaker playing conspecific song ($\chi^2_1 = 0.42$, P = 0.515, Figure 2.3). In the repeatability trials, 59 *G. bimaculatus* females were tested, though data for 4 of these females were not included as they were trialled fewer than 4 times each. When measured repeatedly, *G. bimaculatus* females were somewhat repeatable in their song choice (R = 0.056, SE = 0.033, P = 0.023), more often choosing heterospecific song (Sign test: 13 successes / 48 trials, P = 0.029, Figure 2.4). They were also repeatable in their latencies to reach the chosen speaker (R = 0.425, SE = 0.054, P <0.001).

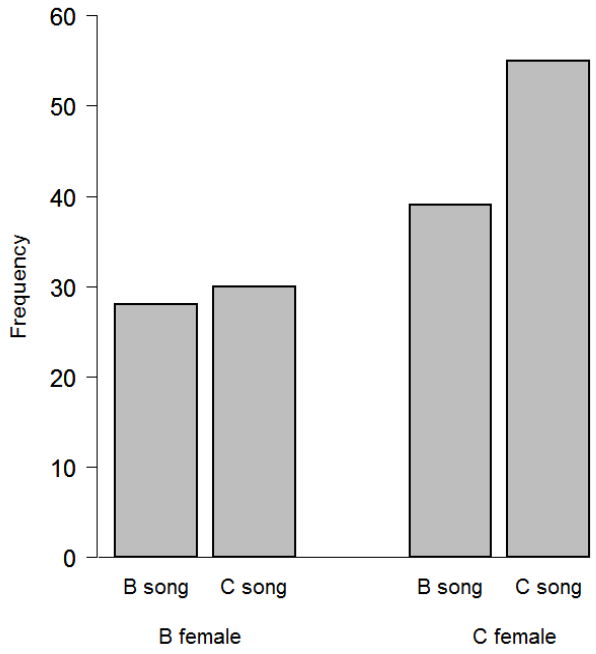


Figure 2.3. The frequencies with which conspecific and heterospecific songs were chosen by *G. bimaculatus* (B) and *G. campestris* (C) females in two-choice trials.

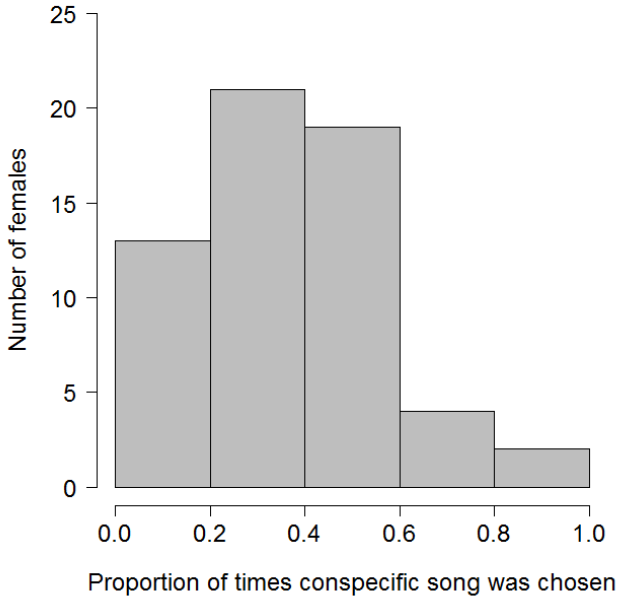


Figure 2.4. The proportion of times each *G. bimaculatus* female chose conspecific song when repeatedly trialled in two-choice tests. If females were inconsistent in their choice of songs we would expect to find a normal distribution centred around 0.5, however we find a skew away from 0.5, indicative of females repeatedly choosing one song over another (in this case heterospecific over conspecific).

2.4.2 Response to inter-pulse interval

A total of 73 *G. bimaculatus* and 117 *G. campestris* females were trialled. Data from one female of each species were removed from the dataset due to these females not responding during any of their trials. There was a significant interaction between IPI and species of female in latency to reach the speaker playing song ($Chi_{1,6} = 11.73$, P = 0.0006, Figure 2.5). Overall *G. campestris* females were faster to reach the speaker, however this did not vary with IPI. The latency of *G. bimaculatus* females to reach the speaker playing song became shorter as IPI increased.

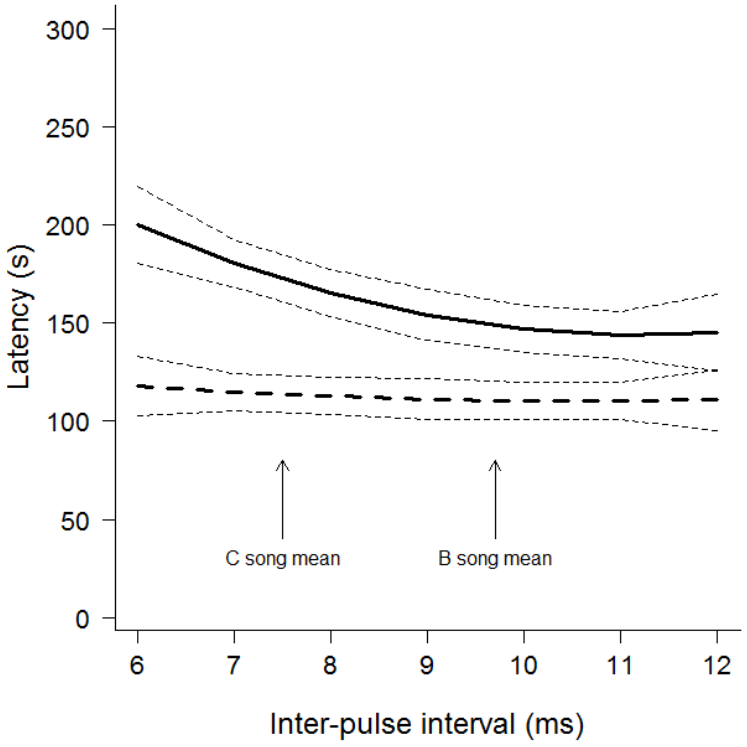


Figure 2.5. Female response (measured as latency to reach speaker) to songs with different IPIs. The response of *G. bimaculatus* is shown as a solid line, *G. campestris* as a broken line. 95% confidence intervals are shown by dotted lines. Note that lower latency values are indicative of stronger preferences. For reference, the mean IPIs of male songs that we calculated from recordings are shown with labelled arrows.

2.5 Discussion

We find that when trialled in an arena, neither *G. bimaculatus* nor *G. campestris* females show preferences for conspecific songs. In *G. bimaculatus* this is not due to females erratically choosing song, rather they are repeatable in their preferences, and are also repeatable in their latency to respond phonotactically. Contradictory to the first study, in the second we find a preference for heterospecific song. Finally, we demonstrate that the females of these species respond differently to songs varying in IPI; while overall *G. campestris* females move faster towards songs, they show no change in response to differing IPIs, whereas *G. bimaculatus* show increasingly strong preferences as IPI becomes longer.

The first two results are intriguing. Our initial finding that females of neither species show preference for conspecific song contradicts previous research. Using the same synthetic songs, Veen *et al.* (2013) found that *G. bimaculatus* preferred conspecific song in two-choice tests. The key difference between the previous and present studies is the method of measuring phonotactic response. Here we used an arena that allowed the females to move freely and orientate towards a particular song, and in doing so change the received sound pressure level of the songs. On the other hand, Veen *et al.* (2013) used a track-ball system, in which females were suspended in place above a polystyrene sphere. This enabled the measurement of turning effort and forward motion without the female moving any closer to the sound source, thereby keeping the female's received sound pressures levels of the songs the same throughout the trial.

Perhaps more difficult to explain is the result found in our second experiment, in which we found not only a preference for song, but a repeatable preference for heterospecific song. This contradicts both Veen *et al.* (2013) and our first experiment. Our sets of trials were carried out two months apart, using females taken from the same stock population, so it is unlikely that these contradictory results are due to genetic differences. Subtle differences in environmental conditions may have had some effect on female response. For example, female *Teleogryllus oceanicus* reared in silent conditions show little discrimination between calling songs compared to those exposed to song during rearing (Bailey & Zuk, 2008). The females used in our trials may have been subjected to different acoustic environments during rearing, depending on the density of males in the laboratory population at that time. The variation we observe between our results suggest that great care needs to be taken when interpreting data from phonotactic trials, and that subtle differences between experiments may have marked impacts on the outcomes.

Although we cannot explain why the results differ between these experiments, the preference for heterospecific song that we find in our second experiment might be explained by the

construction of the synthetic songs, which may not accurately represent natural song. The mean values of parameters were calculated from a relatively small sample of males (Veen *et al.*, 2013), and while the pulse numbers, chirp lengths and inter-chirp intervals were manipulated to match these means, IPI was not. This resulted in the *G. bimaculatus* synthetic song having a very long IPI, around 3 standard deviations away from the mean value that we calculated in our study. If the female preference function is unimodal, then we might expect females to be less responsive to such extreme values. Theory predicts that females should be strongly attracted to songs with traits most typical of their own species, i.e. those that fall within the centre of the distribution of a trait, and less attracted to those that at the extremes, creating a unimodal preference function, that ultimately leads to the avoidance of potentially costly heterospecific matings (Gerhardt, 1991). Since our investigation of IPI only used values within 1 standard deviation of the mean we are not able to determine whether the pattern we observe is part of a unimodal preference function, or whether *G. bimaculatus* preference is open ended. Even if the preference function is unimodal, it does not appear to be sharply tuned to the mean value produced by conspecific males. Females show relatively strong phonotactic responses over a range of IPIs, a range which encompasses both conspecific and heterospecific values.

G. campestris females appear to show no difference in response to IPI. This is surprising since previous research has shown IPI to be important in preference in this species. Thorson *et al.* (1982) showed these females to have a unimodal preference function for this trait (referred to as syllable repetition interval), though they used an extreme range of values to demonstrate this. It is likely that the range of values used in our study represent the centre of the curve, and that we would have also had to expose females to extreme values before observing a marked difference in response. Furthermore we presented the songs to the females in no-choice tests as opposed to two-choice tests. *G. bimaculatus* become much more selective when they are presented with two songs rather than one (Popov & Shuvalov, 1977; Veen *et al.*, 2013), and this might be the case for *G. campestris* also. Regardless of the values of the manipulated parameters, all songs may have been sufficient to trigger a strong phonotactic response, making latency to reach a sound source an unsuitable method of measuring preference.

Observations such as these that females demonstrate little or no species recognition, or even express preferences that oppose predictions, may be due to other cues of male quality in song being more important to females that those indicating species differences. It has recently been argued that the concept of 'species recognition' is flawed, and instead females should be viewed as simultaneously assessing many aspects of male quality, one of which being reproductive

compatibility (Sullivan, 2009; Mendelson & Shaw, 2012). There is likely a trade-off in choosing between cues indicative of compatibility and those indicative of other aspects of male quality. Ultimately females should make mating decisions that maximise their fitness, regardless of how we have taxonomically categorised potential mates. The females in these experiments may have been responding to various components of the synthetic songs, and perceived the song that we have designed to be 'heterospecific' as a high quality song.

Whatever the explanation for the females' preferences running counter to predictions, the consistent choice of heterospecific song was highly repeatable. This suggests that song preference may be a heritable trait, and may therefore be important in the formation of barriers to interbreeding, as well as exerting sexual selection on male traits. Females were also repeatable in their latencies to reach the sound source. These consistent individual differences in behaviour lend support to the idea of 'personality' being found in these species (Dall *et al.*, 2004).

While species recognition may play a role in the maintenance of reproductive isolation in these species, recognition of song is unlikely to be crucial. The weak discrimination between long-range call songs found in both the present study and by Veen *et al.* (2013) suggests that species recognition of song is unlikely to represent a strong pre-copulatory barrier to interbreeding. A combination of close-range cues (Veen *et al.*, 2013) and post-mating mechanisms (Tyler *et al.*, 2013), combined with differences in habitat preference are more likely involved in the maintenance of reproductive isolation between these species. Calling song may be more important for the location of potential mates, or may convey information about the overall quality of the male rather than species identity. This study highlights the need for caution when interpreting data from phonotactic trials, and shows that females may not exhibit well defined preference functions when tested within the natural range of song traits.

Chapter 3 Cuticular hydrocarbons as potential cues for species recognition in field crickets

3.1 Abstract

In crickets cuticular hydrocarbons (CHCs) are important in many aspects of mate choice, and may be used for species recognition if CHC profiles differ between species. *Gryllus campestris* females are highly aggressive towards heterospecific *G. bimaculatus* males, and may use CHCs to determine their species identity. We firstly assess the potential of CHCs to be used for species recognition in these crickets through comparison of profiles using gas chromatography. We then manipulate females' ability to detect CHCs through chemical ablation of the antennae, and measure changes in aggressive responses to heterospecific males. We show that there are significant species differences in CHC expression for both sexes, and that females incapable of receiving chemosensory information via their antennae reduce aggressive behaviour towards heterospecific males.

3.2 Introduction

Mate choice decisions are based upon the traits of potential partners. If gene flow is restricted between two populations, mating traits and preferences may diverge. Eventually individuals from divergent populations may no longer be recognised as potential mates, at which point speciation has occurred. Information about the traits used in species recognition can be received through a range of sensory modalities, for example using auditory, visual or tactile cues, likely in combination (Hebets & Papaj, 2005). Chemoreception (comprised of olfactory and gustatory systems) is thought to be the most ubiquitous of the sensory modalities (Ache & Young, 2005), and is used for species recognition across a broad range of taxa (reviewed by Smadja & Butlin, 2008). For example, swordtail fish can scent the water around them with olfactory cues, with female *Xiphophorus nigrensis* more attracted to the cues from conspecific males than to those from heterospecific *X. cortezi* or *X. montezumae* (McLennan & Ryan, 1999). In mice, olfactory signals found in urine are used to discriminate between individuals, with *Mus musculus musculus* preferring the urine of conspecifics to urine of heterospecific *M. m. domesticus* (Smadja *et al.*, 2004). Wall lizards investigate chemical signals through tongue flicks, with males of both *Podarcis bocagei* and *P. hispanica* preferentially investigating the signals of conspecific females (Barbosa *et al.*, 2006). The cuticles of flies are covered with molecules that can be used in chemoreception. Male *Drosophila mauritania* reduce their efforts to court conspecific females that carry chemical signals transferred from heterospecific *D. sechellia* females (Coyne & Charlesworth, 1997).

Surprisingly, species of true crickets, which have been intensively studied in the context of reproductive isolation (reviewed by Veen *et al.*, 2013), are rarely reported to use chemoreception for species recognition. Like *Drosophila* and all other insect species, the bodies of crickets carry lipid molecules known as cuticular hydrocarbons, or CHCs. Primarily functioning to prevent desiccation (Lockey, 1976), they also serve as cues for short-range chemical signalling (Howard & Blomquist, 1982). There are numerous molecular forms of CHCs, and individuals can gain information about one another through the relative expression of these molecules, primarily detected through the chemosensory hairs covering the antennae. In crickets, CHCs are involved in many aspects of mate choice. Female *Gryllodes sigillatus* can detect their own chemical signature on males they have already mated with, and can use this self-referent cue to mate polyandrously (Ivy *et al.*, 2005). Male *Telelogryllus oceanicus* can detect the mating status of females, with non-virgin females carrying the CHCs of males previously mated with (Thomas & Simmons, 2009). *Gryllus bimaculatus* and *T. oceanicus* both use CHCs to determine the genetic relatedness of potential mates (Simmons, 1989; 1990; Thomas & Simmons, 2011a). *G. bimaculatus, Acheta domesticus, G. integer, T. oceanicus* and *T. commodus* respond to sex differences in chemosensory cues (von Hörmann-Heck, 1957; Otte & Cade, 1976; Rence & Loher, 1977; Hardy & Shaw, 1983; Tregenza & Wedell, 1997; Nagamoto *et al.*, 2005; Leonard & Hedrick, 2009) and in some cases CHC profiles have been shown to differ between the sexes (Warthen & Uebel, 1980; Tregenza & Wedell, 1997; Thomas & Simmons, 2008). Given their general importance in mate choice in crickets, we might also expect CHCs to play an important role in species recognition. There are a few studies suggesting that this may be the case in *G. integer*, *G. lineaticeps* and *Allonemobius* species, (Otte & Cade, 1976; Paul, 1976; Leonard & Hedrick, 2009), and differences in CHC profiles have been found among species of rapidly diverging *Laupala* (Mullen *et al.*, 2007; Mullen *et al.*, 2008). Here, we investigate the role of CHCs in species recognition between a pair of European field crickets.

G. campestris and *G. bimaculatus* have overlapping distributions through southern Europe and further east (Popov & Shuvalov, 1977; Pardo *et al.*, 1993; Gorochov & Llorente, 2001). Like many crickets, the males of these species produce long-range calling song to attract mates, and characteristics of these songs differ between the species. Despite these differences, females only show weak (or no) preference for conspecific song (Veen *et al.*, 2013). Instead, the strength of close-range species recognition mechanisms has been highlighted (Veen *et al.*, 2011; Veen *et al.*, 2013). Though *G. bimaculatus* females preferentially mate with conspecific males, they will frequently engage in hybrid matings in the laboratory, whereas *G. campestris* females will very rarely mate with heterospecifics (Cousin, 1933; von Hörmann-Heck, 1957; Veen *et al.*, 2011; Veen *et al.*, 2013). Despite regular attempts by *G. bimaculatus* males to court *G. campestris* females, we have only ever

observed the females responding indifferently or aggressively (Veen *et al.*, 2011; Veen *et al.*, 2013). In contrast, when presented with a conspecific male, these females will readily mount and mate. This discrimination at close-range may be achieved through a number of sensory modalities, with detection of species-specific CHC profiles a likely contender.

We firstly assess the potential of CHCs to be used for species recognition in these crickets through comparison of CHC profiles using gas chromatography, predicting both sex and species differences among individuals. Given that females are the choosy sex in these crickets, sexual selection is likely to be acting on the males. If sexual selection has driven divergence, we might expect to find greater species differences between the males than the females. We then aim to confirm that *G. campestris* females discriminate between conspecific and heterospecific males using aggressive behaviour to indicate species recognition, and finally, determine whether their ability to detect CHCs alters the extent of aggression towards heterospecific males. We manipulate females' ability to detect CHCs through chemical ablation of the antennae, a method that prevents chemoreception without impairing mechanoreception (Balakrishnan & Pollack, 1997).

3.3 Methods

3.3.1 Study population

CHCs were sampled from 10 wild-caught *G. campestris* (5 male, 5 female), and from 10 lab-reared *G. bimaculatus* (5 male, 5 female). *G. campestris* were collected near Gijon, northern Spain, in spring 2010 as last instar nymphs. They were transferred to the laboratory where they were kept at 28 ^{o}C under a 16:8 light dark cycle, with food and water provided *ad libitum.* *G. bimaculatus* were from a population that had been maintained under these laboratory conditions for 5 years, originally collected near Valencia, southern Spain. All individuals were kept until death, after which they were preserved at -20 ^{o}C. Behavioural assays were carried out with wild-caught *G. campestris* collected in spring 2012, transferred to the laboratory at least 2 weeks prior to use in trials. *G. bimaculatus* were from a population that had been maintained in the laboratory for ~4 generations, having been collected in autumn 2011. All individuals were sexually mature, and had not mated for at least 7 days.

3.3.2 CHC extraction & analysis

To extract the cuticular hydrocarbons we completely submerged each cricket in 4 ml of HPLC grade hexane. The hexane contained pentadecane as an internal standard at a concentration of 10 ppm. The crickets were soaked for 5 min before being removed. 2 µl of each sample was injected into a GCMS (Agilent 7890 Gas Chromatograph coupled with an Agilent 5975 Mass spectrometer). This was fitted with a DB-1 ms column with an internal diameter of 30 m x 0.25 mm. Helium was used as a carrier gas. The inlet was set at 250 ^{o}C, and the injection was in pulsed splitless mode. Separation of the extract was optimised by using a column profile starting at 100 ^{o}C for 1 min, increasing at 20 ^{o}C/min to 250 ^{o}C, then increasing at 5 ^{o}C/min to 320 ^{o}C. It was then held at 320 ^{o}C for 2 min. The MS transfer line was kept at 250 ^{o}C. Data were analysed using MSD Chemstation software version E.02.00.493 (Agilent Technologies).

Males and females were analysed separately as their CHC profiles differ. Expression is known to be sexually dimorphic in *G. bimaculatus* (Tregenza & Wedell, 1997), and visual inspection of the *G. campestris* chromatograms showed differences between males and females in the number of peaks. 30 CHCs were quantified for each male, and 59 for each female. Relative peak size was calculated by dividing the peak areas of a given sample by the peak area of the internal standard (pentadecane) in that sample. These relative peak sizes were then normalised with a log transformation. Separate principal components analyses (PCA) were then run for the female and male data (SPSS v19). We used a correlation matrix to extract PCs with eigenvectors greater than 1 (Norman & Streiner, 1984). Before testing for species differences, we further reduced the dimensionality of the data. Without doing so our ability to run statistical tests would be compromised by limited degrees of freedom. We decided to restrict the number of PCs to cumulatively explaining ~80% of the variance, a value still widely regarded as successfully explaining variance (for example in studies by Peterson *et al.* (2007), Thomas & Simmons (2008), and Sharma *et al.* (2012), PCs with eigenvectors greater than 1 cumulatively explained 80 – 85 % of the variance). PC factor loadings greater than 0.25, or less than -0.25, were considered biologically important (Tabachnick & Fidell, 1989). We tested for species differences in CHC expression using a multivariate analysis of variance (MANOVA) in R v2.14.1. Males and females were tested separately. In each case, the 4 respective PCs of CHC expression were entered as response variables, and species entered as the fixed effect.

3.3.3 Behavioural assays

Males were silenced prior to use in behavioural trials so that the females had to use cues other than song to assess species identity of the males. To do this they were anaesthetised by exposing them to CO_2 gas until they stopped moving (~20 s), and sections of the wings where the file and scraper are found were removed with small dissection scissors (Figure 3.1). The procedure was carried out at least 24 hours before use in trials.

Figure 3.1. A *G. bimaculatus* male, the section of the upper wing removed shown in red. A similar section of the lower wing was also removed.

Behavioural trials were carried out in 20 cm Ø plastic arenas, which were cleaned with 100% ethanol between trials to remove traces of CHCs left by previous occupants. A silenced male and a *G. campestris* female were placed either side of a temporary divider. They were allowed to settle for at least 1 min before the divider was removed. The trial started when the pair made first physical contact (including antennal contact), after which they were observed for 5 min (trials were terminated if the female mounted the male, and the pair immediately separated to prevent mating). The number of times the female flared her mandibles at the male was recorded, as well as the number of seconds spent performing aggressive behaviour. Aggressive behaviour was defined as antennal fencing, mandible flaring, biting, or attempts to attack the male. For each female two pre-treatment trials were carried out, one with a heterospecific male and one with a conspecific male, in either order, allowing us to confirm that female aggression is species specific rather than being directed towards all males. Finally, a post-treatment trial was carried out using a heterospecific male so that we could measure the change in female response towards heterospecific males, and how this change differed between treatment groups. Where possible, the heterospecific male used

in the post-treatment trial was the same as for the pre-treatment trial. Some males were paired with more than one female.

After the pre-treatment trial, females were assigned to ablation or control treatment groups. To ensure that both groups included a similar range of aggressive behaviour, we matched individuals according to the number of flares performed during their pre-treatment trial with the heterospecific male, and divided these pairs across the groups. Females were anaesthetised using CO_2 gas to prevent them from moving during antennal immersion. They were held upside down in a plastic tube and suspended so that their antennae were immersed in the solution, but it did not touch their head or body. The antennae of the females in the ablation group were immersed in 0.4 mol/litre of zinc sulphate (ZnSO·7H2O, Sigma Aldrich) dissolved in 0.3% Triton-X (Sigma Aldrich). Triton-X reduces surface tension, allowing penetration though the fine hairs that cover the antennae. The antennae of females in the control group were immersed in 0.3% Triton-X (Balakrishnan & Pollack, 1997; Ryan & Sakaluk, 2009). Antennae were soaked for 10 minutes, after which they were washed clean with water. The post-treatment trial was not carried out until the next day to allow the female to recover.

Any female that did not behave aggressively towards the male during her pre-treatment trial was removed from the dataset. One of the females was extremely aggressive, and her trials were terminated early to prevent the male from being killed. Data for this female was also removed from the dataset. A comparison of females' pre-ablation responses (number of mandible flares) to conspecific and heterospecific males was made using a Wilcoxon rank sum test. The differences in females' aggression between the pre- and post-treatment trials were normally distributed, confirmed using a Shapiro-Wilks test. The change in aggression in response to treatment was analysed using a paired t-test. Two different measures of aggression were analysed in separate tests, mandible flares, and the number of seconds performing aggressive behaviour.

3.4 Results

3.4.1 CHC profiling

The male PCA returned 6 PCs, with eigenvectors that cumulatively explained 95.1% of the variance. The female PCA returned 8 PCs, with eigenvectors that cumulatively explained 99.1% of the variance. To further reduce the dimensionality of the data, we restricted the number of PCs to cumulatively explaining ~80% of the variance. Using this criteria we kept 4 PCs for the males (87.1 cumulative %) (Table 3.1), and 4 PCs for the females (80.9 cumulative %) (Table 3.2). Each of the PCs

removed from either sex explained less than 7% of the variance. For males the majority of factor loadings in PC1 were of a magnitude that is likely to be biologically important (Tabachnick & Fidell, 1989) and positively loaded, suggesting that overall investment in CHCs is important in males (Table 3.3). For females, there were biologically important factor loadings spread over PC1 and PC2, with no clear pattern in positive or negative loadings, suggesting that investment in particular combinations of CHCs is more important than total investment (Table 3.4).

Overall, there were species differences in the expression of CHCs in both males (MANOVA: $Pillai_{1,8} = 0.966$, P = 0.0007, Table 3.5) and females (MANOVA: $Pillai_{1,8} = 0.947$, P = 0.022, Table 3.6). The difference between male *G. bimaculatus* and *G. campestris* was driven by PC1 ($F_{1,8} = 15.92$, P = 0.004, Table 3.5, Figure 3.2). This is suggestive of species differences in total investment in CHCs, with *G. campestris* producing more of the CHCs that feature in PC1 (Figure 3.2). The difference between female *G. bimaculatus* and *G. campestris* was driven by PC2 ($F_{1,8} = 36.55$, P = 0.0003, Table 3.6, Figure 3.2).

Table 3.1. Variance explained in male data. 6 PCs extracted with eigenvectors >1. PCs 1 – 4 (in bold) cumulatively explain at least 80% variance.

	Eigenvalue	% of Variance	Cumulative %
PC1	**12.281**	**40.938**	**40.938**
PC2	**7.068**	**23.559**	**64.498**
PC3	**3.715**	**12.383**	**76.88**
PC4	**3.074**	**10.248**	**87.129**
PC5	1.385	4.618	91.746
PC6	1.007	3.355	95.102

Table 3.2. Variance explained in female data. 8 PCs extracted with eigenvectors >1. PCs 1 – 4 (in bold) cumulatively explain at least 80% variance.

	Eigenvalue	% of Variance	Cumulative %
PC1	**23.333**	**39.547**	**39.547**
PC2	**11.018**	**18.675**	**58.221**
PC3	**7.752**	**13.139**	**71.36**
PC4	**5.623**	**9.531**	**80.891**
PC5	4.045	6.856	87.746
PC6	3.063	5.192	92.938
PC7	2.534	4.295	97.233
PC8	1.099	1.863	99.096

Table 3.3. Male CHC profile, displaying hydrocarbon identity and factor loadings in each of the PCs. Hydrocarbons are ordered in increasing chain length. Factor loadings of >0.25 are highlighted in bold.

Retention time	Hydrocarbon	Molecular weight	Compound class	PC1	PC2	PC3	PC4
10.452	C23H48	324	Alkane	**0.286**	-0.236	**-0.638**	**-0.371**
11.214	C25H50	350	Alkene	**0.468**	**0.253**	**0.477**	0.07
11.363	C25H52	352	Alkane	**0.545**	**0.473**	**0.459**	-0.178
11.447	C25H52	352	Alkane	0.065	**-0.378**	**0.827**	**-0.252**
11.643	C26H54	366	Alkane	**0.502**	**0.682**	**0.497**	-0.152
11.897	C25H52	352	Alkane	**0.967**	0.013	0.136	-0.069
11.961	C26H54	366	Alkane	**0.324**	**-0.656**	0.246	**0.278**
12.173	C26H54	366	Alkane	**0.764**	0.246	**0.254**	**0.485**
12.745	C27H56	380	Alkane	**0.793**	**-0.573**	-0.088	-0.003
12.903	C27H54	378	Alkene	**0.621**	**0.759**	0.02	-0.099
12.988	C27H54	378	Alkene	**0.404**	**-0.895**	0.007	0.065
13.099	C27H56	380	Alkane	**0.321**	**0.908**	0.108	0.001
13.396	C28H58	394	Alkane	**0.577**	**-0.783**	0.025	0.102
13.714	C28H58	380	Alkane	**0.322**	**0.439**	-0.109	**-0.604**
13.804	C28H58	394	Alkane	**0.713**	**0.52**	0.046	**0.333**
13.867	C28H58	394	Alkane	**-0.389**	**0.804**	-0.027	-0.201
14.063	C28H58	394	Alkane	**-0.509**	**0.695**	0.026	**0.46**
14.709	C29H56	404	Alkadiene	**0.499**	**-0.301**	-0.232	**0.571**
14.804	C29H56	404	Alkadiene	**0.955**	0.165	-0.159	0.017
14.932	C29H58	406	Alkene	**0.966**	0.03	-0.242	0.016
15.138	C29H60	408	Alkane	**0.939**	0.008	-0.062	0.207
15.832	C30H62	422	Alkane	**0.937**	-0.053	0.022	0.223
15.948	C30H62	422	Alkane	-0.168	**0.552**	-0.118	**0.718**
16.954	C31H60	432	Alkadiene	**0.943**	0.059	**-0.311**	0.053
17.05	C31H60	432	Alkadiene	**0.286**	0.225	**-0.837**	**0.352**
17.182	C31H62	434	Alkene	**0.572**	**0.393**	**-0.592**	-0.228
17.219	C31H62	434	Alkene	**0.844**	0.066	**0.257**	-0.061
17.389	C31H64	436	Alkane	**0.752**	0.18	0.045	**-0.58**
18.665	C32H66	450	Alkane	0.23	-0.127	**0.622**	**0.426**
21.058	Unknown			**-0.811**	**0.449**	-0.008	**0.33**

Table 3.4. Female CHC profile, displaying hydrocarbon identity and factor loadings in each of the PCs. Hydrocarbons are ordered in increasing chain length. Factor loadings >0.25 are highlighted in bold.

Retention time	Hydrocarbon	Molecular weight	Compound class	PC1	PC2	PC3	PC4
11.32	C25H52	352	Alkane	**-0.444**	**0.506**	**-0.601**	**-0.387**
11.368	C25H52	352	Alkane	0.195	0.185	**-0.699**	**0.418**
11.452	C23H48	324	Alkane	**-0.407**	0.02	**0.424**	**0.515**
11.553	Unknown			0.226	-0.119	**-0.763**	**-0.558**
11.648	C26H54	366	Alkane	**0.6**	-0.16	**-0.466**	**0.45**
11.776	Unknown			**0.646**	-0.179	**-0.42**	**0.288**
11.908	C26H54	366	Alkane	**0.395**	-0.116	**-0.576**	**0.594**
11.971	C26H54	366	Alkane	**0.747**	**-0.61**	-0.188	0.153
12.056	C26H54	366	Alkane	0.186	**0.529**	**-0.323**	-0.14
12.189	C26H54	366	Alkane	**0.715**	**0.377**	-0.196	**0.391**
12.294	C27H56	380	Alkane	**0.813**	-0.239	**-0.404**	0.158
12.485	C26H54	366	Alkane	**0.8**	**-0.563**	0.011	0.161
12.549	C27H56	380	Alkane	**0.842**	**0.27**	-0.099	**-0.365**
12.591	Unknown		Alkane	**0.776**	0.127	0.235	**0.549**
12.633	C27H56	380	Alkane	-0.053	**-0.832**	**-0.413**	-0.029
12.702	C27H56	380	Alkane	**0.936**	0.066	-0.191	**0.265**
12.755	C27H56	380	Alkane	0.132	**0.591**	0.202	**0.677**
12.888	C27H56	380	Alkane	-0.2	**0.897**	-0.223	-0.063
13.02	C27H56	380	Alkane	**-0.673**	-0.177	**0.318**	0.04
13.131	C28H58	394	Alkane	**0.392**	**0.824**	**-0.256**	-0.008
13.306	Unknown			**0.859**	**0.306**	0.011	**-0.3**
13.465	C28H58	394	Alkane	**0.885**	0.065	0.015	**0.329**
13.576	C27H56	380	Alkane	**0.91**	**-0.393**	0.08	0.067
13.692	C28H58	380	Alkane	0.033	**0.839**	0.24	**0.264**
13.793	C28H58	394	Alkane	**0.783**	0.148	0.138	**0.301**
13.888	C28H58	394	Alkane	**0.753**	**0.609**	-0.059	-0.084
14.047	C28H58	394	Alkane	**-0.917**	-0.156	0.232	0.012
14.164	C28H58	394	Alkane	**0.893**	**-0.414**	0.144	0.056
14.18	C28H58	394	Alkane	**0.942**	0.219	-0.145	-0.054
14.211	C29H60	408	Alkane	0.242	0.048	0.099	**0.359**
14.312	Unknown			**-0.269**	**-0.843**	0.185	**0.299**

14.402	C28H58	394	Alkane	**0.926**	0.064	-0.175	**0.32**
14.471	C29H60	408	Alkane	**0.805**	0.153	**0.308**	**-0.439**
14.54	C29H60	408	Alkane	**0.946**	0.227	-0.102	-0.023
14.688	C29H56	404	Alkadiene	-0.096	**0.38**	**0.723**	**0.542**
14.73	C29H60	408	Alkane	0.199	**0.694**	0.147	**-0.289**
14.863	C29H60	408	Alkane	-0.002	**0.77**	0.048	0.086
14.937	C29H58	406	Alkene	**-0.732**	0.213	**0.506**	-0.109
15.022	C29H60	408	Alkane	**0.859**	**0.336**	0.113	-0.235
15.106	C29H60	408	Alkane	**0.935**	0.218	-0.096	0.031
15.35	Unknown			**0.758**	**0.386**	**0.441**	0.008
15.466	C29H60	408	Alkane	**0.784**	**-0.519**	**0.318**	0.034
15.662	C30H62	422	Alkane	**0.614**	-0.158	**0.671**	-0.016
15.821	C30H62	422	Alkane	-0.066	**0.587**	**0.598**	0.018
15.927	C30H62	422	Alkane	**-0.727**	-0.028	**-0.266**	**0.568**
15.938	C30H62	422	Alkane	**0.81**	-0.043	**0.328**	**-0.252**
16.033	C30H62	422	Alkane	**0.859**	**0.337**	0.113	-0.234
16.218	C30H62	422	Alkane	**0.79**	**-0.56**	0.171	-0.088
16.351	C31H64	436	Alkane	**0.672**	-0.091	**0.425**	**-0.559**
16.684	C29H56	404	Alkadiene	**0.584**	**-0.36**	**0.463**	**-0.331**
16.706	C31H64	436	Alkane	**0.35**	**0.271**	0.206	0.016
16.758	C31H64	436	Alkane	-0.142	**0.753**	**0.354**	-0.066
16.949	C31H60	432	Alkadiene	**-0.36**	**0.523**	0.033	**0.36**
17.251	C31H62	434	Alkene	**-0.395**	**0.395**	**-0.48**	-0.121
17.394	C31H64	436	Alkane	**0.509**	**0.259**	0.233	-0.219
17.759	C31H64	436	Alkane	**0.474**	-0.144	**-0.564**	-0.017
17.833	C31H64	436	Alkane	0.136	**-0.516**	**0.641**	-0.051
18.596	C32H66	450	Alkane	0.225	**-0.427**	0.114	**-0.438**
21.058	Unknown			-0.123	0.23	**-0.664**	**-0.509**

Table 3.5. Analysis of male data. Species differences in CHC expression, shown as output from the MANOVA, as well as the univariate contribution of each principal component.

		df	SS	Test statistic	P
Multivariate	Model	1		0.966 (Pillai)	**0.0007**
	Error	8			
PC1	Model	1	5.990	15.918 (F)	**0.004**
	Error	8	3.010		
PC2	Model	1	1.701	1.865 (F)	0.209
	Error	8	7.299		
PC3	Model	1	0	0 (F)	0.999
	Error	8	9		
PC4	Model	1	1.002	1.003 (F)	0.346
	Error	8	7.998		

Table 3.6. Analysis of female data. Species differences in CHC expression, shown as output from the multivariate ANOVA, as well as the univariate contribution of each principal component.

		df	SS	Test statistic	P
Multivariate	Model	1		0.947 (Pillai)	**0.002**
	Error	8			
PC1	Model	1	0.156	0.141 (F)	0.717
	Error	8	8.844		
PC2	Model	1	7.384	36.547 (F)	**0.0003**
	Error	8	1.616		
PC3	Model	1	0.979	0.9765 (F)	0.352
	Error	8	8.021		
PC4	Model	1	0.0001	<0.0001 (F)	0.992
	Error	8	8.999		

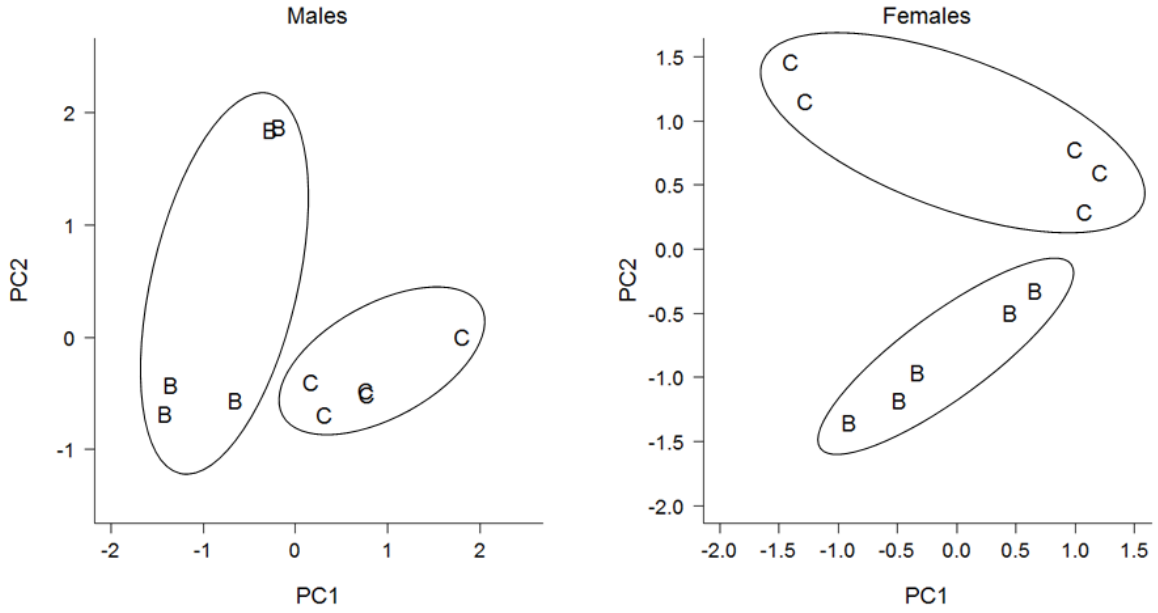


Figure 3.2. Separation of *G. bimaculatus* (B) and *G. campestris* (C) cuticular hydrocarbon extracts based on the first and second principal components (PC) taken from the principal components analysis. Data for males are shown on the left (showing species clustering along PC1), and females on the right (showing species clustering along PC2). Ellipses are for ease of visualising clusters only.

3.4.2 Behavioural assays

A total of 44 *G. campestris* females, 24 *G. campestris* males and 32 *G. bimaculatus* males were used in trials. 16 of the females did not flare their mandibles during their pre-treatment trial with the heterospecific male, and so data associated with these females were removed from the dataset. *G. campestris* females behaved far more aggressively towards heterospecific than conspecific males, with mandible flares rarely being directed towards conspecific males (Wilcoxon; W = 643.5, n = 27, P < 0.001, Figure 3.3). Females with chemically ablated antennae reduced the number of times they flared their mandibles at heterospecific males, whereas control females did not (Paired t-test; t = 2.13, df = 27, P = 0.043, Figure 3.4). When aggression was measured as the number of seconds spent performing aggressive behaviour, the difference between the control and ablated group was non-significant (t = 1.77, df = 27, P = 0.088).

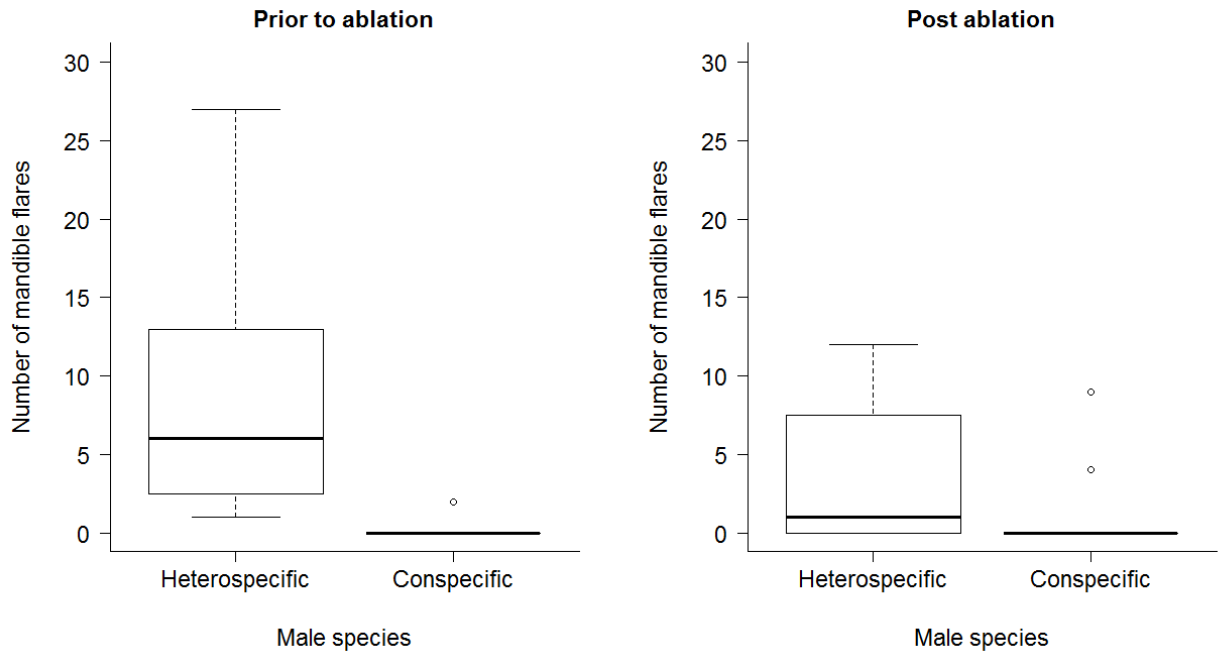


Figure 3.3. Aggression towards heterospecific (*G. bimaculatus*) and conspecific (*G. campestris*) males, prior to chemical ablation of antennae (left) and post chemical ablation of antennae (right). Data are presented as medians and interquartile ranges.

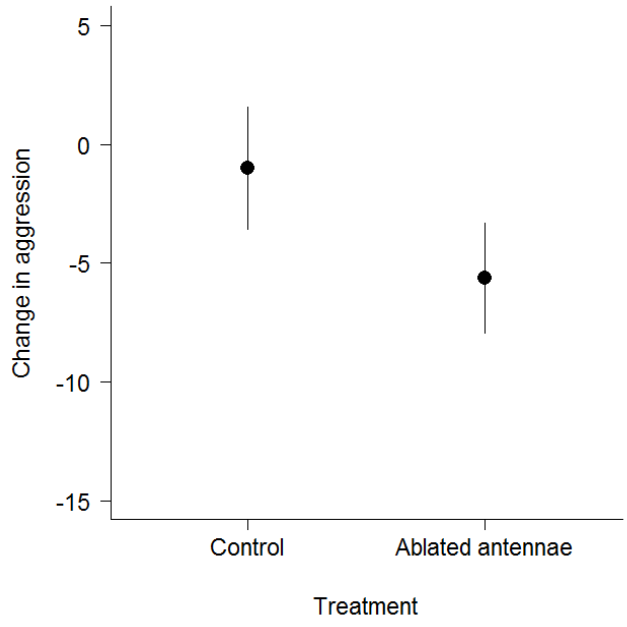


Figure 3.4. The change in aggression towards a heterospecific male after chemical ablation of antennae. Aggression was measured as the number of mandible flares performed by a *G. campestris* female paired with a *G. bimaculatus* male. Data are presented as means ± standard errors.

3.5 Discussion

We show that there are significant species differences in CHC expression for both sexes. In males this difference is driven by *G. campestris* individuals investing more in overall CHC expression, whereas in females the species difference is driven by the combinations of CHCs being expressed. We confirm that female *G. campestris* behave more aggressively towards heterospecific than conspecific males, and that females incapable of receiving chemosensory information via their antennae reduce aggressive behaviour towards heterospecific males.

Since these species tend to be found in different habitats, it is possible that initial divergence in CHC expression was due to adaptation to the environment through natural selection, or drift. This may have been a relatively fast process. Divergence in CHCs among *Laupala* populations is rapid, with cryptic species on different islands having different profiles (Mullen *et al.*, 2007). Sexual selection may also have since driven divergence of, and preferences for, CHC expression (Chenoweth & Blows, 2005; Rundle, 2005). We predicted that males, as the less choosy sex, might be subject to sexual selection and have greater differences in CHC expression than females. We found strong species differences between the males, particularly that *G. campestris* invest more in overall production of CHCs. This might be due to assessment of CHCs being more important in *G. campestris* mate choice than *G. bimaculatus*. Despite showing weak (or no) discrimination between long-range calling songs (Veen *et al.*, 2013), *G. campestris* females almost never accept heterospecific mating opportunities (Cousin, 1933; von Hörmann-Heck, 1957; Veen *et al.*, 2011; Veen *et al.*, 2013). This suggests that short range cues are important to females in assessing potential mates, placing selection pressure on the males to strengthen these cues.

Our samples of *G. campestris* were taken from wild-caught individuals, whereas samples of *G. bimaculatus* were taken from a population maintained in the laboratory. Since CHC expression is a product of environment as well as genotype (Kent *et al.*, 2008), the species differences that we observe could be driven by the differences in environment that these individuals were exposed to as nymphs. However, CHC expression is plastic over relatively short time scales (Thomas & Simmons, 2011b), and since the wild-caught individuals were kept in the laboratory for the entirety of their adult lives, the environmental component of expression should be similar for both species. Even if there are differences generated by conditions experienced in early life, this is unlikely to be important in the interpretation of genotypic species differences. In *Laupala* an analysis comparing CHC profiles found that wild-caught and lab-reared conspecifics clustered together, with both differing from heterospecifics (Mullen *et al.*, 2007). It is likely that this is also the case in our species.

There is uncertainty as to whether the reduction in aggression was due to an impaired ability to assess male species, or whether the loss of chemosensory information as a whole led females to become indifferent to any cricket. If the latter then we might expect ablated females to respond indifferently to conspecific males, as well as reducing aggression to heterospecific males. Although not explicitly studied, we observed some of these females mating with conspecific males after the trials, indicating that they were not indifferent to them. Ryan & Sakaluk (2009) found that ablated female *G. sigillatus* took longer to mount courting males, indicating that while there is a suppression of response to males, their normal behaviour is not completely lost. This lends support to the notion that the reduced aggression is perhaps partly, but not entirely due to indifference towards the males, instead reduced aggression is more likely due to ablated females no longer sensing male species.

The discrepancy in the results obtained from the two measures of aggression is intriguing. While counts of mandible flares revealed a significant difference between the treated and control groups, this difference was only a trend when measured as time spent performing aggressive behaviours. The discrepancy is likely due to the inaccuracy of the latter measurement. A cricket performs each behaviour for only a short time, making the capture of this information difficult and so introducing measurement error. Additionally, the interpretation of the behaviours was not always clear. Though we had a pre-defined list of behaviours categorised as aggressive, there is variation in how each of these behaviours is performed, and there may be cases of non-aggressive behaviour being considered as aggressive and *vice versa*. Of all the behaviours categorised as aggressive, mandible flaring was the least ambiguous. There are no similar behaviours with which to confuse flaring, and, as far as we are aware, flares are not used in other behavioural contexts.

Although antennal ablation reduced the aggression of *G. campestris* females towards heterospecific males, aggression was not eliminated and females did not begin to accept courtship attempts from heterospecific males. This suggests that either our method of chemical ablation was only moderately successful, or that additional cues are informing species recognition. Zinc sulphate is known to be effective in the chemical ablation of antennae, disabling the electrophysiological response of the chemosensory sensilla that are found along the length of antennae (Balakrishnan & Pollack, 1997). It is possible that some of the chemosensory sensilla closest to the cricket's head, which we avoided immersing in the solution, remained active. Also, there are other areas of the body such as the palps that carry chemosensory sensilla (Klein, 1981), and may therefore detect CHCs. These might be particularly important for the detection of volatile airborne compounds, cues that can be passed between individuals without physical contact. Females are likely using additional

cues to assess the males, which may explain why aggression was not entirely eliminated. While we disabled chemoreception of their antennae, mechanoreceptors were unaffected by the zinc sulphate. Females could therefore still detect any tactile differences between the males, such as differences in morphology, or in movements made by the antennae or body. Though the males were silenced to prevent females from using auditory cues, there may be characteristic movements of the wings made by singing males that could be used to differentiate between species, or other visual cues.

The change in behaviour attributed to loss of chemoreception suggests that CHCs are involved in species recognition between *G. campestris* and *G. bimaculatus*. While females of many species use characteristics of male advertisement calls to distinguish between conspecifics and heterospecifics, in these crickets calling song represents a relatively weak barrier to hybridisation. Cues provided through other sensory modalities received at short range, such as chemoreception of CHCs, may therefore be important in the maintenance of species boundaries. The use of CHCs for species recognition may well be important between other species of interbreeding crickets, and we encourage studies explicitly investigating this.

Chapter 4 Multiple post-mating barriers to hybridisation in field crickets

4.1 Abstract

Mechanisms that prevent different species from interbreeding are fundamental to the maintenance of biodiversity. Barriers to interspecific matings, such as failure to recognise a potential mate, are often relatively easy to identify. Those occurring after mating, such as differences in the how successful sperm are in competition for fertilizations, are cryptic, and have the potential to create selection on females to mate multiply as a defence against maladaptive hybridisation. Cryptic advantages to conspecific sperm may be very widespread and have been identified on the basis of observations of higher paternity of conspecifics in several species. However, a relationship between the fate of sperm from two species within the female and paternity has never been demonstrated. We use competitive microsatellite PCR to show that in two hybridizing cricket species, *Gryllus bimaculatus* and *G. campestris*, sequential cryptic reproductive barriers are present. In competition with heterospecifics, more sperm from conspecific males is stored by females. Additionally, sperm from conspecific males has a higher fertilization probability. This reveals that conspecific sperm precedence can occur through processes fundamentally under the control of females, providing avenues for females to evolve multiple mating as a defence against hybridisation, with the counterintuitive outcome that promiscuity reinforces isolation and may promote speciation.

4.2 Introduction

Reproductive isolation between species has long been studied within the classic dichotomous framework of barriers occurring before insemination, such as availability or recognition of potential mates, and those occurring after zygote formation, such as viability and fertility of hybrid offspring (Dobzhansky, 1937). Only relatively recently has attention been paid to the role of cryptic selection mechanisms acting between mating and the fertilization of eggs. This category of mechanisms, termed postmating-prezygotic (Howard *et al.*, 2009) will reduce gene flow between distinct populations of individuals or species, and thus act to maintain species boundaries if the success of conspecific matings is relatively greater than that of heterospecific matings. These barriers are now acknowledged to be important contributors to reproductive isolation, and there are a growing number of studies showing that in closely related species where females will mate to both conspecific and heterospecific males, the heterospecific males do not sire as many offspring, a

phenomenon known as conspecific sperm precedence (CSP). Examples have been recorded across a broad range of taxa, with insect and marine invertebrate species most prevalent in the literature (Howard *et al.*, 2009). Traits associated with postmating-prezygotic processes have been shown to have the potential to diverge rapidly, suggesting they could play an important role in speciation (Civetta & Singh, 1995; Pitnick *et al.*, 2003; Andrés *et al.*, 2006). What we are missing is evidence for the mechanism by which conspecific sperm gain a greater share of fertilizations. This is particularly interesting because if it is something that females can influence, then selection can increase reproductive isolation which would tend to increase the rate of speciation.

Although widely reported, little is known of the underlying processes involved in CSP (reviewed by Howard, 1999). Studies have followed the progress of ejaculates through the female tract without relating this to siring success in the same female (Price *et al.*, 2001), or have relied upon counts of offspring displaying phenotypic markers without elucidating the cryptic processes determining the success of ejaculates within the same female (e.g. Fricke & Arnqvist, 2004). It is also difficult to demonstrate that CSP is due to competition between gametes rather than differential fitness of hybrid embryos or offspring (but see Price, 1997).

To overcome the problems usually associated with the study of CSP, we use a competitive microsatellite PCR (CM-PCR) technique (Wooninck *et al.*, 2000; Bussière *et al.*, 2010), which enables us to determine the relative contribution of an individual to mixed DNA samples. To date this technique has successfully been used to investigate patterns of sperm storage in twice mated dung flies, *Scathophaga stercoraria*, (Bussière *et al.*, 2010), the relationship between spermatophore attachment time and sperm storage in twice mated crickets, *Teleogryllus commodus* (Hall *et al.*, 2010), and the effect of relatedness of mating partners on sperm storage and paternity in twice mated *Gryllus bimaculatus* (Bretman *et al.*, 2009). We apply this technique to study the hybridising field crickets *G. bimaculatus* and *G. campestris*, species in which CSP potentially acts as a reproductive barrier. We firstly determine the representation of sperm from competing males in the spermathecae of doubly mated females, and secondly relate this to the success of each ejaculate in siring nymphs. The ability to directly observe sperm storage translating to siring success within the same female makes CM-PCR a powerful tool in the study of CSP, and to our knowledge this is the first time that it has been employed in this context.

G. bimaculatus and *G. campestris* live in grazed or mown grassland habitats and have overlapping ranges in southern Europe (Pardo *et al.*, 1993; Gorochov & Llorente, 2001). The two species will interbreed in captivity (Cousin, 1933; von Hörmann-Heck, 1957; Veen *et al.*, 2011). Interbreeding is unidirectional, with *G. campestris* females almost never accepting *G. bimaculatus*

males as mates (but see Cousin, 1933). Although mate choice in *G. bimaculatus* is well studied, less is known of mate choice in the context of reproductive isolation between species, i.e. the relationship between intra- and interspecific mate choice. While recent work has revealed reproductive barriers between *G. bimaculatus* and *G. campestris* in terms of mate choice before mating, as well as hybrid viability and sterility (Veen *et al.*, 2011; Veen *et al.*, 2013), to date, nothing is known of potential cryptic barriers in this system. This is a recurrent situation in the study of Gryllidae. Despite intensive study of reproductive isolation at several hybrid zones around the globe (reviewed in Veen *et al.*, 2013), to our knowledge CSP has only previously been examined in *Allonemobius* species where there is strong CSP (Gregory & Howard, 1994; Marshall, 2004), and between *G. pennsylvanicus* and *G. firmus*, where no evidence of CSP was found (Larson *et al.*, 2012).

The mating systems of *G. bimaculatus* and *G. campestris* are similar. Prior to mating, a male provisions a spermatophore with sperm. The number of sperm that a male invests into each spermatophore does not decline over at least the first five matings in *G. bimaculatus* (Simmons, 1986; 1987), however they may alter their investment depending on the perceived quality of potential mates (Hall *et al.*, 2000). Both species are polyandrous with females mating with a number of males during their lifetime (Bretman & Tregenza, 2005; Rodríguez-Muñoz *et al.*, 2011). Mating takes a few seconds, consisting of the female mounting the male and the male externally attaching a spermatophore to her. After mating sperm begin to transfer from the spermatophore to the female reproductive tract. This process takes around an hour, and is occasionally terminated by early removal of the spermatophore (Simmons, 1986), although removal is often prevented by the male through guarding behavior (Simmons, 1991a). *G. bimaculatus* females can also exert cryptic control, biasing the paternity of offspring through differential uptake of conspecific sperm (Bretman *et al.*, 2009), potentially through muscular control (Simmons & Achmann, 2000). Transferred sperm are stored in the spermatheca, and once in storage are not displaced by subsequent matings; rather the spermatheca expands to store multiple ejaculates (Simmons, 1986). It is spherical in form, a shape which is likely to promote mixing of ejaculates rather than stratified sperm storage (Walker, 1980; Simmons, 1986). The lack of stratified storage means there is no last male sperm precedence in this species (Simmons, 1987; Bretman *et al.*, 2009). Instead, success in siring offspring is likely determined as a raffle (Parker, 1982), whereby the more sperm a male has in storage, the greater the chance his sperm will be used to fertilise eggs. Indeed, Bretman *et al*. (2009) found a direct relationship between the amount of sperm individual males had in storage, and their subsequent paternity when *G. bimaculatus* females were mated to both a related and an unrelated male.

As pre-copulatory barriers to hybridisation are relatively weak between *G. bimaculatus* females and *G. campestris* males (Veen *et al.*, 2011), it is possible that post-copulatory barriers play a role as reproductive isolating mechanisms between the two species. Coupled with the knowledge that *G. bimaculatus* females are capable of cryptic female choice in terms of uptake and storage of sperm (Simmons & Achmann, 2000; Bretman *et al.*, 2009), we predict that conspecific sperm precedence will be present in this system, and so expect to find a greater representation of conspecific sperm in the spermathecae of multiply mated females.

While an increasing number of studies have considered cryptic barriers in terms of the overall sperm competition success of males of one species versus another, little attention has been paid to the repeatability of success of individual males. This is an important issue because such repeatability would indicate that success in these contexts is at least partly a male trait. (Tregenza *et al.*, 2009). Additionally, if the same traits are associated with success whether sperm competition is intra- or interspecific, this would indicate that mechanisms of sperm competition are conserved across species. In our experimental design we mate each of the males twice, allowing within-individual success to be compared when competing intra- and interspecifically.

Finally, based upon the assumption that sperm mixing occurs within the spermatheca (Walker, 1980; Simmons, 1986) and Parker's 'raffle principle' of sperm competition (Parker, 1982), we predict a direct relationship between representation in the spermatheca and subsequent paternity. Deviation from this predicted relationship could occur through biased success in post-storage sperm competition or ability to fertilise eggs, or through differential mortality of hybrid offspring. To disentangle these potential mechanisms, we monitor egg laying and hatching success.

4.3 Methods

4.3.1 Study animals

G. campestris were collected from near Gijon, northern Spain (N43 27.193 W5 50.407) as nymphs, and the majority were reared to adulthood in the laboratory. Those that became adult before reaching the laboratory were allowed to adjust to standard laboratory conditions for at least 8 days prior to use in trials. We used wild caught individuals because this species has an obligatory diapause which makes them difficult to rear in larger numbers in the lab. *G. bimaculatus* were collected from Valencia, southern Spain (N39 35.936 W0 34.087), and have subsequently been reared for 6 years in the laboratory. Crickets were provided with food and water *ad libitum*, and

maintained under a 16L:8D photoperiod at 28 ^{o}C. Individuals were separated into small plastic tubs prior to becoming adult to ensure virginity, and were a minimum of 7 days old post-eclosion before being used in mating trials. Mating trials were conducted over a period of 2 years.

4.3.2 Mating trials

Prior to mating trials, almost all males (75%) (see section S4, Supporting information) were exposed to non-experimental conspecific females to stimulate spermatophore development and courtship behaviour. These individuals were separated by wire mesh so that the female could be detected but not mated with. Males were monitored for the onset of courtship behaviour, indicating that a spermatophore has been produced and is ready to be transferred. Mating trials were carried out in 11 x 11 cm plastic containers lined with paper for traction. Only *G. bimaculatus* females were used, as they mate both intra- and interspecifically (Veen *et al.*, 2011). Each pair was given 2 h to mate, if they had not done so within this time the pair were trialled again on subsequent days (including re-exposing the male to a non-experimental conspecific female) for a maximum of five days before being discarded. Mating pairs were observed following successful mating, and spermatophore attachment time was standardised to 1 h, the period of time required for almost all contents of the spermatophore to be transferred to the female (Simmons, 1986). Females can bias paternity through early removal of the spermatophore (Simmons, 1986), but this was prevented through male guarding behaviour (Simmons, 1991a). If the male's behaviour was not sufficient to prevent attempts by the female at early spermatophore removal the female was moved into a small vial to restrict her movement.

Only *G. bimaculatus* females were used. They were mated twice, to a conspecific *G. bimaculatus* (B) and a heterospecific *G. campestris* (C) in either order (BC/CB) so that competition between males was interspecific, or mated to two males of the same species (BB or CC) so that competition between males was intraspecific. We aimed to pair each male twice, each time facing a different competitive treatment (either intra- or interspecific), but in the same order as first or second male to mate on both occasions. No male was used more than once in either intra- or interspecific treatments (Table 4.1). In all, 70 triads of individuals were mated.

After mating, males were preserved in 100% ethanol, or frozen at -20 ^{o}C, until DNA extraction. After their second mating, females were allowed to lay eggs in a small container of damp sand for 48 h before preservation in ethanol. Eggs were removed from the sand and counted. A random sample of 100 of the eggs (or fewer if the total number laid was less than 100) was

incubated at 28 ^{o}C on damp cotton wool. Upon hatching, nymphs were counted and collected twice daily, and either frozen or stored in ethanol.

Table 4.1. Example triad design. Only *G. bimaculatus* females were used, each of which was mated twice, to a either two conspecifics, two heterospecifics or one of each. We aimed to mate each male twice, so that he appeared in both interspecific and intraspecific competitive contexts. B males were conspecific to the female whereas C males were heterospecific. Competition between BC or CB pairs of males was interspecific, competition between BB or CC males was intraspecific.

Triad	*G. bimaculatus* female	1st Male to mate	2nd Male to mate	Competition between males
BC	1	B.1	C.2	Interspecific
CB	2	C.1	B.2	Interspecific
BB	3	B.1	B.2	Intraspecific
CC	4	C.1	C.2	Intraspecific

4.3.3 Molecular analysis

DNA was extracted from adult legs and whole nymphs using a salt extraction protocol (see Bretman & Tregenza, 2005 for details). 30 nymphs (or fewer depending on hatching success) were sampled from each triad, a number chosen to maximise accurate representation of each male's siring success, without becoming an unmanageable amount of tissue to extract DNA from. Extractions carrying pigment from the cuticle were cleaned prior to PCR using a DNA clean-up kit (Genomic DNA Clean & Concentrator, Zymo research). To estimate the amount of sperm stored by both males, the spermathecae (containing DNA from the female as well as from each male's sperm) were dissected from females, and the DNA extracted using a chelex protocol (see Bretman & Tregenza, 2005). DNA from adult legs was standardised to 10 ng/µl using a NanoVue (GE Healthcare).

The CM-PCR technique requires the identification of a unique microsatellite allele marker in each of the two males that comprise a mating triad, i.e. one not shared by the other male or the female. To identify unique alleles, adults were genotyped at up to 10 microsatellite loci [*Gbim*04, 15 (Dawson et al 2003); *Gbim*21, 29, 48, 49, 52, 57, 66 and 72 (Bretman *et al.*, 2008)] (MJ Research Thermal Cycler PTC-200) on an ABI 3130XL sequencer (Applied Biosystems), and allele sizes scored

using GENEMAPPER v3.7 (Applied Biosystems). For details of PCR conditions for these microsatellite loci see Section S1, Supporting Information. Unique alleles were identified for 55 of the 70 triads. 32 triads (out of the 55) were made up of females mated to interspecifically competing males (BC/CB), 12 to two *G. bimaculatus* males (BB), and 11 to two *G. campestris* males (CC). Of the 55 triads, 17 did not produce nymphs. A total of 76 individual males were used, with equal numbers of each species. Of these males, 36 featured in both an intraspecific and an interspecific competition triad.

A standard curve was made for each of the 55 triads (following Bretman *et al.*, 2009), from which to determine a male's representation in the spermatheca and nymph samples. Each standard contained a mix of DNA from the two males in varying proportions, such that the focal male (the B male in BC/CB triads, the first male to mate in BB or CC triads) accounted for 6.25%, 12.5%, 25%, 50%, 75%, 87.5% and 93.75% of the mix. As female DNA will be present in the nymphs and could potentially contaminate the sperm samples, we made a second set of standards following Bretman *et al.* (2009), including the DNA from the female in a 1:1 ratio with the DNA mixture from the 2 males. The standards, as well as the spermatheca and nymph samples corresponding to each triad, were then genotyped at the relevant locus identified for that triad as possessing unique male alleles. The use of a unique standard curve for each triad, rather than for all the samples as a whole (as in Bussière *et al.*, 2010; Hall *et al.*, 2010), avoids potential problems such as preferential amplification of smaller alleles, and so does not require any statistical adjustment for such effects.

We scored the unique alleles for each triad in GENEMAPPER and extracted their total peak areas. The relative peak area of the focal male was then calculated as (area of focal male / area of focal male + area of other male), and then plotted against the proportion of focal male DNA in the standard mix to create a standard curve for each triad. We repeated this process for the standard samples containing 50% female DNA. The inclusion of female DNA in the standard mixes made a marked difference to relative peak heights (in most cases changing the fit of the standard curve from linear to non-linear), so it was these values that were used to create the standard curves. Curves were fitted as linear, logarithmic or polynomial. Best fit could not be chosen based upon ANOVA, as the linear and logarithmic models contain the same number of parameters (comparisons require models to differ in the number of parameters they contain, see *Statistical analyses)*. Instead, best fit was selected based upon AIC, whereby the AIC delta scores of each model were compared and considered to be different if greater than 2 (Burnham & Anderson, 2002), with the requirement that the curve must increase and not asymptote through the range of the data. The fit of the standard curve to each of the sets of standards was high (mean R^2 = 0.976 +/- SE 0.004). The relative peak

area of the allele from the focal male in the spermatheca and nymph samples was calculated using the formula from the standard curve to determine that male's representation in both samples.

Repeatability of the quantification of the proportion of male DNA in spermatheca and nymph samples was assessed by randomly selecting a subset of samples (8 spermathecae, 9 nymphs and 7 standards), and repeating the PCR and genotyping to yield a duplicate estimate of proportion of DNA. The repeatability of the selection of samples re-amplified and genotyped was high (R^2 = 0.982, see Section S2, Supporting information. An outlier in the dataset, a *G. bimaculatus* male featuring in only one triad, where almost all of the sperm in the spermatheca were his but where he sired none of the offspring, was excluded prior to the analysis on the grounds this male was almost certainly infertile.

4.3.4 Statistical analyses

All analyses were carried out using R v2.14.1 (R, 2011). We used the package 'lme4'(Bates *et al.*, 2011) to fit generalised linear mixed models (GLMM) to assess factors affecting success in sperm storage, the relationship between representation in the spermatheca and success in siring nymphs, and individual male success across contexts. In analyses where data from a focal male from each triad was used, the focal male was taken to be the *G. bimaculatus* males in interspecific triads, or chosen haphazardly to randomly include equal numbers of first and second position males in intraspecific triads (note the difference in choice of focal male relative to the molecular analyses). Hybrid offspring have reduced hatching success; hence measures of fertilization success based on counts of nymphs need to be adjusted appropriately. To do this, we multiplied the proportion of offspring observed from heterospecific males by a correction factor based on the mean observed hatching rate of pure and hybrid offspring from eggs laid by females that only mated to one type of male (correction factor = the ratio of the hatching success of purebred offspring (from BB triads), to the hatching success of hybrid offspring (from CC triads)). Significance of terms was assessed by likelihood ratio tests between nested models (one containing the term of interest and one with that term removed) (Crawley, 2007). General linear models (GLM) using ANOVA based model selection were used to analyse the differences in egg laying across triad types, the differences in hatching success across triad types, and the relationship between the amount of *G. campestris* sperm in storage and hatching success. For detailed analytical methods and model output see section S3, Supporting information.

4.4 Results

4.4.1 Representation of competing males in the spermatheca

The contribution of sperm from a particular male to spermathecal storage depended upon both competition type (intraspecific or interspecific) and male species, but there was no effect of whether a male was first or second to mate (lmer; competition x species interaction; $\chi^2_{1,7} = 27.85$, $P < 0.001$, mating order; $\chi^2_{1,8} = 0.34$, $P = 0.562$). When competition was interspecific, much more sperm was stored from the *G. bimaculatus* male (Fig. 4.1).

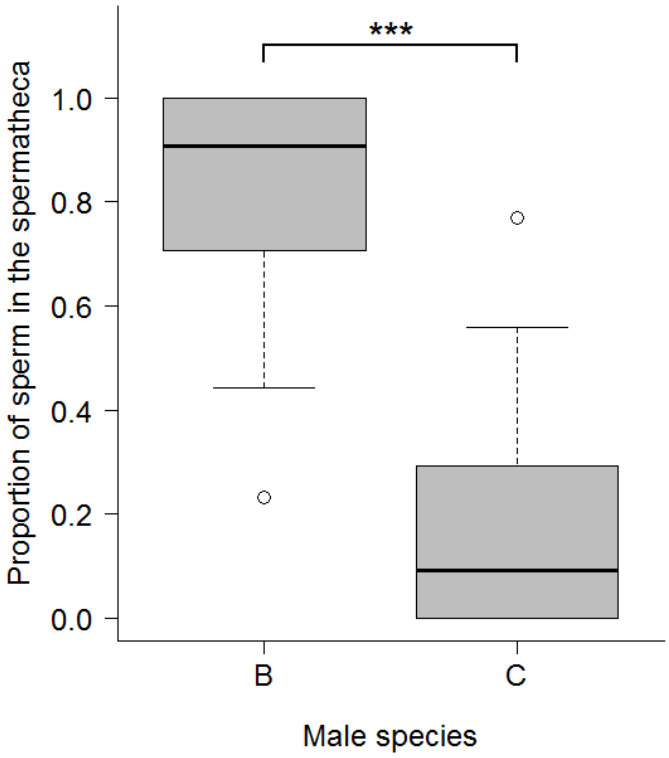


Figure 4.1. Success of *G. bimaculatus* and *G. campestris* males competing interspecifically, in terms of the proportion of sperm stored in the spermatheca. Boxes show the upper and lower quartiles, and central lines show medians. Statistical significance: *** $P < 0.001$.

4.4.2 Individual male success across contexts

Individual male success in sperm storage across contexts (intraspecific vs interspecific competition) was repeatable; *G. campestris* males that were more successful in having their sperm stored when

competition was intraspecific were also more likely to be successful in having their sperm stored when competition was interspecific (lmer; $\chi^2_{1,6} = 3.90$, $P = 0.048$). As already shown in earlier analyses, overall *G. bimaculatus* males did much better than *G. campestris* males (lmer; $\chi^2_{1,6} = 22.63$, $P < 0.001$, Fig. 4.2).

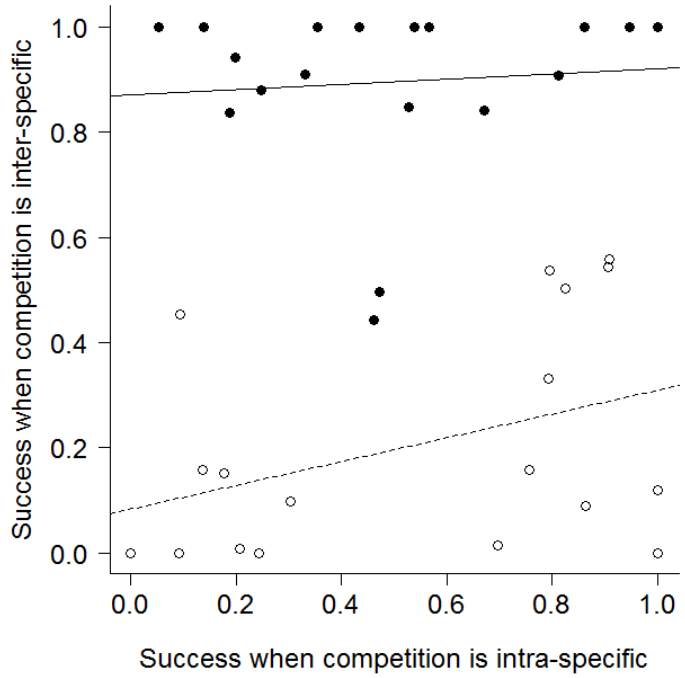


Figure 4.2. Individual male success in sperm storage across different mating contexts, plotted as the proportion of the sperm stored by a female that came from a male when competition was intraspecific versus success when competition was interspecific. Filled points and solid line show *G. bimaculatus* males, open points and dashed line show *G. campestris* males.

4.4.3 Success of competing males in siring nymphs

When competition was intraspecific, a male's success in siring nymphs was dependent upon his representation in the spermatheca. However when competition was interspecific, almost all nymphs were sired by the *G. bimaculatus* male, regardless of representation in the spermatheca (lmer; competitor type x sperm storage interaction $\chi^2_{1,6} = 3.96$, $P = 0.047$, Fig. 4.3). Neither species identity of the focal male or mating order had an effect (lmer; species $\chi^2_{1,11} = 0.17$, $P = 0.681$, mating order; $\chi^2_{1,7} = 2.05$, $P = 0.153$).

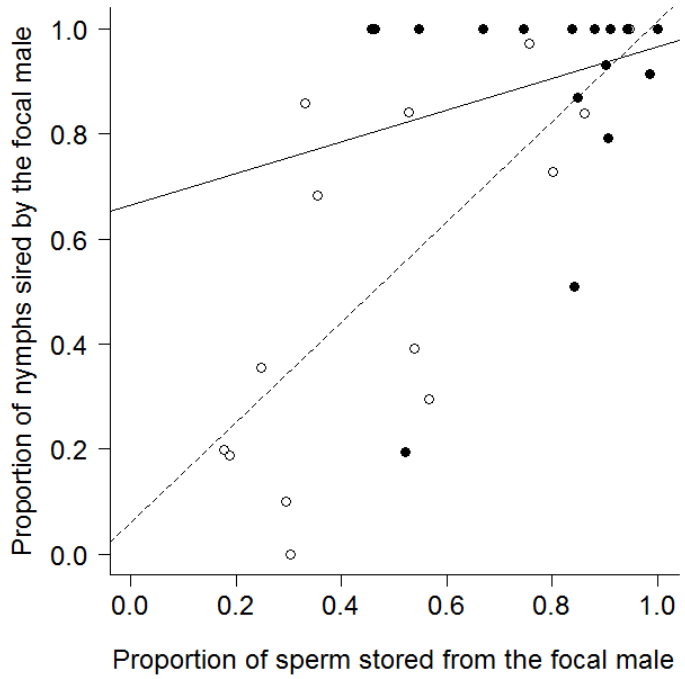
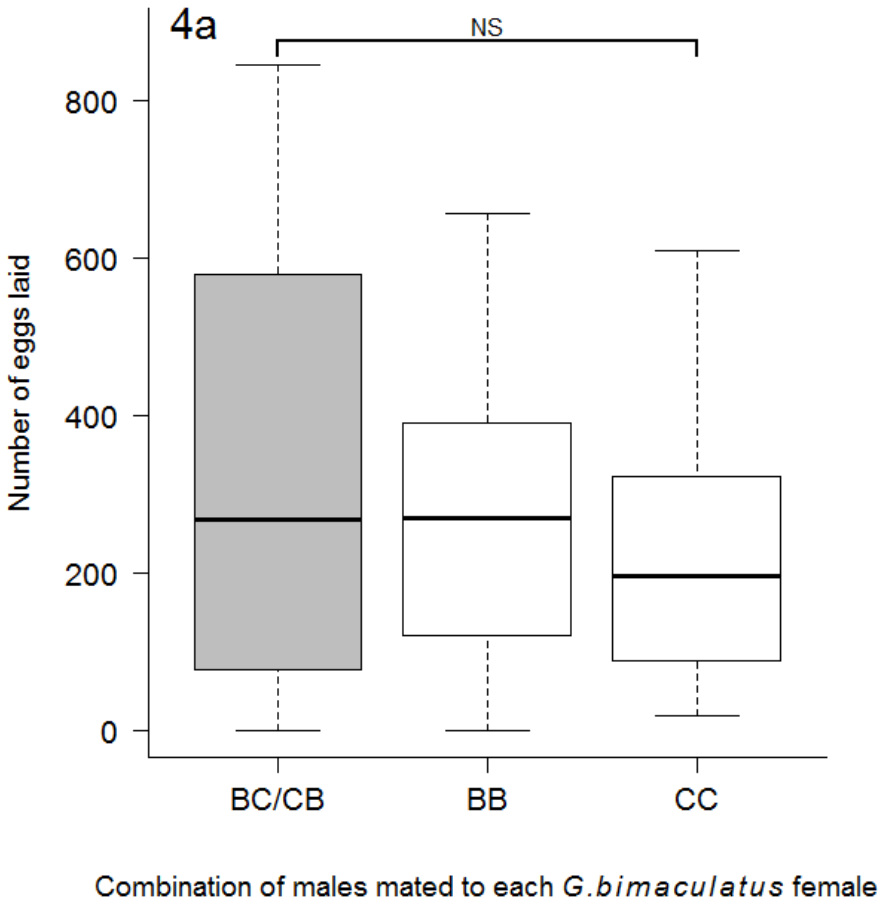


Figure 4.3. The relationship between the proportion of sperm in storage and the subsequent proportion of nymphs sired by each focal male. The proportion of nymphs sired by *G. campestris* males was corrected to account for the lower hatching success of hybrid offspring (see text). In interspecific pairings, the focal male was always *G. bimaculatus.* Open points and dashed line show males competing intraspecifically, filled points and solid line show males competing interspecifically.

4.4.4 Egg laying and hatching success

We found no evidence for differing success in the number of eggs laid among triad combinations (females mating to one male from each species, or to two males of either species) (GLM; $F_{2,52} = 0.77$, $P = 0.47$, Fig. 4.4a). However, egg hatching success differed greatly among triads (GLM; $F_{2,48} = 6.99$, $P = 0.002$, Fig. 4.4b). Post-hoc model comparison showed that this difference was due to lower hatching success in the CC triads (GLM; $F_{1,48} = 12.87$, $P < 0.001$) – there was no difference between the BB and BC/CB triads in the proportion of nymphs (GLM; $F_{1, 47} = 1.06$, $P = 0.308$). In triads where competition was interspecific, the proportion of *G. campestris* sperm present in storage did not predict hatching success ($F_{1,26} = 0.58$, $P = 0.452$, Fig. 4.5). Of the four females that did not lay eggs, all but one had genetic material from the male in the spermatheca.


4a
NS
800
600
400
200
0
Number of eggs laid
BC/CB
BB
CC
Combination of males mated to each G.bimaculatus female

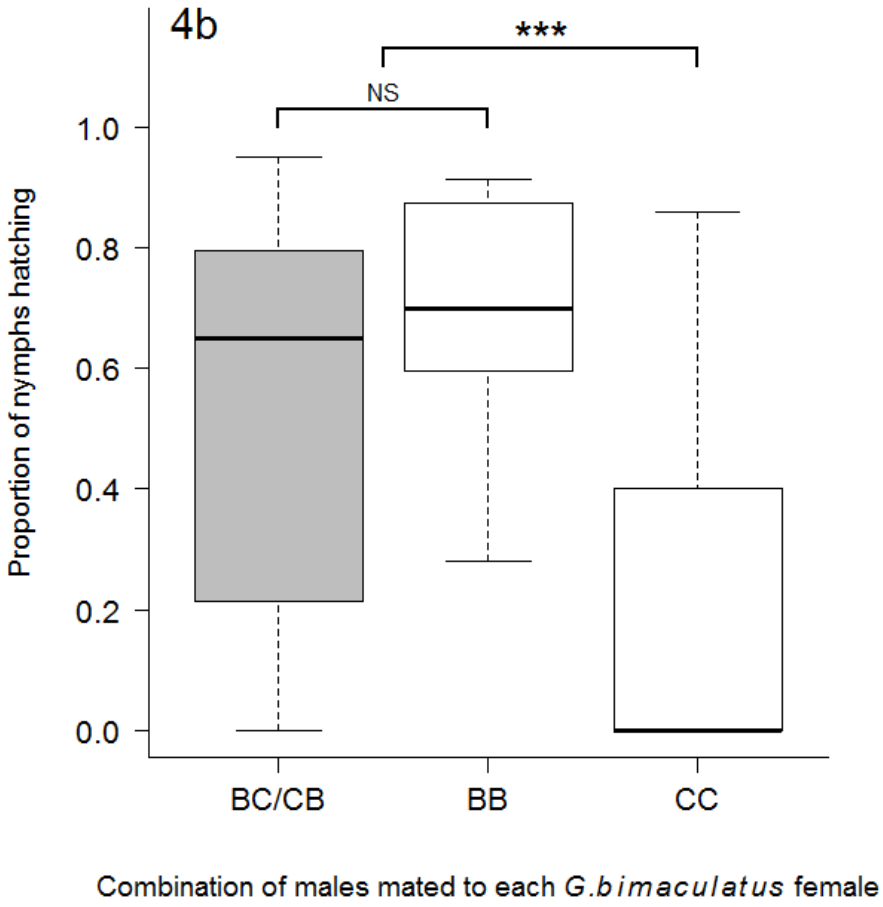

4b
***
NS
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of nymphs hatching
BC/CB
BB
CC
Combination of males mated to each G.bimaculatus female

Figure 4.4. The total number of eggs laid by females in the different triad types (4a) and the proportion of nymphs hatching from a sample of eggs laid by females in the different triad types (4b). BC/CB triads (shaded in grey) are those comprised of a *G. bimaculatus* female mated to a *G. bimaculatus* male and a *G. campestris* male in either order, competing interspecifically. BB triads are those comprised of competing *G. bimaculatus* males, CC comprised of *G. campestris* males. Boxes show the upper and lower quartiles, and central lines show medians. There were no differences among groups in the total number of eggs laid (4a). Fewer nymphs hatched in the CC triads than the other triad types, between the BB and BC/CB triads there was no difference (4b). Statistical significance: NS $P > 0.05$; *** $P < 0.001$.

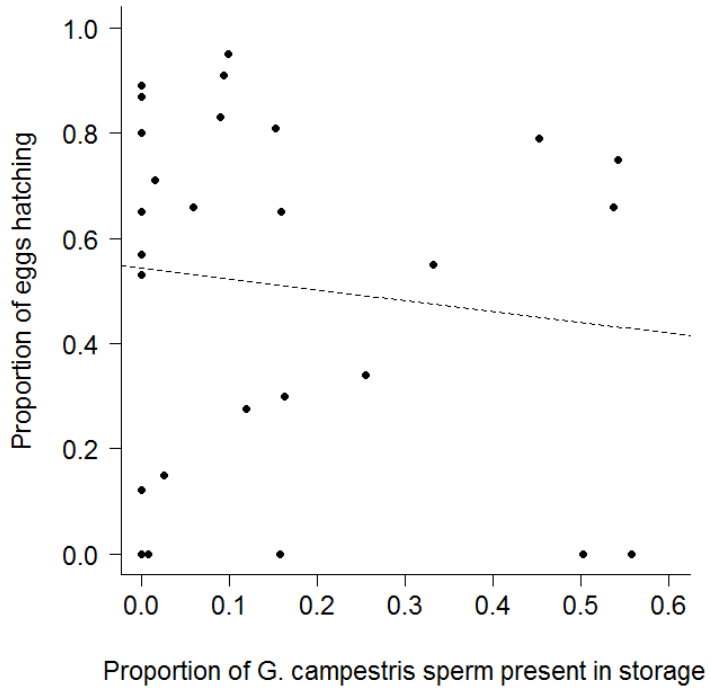


Figure 4.5. The relationship between the proportion of eggs hatching and the proportion of *G. campestris* sperm present in storage for intra- and interspecific triads.

4.5 Discussion

We demonstrate strong conspecific sperm precedence in these closely related species, with obvious potential to create a significant postmating-prezygotic reproductive barrier. When males of both species competed, there was a strong bias in sperm stored by *G. bimaculatus* females in favour of the *G. bimaculatus* male. Additionally we found that individual male success in getting sperm into storage was repeatable whether they were competing with a conspecific or a heterospecific. When males competed intraspecifically, representation in the spermatheca predicted success in siring offspring, however this was not the case for males competing interspecifically. In these triads the conspecific male sired almost all of the emerging offspring regardless of representation in the spermatheca, suggesting there are also mechanisms determining CSP post-storage.

There are a number of possible mechanisms that could create CSP. Bias in storage could occur as a result of differential investment of sperm into the spermatophore by males in response to the perceived quality of available mating partners (Gage & Barnard, 1996). Although crickets may engage in this sort of manipulation (Hall *et al.*, 2010), our study was designed to prevent such effects by housing males with a conspecific female during spermatophore production in all but a minority of cases (see section S4, Supporting information). In our study, it is more likely that bias in spermathecal representation is mediated by a female response such as assistance or inhibition of ejaculate uptake through female muscular control (Simmons & Achmann, 2000), acting as a form of cryptic female choice (Hall *et al.*, 2010). Or that the low representation of *G. campestris* sperm in the spermatheca is due to morphological incompatibility between the spermatophore and the female reproductive tract, inhibiting sperm transfer (Dufour, 1844), or differences between ejaculates in stimulating uptake by the female. *G. campestris* sperm might be less able to traverse the long spermathecal duct due to poor motility in an environment which they have not evolved with (Gregory & Howard, 1994), or ejaculate components may actively inhibit rival sperm (Price, 1997).

While overall *G. bimaculatus* males had greater success in sperm storage than *G. campestris*, each species showed variation in success among individual males. Interestingly, we found that individual success in gaining representation in the spermatheca was moderately repeatable, even across competitive contexts. Those that were successful when competing against a male of their own species were more likely to be successful when competing interspecifically. This suggests that traits that confer a competitive advantage in sperm competition when competing intraspecifically may also increase the chances of success when competing interspecifically. Examples of repeatability in

reproductive success are scant in the literature (but see Tregenza *et al.*, 2009) and we encourage research to explicitly investigate this across a range of species.

We found mating order to have no effect on representation in sperm storage, or secondly on subsequent success in siring nymphs. The first observation suggests that last male precedence, a phenomenon recorded in many other insect species (Simmons & Siva-Jothy, 1998), is not found in *G. bimaculatus* in line with previous studies (Simmons, 1987; Bretman *et al.*, 2009). Females may be equally motivated to store sperm when virgin as when already mated, and sperm displacement by competing males does not occur (Simmons, 1986). The second observation supports the idea that sperm storage is not stratified to create a 'last in, first out' system, rather sperm mixing occurs in the spermatheca (Walker, 1980; Simmons, 1986).

Based upon Parker's 'raffle principle' (1982), and the assumption of sperm mixing in the spermatheca, we predicted that success in siring nymphs would directly relate to the amount of sperm in storage. When a male competed against another of the same species, we found this prediction to hold true. However, when males of the two species competed we found that almost all nymphs were sired by the conspecific male, regardless of their sperm representation in the spermatheca. This 'post-storage' bias against heterospecific males suggests that success in sperm competition in these crickets is not simply a 'raffle' determined by sperm number, instead CSP may act at multiple stages in this system; firstly at the stage of sperm uptake and storage, and secondly after sperm have left the spermatheca.

In the cases of interspecifically competing males, deviation from our prediction that representation in the spermatheca determines siring success may be driven by a number of factors. Although heterospecific sperm are able to traverse the reproductive tract as far as the spermatheca, they may be less able to survive storage than conspecific sperm. Further work, in which spermathecal contents are stained to differentiate between live and dead sperm (Damiens *et al.*, 2002), might allow us to assess the survival of heterospecific sperm in storage. However, to replicate the disadvantage that heterospecific sperm experience when competing interspecifically, conspecific ejaculate would also need to be present, perhaps through artificial introduction of seminal fluids to the spermatheca. Another potential driver of post-storage bias against heterospecific sperm might be their ability to leave storage and traverse the reproductive tract to the eggs. If they are able to reach the site of fertilisation, they may be less able to attach to and penetrate the eggs (Shaw *et al.*, 1994). Eggs could be stained soon after laying to assess presence or absence of sperm (Sarashina *et al.*, 2005).

Alternatively, the failure to predict a male's success in siring nymphs from his representation in the spermatheca may be due to post-zygotic hybrid mortality. Although not often reported, instances of hybrid embryo mortality have been found across a range of species (for example, Kinsey, 1967; Elinson, 1981; Álvarez & Garcia-Vazquez, 2011). Arrest of embryogenesis occurs at a range of developmental stages and may be driven by genetic incompatibilities, for example differences in chromosomal rearrangements, alleles not functioning together, or infection by different endosymbionts (Coyne & Orr, 2004). However, if the differences in offspring sired that we observed were due to hybrid embryonic mortality, we would predict that females storing more *G. campestris* sperm should have lower egg hatching success. We found no such relationship within the interspecific triads suggesting that CSP is determined earlier than embryonic development.

In *Drosophila* species, egg laying is stimulated by seminal proteins present in the ejaculate (Gillott, 2003b), potentially acting as a species isolating mechanism if heterospecific ejaculate fails to stimulate laying, especially as seminal proteins evolve very rapidly (Swanson *et al.*, 2001). However, this is unlikely to play a role in this system; we observed no difference in the number of eggs laid among triads of different species combinations, corroborated by Veen *et al.* (2013), who found no difference in the number of eggs laid by female *G. bimaculatus* singly mated to *G. bimaculatus* or *G. campestris* males.

Our *G. bimaculatus* crickets were from a laboratory stock, reared over many generations, and it is likely that this population had lower genetic variability than the wild population. Our difficulty in identifying allelic mis-matches among *G. bimaculatus* individuals is consistent with this suggestion. Despite the costs to offspring fitness usually associated with inbreeding (Charlesworth & Charlesworth, 1987; Tregenza & Wedell, 2002), we found a strong bias in sperm storage and paternity in favour of the *G. bimaculatus* males.

CSP acts as a strong but not complete barrier to hybridisation in this system, and is likely to be complemented by other barriers. Prior to mating, females can choose mates based upon cues such as calling song, courtship song, or pheromones (Tregenza & Wedell, 1997; Veen *et al.*, 2011; Veen *et al.*, 2013). *G. campestris* females strongly discriminate against *G. bimaculatus* males, almost never interbreeding (Cousin, 1933; von Hörmann-Heck, 1957; Veen *et al.*, 2011). *G. bimaculatus* females, however, are less choosy and are known to interbreed in captivity, although less readily so than to males of their own species (Veen *et al.*, 2011). This difference between the species in female response to heterospecific mating partners may be indicative of differential costs of interbreeding, and it is possible that the relative strength of barriers to interbreeding differ also. It is possible that CSP acts to strengthen the relatively weak pre-copulatory barriers observed in *G. bimaculatus*. Traits

associated with postmating-prezygotic sexual selection can evolve relatively quickly (Civetta & Singh, 1995; Pitnick *et al.*, 2003; Andrés *et al.*, 2006). These traits may diverge in allopatry and subsequently act to isolate species upon secondary contact. Alternatively these traits may have diverged following isolation owing to other barriers – the current strength of isolating mechanisms does little to inform us of their historical significance in speciation (Schluter, 2001; Coyne & Orr, 2004). The mechanisms involved in CSP can only act as barriers if a female mates with a conspecific as well as a heterospecific male. Females may have evolved multiple mating to prevent interbreeding, and so promiscuity might, counter intuitively, reinforce isolation and promote speciation. Both *G. bimaculatus* and *G. campestris* are highly polyandrous in the wild. Bretman and Tregenza (2005) found that the mean number of males represented in the spermatheca of each female in a Spanish population of *G. bimaculatus* was 4.5, and video observation of a natural population of *G. campestris* (Rodríguez-Muñoz *et al.*, 2011) revealed frequent polyandry in that species as well. Therefore, it is likely that in natural populations, a heterospecific ejaculate might compete with multiple conspecific ejaculates, leading to an even stronger precedence than reported here.

Since the introduction of concepts such as sperm competition (Parker, 1970) and cryptic female choice (Thornhill, 1983; Eberhard, 1996), there has been a growing interest in cryptic processes, and the development of molecular techniques has allowed these processes to be more rigorously investigated. Through the use of such techniques we come closer to understanding which of the many processes involved in insemination, sperm movement and fertilization govern CSP in *Gryllus*. We suggest that CSP acts at multiple cryptic stages, potentially acting as a strong but not complete barrier to hybridisation in this system, with potential to have been involved in the process of speciation.

S1, Supporting Information: PCR conditions for microsatellite loci

Table S1.1. PCR conditions for microsatellite loci

	Multiplex of *Gbim*04, 15, 29, 52, 66	***Gbim*21**	***Gbim*48**	***Gbim*49**	***Gbim*57**	***Gbim*72**
T_a (^{0}C)	65	65	60	65	65	65
H2O	4.8	5.55	4.9	4.3	6.1	5.8
Buffer	1.0	1.0	1.0	1.0	1.0	1.0
dNTPs	1.2	1.0	1.2	1.2	1.2	1.2
$MgCl_2$	0.4	0.4	0.4	0.4	0.4	0.4
Taq	0.1	0.05	0.1	0.1	0.1	0.1
F / R primer **04**	0.21	0.5	0.7	0.5	0.1	0.25
F / R primer **15**	0.21					
F / R primer **29**	0.21					
F / R primer **52**	0.16					
F / R primer **66**	0.21					

T_a annealing temperature (^{0}C)

For characterization of microsatellite loci and PCR profile see Dawson *et al*. (2003) and Bretman *et al.* (2008).

S2, Supporting Information: Repeatability of CM-PCR

A selection of samples were re-amplified and sequenced to assess the repeatability of the CM-PCR process. Repeatability was high; proportional peak area calculated from the second PCR was predicted by the first (linear regression; $R^2 = 0.98$, $F_{1,22} = 471$, $P < 0.0001$, Fig. S2.1).

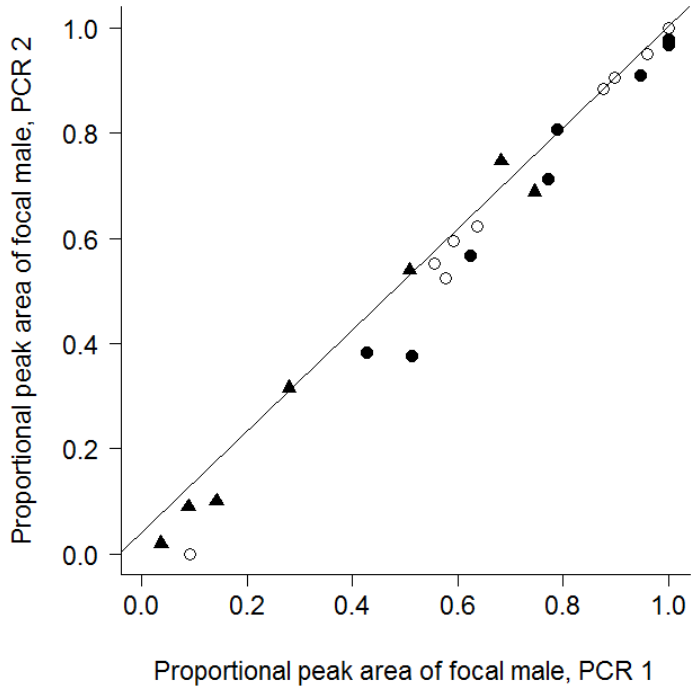


Figure S2.1. The proportional peak area of a focal male calculated from the second PCR against the proportional peak area of the first. Filled dots show spermatheca samples, open dots show nymph samples, and triangles show standards (including female DNA).

S3, Supporting Information: Detail of statistical analyses and model output

S3.1 Representation of competing males in the spermatheca

All analyses were carried out using R v2.14.1 (R, 2011). To estimate how success in sperm storage is determined, we fitted a generalised linear mixed model (GLMM) (lmer: R lme4 library (Bates *et al.*, 2011; R, 2011). The proportion of sperm stored was logit transformed (advocated by Warton & Hui, 2010, an alternative to the arcsine square root transformation) and entered into the model as the response variable. Male species ('Species'; B or C), mating order ('Order'; 1 or 2) and the competition type faced by the males ('Competition'; inter or intra) were entered as explanatory variables, as well as all interactions between these variables. ID, a unique number for each individual, was included as a random effect to control for some individuals being used twice as described above. Model selection was carried out through sequential removal of explanatory variables and their interactions, until only significant terms remained in the model, their significance determined through likelihood ratio tests (Crawley, 2007). Parameter estimates and associated standard errors (s.e.) from the model are presented in Table S3.1. R takes the first level of a factor as its reference – estimates presented are for the named level relative to the reference level. Test statistics, degrees of freedom (df), P-values and the order in which explanatory variables were excluded from the model selection process (#) are also presented.

Table S3.1. Representation of competing males in the spermatheca

	GLMM			Model selection			
Full model	Estimate	s.e.	t	χ^2	df	Pr(>χ^2)	#
Intercept	0.35	1.90	0.19				
Species C	-0.56	2.74	-0.20				
Competition inter	3.37	2.29	1.47				
Order 2	-0.37	1.20	-0.31	0.34	1,8	0.562	4
Species C : Competition inter	-6.03	3.21	-1.88	27.85	1,7	***<0.001***	5
Species C : Order 2	0.01	1.73	0.01	0.01	1,9	0.928	3
Competition inter : Order 2	0.12	1.42	0.08	<0.01	1,10	0.984	2
Species C : competition inter : Order 2	-0.21	2.02	-0.10	0.01	1,11	0.920	1

S3.2 Success of competing males in siring nymphs

The relationship between representation in the spermatheca and success in siring nymphs was likewise analysed using GLMM. To account for the lower hatching success of hybrid offspring, the proportion of nymphs sired by the heterospecific male was multiplied by a correction factor based on the mean observed hatching rate of pure and hybrid offspring from eggs laid by females that only mated to one type of male (correction factor = the ratio of the hatching success of purebred offspring to the hatching success of hybrid offspring). The proportion of nymphs sired by the focal male (the focal male being *G. bimaculatus* in interspecific triads, or chosen haphazardly to randomly include equal numbers of first and second position males in intraspecific triads) was logit transformed and entered as the response variable. The proportion of sperm in the spermatheca ('Sperm'), the type of competition faced by the focal male ('Competition'; inter or intra), mating order ('Order'; 1 or 2), and the species of the focal male were included as fixed effects. Interactions were fitted between Sperm, Competition and Order. ID was entered as a random effect. See Table S3.2 for model output.

Table S3.2. Success of competing males in siring nymphs

	GLMM			Model selection			
Full model	Estimate	s.e.	t	χ^2	df	Pr(>χ^2)	#
Intercept	-7.83	5.48	-1.43				
Sperm	12.40	8.46	1.46				
Competition inter	4.34	9.45	0.46				
Order 2	2.09	3.07	0.68	2.05	1,7	0.153	5
Species C	-0.61	1.48	-0.41	0.17	1,11	0.681	1
Sperm : Competition inter	-3.78	12.39	-0.31	3.96	1,6	***0.047***	6
Sperm : Order 2	-1.25	5.26	-0.24	0.63	1,8	0.430	4
Competition inter : Order 2	2.80	5.67	0.49	0.11	1,9	0.746	3
Sperm : Competition inter : Order 2	-3.28	7.65	-0.43	0.12	1,10	0.733	2

S3.3 Egg laying & hatching success

Differences in egg counts across triad types ('Triad'; BB, BC/CB, or CC) were analysed using a general linear model (GLM). As the data were overdispersed, the model was fitted with quasipoisson error structure. Model selection was based on ANOVAs using the F distribution. See Table S3.3 for model output. Differences in proportional hatching success across triad types was similarly analysed, but with quasibinomial error structure. See Table S3.4 for model output.

Table S3.3. Egg laying

	GLM			Model selection			
Full model – Egg laying	Estimate	s.e.	t	F	df	Pr(>F)	#
Intercept	5.60	0.018	319.20				1
Triad BC/CB	0.21	0.02	10.59				1
Triad CC	-0.15	0.03	-5.63	0.77	2,52	0.47	1

Table S3.4. Hatching success

	GLM			Model selection			
Full model - Hatching	Estimate	s.e.	t	F	df	Pr(>F)	#
Intercept	0.75	0.37	2.05				1
Triad BC/CB	-0.44	0.43	-1.02				1
Triad CC	-1.92	0.57	-3.35	6.99	2,48	***0.002***	1

S3.4 Individual male success across contexts

Individual male success across contexts was analysed using GLMM. A model was created with logit transformed proportion of sperm in storage from a given male when competing interspecifically as the response variable. Proportion of sperm when competing intraspecifically ('Intra.success'), male species ('Species'; B or C) and their interaction were entered as fixed effects. ID was included as a random effect. See Table S3.5 for model output.

Table S3.5. Individual male success across contexts

	GLMM			Model selection			
Full model	Estimate	s.e.	t	χ^2	df	Pr(>χ^2)	#
Intercept	3.42	1.23	2.77				2
Intra.success	1.71	2.21	0.77	3.90	1,6	***0.048***	2
Species C	-7.62	1.62	-4.71	22.63	1,6	***<0.001***	2
Intra.success : Species C	1.47	2.69	0.55	0.30	1,7	0.587	1

S3.5 Relationship between the amount of G. campestris sperm in storage and hatching success

The relationship between the proportion of eggs hatching and the proportion of *G. campestris* sperm in storage was analysed using GLM. Proportion of *G. campestris* sperm ('C.sperm') and triad type ('Triad'; BB, BC/CB, or CC) were fitted as an interaction. A quasibinomial error structure was fitted. See Table S3.6 for model output.

Table S3.6. Relationship between the amount of *G. campestris* sperm in storage and hatching success

	GLM			Model selection			
Full model	Estimate	s.e.	t	F	df	Pr(>F)	#
Intercept	0.47	0.32	1.48				1
C.sperm	-0.97	1.27	-0.76	0.58	1,26	0.452	1

S4, Supporting Information: Exposure to non-experimental females prior to mating trials

Prior to their first mating, we presented males with a conspecific non-experimental female to stimulate spermatophore production and courtship behaviour. Individuals were separated by wire mesh so that the female could be detected, but not mated with. A potential criticism of our study is that, depending on individuals' propensities to mate, some second matings were immediate, and so males were not re-exposed to a conspecific female, whereas others were delayed, in which case males were re-exposed to conspecific non-experimental females at the beginning of each day. As a consequence of this, some spermatophores from *G. campestris* males were made during or following exposure to *G. campestris* females, others in association with *G. bimaculatus* females. We investigated whether exposure to different species of females had an effect on the proportion of sperm stored using GLMM. The proportion of sperm stored was logit transformed and entered as the response variable. The species of female ('Female'; B or C) and the type of competition faced by the male ('Competition'; intra or inter), as well as their interaction were included as fitted effects, and individual ID entered as a random effect (see Table S4.1 for model output). The species of female that *G. campestris* males were exposed to prior to spermatophore production had no effect on the proportion of sperm stored (lmer; $\chi^2_{1,6} = 0.59$, $P = 0.44$), suggesting that representation of a male in the spermatheca is due to female mediated uptake of ejaculate (Hall *et al.*, 2010) rather than bias in sperm numbers invested by the male. Given that males have the capability to alter their investment in spermatophores (Hall *et al.*, 2000), either they do not alter their investment in response to female species, or any bias is overridden by the strength of female choice.

Table S4.1. Exposure to non-experimental females prior to mating trials

	GLMM			Model selection			
Full model	Estimate	s.e.	t	χ^2	df	Pr(>χ^2)	#
Intercept	-0.37	0.98	-0.38				3
Female C	-1.98	1.45	-1.37	0.59	1,6	0.444	2
Competition inter	-3.32	1.59	-1.59	4.79	1,5	***0.029***	3
Female C : Competition inter	2.39	2.09	2.09	1.30	1,7	0.255	1

Chapter 5 Fertilisation and early developmental barriers to hybridisation in field crickets

5.1 Abstract

Interactions between the female reproductive tract and sperm, and between gametes, are cryptic and potentially complex, providing multiple opportunities for reproduction to go awry. These cryptic mechanisms have the potential to act as barriers to gene flow between species, and may be important in the process of speciation. There are multiple post-mating barriers to interbreeding between the hybridising field crickets *Gryllus bimaculatus* and *G. campestris.* Female *G. bimaculatus* preferentially store sperm from conspecific males when mated to both conspecific and heterospecific partners. Additionally, conspecific males sire an even greater proportion of offspring than would be predicted from their sperm's representation in the spermatheca. The nature of these post-sperm-storage barriers to hybridisation are unknown. We use a fluorescent staining technique to determine whether barriers occur prior to, or during embryo development.

We show that eggs laid by *G. bimaculatus* females mated to *G. campestris* males are less likely to begin embryogenesis than eggs from conspecific mating pairs. Of the eggs that are successfully fertilised and start to develop, those from heterospecific mating pairs are more likely to arrest early, prior to blastoderm formation. Having reached advanced stages of embryogenesis, hybrid survival through to hatching is equal to that of embryos from conspecific mating pairs.

Post-sperm-storage barriers to hybridisation are sufficiently large to play a role in the maintenance of reproductive isolation between *G. bimaculatus* and *G. campestris*. The number of eggs that fail to develop represent a substantial cost to *G. bimaculatus* females, and this cost could reinforce the evolution of barriers occurring earlier in the reproductive process.

5.2 Introduction

The evolution and maintenance of new species requires reproductive isolation, whereby barriers to interbreeding prevent gene flow between incipient species (Mayr, 1963; Mayr, 1969). There has been an emphasis on studying barriers that occur prior to mating, and those that affect the viability and fertility of hybrid offspring (Dobzhansky, 1937). Less attention has been paid to the barriers occurring between these stages, presumably because of the difficulty of studying cryptic mechanisms. New techniques are now allowing important insights to be gained, and there is increasing interest in how cryptic post-mating mechanisms might act to maintain reproductive isolation (for example, Matute & Coyne, 2010; Sagga & Civetta, 2011; Larson *et al.*, 2012).

Instances of mixed-species pairings producing fewer offspring than pure-species pairings have been recorded in a number of species, and are often attributed to differences in uptake and storage of sperm (Katakura, 1986; Price *et al.*, 2001), or the capacity of sperm to reach and fertilise eggs (Gregory & Howard, 1994; Palumbi, 1998). Although the genetics of hybrid inviability have been studied extensively (reviewed by Coyne & Orr, 2004), there are few studies of animal species in which reduced reproductive output is directly attributed to embryonic mortality of hybrids. Even so, reports of this phenomenon come from a broad range of taxa (Kinsey, 1967; Sellier *et al.*, 2005; Álvarez & Garcia-Vazquez, 2011), suggesting that embryonic mortality may be a common feature of hybridising systems.

Here we aim to assess the importance of cryptic barriers occurring prior to and during embryogenesis in the hybridising field crickets *Gryllus bimaculatus* and *G. campestris*. These species have overlapping distributions through central Spain (Pardo *et al.*, 1993; Gorochov & Llorente, 2001), and while *G. campestris* females almost never interbreed, *G. bimaculatus* females hybridise readily with *G. campestris* males in the laboratory (von Hörmann-Heck, 1957; Veen *et al.*, 2011). Female *G. bimaculatus* respond readily to mating signals from male *G. campestris* and hybrid offspring are both viable and fertile (Veen *et al.*, 2011). The lack of premating barriers suggests that cryptic barriers may play a role in the maintenance of reproductive isolation between these species.

The uptake and storage of sperm has been shown to be a significant barrier to interbreeding in this system. A recent study identified strong conspecific sperm precedence, whereby *G. bimaculatus* females doubly mated to both *G. bimaculatus* and *G. campestris* males preferentially stored sperm from the conspecific male (Tyler *et al.*, 2013). The spermatheca in this species is approximately spherical, a shape which is likely to promote sperm mixing rather than stratified storage (Walker, 1980; Simmons, 1986). The representation of a male in the spermatheca is therefore expected to directly predict his success in siring offspring (Parker, 1982). However this relationship did not hold. Conspecific males sired an even greater proportion of offspring than predicted from patterns of sperm storage indicating the presence of additional post-mating barriers to hybridisation. Here we use a fluorescent staining technique to identify when these subsequent barriers occur. We firstly assess whether eggs from interspecies pairings are less likely to be fertilised and start developing, and secondly assess whether developing embryos arrest before hatching. We aim to establish the potential for fertilisation and embryogenesis to act as barriers to interbreeding, providing insights into the mechanisms of reproductive isolation.

5.3 Methods

5.3.1 Study animals

G. bimaculatus were collected from Valencia, Spain in 2011 and reared in the lab for ~4 generations. Crickets were housed at 28 °C under a 16:8 light:dark cycle, with food and water provided *ad libitum*. Last instar nymphs were isolated to ensure virginity upon adult emergence. *G. campestris* were collected near Gijon, Spain in spring 2012 as last instar nymphs or adults. These wild caught individuals were kept in the laboratory for at least 7 days prior to use in trials. All individuals were a minimum of 7 days old post-emergence before use in experimental trials to ensure sexual maturity.

5.4.2 Matings and oviposition

Prior to heterospecific mating trials, males were exposed to non-experimental conspecific females to stimulate spermatophore production and to encourage courtship behaviour. These stimulating females were separated from the males by wire mesh so that the female could be detected, but not mated with. Virgin *G. bimaculatus* females were paired with either a conspecific (BB pairing) or a heterospecific (BC pairing) male. Those *G. campestris* males successfully mated to a heterospecific female were subsequently mated to a conspecific female (CC pairing), to confirm that any failure to fertilise *G. bimaculatus* eggs was due to post-mating reproductive barriers rather than infertility. Virgin *G. campestris* females were only paired with conspecific males as they will almost never interbreed (Cousin, 1933; von Hörmann-Heck, 1957; Veen *et al.*, 2011). Mating pairs were placed in a 11 x 11 cm arena lined with paper for traction and observed. If courtship or mating did not occur within approximately 1 h the male was replaced with another or trialled on subsequent days. Successful mating was confirmed by the presence of a spermatophore attached to the female. Post-mating, the pair were left in the arena for around 1 h, the time required for most of the spermatophore contents to be taken-up by the female (Simmons, 1986).

Females were then housed individually, and provided with a small dish of damp sand to oviposit in for ~48 h. These dishes were replaced at intervals so that each female was provided with 4 dishes over the ~48 h period. After removal from the female, dishes were incubated at 28 ^{o}C for a minimum of 10 h, up to ~24 h, before being processed. Eggs were then removed from the sand and counted. If fewer than 20 eggs were laid in each dish then all were processed for assessment of early stage embryogenesis. If a large number of eggs were laid, then 20 were randomly selected for processing, and the rest were incubated on damp cotton wool to assess late stage embryogenesis and hatching.

5.3.3 Assessment of early stage embryogenesis

Soon after laying (within 3 h), a fertilised egg begins meiosis, and divisions can be seen as a female pronucleus and polar bodies (≤4 nuclei) on the dorsal side of the egg. The pronucleus then migrates to the ventral side of the egg where sperm enter through micropyles. Here, male and female pronuclei fuse, and mitotic division begins (Sato & Tanaka-Sato, 2002). After 9 h, more than 100 nuclei can be seen on the surface of the egg, and after 12 h, around 500 nuclei will be uniformly distributed on the surface, forming the blastoderm (Sarashina *et al.*, 2005). Unfertilised eggs will often undergo initial meiotic division, but will never progress to have more than 4 nuclei (Sarashina *et al.*, 2003). Without sampling eggs within 2 min of laying when sperm might still be seen (Sarashina *et al.*, 2005), it is not possible to tell whether eggs that only ever have ≤4 nuclei have not been penetrated by sperm, whether fusion between gametes has not occurred, or they were fertilised but development has arrested before the onset of mitosis. In attempting to assess this we might risk missing the sperm, leading us to draw false conclusions about fertilisation success. In addition to this, regular disturbance of females deters them from ovipositing, further hindering the ability to assess fertilisation in newly laid eggs. We therefore made no attempt to investigate this, and instead categorised any egg with ≤4 nuclei as 'undeveloped', while any egg with more than 4 nuclei was considered to have started embryogenesis (Matute & Coyne, 2010; Larson *et al.*, 2012). Since we processed eggs after a minimum development time of 10 h, we conservatively expected at least 100 nuclei to be seen if an egg were developing normally, or fewer if embryogenesis had started and subsequently arrested. These were categorised as 'normally developing' (>100) and 'partially developed' (5 - 100), respectively (Figure 5.1).

The protocol for preparing and staining eggs is adapted from the methodologies of Sarashina *et al*. (2005) and Larson *et al*. (2012). To remove the thick opaque chorion, the eggs were firstly soaked in 50 % bleach for 5 min at 22 ^{o}C and gently shaken. They were then washed 3 times with phosphate buffered saline (PBS) solution, and fixed in equal parts paraformaldehyde (4% in PBS) and heptane for 20 min at 22 ^{o}C, with gentle shaking. Eggs were washed again and then stored in methanol at 4 ^{o}C until staining. Eggs were stained with 4',6-diami-2-phenylindole (DAPI) for 20 min at 22 ^{o}C with gentle shaking, then transferred to a microscope slide and viewed using a fluorescent microscope (Olympus BX61) and analySIS^{D} software. Each egg was visually inspected for nuclei, seen as fluorescent blue dots, and categorised as undeveloped (≤4), partially developed (5 - 100), or normally developing (≥100) depending on the number of nuclei seen. Any captured images were colour and contrast optimised in analySIS^{D}. Figure 5.1 was created by cropping 3 separate images and placing them alongside each other using Microsoft PowerPoint software.

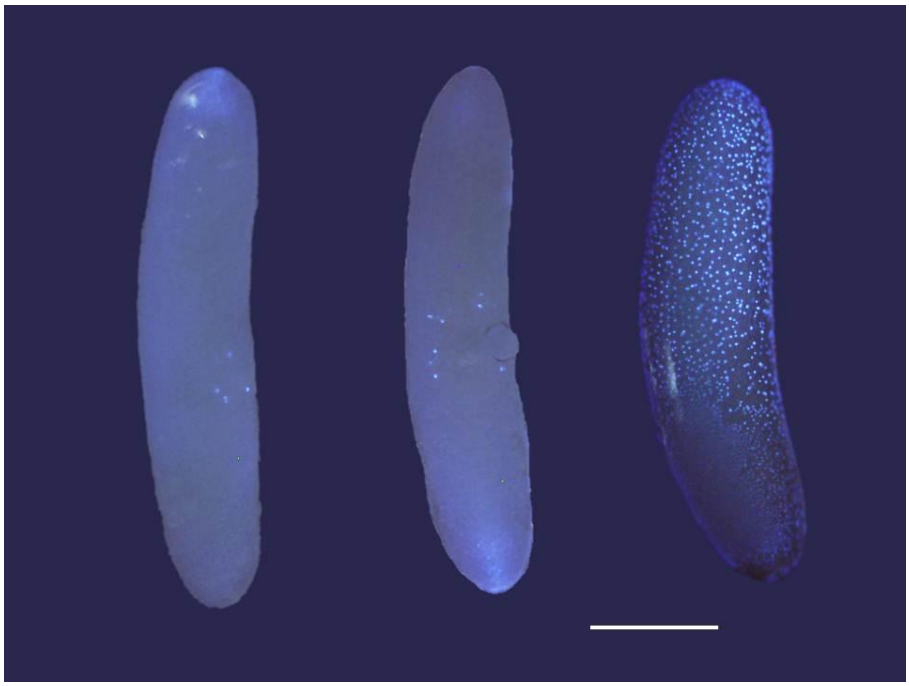


Figure 5.1. Examples of eggs stained with DAPI, all ~48 h post laying. From left to right: undeveloped, partially developed and normally developing eggs. Bar = 0.5 mm.

5.3.4 Assessment of late stage embryogenesis

If early stage embryogenesis is successful, then embryos will continue to grow, passing through several developmental stages until the embryo undergoes segmentation and the organs form distinct structures. At this stage the eyes of the embryo can be seen by the naked eye through the chorion. The eggs that were not processed for assessment of early stage embryogenesis were incubated on cotton wool, and after 8 days of incubation, the number of eggs in each clutch with visible eyespots was counted, as well as the number that subsequently hatched.

5.3.5 Statistical analyses

To quantify early stage embryogenesis we calculated 3 proportions: 'prop.undeveloped'; the proportion of all eggs that completely failed to develop (≤4 nuclei), 'prop.partial'; of the eggs that began to develop, the proportion that only partially developed (5 – 100 nuclei), and 'prop.developed'; the proportion of all eggs that continued to develop successfully (≥100 nuclei). To quantify late stage embryogenesis we calculated 2 more proportions: 'prop.eyespots'; of the eggs

incubated, the proportion that contained embryos with eyespots, and 'prop.hatch'; of the eggs incubated, the proportion that hatched.

The relationships between the mating pair combinations (BC, BB, or CC) and each of the proportions prop.undeveloped and prop.partial were analysed using generalized linear mixed models (lme4 package (Bates *et al.*, 2011), R v 2.14.1 (Development Core Team, 2011)), fitted with binomial error structures. The proportion of eggs (prop.undeveloped or prop.partial) was entered as the response variable. Mating pair combination was entered as the explanatory variable. Male ID, a unique number assigned to each individual, was entered as a random effect to control for multiple use of individuals. The overall significance of the explanatory term was determined through model comparison using likelihood ratio tests (Crawley, 2007). Any post-hoc comparisons between levels of the explanatory variable were likewise made through model comparison using likelihood ratio tests.

The relationship between the proportion of normally developing eggs at early embryogenesis (prop.developed) and the proportion of eggs that contained late stage embryos (prop.eyespots) was analysed using a generalized linear model. Data were overdispersed so the model was fitted with quasibinomial error structure. Prop.eyespots was fitted as the response variable. Prop.developed and the mating pair combination, as well as their interaction were entered as explanatory variables. The significance of the explanatory variables was determined through model comparison using F tests. The relationship between the proportion of eggs that contained embryos (prop.eyespots) and the proportion that subsequently hatched (prop.hatch), interacting with mating pair combination, was likewise analysed using a generalized linear model.

5.4 Results

5.4.1 Early stage embryogenesis

There were three cases of *G. campestris* males failing to produce any normally developing eggs when mated to either a heterospecific or conspecific female. These cases were assumed to be due to infertility, and any data associated with these individuals were removed from the dataset prior to analyses. Of the remaining females that laid eggs, 13 were in BB mating pairs, 16 in BC mating pairs, and 16 in CC mating pairs.

The proportion of eggs failing to develop differed among the mating pair combinations (lmer; $\chi^2_{2,4} = 19.11$, $P < 0.0001$, Figure 5.2), with *G. bimaculatus* females mated to heterospecific males (BC) laying the greatest number of undeveloped eggs, and *G. bimaculatus* females mated to conspecific males

(BB) laying the fewest. Of the eggs that started to develop, the proportion that only partially developed differed significantly among the mating pair combinations (lmer; $\chi^2_{2,4} = 12.14$, $P = 0.0023$). Post hoc tests revealed that this difference was due to eggs from heterospecific pairings (BC) being more likely to only partially develop than eggs from the conspecific pairs (BB & CC) (lmer; $\chi^2_{1,3} = 9.75$, $P = 0.0018$, Figure 5.3). There was no difference between the conspecific pairs (BB & CC) in the proportion of eggs that only partially developed (lmer; $\chi^2_{1,4} = 2.39$, $P = 0.122$). The cases of eggs only partially developing tended to be concentrated within a few clutches, rather than being equally spread across clutches (Figure 5.4, see (c) and (d)).

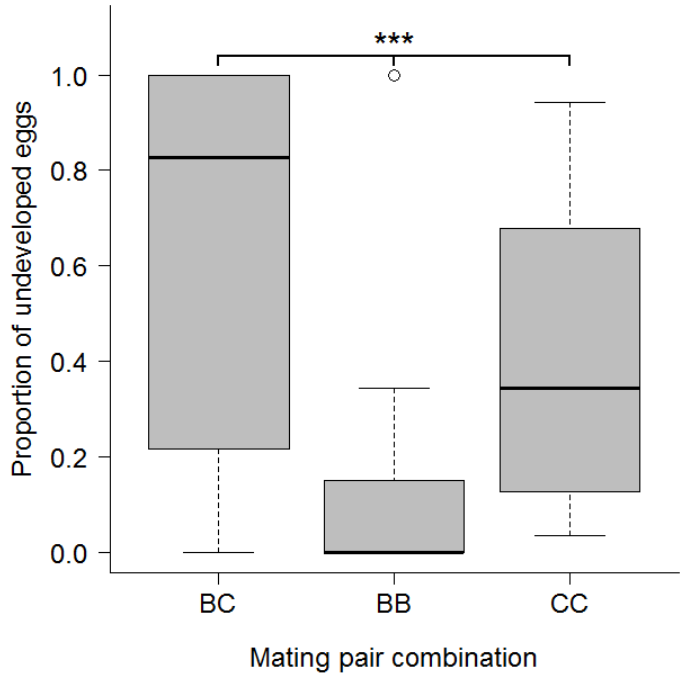


Figure 5.2. The proportion of eggs that failed to develop across the mating pair types. In each combination, female species is denoted by the first letter, and male species by the second. Data are displayed as medians (thickened line) and inter-quartile ranges (grey boxes), circles are outlying values. There are significant differences between all mating pair combinations. Statistical significance from glm (see text): *** $P < 0.001$.

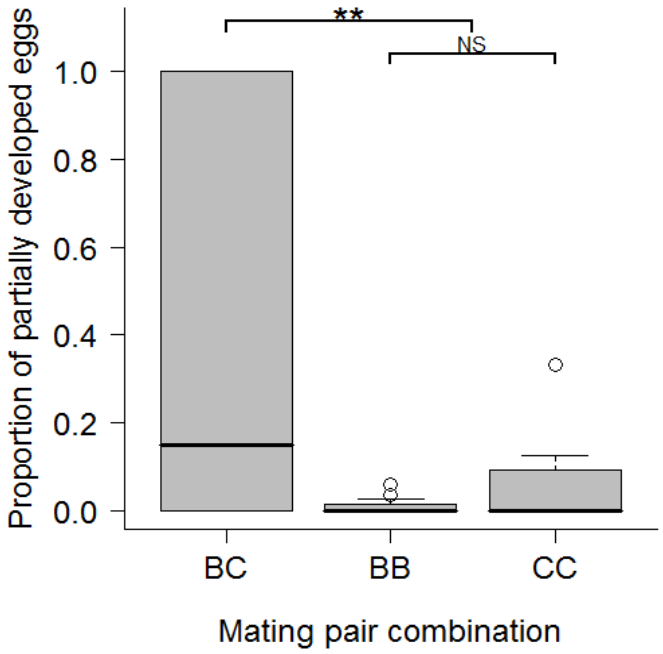


Figure 5.3. The proportion of eggs that only partially developed across the mating pair types. In each combination, female species is denoted by the first letter, and male species by the second. Data are displayed as medians (thickened line) and inter-quartile ranges (grey boxes), circles are outlying values. While BB and CC mating pairs do not differ in the proportion of eggs that only partially develop, heterospecific (BC) mating pairs differ from the conspecific (BB & CC) mating pairs. Statistical significance: NS $P > 0.05$; ** $P < 0.01$.

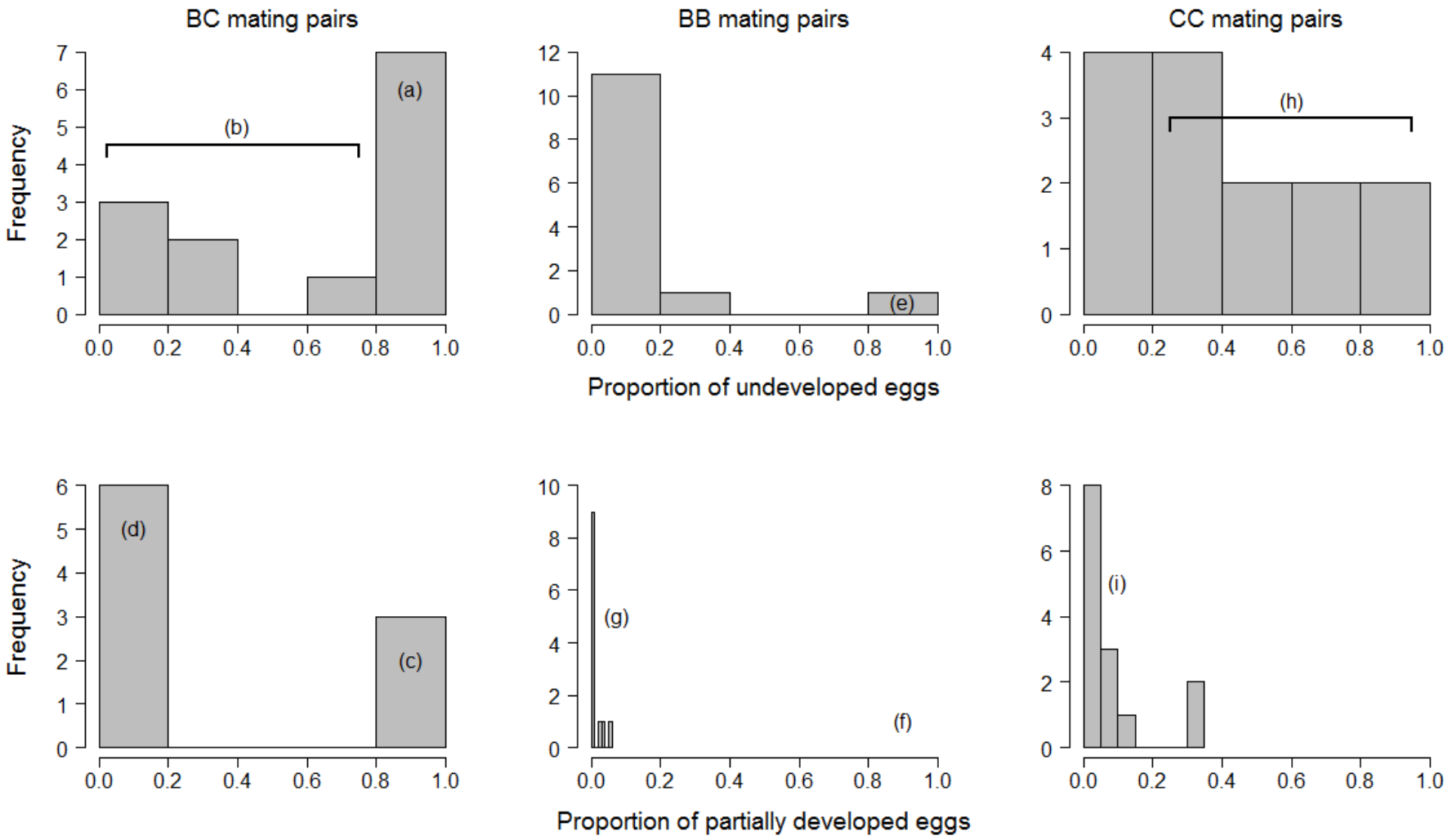

BC mating pairs
BB mating pairs
CC mating pairs
Frequency
(a)
(b)
(e)
(h)
(d)
(c)
(g)
(f)
(i)
Proportion of undeveloped eggs
Proportion of partially developed eggs

Figure 5.4. Egg development across mating pair combinations. Histograms showing the frequency distributions of the proportion of eggs that did not develop (upper row), and the proportion of partially developed eggs (lower row), across each mating pair combination (by column left to right; BC, BB and CC). For BC mating pairs, more than half the clutches had a majority of undeveloped eggs (a). The remaining clutches varied in the proportion of undeveloped eggs and eggs beginning embryogenesis (b). Of these clutches showing signs of development, there was a bimodal distribution of either a majority of eggs partially developing (c), or eggs developing normally (d), rather than a normal distribution of development success. BB clutches rarely consisted of undeveloped eggs (e) or partially developed eggs (f). Instead, clutches had a majority of eggs that developed normally (g). The majority of CC clutches consisted of at least some undeveloped eggs (h). Of the eggs beginning embryogenesis, almost all developed normally (i).

5.4.2 Late stage embryogenesis

Among clutches, the proportion of eggs that showed successful early development predicted the proportion of eggs that contained late stage embryos with eyespots (glm; $F_{1,14} = 25.31$, $P < 0.001$). This relationship did not differ among the mating pair combinations (glm; $F_{2,13} = 0.31$, $P = 0.740$, Figure 5.5). Almost all eggs containing late stage embryos with eyespots went on to hatch (glm; $F_{1,14} = 193.65$, $P < 0.001$), and likelihood of death did not differ among the mating pair combinations (glm; $F_{2,13} = 0.33$, $P = 0.726$, Figure 5.6).

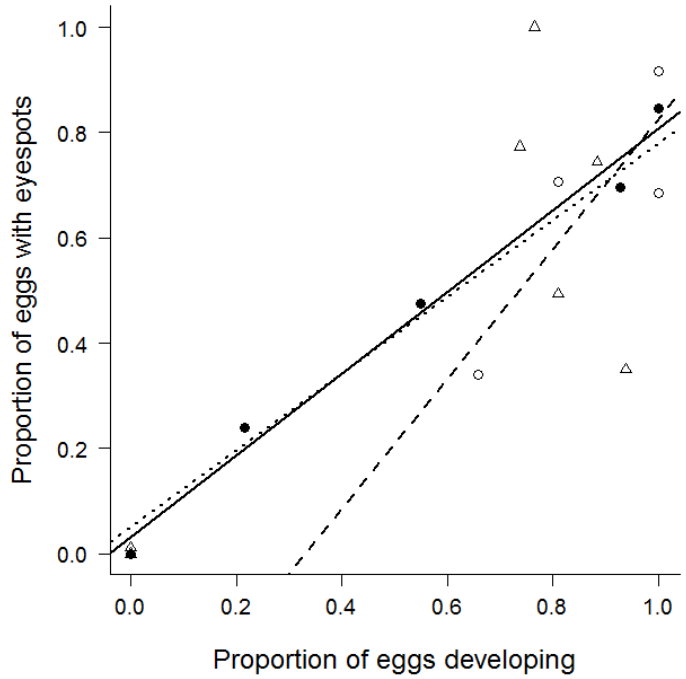


Figure 5.5. The relationship between early and late stage embryogenesis. The proportion of eggs developing normally at early embryogenesis predicts the proportion that contained late stage embryos with eyespots. Clutches from BC mating pairs are shown by closed dots and solid line. BB mating pairs are shown by open dots and broken line. CC pairs are shown by open triangles and dotted line.

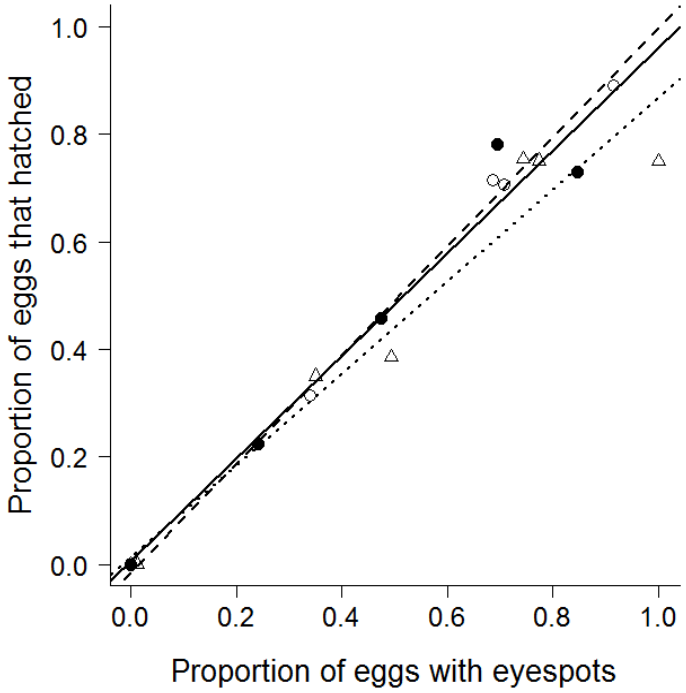


Figure 5.6. The relationship between late stage embryogenesis and hatching success. The proportion of eggs containing embryos with eyespots predicts the proportion that subsequently hatched. Clutches from BC mating pairs are shown by closed dots and solid line. BB mating pairs are shown by open dots and broken line. CC pairs are shown by open triangles and dotted line.

5.5 Discussion

There are multiple post-mating barriers to hybridisation in this system. A strong isolating mechanism at the stage of uptake and storage of sperm has already been demonstrated, and the inferred presence of further mechanisms between sperm storage and the hatching of eggs highlighted (Tyler *et al.*, 2013). Here we show that another potential prezygotic barrier occurs prior to embryogenesis, and another postzygotic barrier during early embryogenesis.

Eggs from mixed-species pairs were far less likely to begin embryogenesis than those from pure-species pairs. This has likewise been demonstrated in other interbreeding species. Almost all eggs from heterospecific crosses between *Gryllus firmus* and *G. pensylvanicus* go unfertilised (Larson *et al.*, 2012). Similarly, almost all eggs laid by *Drosophila virilus* females mated to *D. novamexicana* are not fertilised (Sagga & Civetta, 2011). Fewer eggs hatch from mixed species pairings of *Drosophila santomea* and *D. yakuba* than from pure-species pairings, assumed to be due to lower rates of fertilisation. There are a range of mechanisms between storage of sperm and fertilisation

that could be driving this barrier to interbreeding, since interactions between sperm and females are complex. For example, heterospecific sperm may have reduced survival in the spermatheca (Katakura, 1986), or may be less able to traverse the female's reproductive tract (Gregory & Howard, 1994). Upon reaching the site of fertilisation, the sperm may be unable to penetrate the egg wall (O'Rand, 1988), or the eggs may be incorrectly fertilised (Alipaz *et al.*, 2001). The present study does not allow us to resolve which of the cascade of sperm-female or sperm-egg interactions act as isolating mechanisms in the *G. bimaculatus* and *G. campestris* system, however it is likely that a combination of factors are involved, as has been demonstrated in *Drosophila melanogaster* (2010).

In our system we found a large number of eggs from mixed-species pairs that failed to develop, which, notably, were not equally spread across clutches. While some clutches were dominated by eggs that showed no signs of embryogenesis, in others almost all eggs started developing. The cases of entire clutches failing cannot be explained by male infertility, since this was ruled out in our methodology. They are more likely due to incompatibilities in traits between particular mating partners. If there are individual differences in the traits of sperm, then we might expect variation among males in the success of sperm-female or sperm-egg interactions. In cases of heterospecific crosses, this variation might translate into extreme differences in individual success, whereby the traits exhibited by some males are complementary to those of the female, whereas others are not. This could produce the binary success and failure of hybrid clutch development observed in this study.

We would expect the proportion of eggs that failed to develop to be similar between the pure-bred *G. campestris* eggs and the pure-bred *G. bimaculatus* eggs, however we found a significant difference between these groups. While this might be explained by differences between the species in egg viability, it is perhaps more likely due to difference in responses to the laboratory environment. In the wild *G. bimaculatus* are found in hot, arid environments, whereas *G. campestris* are found in more temperate regions (Gorochov & Llorente, 2001). The relatively high temperature maintained in the laboratory may therefore be sub-optimal for egg development of *G. campestris*. Furthermore, unlike the *G. campestris* which were wild-caught, our *G. bimaculatus* crickets had been reared in the laboratory for a number of generations, so might possibly have become somewhat lab adapted. This likely difference in the optimum temperature for egg incubation might have also had an influence on the developmental success of hybrids, but the existence of differences between the hybrid eggs and eggs from both conspecific pairings demonstrates that their low success is not just a temperature artefact.

As well as the strong barrier prior to embryogenesis (≤4 nuclei), we have also identified a difference in developmental success after the onset of mitosis. Eggs were all a minimum of 10 hours post-laying, by which time the nuclei of a developing egg would be expected to be uniformly distributed over the surface, soon to form the blastoderm (Sarashina *et al.*, 2005). Of the eggs that began embryogenesis, we found that those fertilised by heterospecific sperm were more likely to arrest during early development. Most of these partially developed eggs contained fewer than 20 nuclei, suggesting that developmental arrest occurred within the first few mitotic divisions, long before blastoderm formation. We found no evidence for a barrier late in development - the relationship between the number of eggs with eyespots and the number that successfully hatched was strong, regardless of the species identities of the parents.

While we find hybrid arrest in field crickets occurring during very early development, prior to blastoderm formation, the few examples in the literature from other animal species report a range of stages at which arrest may occur, notably around the time of gastrulation, the stage at which three distinct germ layers are formed. Hybrid eggs laid by female *Drosophila pseudoobscura* mated to male *D. miranda* degenerate within a few hours of fertilisiation (Kaufmann, 1940), and likewise eggs from crosses between *D. virilis* and *D. littoralis* arrest during the first few divisions of cells (Mitrofanov & Sidorova, 1981). Hybrid eggs produced by females of the common duck, *Anas platyrhnchos*, inseminated by the Muscovy duck, *Cairina moschata*, are likely to arrest early in development, prior to blastoderm formation (Sellier *et al.*, 2005). Crosses in which the eggs of the sea urchin *Heliocidaris tuberculata* are fertilised by *H. erythrogramma* sperm result in arrest at gastrulation, due to differences between the parental species in how axes of asymmetry are determined (Raff *et al.*, 1999). Among a number of the nematode genus *Caenorhabditis*, hybrid embryos arrest due to defects in the initiation of gastrulation, or later, during compaction or elongation of the embryo (Baird & Yen, 2000). The hybrid embryos produced by female *Rana catesbeiana* and male *R. clamitans* frogs develop an abnormal elongated gastrula, and are unable to develop further (Elinson, 1981). In hybrid toads, abnormalities occur later in development, with embryos from crosses between female *Bufo fowleri* and male *B. americanus* often failing during body elongation and development of the tail bud (Volpe, 1955). Hybrids between female brown trout, *Salmo trutta*, and male Atlantic salmon*, S. salar*, die even later in development, mainly between hatching and complete yolk absorption (Álvarez & Garcia-Vazquez, 2011). Hybrids between populations of *Podisma pedestris* grasshoppers cease to develop at a range of embryonic stages (Barton & Hewitt, 1981). Likewise, embryos from crosses between five lamprey species vary in the stages at which fatality occurs, ranging from four cells, through to the hatching of larvae. The stage

of fatality depends upon the parental species, occurring earlier with increasing genetic distance between dam and sire (Piavis *et al.*, 1970).

Although the genetics of hybrid inviability have been well studied (for example, Wu & Davis, 1993; True *et al.*, 1996; Turelli & Orr, 2000; Noor *et al.*, 2001), many of these studies refer vaguely to 'hybrid lethality' without verifying when this occurs. And despite the widely recognised importance of studies of hybrid embryos in the field of developmental biology, there has been surprisingly little attention paid in the context of reproductive isolation, with only a handful of reports of failed embryogenesis in hybrid animals. Despite this, reports come from a broad range of taxa, suggesting this may be a common phenomenon in hybridising systems. As well as acting at a variety of stages among species, arrest is sometimes unidirectional, only affecting one cross, and often only affecting one of the sexes. This indicates there is no common underlying mechanism to hybrid embryo mortality, and has led to a number of genetic modes being implicated (Coyne & Orr, 2004).

5.6 Conclusions

We have demonstrated that there are multiple mechanisms occurring after sperm storage that reduce the reproductive success of crosses between *G. bimaculatus* females and *G. campestris* males. Eggs from this heterospecific cross were less likely to begin embryogenesis, and if they did begin developing, they were more likely to arrest than eggs from conspecific mating pairs. These post-storage barriers to hybridising are potentially important in the maintenance of reproductive isolation between *G. bimaculatus* and *G. campestris* and they may have also played a historical role in the initial divergence of the populations. Not only do these cryptic mechanisms act as a method to reduce gene flow, they also create a cost to interbreeding. Despite the viability and fertility of hybrids that do hatch in this system (Veen *et al.*, 2011), the number of eggs that fail to develop represent a substantial cost to *G. bimaculatus* females. Eggs are energetically expensive to produce, and so females should avoid laying clutches of eggs that don't yield offspring, and thus avoid interbreeding. This cost could reinforce the evolution of barriers occurring earlier in the reproductive process.

Chapter 6 The influence of conspecific and heterospecific ejaculates on female post-mating behaviour

6.1 Abstract

In polyandrous mating systems the reproductive success of a male may be reduced if a female they have mated with goes on to mate with another male and invests in his offspring, rather than those of the original mate. Hence it is in the interests of males to suppress re-mating in females they have mated with. This may be achieved through transferring manipulative compounds to the female within the ejaculate, triggering changes in female post-mating behaviour. These compounds are likely to evolve relatively quickly, and so their effectiveness may be dependent on whether a male has mated to a conspecific or heterospecific female. We mated *Gryllus bimaculatus* females to either conspecific males or heterospecific *G. campestris* males and measured their post-mating change in response to male calling song. We found suppression and subsequent recovery of phonotaxis, but this pattern did not differ between females mated to conspecific or heterospecific males. Compounds transferred by males also stimulate other post-mating female behaviours such as oviposition. We investigated the relationship between the number of eggs laid and post-mating phonotaxis, and found that females that laid more than the median number of eggs followed the pattern of suppression and subsequent recovery of phonotaxis, whereas those female that laid fewer than the median number of eggs showed no change in phonotaxis. We speculate that if a female detects that she has received sufficient sperm to fertilise a large number of eggs, then she may cease mate search behaviour until sperm stores have depleted.

6.2 Introduction

Females produce larger gametes than males, meaning there is a fundamental difference between the sexes in their initial investment in offspring. Consequently their mating strategies differ, and conflict arises when the strategy of one sex is detrimental to the reproductive success of their mate. The primary areas for such conflicts are over how much of her resources the female invests in the offspring of any one of what may be several mates, and whether or not, and how quickly she mates with another male.

Females frequently choose to mate with a new male before sperm stored from a previous mating have depleted. There are benefits to be gained from rapid re-mating, for example the receipt of nutritious nuptial gifts (Gwynne, 1984), the potential to reduce inbreeding (Cornell &

Tregenza, 2007) or improving embryo viability (Arnqvist & Nilsson, 2000). However, the presence of multiple ejaculates within a female creates the potential for sperm competition between males (Parker, 1970). A male may improve his reproductive success through active removal of a rival's sperm using genitalic adaptations (Arnaud *et al.*, 2001), or through inhibition of rival sperm survival through the transfer of chemical compounds in the ejaculate (Price, 1997).

Alternatively, a male may improve his success through manipulation of female post-mating behaviour. Males can guard females to fend off would-be competitors or prevent mate-searching (Alcock, 1994), insert copulatory plugs into the reproductive tract of females to prevent intromission by novel males (Devine, 1975), or transfer manipulative compounds to females, which can trigger changes to a suite of post-mating behaviours (Gillott, 2003a). Females are expected to evolve counter-adaptations to counter the effects of male exploitation. This conflict between the sexes may lead to a cycle of adaptation and counter-adaptation (Holland & Rice, 1998).

The genes encoding proteins associated with sexual selection, and potentially those involved in manipulations, evolve relatively quickly (Swanson *et al.*, 2001; Andrés *et al.*, 2006) and so are likely to be divergent between closely related species. The capacity of manipulative compounds to trigger changes in females may therefore differ depending on the evolutionary histories of mating individuals. The evolution of female resistance has been demonstrated by artificial introduction of manipulative compounds across distantly related species (Sakaluk *et al.*, 2006). In contrast, here we examine post mating changes in female behaviour in response to natural matings between sister species. We investigate changes to mate-searching behaviour, expecting to find a difference between those females mated to conspecific males and those mated to heterospecific males.

As well as mate-searching, there may be other behaviours that change after mating. Compounds in ejaculates are likely to have multiple targets (Stanley-Samuelson & Loher, 1985), and those males successful in manipulating changes to mate-searching may also be successful in manipulating other behaviours such as egg laying. We might therefore expect to find a relationship between the strength of change in one behaviour and the strength of change in another.

As well as viewing changes in female post-mating behaviour as manipulations imposed by males, these changes might also be thought of as the female responding to cues received from mating. If searching out mates or mating bear significant costs (Magnhagen, 1991; Rowe, 1994), then these costs might trade-off against the benefits of polyandry. Once a female has detected that she has sufficient sperm to fertilise a number of eggs, then it may be in her interests to cease mate-search behaviour until stored sperm have depleted and need to be replenished. Since the act of

mating alone may not reliably indicate to the female that she has received sperm, the detection of seminal proteins in the ejaculate may be a better cue (Loher & Edson, 1973). Using egg laying as an indication that the copulation has been successful, we expect those females that lay many eggs after mating to be less likely to seek out further mates, whereas we expect those females laying few or no eggs to continue to search for potential mates.

We use two species of field crickets, *Gryllus bimaculatus* and *G. campestris.* These sister species are widely distributed in Europe. Their distributions are known to overlap in central Spain (Pardo *et al.*, 1993; Gorochov & Llorente, 2001), and zones of contact elsewhere north of the Mediterranean are likely. Hybridisation in the laboratory is asymmetrical; with only the females of *G. bimaculatus* willing to interbreed (*G. campestris* females have been observed to hybridise (Cousin, 1933)) but such matings are very rare and in our experience, *G. bimaculatus* males are invariably subject to aggression from *G. campestris* females (von Hörmann-Heck, 1957; Veen *et al.*, 2011). Females of both species are polyandrous in the lab and the field (Tregenza & Wedell, 1998; Bretman & Tregenza, 2005; Rodríguez-Muñoz *et al.*, 2011). Male crickets produce calling song, a signal used by females to find potential mates. The process of orientation and movement towards song, known as phonotaxis, is suppressed post-mating in *G. bimaculatus*. This loss of phonotaxis recovers naturally over a number of days, or can be artificially reinstated by preventing the female from receiving signals from the spermatheca, either through its removal or by severing the ventral nerve cord (Loher *et al.*, 1981; Loher *et al.*, 1993). Although the suppression mechanism is unclear, the transfer of manipulative seminal proteins to the female is at least partly involved (Green & Tregenza, 2009). Detection of mechanical filling of the spermatheca may also be important (Loher *et al.*, 1993). As well as phonotaxis, oviposition is also affected by the transfer of seminal proteins (Bentur *et al.*, 1977).

6.3 Methods

6.3.1 Rearing

G. bimaculatus were collected from Valencia, Spain in summer 2011 and reared in the lab over subsequent generations. Crickets were housed at 28 ^{o}C under a 16:8 light:dark cycle, with food and water provided *ad libitum*. Last instar nymphs were isolated to ensure virginity upon becoming adult. *G. campestris* were collected from near Gijon, Spain in late spring 2012 as last instar nymphs or adults. These wild caught individuals were kept in the laboratory for at least 7 days prior to use in

trials in early summer 2012. All individuals had matured a minimum of 7 days before use in experimental trials to ensure sexual maturity.

6.3.2 Song construction and phonotaxis

Measurements of song parameters and the artificial songs presented here are taken directly from Veen *et al.* (2013). The calling songs of 26 *G. bimaculatus* males were recorded at 28 ^{o}C +/- 1 ^{o}C, and temporal parameters were measured using a custom program written in JAVA that calculates distances between peaks from .wav audio files. Means were calculated for pulses per chirp, chirp length, and inter-chirp interval (see Fig. 6.1) for individual males, and subsequently for group means. Using Audacity software (http://audacity.sourceforge.net) synthetic song was constructed by randomly selecting a chirp and manipulating the song parameters to match those of the calculated group means (Pulses per chirp; 3.24 ± 0.54, Chirp length (ms); 74.94 ± 9.93, Inter-chirp length (ms); 278.19 ± 85.87). This chirp was then repeated to create a 10 min long song, to be played to females during phonotactic trials.

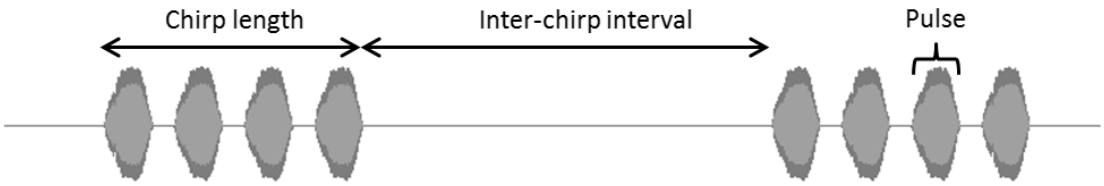


Figure 6.1. Parameters of calling song

Prior to phonotactic trials, food, water and debris were removed from the boxes in which the females were housed, the lids loosely replaced, and the boxes inverted to leave the females standing on the inside of the lids. These were then kept in a polystyrene container and left for at least 15 min to allow the females to settle. Trials were conducted at 28 ^{o}C +/- 1 ^{o}C under red light within a 126 x 67 x 29 cm wooden arena, constructed with two speakers in adjacent corners angled towards the centre of the arena at 70 degrees. A semi-circle with a radius of 5 cm was marked out in front of each speaker. Each female was transferred in turn to the arena while still within her upturned box, and the body of the box was removed to leave the female in the centre of the arena. Song was then played to the female at ~65 dB (re 20 μPa) from one of the speakers, either left or right, and her response to the song recorded as latency to reach that speaker. The trial was

terminated when the female's entire body was within the marked semi-circle, or after 10 min. If the female did not reach the speaker within the initial 10 min trial, they were excluded from the experiment. Following the initial phonotactic trial, each female was mated (see below) and then trialled the day after mating, and each day for 5 subsequent days. Each female was played song from both the left and right speakers, alternating between trials. During these post-mating trials, if the female did not reach the speaker within the 10 min, their latency was recorded as 10 min. If the female died during this 6 day post-mating period, all data from her trials were removed from the dataset, as there is likely a change in phonotactic response prior to death.

6.3.3 Matings

Prior to mating trials, *G. campestris* males were exposed to non-experimental conspecific females to encourage courtship behaviour. They were divided by wire mesh so that the female could be detected but not mated with. Only *G. bimaculatus* females were used in the trials, as they mate both intra- and interspecifically. Virgin females were paired with either a conspecific or heterospecific male. Each male was only used once. Mating pairs were placed in 11 x 11 cm plastic containers lined with paper for traction, and observed. If courtship or mating did not occur within 1 h the male was replaced with another or trialled on subsequent days. Successful mating was confirmed by the presence of a spermatophore attached to the female. Post-mating, the pair was left in the arena for 1 h, the time required for most of the spermatophore contents to be taken up by the female (Simmons, 1986). During this time the male has the opportunity to engage in mate guarding behaviour (Simmons, 1991b), which includes preventing the female from prematurely removing the spermatophore (Simmons, 1986). After mating the female was provided with a small dish of damp sand in which to oviposit. After ~48 h these dishes were removed, and the number of eggs laid was counted.

A total of 42 females were mated. 8 females from the conspecific and 2 females from the heterospecific treatment groups were excluded from the dataset as they died during the 6 days of post-mating trials, leaving 16 in the conspecific and 18 in the heterospecific treatment groups.

6.3.4 Statistical analyses

Statistical analyses were performed using R v2.14.1 (R Development Core Team, 2011). To estimate how phonotaxis is affected by whether a mating partner is conspecific or heterospecific, a generalised linear mixed model (GLMM) was fitted (lmer: R lme4 library (Bates *et al.*, 2011)) with

latency to reach the speaker as the response variable. Day (coded as numbers 0 – 6, Day 0 being the trial carried out prior to mating, and Day 1 – 6 being trials carried out post-mating), male species and the number of eggs laid by the female were entered as explanatory variables, as well as all interactions between these variables. Female ID, a unique number for each individual, was entered as a random effect to control for repeated measures from individuals. Model selection was carried out through sequential removal of explanatory variables and their interactions, until only significant terms remained in the model, their significance determined through likelihood ratio tests (Crawley, 2007). A comparison was made between the model including Day fitted linearly, or including day fitted as a quadratic polynomial, the difference determined through a likelihood ratio test.

6.4 Results

Female phonotaxis, measured as latency to reach a speaker playing synthetic male advertisement call, showed a reduction post-mating. Phonotactic response continued to decay for approximately 3-4 days post-mating, after which responses appeared to strengthen again. This pattern of loss and subsequent recovery of phonotaxis is substantiated by the finding that female response was better described by a quadratic polynomial fit of day than a linear fit (lmer; $\chi^2_{2,9} = 13.15$, $P = 0.0014$). There was no difference in phonotactic response between females mated to conspecific or heterospecific males (lmer; male species; $\chi^2_{1,10} = 0.010$, P = 0.919, Fig. 6.2). Each female laid between 0 and 545 eggs within ~48 h post-mating, the median being 18 eggs. The more eggs that a female laid, the more likely she was to follow the pattern of loss and subsequent recovery of phonotaxis, regardless of the species of male she mated to (lmer; day^2 x egg count interaction; $\chi^2_{2,9} = 6.23$, P = 0.044, Fig. 6.3).

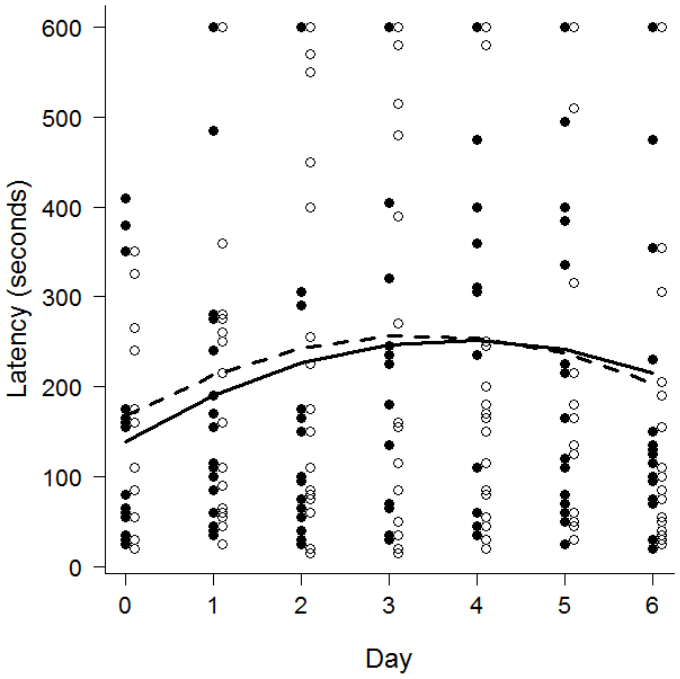


Figure 6.2. Phonotactic response of females mated to conspecific and heterospecific males. Female phonotaxis, measured as latency to reach a speaker playing synthetic male calling song, over a period of 7 days. The day 0 trial occurred prior to mating. Days 1 – 6 were carried out post-mating. Closed dots and solid line correspond to females mated to a conspecific male. Open dots and broken line correspond to females mated to a heterospecific male. (NB; high latency values correspond to weak phonotaxis, and *vice versa*).

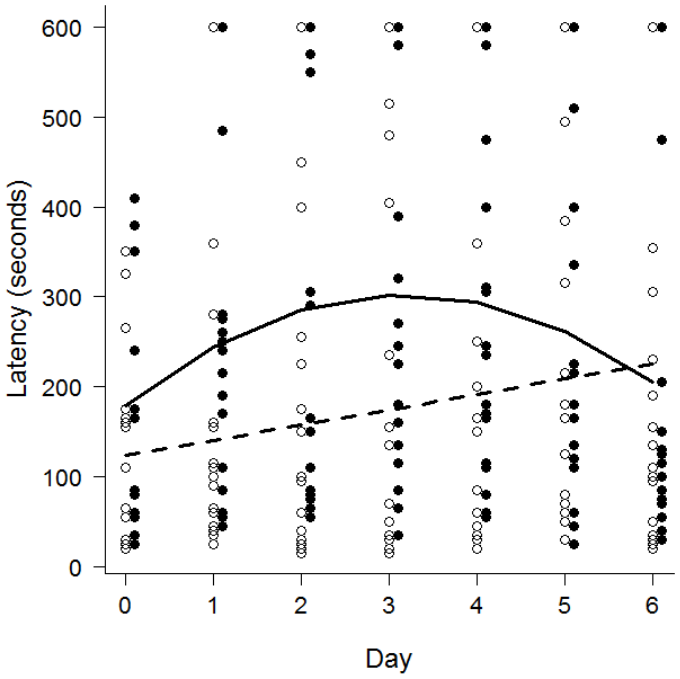


Figure 6.3. Phonotactic response of females laying more than and less than the median number of eggs. Female phonotaxis, measured as latency to reach a speaker playing synthetic male calling song, over a period of 7 days. The day 0 trial occurred prior to mating. Days 1 – 6 were carried out post-mating. Closed dots and solid line correspond to females laying more than the median number of eggs. Open dots and broken line correspond to females laying fewer than the median number of eggs. (NB; high latency values correspond to weak phonotaxis, and *vice versa*).

6.5 Discussion

We have demonstrated post-mating suppression and subsequent recovery of phonotaxis, indicative of a refractory period for mate search behaviour in *G. bimaculatus* females. This pattern did not differ between females mated to conspecific or heterospecific males. We also found that there was a significant relationship between phonotactic response and the number of eggs a female laid within ~48 hours post-mating.

The pattern of suppression and recovery of phonotaxis found here was likewise reported in *G. bimaculatus* by Loher *et al.* (1993), finding a post-mating loss for some females, which sometimes recovered naturally over a number of days, or could be artificially reinstated by removal of the spermatheca or severing the ventral nerve cord. The authors speculated that the loss of phonotaxis

was mediated through detection of mechanical filling of the spermatheca. More recently it has been shown that seminal proteins found in the ejaculates of *G. bimaculatus* males may be important. Sperm were removed from ejaculate, and the remaining seminal proteins injected into the abdominal cavity of females. Movement of injected females was reduced, though phonotaxis *per se* remained unaffected (Green & Tregenza, 2009). A combination of multiple cues may therefore stimulate changes in post-mating behaviour in this species.

We found no significant difference in phonotactic responses between females mated to conspecific males and those mated to heterospecifics. A possible explanation for the similarity of these species in their capability to manipulate female post-mating behaviour is that the seminal proteins associated with manipulation have not diverged substantially post speciation. This explanation runs counter to the finding that genes encoding seminal proteins evolve rapidly in a number of *Gryllus* species (Andrés *et al.*, 2006). An alternative explanation might be that even if these proteins have diverged, the receptors or targets within the female upon which they act have low specificity, and may be triggered by a diverse range of proteins. Alternatively, if the detection of mechanical filling is of greater importance to changes in phonotaxis than seminal proteins, then, assuming the ejaculates of each species are of a similar size, the female would receive the same sensory stimulus regardless of the species of her mate. There is a possibility that the changes to female post-mating behaviour are stimulated by the act of mating itself (for example in fowl, Løvlie *et al.*, 2005). However the work of Loher *et al.* (1993) suggests that this is unlikely to be the case, since they were able to make changes to female mate search behaviour through physiological manipulations.

It is perhaps surprising that females show no difference in refractory response to the ejaculates of heterospecifics compared to conspecifics. Interspecific matings are costly, since clutches of hybrid eggs are far less likely to hatch than those of pure-bred clutches (Veen *et al.*, 2013), and hybrid offspring often have reduced survivorship and fecundity compared to pure bred offspring (Dobzhansky, 1936; Orr, 1996; Hatfield & Schluter, 1999). Following an interspecific mating it would be beneficial to the female to continue to respond to calling song, and to search out and re-mate with a conspecific male. In doing so she may eliminate the potential cost of the interspecific mating, by establishing competition between the heterospecific and conspecific ejaculates. Conspecific sperm precedence (Howard, 1999) is relatively strong in this system, meaning that sperm from a heterospecific is highly unlikely to fertilise a female's eggs if sperm from a conspecific male is simultaneously present within the female reproductive tract (Tyler *et al.*, 2013). The absence of this

strategy may lead to a greater number of hybrid clutches being laid, and the potential introgression of genetic material from *G. campestris* into *G. bimaculatus*. It is perhaps plausible that females do actively search for novel conspecific males after mating with a heterospecific, however, under a scenario of male manipulation, the seminal fluid proteins delivered by heterospecific males may be more effective in suppressing mate-searching than the seminal fluids of conspecific males. These opposing forces could well cancel each other out, generating the lack of difference in mate search behaviour that we observe between the two experimental groups.

We found a significant relationship between the number of eggs that a female laid and their post-mating response to calling song. Seminal proteins may have multiple targets, affecting both phonotaxis and egg laying. Stanley-Samuelson & Loher (1985) injected female *Teleogryllus commodus* with radioactive seminal compounds, and followed their progress to a range of tissues including hindgut, ovaries and ventral nerve cord. These are likely to target a similar range of tissues in *G. bimaculatus*, including targets controlling oviposition (Bentur *et al.*, 1977; Bretman *et al.*, 2006). It is possible that variable transfer of ejaculate to females would lead to phonotaxis and egg laying to vary similarly to each other as a dose-dependent effect, creating the relationship observed in this study. However, to explain the complete lack of egg laying and refractory period in many of the females in this study, we would have to assume that no ejaculate had been transferred to these individuals. This is unlikely in this system, since in previous instances of females not laying eggs within a 48 hour post-mating period, genetic material from a male was almost always found within the spermatheca (Tyler *et al.*, 2013). Cases of females laying no or very few eggs were remarkably common in this study, a finding that contrasts with those of Veen *et al.* (2013) and Tyler *et al*. (2013). The main difference between these studies was the amount of time that the stock population of *G. bimaculatus* had been maintained in the laboratory prior to use in experiments. The earlier studies used crickets collected from the wild at least 5 years previously, whereas in the present study the crickets had only been in the laboratory for 8 months, and were perhaps less adapted to laboratory conditions.

Another possible explanation for the observed relationship is that males vary in their abilities to manipulate female post-mating behaviour through quantity or quality of seminal proteins within an ejaculate, rather than the volume of ejaculate itself. Bretman *et al.* (2006) observed that females mated to dominant males laid more eggs, and suggested that this may be due to variation in manipulative ability. Though all males may provision their spermatophores with a similar volume of

ejaculate, some males may transfer more, or more potent, seminal proteins, and those males that are better able to supress phonotaxis may be better able to stimulate egg laying also.

Manipulations by males, such as those discussed here, are often thought to oppose the interests of polyandrous females, setting the scene for sexual conflict over female re-mating rate (Arnqvist & Nilsson, 2000) and evolution of resistance to the manipulation (Sakaluk *et al.*, 2006). However, less attention has been paid to the potential benefits to females of reduced mate search behaviour or re-mating rate. If the costs of searching out new mates outweigh the benefits of polyandry, then those females that have already acquired sufficient sperm to fertilise eggs may benefit from suspending further mate searches until sperm have depleted. There are many ways in which mating behaviours may incur costs, for example time and energy (Dewsbury, 1982; Forsyth *et al.*, 2005), exposure to pathogens and parasites (Sheldon, 1993; Thrall *et al.*, 2000), or an increased chance of being predated upon while performing these conspicuous behaviours (Magnhagen, 1991; Rowe, 1994). For example, in *G. campestris*, females have been shown to be several times less likely to be predated if they remain at a burrow with a male, whereas males sharing a burrow experience higher predation rates (Rodríguez-Muñoz *et al.*, 2011). A post-mating reduction in mate search behaviour, or movement in general, would therefore be beneficial to these females if it kept them at the burrow with their 'chivalrous' mating partners.

There may also be indirect fitness benefits to be gained from mating with manipulative males; if the ability to manipulate female post-mating behaviour is heritable, then the female will benefit through investing in the production of manipulative sons (Pizzari & Birkhead, 2002). Care therefore needs to be taken when attributing changes in post-mating behaviour to male 'manipulations'. Rather than conflicting with a female's best interests, these changes may reflect a female opting to respond to cues conveyed during mating, representing sexual co-operation rather than conflict.

Chapter 7 General Discussion

I have identified multiple barriers to interbreeding between the crickets *Gryllus bimaculatus* and *G. campestris*, which have the potential to reduce gene flow between these species. These barriers can occur at a range of stages through the reproductive process, from pre-mating advertisement through to post-zygotic egg development. They vary in strength, and are asymmetric.

Female *G. bimaculatus* only show weak preference for conspecific calling song, often responding phonotactically to songs typical of heterospecific males (Chapter 2). When paired with heterospecific males they will mate, though less readily than to conspecific males (Cousin, 1933; von Hörmann-Heck, 1957; Veen *et al.*, 2011). When mated to both conspecific and heterospecific males *G. bimaculatus* females will preferentially take up and store sperm from the conspecific male, and sperm from conspecific males is more likely to sire offspring than would be predicted from the proportion of sperm in storage (Chapter 4). Eggs laid by *G. bimaculatus* females mated to heterospecific males are less likely to begin embryogenesis, and are more likely to arrest during early stages of embryogenesis. Those hybrid embryos that survive to late stages of development have hatching success equal to that of pure-bred embryos (Chapter 5). After mating, phonotaxis follows a pattern of suppression and subsequent recovery, though this pattern does not differ between females mated to conspecific or heterospecific males (Chapter 6).

While partial barriers to interbreeding expressed through *G. bimaculatus* females are numerous and occur at a range of stages, in *G. campestris* there is a strong barrier acting early in the reproductive process (Veen *et al.*, 2011). Though they show no preference for calling song typical of conspecific males (Chapter 2), upon meeting there is a strong pre-mating barrier to interbreeding. This is likely driven by females responding to close range species recognition cues, one of them being recognition of species differences in cuticular hydrocarbon expression (Chapter 3).

7.1 Multiple reproductive barriers

It is unsurprising that there are multiple pre-mating barriers to gene flow between these species, since they have gone through significant divergence in adapting to their distinct niches, and there have likely been differences in sexual selection pressures on traits that are used in mate choice. It is also unsurprising that cryptic post-mating mechanisms strongly affect the success of inter-species matings, since traits associated with these processes have been shown to have the potential to diverge rapidly (Civetta & Singh, 1995; Pitnick *et al.*, 2003; Andrés *et al.*, 2006). This is not unusual,

with multiple interacting barriers also found in other cricket species such as in *Laupala* (Mendelson & Shaw, 2006; Mullen et al., 2007), between *G. rubens* and *G. texensis* (Gray & Cade, 2000; Gray, 2005), and in *G. integer* (Leonard & Hedrick, 2009), and across other taxa for example in birds (Veen *et al.*, 2001; Sætre & Sæther, 2010) and frogs (Lemmon, 2009). In each case the contribution of barriers varies, with some species having many barriers of weak effect, and others having few barriers of strong effect. Since there is no evidence for any one mechanism representing a total barrier to interbreeding between *G. bimaculatus* and *G. campestris*, it is likely that a combination of multiple barriers act cumulatively, particularly in *G. bimaculatus*.

The strength of barriers may differ depending on whether females encounter males in isolation, or whether they encounter multiple males simultaneously. *G. bimaculatus* females demonstrate stronger preference for conspecific song when exposed to both conspecific and heterospecific song than when exposed to songs sequentially (Popov & Shuvalov, 1977; Veen *et al.*, 2013). This pattern may also apply to other cues used for pre-mating species recognition. Changes in barrier strength depending on mating context are similarly found in post-mating barriers to interbreeding. While we found that *G. bimaculatus* females will take up sperm from heterospecific males and use this sperm to fertilise eggs, when there was competition from a *G. bimaculatus* male, the success of the heterospecific male was greatly reduced, providing an example of conspecific sperm precedence (Chapter 4). If these species do encounter one another in the wild then the likelihood and success of interbreeding might rely heavily on the densities of males, and the ratio of conspecific to heterospecific males in a given location.

In cases such as these where there are multiple mechanisms that might limit gene flow, it is difficult to determine the most important mechanisms that prevent gene flow, and whether all of the major potential barriers have been identified. Since many aspects of morphology and behaviour will diverge during speciation, it is likely that there are traits important in mate choice and reproductive success that have been overlooked. It may also be important to consider how these barriers act when studied concurrently rather than in isolation. For example, when examined as discrete mechanisms, late acting barriers may reveal the potential to limit gene flow, but in nature, they may not play a major role in limiting gene flow if earlier acting barriers prevent mating.

7.2 Asymmetry of reproductive barriers

The asymmetry found in the relative strength of barriers between *G. bimaculatus* and *G. campestris* is not unusual, as there are many reported cases of hybridising systems in which one species is less

likely to interbreed than the other (Wirtz, 1999; Coyne & Orr, 2004). In this case, *G. bimaculatus* females differ from *G. campestris* in their propensity to interbreed, as well as in the strength and range of barriers that are involved (Veen *et al.*, 2011).

This asymmetry may be due to differences in selection pressures experienced by *G. bimaculatus* and *G. campestris* during speciation. Species diverging in allopatry experience different environments and so different selection pressures (Mayr, 1963). Males may adapt to their local environments through natural selection, driving differentiation in traits that are also used in female choice. If female preference for a trait is open ended, preference may not diverge to match the distribution of the male trait. This would ultimately lead to a mismatch between trait and preference (Panhuis *et al.*, 2001). Depending on the differences in selection pressures experienced by the species in their respective environments, this mismatch might be more exaggerated in one species than the other. This could ultimately lead to the females of one species being more receptive to the cues of heterospecific males than the females of the other species, thus creating an asymmetry in the propensity to interbreed.

Asymmetry in pre-mating barriers may also be driven by differences experienced by females in the costs associated with reproduction. As well as the energy invested in producing and laying eggs, there are costs associated with the mating itself. Time and energy is used in searching for potential mates (Dewsbury, 1982; Forsyth *et al.*, 2005), and individuals risk exposure to disease or predation (Magnhagen, 1991; Sheldon, 1993). If these costs are greater for one species than the other, then this may create a difference in the choosiness of the females, impacting on how likely they are to accept inter-species matings. Additionally, if speciation is strengthened in sympatry, then the cost of producing inviable or sterile hybrid offspring may select for improved species recognition mechanisms through reinforcement (Servedio & Noor, 2003). If hybrids produced from one cross are relatively more costly than from the reciprocal cross, then the former will experience stronger reinforcement. This would drive the evolution of stronger pre-zygotic barriers in one species than the other.

As well as asymmetry in the strength of barriers between the species, there is also an asymmetry between the sexes (Svensson *et al.*, 2007). While *G. campestris* females rarely or never accept matings with heterospecifics, *G. campestris* males will often court *G. bimaculatus* females (Veen *et al.*, 2011). This difference between the sexes is consistent with expectations from inequality in reproductive investment. While males experience energetic costs in terms of the production of sperm, the expense of producing eggs is much greater (Trivers, 1972). The sex

investing the most in reproduction should be most choosy, and thus be less likely to mate with heterospecifics.

7.3 Future directions

A key step forwards in the understanding of reproductive isolation between *G. bimaculatus* and *G. campestris* will be the confirmation and mapping of the geographic overlap between their distributions (Pardo *et al.*, 1993; Gorochov & Llorente, 2001), confirmation that they encounter one another at this area of overlap, and determining whether or not hybrid individuals are present in the wild. While I attempted to achieve this, I was unsuccessful (see Appendix A). Sampling of individuals from sympatry will open up numerous avenues of research. For example, genetic analyses of sampled individuals might reveal whether there has been any introgression of genes from *G. campestris* into *G. bimaculatus,* and whether this has contributed to a change in fitness (Wirtz, 1999). Behavioural studies previously performed using allopatric individuals could be repeated using sympatric individuals, and comparisons made. These comparisons might rule out the possibility of reinforcement acting to strengthen barriers between these species. If this were a driving force in speciation, we would expect to find greater divergence of traits used in species recognition in sympatric than allopatric populations, as individuals in sympatry would be more likely to experience the costs of hybridisation leading to selection for stronger barriers (Servedio & Noor, 2003). Instead we predict that comparable barriers would be found between allopatric and sympatric populations, indicating that these species diverged without any strengthening of barriers through reinforcement.

Another potentially rewarding avenue of research would be to investigate the post-mating barriers to interbreeding in *G. campestris* females. If it were possible to coerce these females into mating with heterospecific males, or to artificially inseminate them with heterospecific sperm, we might reveal whether there are any post-mating barriers, their strength relative to pre-mating barriers, and how they compare to analogous barriers in *G. bimaculatus*. Despite attempts to mate *G. campestris* females with *G. bimaculatus* males this was unsuccessful, regardless of the manipulations employed. While artificial insemination has been successful in bees (Baer & Schmid-Hempel, 2000), the development of a similar technique in crickets would be a major undertaking.

My investigations of species recognition by females (Chapters 2 & 3) might be better studied in terms of assessment of overall male quality. Though species are taxonomically categorised into discrete units, in reality there is a continuum of reproductive compatibility between potentially interbreeding individuals, with varying fitness benefits to be gained from matings. Reproductive

compatibility can therefore be viewed as an aspect of male quality, one which is likely traded-off against other aspects of quality during female mate choice (Sullivan, 2009; Mendelson & Shaw, 2012). It is therefore overly simplistic to present females with species recognition cues and measure preferences for them, without considering what other cues females might be using to determine preference. This may be especially true for species that have evolved in allopatry, as these will have diverged without selection for species recognition cues.

Our current understanding of chemosensory cues in pre-copulatory species recognition might benefit from being expanded. I have shown that CHC expression differs between the species, and that recognition of CHCs through the antennae is important in moderating *G. campestris* female aggression towards heterospecific males (Chapter 3). Through chemical ablation of the antennae, aggression was reduced so that females behaved indifferently to the males. The next step would be to see if manipulations of males could lead to these females responding positively to courtship attempts, and perhaps even mount heterospecific males. This could be achieved through 'perfuming' the males with CHCs extracted from *G. campestris* males (Thomas & Simmons, 2009). It would also be interesting to analyse CHC profiles in hybrid individuals, and determine whether expression is intermediate to that of the parent species.

My investigations of cryptic post-mating barriers to interbreeding revealed that there are multiple barriers acting between these species (Chapters 4 & 5). However, the exact nature of these mechanisms remains unknown. Studies following the progress of ejaculates through the reproductive tract may provide insights into this (Damiens *et al.*, 2002; Manier *et al.*, 2010). It would be interesting to determine whether or not the progress of heterospecific sperm is slower than that of conspecifics, and whether heterospecific sperm are less likely to reach the site of fertilisation. Viability studies of sperm exposed to ejaculates of other males might reveal chemical components that actively inhibit rival sperm (Price, 1997). Detailed studies at the point of fertilisation might reveal whether or not heterospecific sperm are less able to penetrate eggs (Shaw *et al.*, 1994), or proceed through the steps required for the formation of a zygote (Sarashina *et al.*, 2005).

7.4 Conclusions

I have demonstrated that there are multiple barriers to interbreeding between these species. While studied in isolation some of these barriers are weaker than others, they cumulatively represent a strong barrier to gene flow. This highlights the need for comprehensive studies which include all stages of mating, post-mating processes within females, and offspring development to understand

reproductive isolation between species. My work also draws attention to the potential conflict between assessment of species identity and assessment of mate quality, and following Sullivan (2009), consideration of these on a continuum of mate quality should be encouraged, with assessment of reproductive compatibility rather than taxonomic identity, especially in systems that may have diverged in allopatry.

Appendix A Investigating sites of potential geographic overlap between *Gryllus campestris* and *G. bimaculatus* in the Castilla-La Mancha region of Spain

A.1 Background

The field crickets *Gryllus campestris* and *G. bimaculatus* are both found in Spain, with distributions that overlap through the centre / south of the country (Gorochov & Llorente, 2001). A detailed study of Orthoptera (Ensifera) was carried out in south-east Spain through 1989 and 1990 (Pardo *et al.*, 1993). Within the Castilla-La Mancha region 179 localities were sampled, and from these 47 species were collected. Of these localities, 6 were home to both *G. campestris* and *G. bimaculatus*.

During May 2010 we returned to these sites and closely surrounding areas in an attempt to confirm areas of sympatry, and to make collections of crickets. We were able to get within 1.5 km (most less than 0.5 km) of all 6 sites identified as having both species by Pardo *et al.* (1993). We also investigated other locations that had suitable habitat for crickets, visiting 16 sites in total. At all sites we listened for calling song, searched under rocks, and looked for *G. campestris* burrows. Unfortunately the weather was unseasonably poor, and the search for crickets at most of the locations was hampered by cool temperatures, rain and even snow. We were not successful in capturing *G. bimaculatus* from any of the sites, and caught only three *G. campestris*. We did however hear male calling song at many of the locations, and although could not reliably identify which species was calling, could infer which species it might be by the time of day (*G. campestris* are predominantly diurnal, whereas *G. bimaculatus* are predominantly nocturnal).

A.2 Maps of locations visited

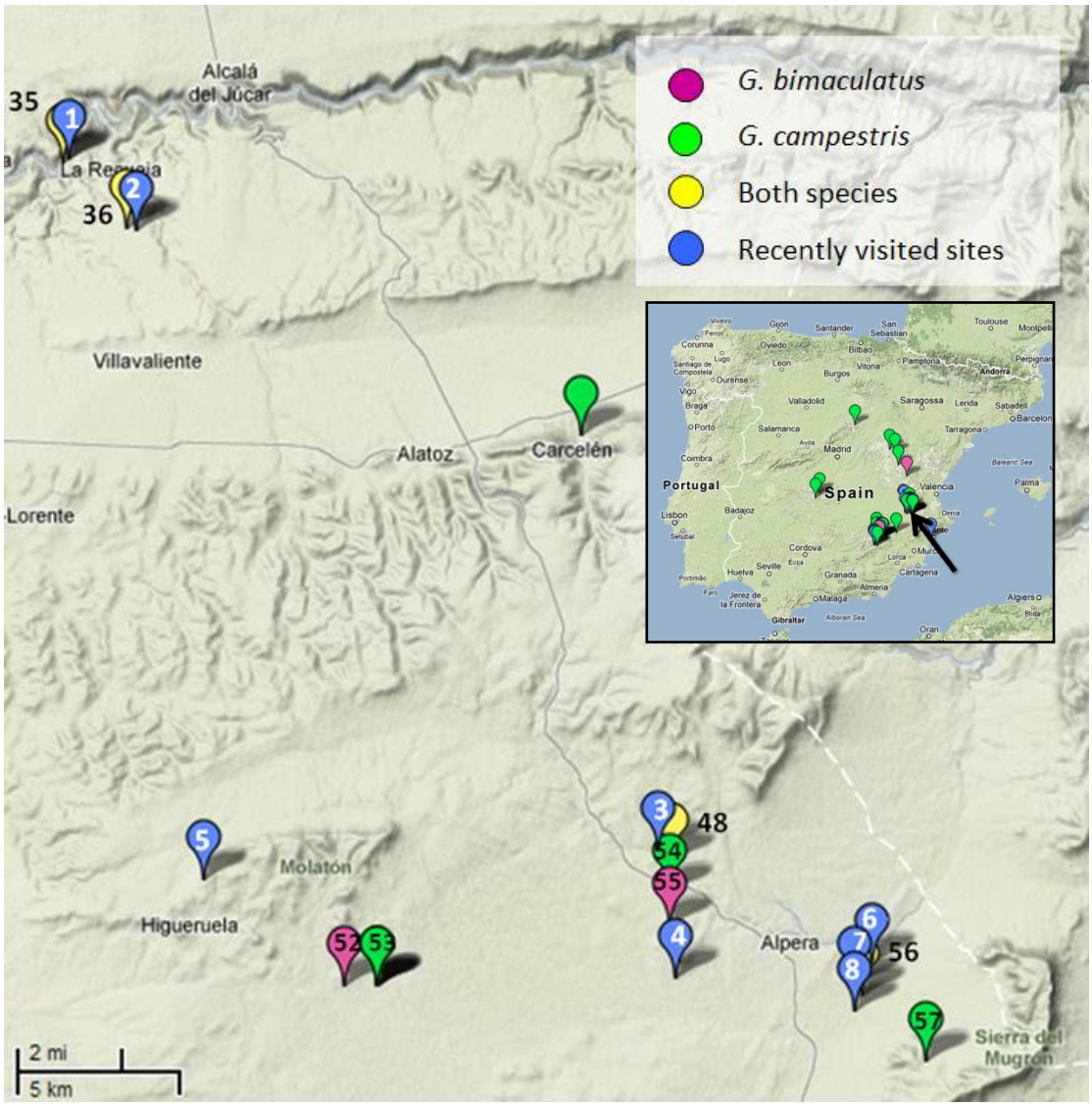


Figure A.1. Sites surrounding Alpera. Locations where Pardo *et al*. found *G. bimaculatus* (pink pins), *G. campestris* (green pins), and both species (yellow pins) are shown, as well as sites that we recently visited (blue pins). Site IDs are given as numbers on, or adjacent, to the pins. The black arrow in the inset map shows where in Spain these locations were found.

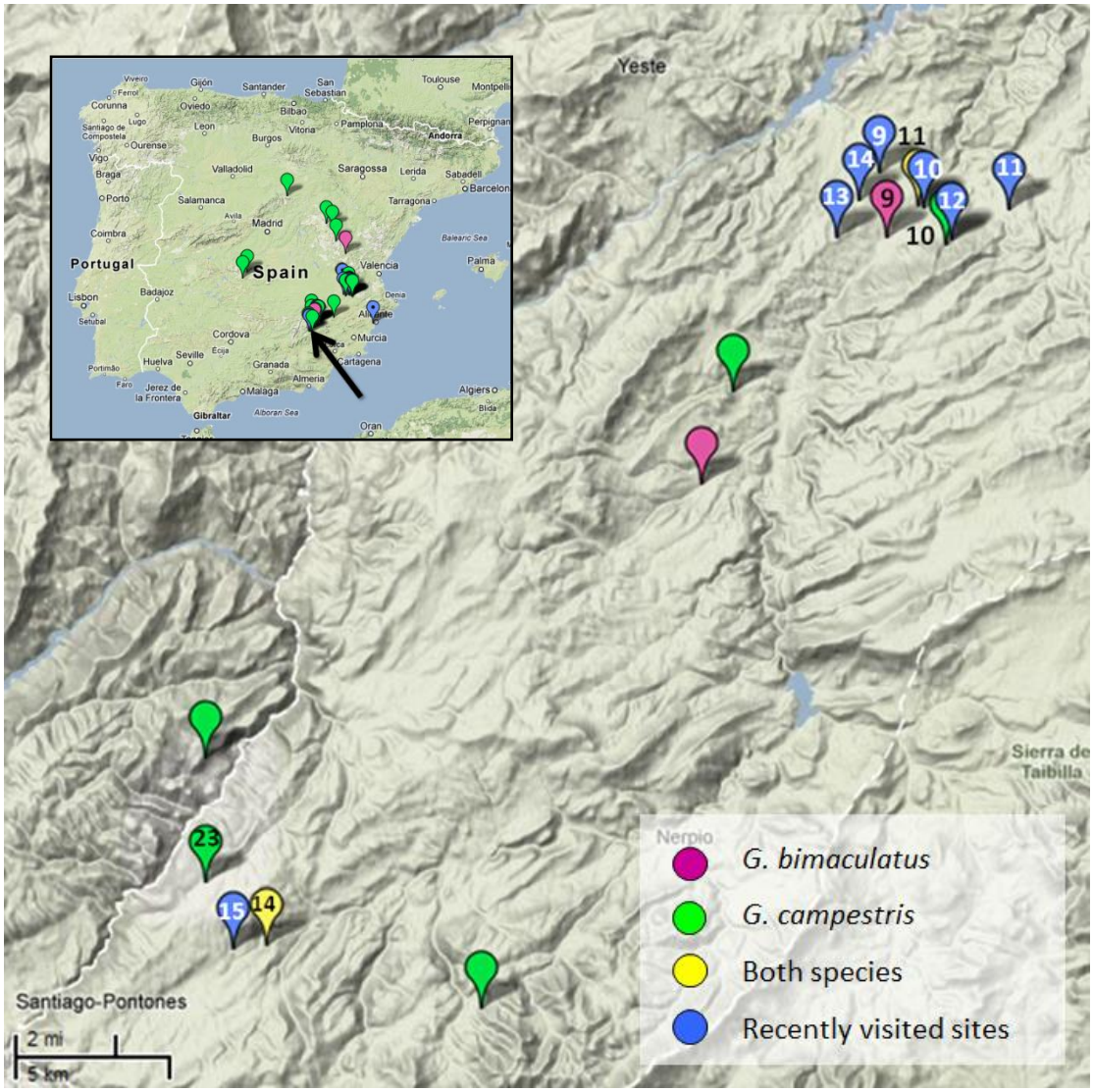


Figure A.2. Sites surrounding Nerpio. Locations where Pardo *et al*. found *G. bimaculatus* (pink pins), *G. campestris* (green pins), and both species (yellow pins) are shown, as well as sites that we recently visited (blue pins). Site IDs are given as numbers on, or adjacent, to the pins. The black arrow in the inset map shows where in Spain these locations were found.

A.3 Detail of sites visited

The sites we visited, the date of the visit, and a brief description are given. Where relevant, detail taken from Pardo *et al*. (1993) for the nearest site is given in blue.

Site 1 **La Recueja** (11/05/10)

The particular location identified in by Pardo *et al.* (1993) as high moist pasture has since been converted to agricultural land, however there is pasture adjacent to this area, on both sides of the river. The slopes of the valley are dry and rocky with little vegetation. Along a 1km stretch we heard several males calling in the mid afternoon.

Site P35

High moist pasture, 540m, UTM:XJ 3037

G. campestris: 2♂, 1♂nymph (27/8/90)

G. bimaculatus: 1♂ (27/8/90)

Site 2 **Medrila** (11/05/10)

A large area of mixed farmland comprised of crops, ploughed fields, vin yards and large piles of stones.

8pm: 10+ males calling over a 2km stretch (often on the edge of ploughed areas or in wheat fields.

10pm: ~30 males calling

The increase in calling from evening to night might suggest *G. bimaculatus* are present. One *G. campestris* male was caught.

Site P36

700m, UTM:XJ 3235 , Low sparse scrub

G. campestris: 1♂, 1♀ (21/5/90)

G. bimaculatus: 2♂, 1♀ (21/5/90)

Site 3 **Laguna** (12/05/10)

A shallow agricultural valley, with ploughed fields, grass and orchards. No calling at midday or late evening, however a local stated that there is usually calling during the day and night.

Site P48, Torro Prado

900m. UTM:XJ 4916 Low dry pasture

G. campestris:3♀ (18/5/90, 28/6/90, 13/11/90)

G. bimaculatus:1♀ nymph (25/9/90)

Site P54, Cruz

930m, UTM:XJ 4915, Low sparse scrub

G. campestris only:2♀ nymphs (28/8/90)

Site 4 **nr Higueruela** (12/05/10)

Wheat and ploughed fields. Many males calling in the wheat field during the mid-afternoon. Two *G.campestris* males were caught in the grass at the edge of a newly ploughed field.

Site P55, Delgado

900m, UTM:XJ 4914 Low dry pasture

G. bimaculatus only:1♀ nymph (28/8/90)

Site 5 **nr Higueruela** (12/05/10)

Grass and scrub in and around a wind farm. Males were heard calling during the mid-afternoon.

Site P52, Guarda

960m, UTM:XJ 3912, Low sparse scrub

G. bimaculatus only:1♀ nymph (28/8/90)

Site P53, Soton

950m, UTM:XJ 4012, Low dry pasture

G. campestris only: 2♀ nymphs (26/9/90)

Site 6 **nr Alpera** (12/05/10)

Scrubby grass field. Surrounding fields of wheat, vin yards etc. During mid-afternoon lots of males were calling from one side of the road, but silent in late evening, suggesting only *G. campestris* present.

Site 7 **nr Alpera** (12/05/10)

Large agricultural area. Mixture of ploughed fields, crops and orchards. No song in the afternoon, but one male calling in the late evening in a recently ploughed field.

Site P56, Moron

830m, UTM:XJ 5512 Low moist pasture

G. campestris:1♂, 1♀ (17/5/90)

G. bimaculatus:1♀ nymph (26/9/90)

Site 8 **nr Alpera** (12/05/10)

Large agricultural area. A mixture of ploughed fields, crops and orchards. No song in the afternoon, and at night only song that didn't sound like *G. campestris* or *bimaculatus*

Site 9 **nr Claras** (13/05/10)

Field of legumes close to solar panels. Lots of calling during the mid-afternoon, considerably less by early evening.

Site 10 **Claras** (13/05/10)

Grassy fields near a river (area very water-logged). In the afternoon, only the calling song of another species.

Site P11, Tobarico

680m, UTM:WH 6842, High moist pasture

G. campestris:1♂, 1♀ nymph (12/9/89, 22/11/89)

G. bimaculatus:1♂ nymph, 1♀ nymph, 1♀ (17/2/89, 14/8/89)

Site 11 (13/05/10)

Orchard with grass meadow next to a wheat field. Lots of males calling in the early evening, but none at night, suggesting only *G. campestris* may be present.

Site 12 (13/05/10)

Steep scrubby fields / orchards following a mountain road. No song heard during the early evening.

Site P10, Tobar

840m, UTM:WH 6941, Low sparse scrub

G. campestris only: 1♂, 1♀ (22/11/89)

Site 13 (13/05/10)

Followed a dirt road running through fields at higher altitude. No song during the evening

Site 14 (13/05/10)

A newly ploughed field with some scrubby patches of grass / weeds, and orchards close by. There were a few males singing during the evening. A *G. campestris* female was caught from under a rock at the edge of the field. At night only 2 males were heard.

Site 15 El Pozo (14/05/10)

Snow covered ploughed fields in a mountain valley. No calling was heard during the afternoon at this location or at any of the stops en-route.

Site P14, El Pozo

1.420m, UTM:WH 4718, Low moist pasture

G. campestris:2♂♂, 3♀♀ (20/5/89, 20/6/89, 14/8/89)

G. bimaculatus:2♀♀ nymphs (20/5/89)

Site 16 nr Alicante (15/05/10)

Arid scrubby / grassy land. Nothing was heard calling during the afternoon.

Appendix B Genes for behaviour: Searching a *Gryllus campestris* transcriptome for candidates

B.1 Background

We are developing the field cricket *Gryllus campestris* as a model system to study divergence and speciation between natural populations utilising a combination of next-generation sequencing and behavioural studies. We present and describe a novel transcriptome and suggest candidate genes involved in mating behaviour. These candidate genes may be used in the future to ask questions about mating behaviour and reproductive isolation, bridging the gap between behavioural traits and the underlying genetic causes. For example, gene expression studies could be used to compare the expression of genes implicated in song, between males that do and don't sing to heterospecific females.

B.2 Methods

B.2.1 cDNA preparation & sequencing

The *G. campestris* used to construct the cDNA library were reared in incubators or temperature controlled rooms (28 ^{o}C), lighting on a 14:10 hr light dark cycle, and food and water provided *ad libitum*. Individuals were separated upon adult eclosion. They were physically but not acoustically isolated. Heads (without antennae) were dissected from 5 adult males and 5 adult females, and used to prepare a normalised cDNA library. These were sequenced using Roche 454 next generation sequencing methods, and the data uploaded to InsectaCentral.

B.2.2 Identifying candidate genes

A candidate gene approach to identify genes associated with behaviour. A list of genes that have been implicated in behaviours associated with courtship and copulation was generated from the *Drosophila melanogaster* literature. This list includes biological processes which may be indirectly involved in courtship and copulation, for example locomotory behaviour, or circadian rhythm. The transcribed protein sequences for these candidates were obtained from the National Centre for Biotechnology Information (NCBI) Entrez gene database (available at http://www.ncbi.nlm.nih.gov/sites/gquery). tblastn similarity searches were then performed against the cricket data using the InsectaCentral BLAST server (available at http://insectacentral.org/). The E value, length and percentage of positive matches from the top search result were recorded. To check that the search returned a genuine ortholog from the cricket data, a protein BLAST against

Insecta was performed on the NCBI server. Gene ontology (GO), Enzyme classifications (EC) and InterPro predicted enzyme functions were performed to classify the *G campestris* ESTs using InsectaCentral.

B.3 Results

We obtained 1,349,510 high quality reads (averaging 523.26bp in length) corresponding to one and a half sequencing plates (Fig B.1). These assembled into 45383 derived contigs, with an average sequence length of 841.34 bp (Fig B.2). 31 candidate genes implicated in courtship and mating behaviours were identified from the *D. melanogaster* literature (Table B.3). Of these, 20 were found to have orthologs in the *G. campestris* transcriptome (Table B.4).

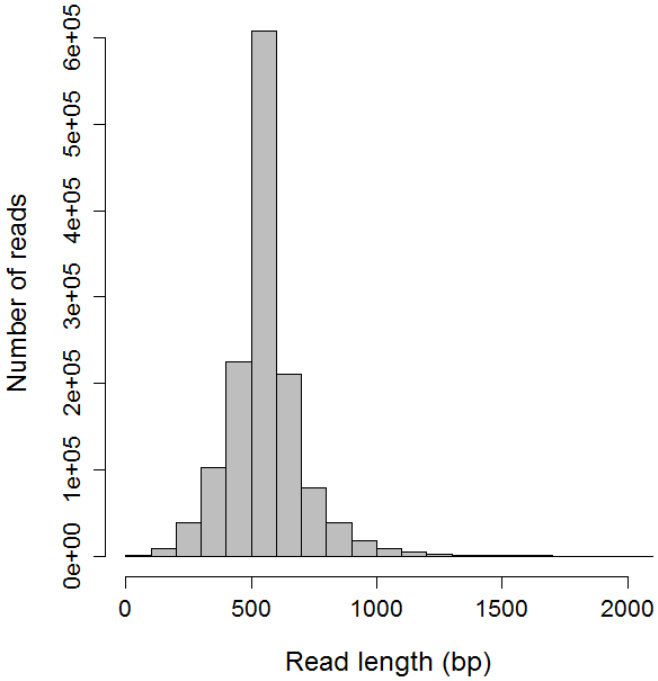


Figure B.1. Characteristics of *G. campestris* 454 transcriptome: Frequency histogram of read lengths

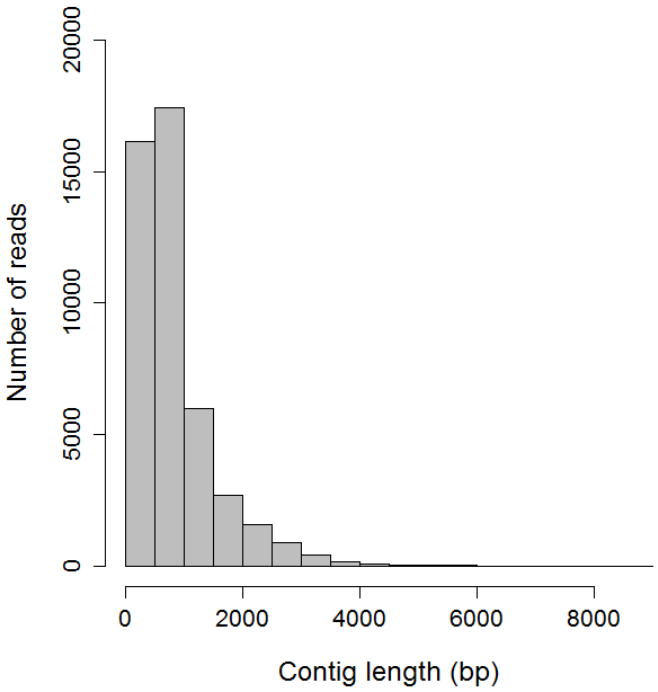


Figure B.2. Characteristics of *G. campestris* 454 transcriptome: Frequency histogram of contig lengths

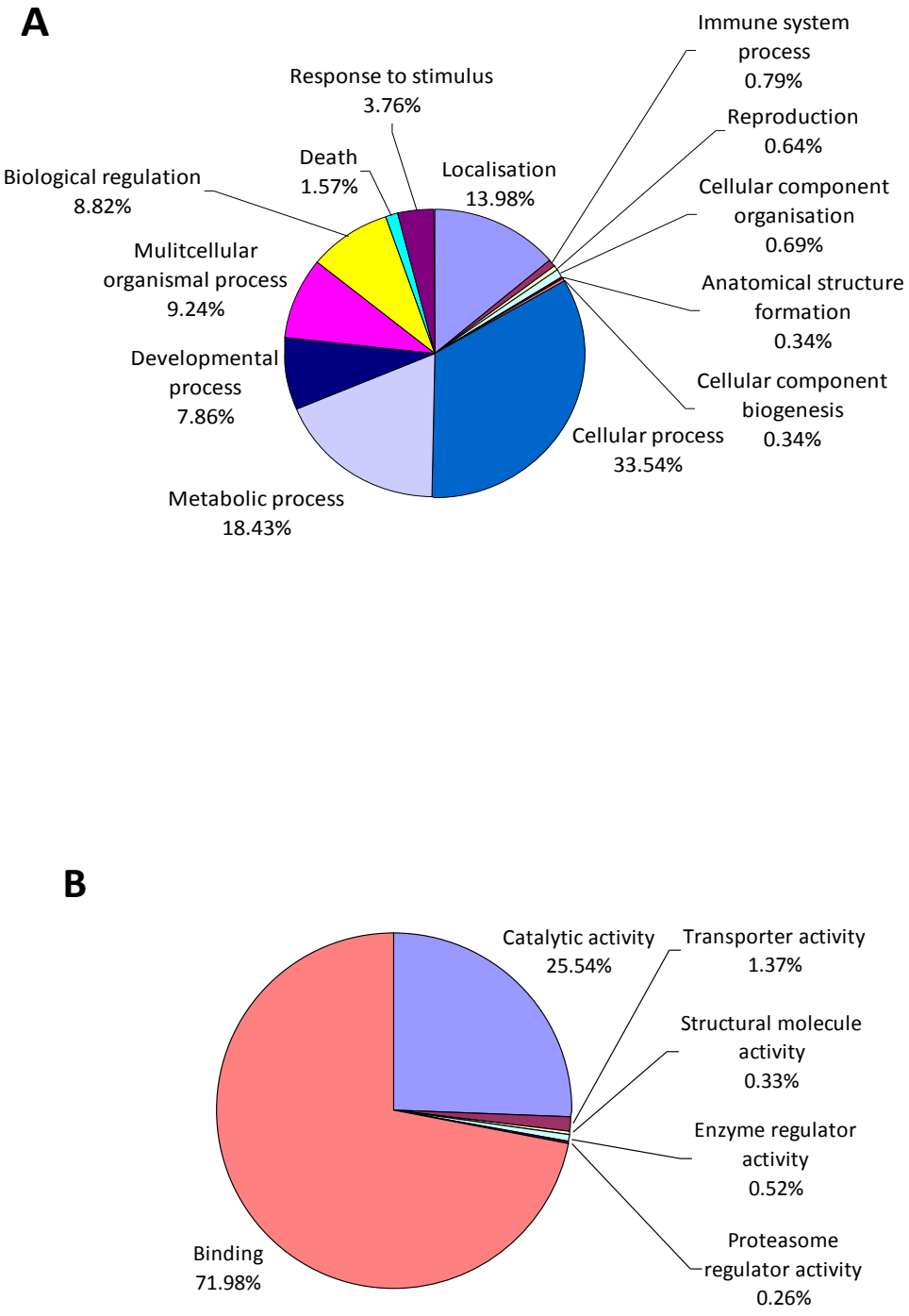


Figure B.3. EST annotation for the *G. campestris* adult head transcriptome. (A) Biological process GO terms at level 2. (B) Molecular function GO terms at level 2.

Table B.1. Summary of the top 20 Enzyme classifications represented in the *G. campestris* transcriptome

Frequency	Enzyme Classification
1905	Non-specificserine / threonineproteinkinase
1096	Proteasome endopepdidase complex
776	Ubiquitin--proteinligase
724	Peptidylprolylisomerase
646	Histon-lysineN-methyltransferase
589	NADH dehydrogenase (quinone)
589	Protein-tyrosine-phosphatase
575	Adenosinetriphosphatase
550	Phosphoproteinphosphatase
537	Dolichyle-diphosphooligosaccharide--proteinglycotransferase
525	Glutathionetransferase
479	Trans-1,2-dihydrobenzene-1,2-dioldehydrogenase
525	Enoyl-CoAhydratase
457	Non-specificprotein-tyrosinekinase
447	Prostaglandin-Dsynthase
437	Proteindisufide-isomerase
407	Butyryl-CoAdehydrogenase
347	Ubiquinol--cytochrome-creductase
331	Pyruvatedehydrogenase (acetyl-transferring)
324	H(+)-transportingtwo-sectorATPase

Table B.2. Summary of the top 20 InterPro families / domains represented in the *G. campestris* transcriptome

InterPro	Frequency	Description
IPR000504	832	RNA recognition motif, RNP-1
IPR015706	776	RNA-directed DNA polymerase (reverse transcriptase, related)
IPR001650	724	DNA/RNA helicase, C-terminal
IPR001353	676	Proteasome, subunit alpha / beta
IPR002110	631	Ankyrin repeat
IPR000717	631	Proteasome component (PCI) domain
IPR012336	603	Thioredoxin-like fold
IPR007087	589	Zinc finger, C2H2-type
IPR001680	575	WD40 repeat
IPR001478	575	PDZ/DHR/GLGF
IPR001395	562	Aldo/keto reductase
IPR015880	550	Zinc finger, C2H2-like
IPR016040	537	NAD(P)-binding domain
IPR001993	513	Mitochondrial substrate carrier
IPR002198	490	Short-chain dehydrogenase / reductase SDR
IPR000608	468	Ubitquitin-conjugating enzyme, E2
IPR016024	457	Armadillo-type fold
IPR001623	417	Heat shock protein DnaJ, N-terminal
IPR002290	407	Serine/threonin-protein kinase domain
IPR001841	398	Zinc finger, RING-type

Table B.3. Candidate genes from the *D. melanogaster* literature

	BIOLOGICAL PROCESS	**MOLECULAR FUNCTION** (based on experimental evidence, **based on predictions or assertions**)
Btk29A	Determination of adult lifespan	**Protein tyrosine kinase activity**
	Imaginal disc-derived male genitalia development	
	Oogenesis	
	Ovarian fusome organisation	
	Ovarian nurse to oocyte transport	
	Ovarian ring canal formation	
cacophony	Adult locomotory behaviour	**Voltage-gated calcium channel activity**
	Courtship behaviour	
	Detection of light	
	Male courtship behaviour	
	Veined wing song production	
courtless	Male courtship behaviour	**Ubiquitin-protein ligase activity**
	spermatogenesis	
cryptochrome	Circadian rhythm	**Blue light photoreceptor activity**
	Entrainment of circadian clock	**FAD binding**
	Gravitaxis	**G-protein couples photoreceptor activity**
	Locomotor rhythm	**Protein binding**
	Magnetoreception	**Transcription repressor activity**
	Phototransduction	
	Response to light stimulus	
eloF	Courtship behaviour	**Fatty acid elongase activity**
	Pheromone metabolic process	
foraging	Habituation	**cGMP-dependent protein kinase activity**
	Long-term memory	
	Short-term memory	
fruitless	Male courtship behaviour	**Protein binding**
	Veined wing song production	**RNA polymerase II transcription factor**

	Mating behaviour	**activity**
	Sex determination	**Transcription factor activity**
	Aggressive behaviour	**Zinc ion binding**
glass	Entrainment of circadian clock	**Nucleic acid binding**
	Entrainment of circadian clock by photoperiod	**Specific RNA polymerase II transcription factor activity**
	Response to red light	**Transcription factor activity**
		Zinc ion binding
latheo	**Learning**	-
	Learning or memory	
	Olfactory learning	
nonA	Male courtship behaviour	**mRNA binding**
	Veined wing song production	**nucleotide binding**
	Phototaxis	**poly-pyrimidine tract binding**
	Visual behaviour & perception	
pale	Adult locomotory behaviour	**Iron ion binding**
	Male courtship behaviour	**Tyrosine 3-monooxygenase activity**
qtc	Male courtship behaviour	-
rdgB	Phototransduction	**Phosphatidylinositol transporter activity**
	Rhodopsin mediated signalling pathway	**Phosphatidylcholine transmembrane transporter activity**
	Sensory perception of smell	
retained	Male courtship behaviour	**DNA binding**
	Muscle organ development	**SUMO binding**
	Oogenesis	**Transcription activator activity**
	Regulation of female receptivity	
sarah	Egg activation	**Protein binding**
	Long-term memory	
	Olfactory learning	
	Regulation of female receptivity	
	Regulation of female receptivity, post mating	
shibire	Learning or memory	**Actin binding**

	Male courtship behaviour	**Microtubule binding**
	Veined wing extension	
	Veined wing generated song production	
	Memory	
	Olfactory learning	
	Short-term memory	
	Sperm individualisation	
spinster	Locomotion	**-**
	Oogenesis	
	Regulation of female receptivity	
timeless	Copulatory behaviour	**Protein binding**
	Copulation	**Protein heterodimerisation activity**
	Locomotor rhythm	
	Mating behaviour	
	Negative phototaxis	
	Photoperiodism	
	Regulation of circadian sleep/wake cycle	
	Regulation of circadian sleep/wake cycle, sleep	
yellow	**Male courtship behaviour, veined wing extension**	**-**
	Male mating behaviour	

Table B.4. Orthologs found in the *G. campestris* transcriptome

Gene (full)	Gene	Isoform	Dmel length	Accession number	Length	e-value	% positive
Btk family kinase at 29A (fickle)	*Btk29A*	A	603	IC58607AbEcon60402	542	8.02E-57	Frameshift
				IC58607AbEcon17896	**995**	**1.06E-32**	**65**
		B	786	IC58607AbEcon60402	542	1.33E-56	Frameshift
				IC58607AbEcon27647	**496**	**4.68E-38**	**71**
		C	603	IC58607AbEcon60402	542	8.02E-57	Frameshift
				IC58607AbEcon17896	**995**	**1.06E-32**	**65**
		D	786	IC58607AbEcon60402	542	1.33E-56	Frameshift
				IC58607AbEcon27647	**496**	**4.68E-38**	**71**
		E	786	IC58607AbEcon60402	542	1.33E-56	Frameshift
				IC58607AbEcon27647	**496**	**4.68E-38**	**71**
		F	603	IC58607AbEcon60402	542	8.02E-57	Frameshift
				IC58607AbEcon17896	**995**	**1.06E-32**	**65**
cacophony	*cac*	A	1848	IC58607AbEcon10133	1258	2.38E-129	Frameshift
				IC58607AbEcon7517	**1169**	**5.24E-88**	**Frameshift**
				IC58607AbEcon59676	**628**	**4.81E-38**	**Frameshift**
				IC58607AbEcon96910	**384**	**2.02E-32**	**Frameshift**
				IC58607AbEcon53669	**424**	**1.21E-14**	**85**
		B	1848	IC58607AbEcon10133	1258	2.40E-129	Frameshift
				IC58607AbEcon7517	**1169**	**3.91E-95**	**Frameshift**
				IC58607AbEcon59676	**628**	**4.69E-38**	**Frameshift**
				IC58607AbEcon96910	**384**	**2.01E-32**	**Frameshift**
				IC58607AbEcon40213	**345**	**9.49E-14**	**Frameshift**
		C	1848	IC58607AbEcon10133	1258	2.58E-131	Frameshift
				IC58607AbEcon7517	**1169**	**5.79E-88**	**Frameshift**
				IC58607AbEcon59676	**628**	**4.43E-38**	**Frameshift**
				IC58607AbEcon96910	**384**	**2.26E-32**	**Frameshift**
				IC58607AbEcon4022	**1059**	**8.61E-16**	**77**
		D	1848	IC58607AbEcon10133	1258	2.58E-131	Frameshift

			IC58607AbEcon7517	**1169**	3.84E-95	**Frameshift**
			IC58607AbEcon59676	**628**	4.32E-38	**Frameshift**
			IC58607AbEcon96910	**384**	2.26E-32	**Frameshift**
			IC58607AbEcon4022	**1059**	8.33E-16	**77**
	E	1851	IC58607AbEcon10133	1258	2.25E-129	Frameshift
			IC58607AbEcon7517	**1169**	**5.75E-88**	**Frameshift**
			IC58607AbEcon59676	**628**	**5.11E-38**	**Frameshift**
			IC58607AbEcon96910	**384**	**2.03E-32**	**Frameshift**
			IC58607AbEcon53669	**424**	**1.24E-14**	**85**
	F	1851	IC58607AbEcon10133	1258	2.63E-131	Frameshift
			IC58607AbEcon7517	**1169**	**4.01E-95**	**Frameshift**
			IC58607AbEcon59676	**628**	**4.51E-38**	**Frameshift**
			IC58607AbEcon96910	**384**	**2.11E-32**	**Frameshift**
			IC58607AbEcon4022	**1059**	**8.14E-16**	**77**
	G	1849	IC58607AbEcon10133	1258	2.65E-131	Frameshift
			IC58607AbEcon7517	**1169**	**3.81E-95**	**Frameshift**
			IC58607AbEcon59676	**628**	**4.54E-38**	**Frameshift**
			IC58607AbEcon96910	**384**	**2.26E-32**	**Frameshift**
			IC58607AbEcon4022	**1059**	**8.26E-16**	**77**
	H	1849	IC58607AbEcon10133	1258	2.38E-129	Frameshift
			IC58607AbEcon7517	**1169**	5.29E-88	**Frameshift**
			IC58607AbEcon59676	**628**	4.98E-38	**Frameshift**
			IC58607AbEcon96910	**384**	2.04E-32	**Frameshift**
			IC58607AbEcon53669	**424**	1.23E-14	**85**
	I	1850	IC58607AbEcon10133	1258	2.25E-129	Frameshift
			IC58607AbEcon7517	**1169**	5.47E-88	**Frameshift**
			IC58607AbEcon59676	**628**	4.94E-38	**Frameshift**
			IC58607AbEcon96910	**384**	2.04E-32	**Frameshift**
			IC58607AbEcon53669	**424**	1.23E-14	**85**
	J	1850	IC58607AbEcon10133	1258	2.25E-129	Frameshift
			IC58607AbEcon7517	**1169**	3.85E-95	**Frameshift**

				IC58607AbEcon59676	**628**	4.54E-38	**Frameshift**
				IC58607AbEcon96910	**384**	2.22E-32	**Frameshift**
				IC58607AbEcon4022	**1059**	8.20E-16	**77**
courtless	*crl*	.	167	IC58607AbEcon1896	862	5.07E-86	94
cryptochrome (D.melanogaster)	*cry*	.	542	IC58607AbEcon13865	1115	2.55E-42	57
				IC58607AbEcon27361	**502**	**1.66E-36**	**60**
				IC58607AbEcon37975	**605**	**1.63E-33**	**55**
cryptochrome 2 (T.castaneum)	*cry2*	.	535	IC58607AbEcon41812	422	8.14E-47	85
				IC58607AbEcon94429	**314**	**2.87E-46**	**93**
				IC58607AbEcon27705	**700**	**7.83E-36**	**57**
doublesex	*dsx*	A	549	No Hit			
		B	427	No Hit			
		C	427	No Hit			
egghead	*egh*	.	457	No Hit			
		B	457	No Hit			
		C	457	No Hit			
elongase F	*eloF*	.	257	IC58607AbEcon2780	2227	2.23E-26	49
female-specific independent of transformer	*fit*	.	121	No Hit			
foraging	*for*	A	1088	IC58607AbEcon2486	3398	1.63E-66	64
				IC58607AbEcon887	**1728**	**3.92E-32**	**52**
		B	742	IC58607AbEcon2486	3398	8.32E-67	59
				IC58607AbEcon887	**1728**	**1.46E-32**	**52**
		C	894	IC58607AbEcon2486	3398	8.81E-67	64
		D	894	IC58607AbEcon2486	3398	8.81E-67	64
		E	934	IC58607AbEcon2486	3398	1.24E-66	64
		F	894	IC58607AbEcon2486	3398	8.81E-67	64
		G	894	IC58607AbEcon2486	3398	8.81E-67	64
		H	1088	IC58607AbEcon2486	3398	1.63E-66	64

				IC58607AbEcon887	**1728**	**3.92E-32**	**52**
		I	1088	IC58607AbEcon2486	3398	1.63E-66	64
				IC58607AbEcon887	**1728**	**3.92E-32**	**52**
		J	934	IC58607AbEcon2486	3398	1.24E-66	64
		K	894	IC58607AbEcon2486	3398	8.81E-67	**64**
fruitless	*fru*	A	516	IC58607AbEcon4914	691	1.95E-50	Frameshift
		B	789	IC58607AbEcon4914	691	4.98E-50	Frameshift
		C	854	IC58607AbEcon4914	691	8.46E-50	Frameshift
		D	665	IC58607AbEcon4914	691	6.70E-50	Frameshift
		E	955	IC58607AbEcon4914	691	8.34E-50	Frameshift
		F	688	IC58607AbEcon4914	691	5.35E-50	Frameshift
		G	796	IC58607AbEcon4914	691	6.55E-50	Frameshift
				IC58607AbEcon108021	**424**	**1.79E-40**	**83**
		H	695	IC58607AbEcon4914	691	6.44E-50	Frameshift
				IC58607AbEcon108021	**424**	**1.29E-40**	**83**
		I	870	IC58607AbEcon4914	691	2.35E-50	Frameshift
		J	906	IC58607AbEcon4914	691	1.38E-49	Frameshift
		K	705	IC58607AbEcon4914	691	5.32E-50	Frameshift
				IC58607AbEcon108021	**424**	**1.24E-40**	**83**
		L	854	IC58607AbEcon4914	691	8.46E-50	Frameshift
		M	854	IC58607AbEcon4914	691	8.46E-50	Frameshift
		N	960	IC58607AbEcon4914	691	9.12E-50	Frameshift
glass	*gl*	A	604	IC58607AbEcon1730	2400	8.09E-34	Frameshift
		B	557	IC58607AbEcon1730	2400	7.91E-30	Frameshift
ken and barbie (okina)	*ken*	.	601	No Hit			
latheo	*lat*	.	721	IC58607AbEcon117029	405	8.85E-30	Frameshift
				IC58607AbEcon44526	356	1.21E-15	Frameshift
lingerer	*lig*	A	1343	No Hit			
		B	1332	No Hit			
		C	1343	No Hit			

		D	1375	No Hit			
		E	1332	No Hit			
		F	1332	No Hit			
		G	1332	No Hit			
lush	*lush*	A	153	No Hit			
		B	153	No Hit			
neither inactivation nor afterpotential B	*ninaB*	.	620	No Hit			
no on or off transient A (dissonance)	*nonA*	A	700	IC58607AbEcon13856	1689	1.77E-79	Frameshift
				IC58607AbEcon12495	**748**	**1.06E-15**	**Frameshift**
		B	742	IC58607AbEcon13856	1689	1.31E-79	Frameshift
				IC58607AbEcon12495	**748**	**5.20E-16**	**Frameshift**
		C	698	IC58607AbEcon13856	1689	1.84E-79	Frameshift
				IC58607AbEcon12495	**748**	**9.16E-16**	**Frameshift**
pale	*ple*	A	508	IC58607AbEcon2390	1692	8.20E-114	71
		B	579	IC58607AbEcon2390	1692	9.02E-114	71
paralytic (smellblind)	*para*	A	2131	No Hit			
		B	2131	No Hit			
		C	2131	No Hit			
		D	2114	No Hit			
period	*per*	.	143	No Hit			
prospero	*pros*	.	1403	No Hit			
		E	1835	No Hit			
		F	1703	No Hit			
		G	1674	No Hit			
quick-to-court	*qtc*	A	721	IC58607AbEcon22690	1348	1.38E-16	77
				IC58607AbEcon37583	**181**	**1.20E-06**	**66**
		B	566	IC58607AbEcon22690	1348	1.11E-16	77
				IC58607AbEcon37583	**181**	**8.54E-07**	**66**

		C	566	IC58607AbEcon22690	1348	1.11E-16	77
				IC58607AbEcon37583	**181**	**8.54E-07**	**66**
		D	598	IC58607AbEcon22690	1348	1.02E-16	77
				IC58607AbEcon37583	**181**	9.76E-07	**66**
		E	690	IC58607AbEcon22690	1348	1.42E-16	77
				IC58607AbEcon37583	**181**	**1.21E-06**	**66**
		F	721	IC58607AbEcon22690	1348	1.38E-16	77
				IC58607AbEcon37583	**181**	**1.20E-06**	**66**
		G	535	IC58607AbEcon22690	1348	1.01E-16	76
				IC58607AbEcon37583	**181**	**7.57E-07**	**66**
retained (dead ringer)	*retn*	A	906	IC58607AbEcon493	2084	9.24E-11	54
		B	911	IC58607AbEcon493	**2084**	9.61E-11	**54**
retinal degeneration B	*rdgB*	A	1259	IC58607AbEcon5724	2578	1.89E-60	62
				IC58607AbEcon82069	**441**	**1.23E-21**	**87**
		B	1250	IC58607AbEcon5724	2578	2.04E-60	62
				IC58607AbEcon82069	**441**	**1.23E-21**	**87**
		C	1259	IC58607AbEcon5724	2578	1.89E-60	62
				IC58607AbEcon82069	**441**	**1.23E-21**	**87**
		D	1259	IC58607AbEcon5724	2578	1.89E-60	62
				IC58607AbEcon82069	**441**	1.23E-21	**87**
		E	1241	IC58607AbEcon5724	2578	1.94E-60	62
				IC58607AbEcon82069	**441**	**1.24E-21**	**87**
		F	1250	IC58607AbEcon5724	2578	2.04E-60	62
				IC58607AbEcon82069	**441**	**1.23E-21**	**87**
		G	1237	IC58607AbEcon5724	2578	2.12E-60	62
				IC58607AbEcon82069	**441**	**1.15E-21**	**87**
		H	1263	IC58607AbEcon5724	2578	2.11E-60	62
				IC58607AbEcon82069	**441**	**1.19E-21**	**87**
sarah	*sra*	.	292	IC58607AbEcon2728	3242	1.03E-62	70
shibire	*shi*	A	830	IC58607AbEcon14936	670	5.75E-93	85

			IC58607AbEcon17959	**687**	**8.74E-89**	**90**
			IC58607AbEcon58283	**698**	**3.83E-59**	**86**
			IC58607AbEcon46538	**473**	**8.92E-46**	**72**
	B	830	IC58607AbEcon14936	670	5.75E-93	85
			IC58607AbEcon17959	**687**	**8.74E-89**	**90**
			IC58607AbEcon58283	**698**	**3.83E-59**	**86**
			IC58607AbEcon46538	**473**	**8.92E-46**	**72**
	C	830	IC58607AbEcon14936	670	5.75E-93	85
			IC58607AbEcon17959	**687**	**8.74E-89**	**90**
			IC58607AbEcon58283	**698**	**3.83E-59**	**86**
			IC58607AbEcon46538	**473**	**8.92E-46**	**72**
	E	830	IC58607AbEcon14936	670	5.75E-93	85
			IC58607AbEcon17959	**687**	**8.74E-89**	**90**
			IC58607AbEcon58283	**698**	**3.83E-59**	**86**
			IC58607AbEcon46538	**473**	**8.92E-46**	**72**
	F	877	IC58607AbEcon14936	670	4.85E-93	85
			IC58607AbEcon17959	**687**	**7.75E-89**	**90**
			IC58607AbEcon58283	**698**	**2.99E-59**	**86**
			IC58607AbEcon46538	**473**	**8.45E-46**	**72**
	G	877	IC58607AbEcon14936	670	4.85E-93	85
			IC58607AbEcon17959	**687**	**7.75E-89**	**90**
			IC58607AbEcon58283	**698**	**2.99E-59**	**86**
			IC58607AbEcon46538	**473**	**8.45E-46**	**72**
	H	830	IC58607AbEcon14936	670	5.75E-93	85
			IC58607AbEcon17959	**687**	**8.74E-89**	**90**
			IC58607AbEcon58283	**698**	**3.83E-59**	**86**
			IC58607AbEcon46538	**473**	**8.92E-46**	**72**
	I	830	IC58607AbEcon14936	670	5.75E-93	85
			IC58607AbEcon17959	**687**	**8.74E-89**	**90**
			IC58607AbEcon58283	**698**	**3.83E-59**	**86**
			IC58607AbEcon46538	**473**	**8.92E-46**	**72**

		J	877	IC58607AbEcon14936	670	4.85E-93	85
				IC58607AbEcon17959	**687**	**7.75E-89**	**90**
				IC58607AbEcon58283	**698**	**2.99E-59**	**86**
				IC58607AbEcon46538	**473**	**8.45E-46**	**72**
		K	877	IC58607AbEcon14936	670	4.85E-93	85
				IC58607AbEcon17959	**687**	**7.75E-89**	**90**
				IC58607AbEcon58283	**698**	**2.99E-59**	**86**
				IC58607AbEcon46538	**473**	**8.45E-46**	**72**
		L	883	IC58607AbEcon14936	670	4.89E-93	85
				IC58607AbEcon17959	**687**	**7.81E-89**	**90**
				IC58607AbEcon58283	**698**	**3.02E-59**	**86**
				IC58607AbEcon46538	**473**	**8.52E-46**	**72**
		M	836	IC58607AbEcon14936	670	5.80E-93	85
				IC58607AbEcon17959	**687**	**8.81E-89**	**90**
				IC58607AbEcon58283	**698**	**3.86E-59**	**86**
				IC58607AbEcon46538	**473**	**9.00E-46**	**72**
spinster	*spin*	A	605	IC58607AbEcon3330	2437	2.37E-155	71
		B	630	IC58607AbEcon3330	2437	1.66E-149	Frameshift
		C	605	IC58607AbEcon3330	2437	2.17E-149	Frameshift
		D	630	IC58607AbEcon3330	2437	1.73E-155	71
		E	402	IC58607AbEcon3330	2437	1.82E-95	Frameshift
timeless	*tim*	A	1389	IC58607AbEcon33168	830	4.43E-32	72
				IC58607AbEcon78538	**450**	3.79E-20	**65**
				IC58607AbEcon40748	**478**	2.37E-05	**90**
		B	1398	IC58607AbEcon33168	830	8.98E-32	71
				IC58607AbEcon78538	**450**	**3.78E-20**	**65**
				IC58607AbEcon40748	**478**	**2.42E-05**	**90**
		C	914	IC58607AbEcon33168	830	3.52E-32	72
				IC58607AbEcon40748	**478**	**1.47E-05**	**90**
		D	1421	IC58607AbEcon33168	830	5.14E-32	72
				IC58607AbEcon78538	**450**	**4.05E-20**	**65**

				IC58607AbEcon40748	**478**	**2.40E-05**	**90**
		E	1122	IC58607AbEcon33168	830	5.00E-32	72
				IC58607AbEcon78538	**450**	**2.96E-20**	**65**
				IC58607AbEcon40748	**478**	**1.87E-05**	**90**
		F	1122	IC58607AbEcon33168	830	5.00E-32	72
				IC58607AbEcon78538	**450**	**2.96E-20**	**65**
				IC58607AbEcon40748	**478**	**1.87E-05**	**90**
		G	1389	IC58607AbEcon33168	830	4.43E-32	72
				IC58607AbEcon78538	**450**	**3.79E-20**	**65**
				IC58607AbEcon40748	**478**	**2.37E-05**	**90**
		H	1389	IC58607AbEcon33168	830	4.43E-32	72
				IC58607AbEcon78538	**450**	3.79E-20	**65**
				IC58607AbEcon40748	**478**	2.37E-05	**90**
transformer	*tra*	A	197	No Hit			
		B	36	No Hit			
yellow	*y*	.	541	IC58607AbEcon16600	676	8.98E-16	51

Appendix C Cuticular hydrocarbon profiles of a wild *Gryllus campestris* population

C.1 Background

We have been studying a small population of *Gryllus campestris* that lives in a meadow in northern Spain. Adults emerge from their burrows in late spring, at which time each individual in the population is caught. Each individual is labelled with an ID tag, and a sample of their cuticular hydrocarbons (CHCs) taken, as well as tissue for genetic analyses. They are then returned to their burrows and are tracked using a network of infrared video cameras through the mating season (early summer). Behavioural data for each individual is recorded from these videos. We intend to look for relationships between CHC expression and other traits, for example reproductive fitness. Here I present the CHC profiling component of this research.

C.2 Methods

A sample of CHC was removed from the crickets by rubbing their body with filter paper. These samples were then processed and injected into a GCMS (Gas Chromatograph coupled with a Mass Spectrometer). Data were analysed using MSD Chemstation software (Agilent Technologies). 58 males and 101 females were successfully analysed. Males and females were analysed separately as their CHC profiles differ. Expression in known to be sexually dimorphic in *G. bimaculatus* (Tregenza & Wedell, 1997), and visual inspection of the *G. campestris* chromatograms showed differences between males and females in the number of peaks. 35 CHCs were quantified for each male, and 52 for each female. Relative peak size was calculated by dividing the peak areas of a given sample by the peak area of the internal standard (pentadecane) in that sample. These relative peak sizes were then normalised with a log transformation. Separate principal components analyses (PCA) were then run for the female and male data (SPSS v19). We used a correlation matrix to extract PCs with eigenvectors greater than 1 (Norman & Streiner, 1984). PC factor loadings greater than 0.25, or less than -0.25, were considered biologically significant (Tabachnick & Fidell, 1989).

C.3 Results

The male PCA returned 7 PCs, with eigenvectors that cumulatively explained 82.9% of the variance (Table C.1). The female PCA returned 11 PCs, with eigenvectors that cumulatively explained 80.6% of the variance (Table C.2). For both males and females almost all factor loadings in PC1 were of a magnitude that is likely to be biologically significant (Tabachnick & Fidell, 1989) and positively

loaded, suggesting that overall investment in CHCs is important. Of the CHCs that had significant factor loadings in PC2, for males the shorter chain molecules tended to be positively loaded and the longer chain molecules negatively loaded (Table C.3), while the opposite was true for females (Table C.4).

Table C.1. Variance explained in male data. 7 PCs extracted with eigenvectors >1 cumulatively explain at least 83% variance.

	Eigenvalue	% of Variance	Cumulative %
PC1	13.403	38.294	38.294
PC2	7.679	21.939	60.233
PC3	2.528	7.224	67.457
PC4	1.814	5.184	72.641
PC5	1.394	3.983	76.624
PC6	1.125	3.215	79.839
PC7	1.087	3.106	82.945

Table C.2. Variance explained in female data. 11 PCs extracted with eigenvectors >1 cumulatively explain 80% variance.

	Eigenvalue	% of Variance	Cumulative %
PC1	17.653	34.614	34.614
PC2	7.135	13.990	48.604
PC3	3.990	7.823	56.427
PC4	2.632	5.160	61.587
PC5	1.832	3.592	65.178
PC6	1.595	3.128	68.306
PC7	1.556	3.051	71.356
PC8	1.359	2.666	74.022
PC9	1.252	2.455	76.477
PC10	1.063	2.085	78.562
PC11	1.036	2.031	80.593

Table C.3. Male CHC profile, displaying factor loadings in each of the PCs. Hydrocarbons are ordered in increasing chain length. Factor loadings of >0.25 are highlighted in bold.

Retention time	PC1	PC2	PC3	PC4	PC5	PC6	PC7
8.694	**0.25**	**0.512**	**0.082**	-0.023	-0.051	**0.119**	**0.15**
8.907	**0.335**	**0.781**	0.017	-0.168	-0.15	-0.151	**0.094**
8.966	**0.431**	**0.87**	0.016	-0.075	-0.146	-0.081	**0.06**
9.014	**0.399**	**0.877**	-0.064	-0.071	-0.111	-0.08	**0.069**
9.068	**0.324**	**0.882**	-0.031	-0.114	-0.157	-0.117	**0.082**
9.277	**0.589**	**0.627**	-0.124	-0.222	**0.05**	-0.099	**0.074**
9.843	**0.531**	**0.782**	-0.002	-0.163	-0.063	-0.044	**0.063**
10.836	**0.185**	**0.61**	**0.18**	-0.079	-0.126	**0.132**	-0.013
11.218	**0.391**	**0.463**	-0.14	**0.432**	**0.477**	-0.202	**0.152**
11.855	**0.688**	-0.034	-0.122	**-0.294**	**0.11**	**0.095**	-0.153
11.977	**0.498**	**0.508**	**0.211**	**0.178**	-0.171	**0.075**	-0.129
12.069	**0.634**	**0.153**	0.019	**-0.282**	**0.197**	**0.081**	**-0.489**
12.560	**0.754**	**0.319**	-0.119	**0.259**	**0.166**	**0.056**	**-0.314**
13.334	**0.348**	**-0.385**	-0.023	**-0.349**	**0.33**	**0.137**	**0.13**
13.594	**0.793**	**0.533**	-0.064	-0.104	0.012	-0.071	**0.034**
13.741	**0.402**	-0.172	**0.88**	-0.087	-0.087	0.006	0.009
14.256	**0.49**	**0.369**	**0.153**	-0.015	**0.349**	**0.414**	-0.076
14.728	**0.705**	-0.155	-0.083	**0.091**	-0.157	**0.497**	-0.109
14.944	**0.822**	-0.017	-0.077	-0.118	-0.075	**0.052**	-0.042
15.183	**0.812**	**-0.447**	-0.068	**-0.305**	**0.106**	-0.112	0.02
15.356	**0.793**	**-0.419**	-0.13	**-0.261**	**0.135**	-0.176	**0.049**
15.481	**0.891**	**-0.343**	-0.153	-0.012	**0.026**	-0.014	0.013
16.133	**0.437**	**0.22**	-0.133	**0.482**	**0.443**	-0.117	**0.036**
16.347	**0.619**	**-0.277**	-0.052	0.013	-0.184	0.025	**-0.488**
16.841	**0.642**	-0.204	-0.101	-0.022	-0.022	**0.402**	**0.492**
17.679	**0.508**	**0.247**	-0.184	**0.599**	-0.017	0.015	-0.123
17.876	**0.475**	-0.115	**0.842**	**0.145**	**0.1**	-0.076	**0.035**
18.587	**0.471**	-0.114	**0.843**	**0.146**	**0.104**	-0.075	**0.034**
19.395	**0.859**	**-0.372**	-0.047	-0.165	0.019	-0.131	**0.099**
19.720	**0.857**	**-0.427**	-0.048	-0.114	-0.01	-0.166	**0.068**
19.943	**0.794**	**-0.519**	-0.063	**0.046**	-0.088	-0.147	**0.068**
20.050	**0.806**	**-0.488**	-0.072	**0.093**	-0.085	-0.125	**0.08**
20.325	**0.805**	**-0.393**	-0.077	**0.239**	**0.027**	-0.04	**0.126**
22.129	**0.701**	-0.188	-0.094	**0.33**	**-0.385**	**0.331**	**0.194**
25.533	**0.53**	**-0.371**	-0.055	**0.23**	**-0.488**	**-0.313**	-0.111

Table C.4. Female CHC profile, displaying factor loadings in each of the PCs. Hydrocarbons are ordered in increasing chain length. Factor loadings >0.25 are highlighted in bold.

Retention time	PC1	PC2	PC3	PC4	PC5	PC6	PC7
8.696	**0.783**	**-0.509**	-0.085	0.116	0.186	0.102	0.019
8.908	**0.816**	**-0.464**	-0.14	0.08	0.158	0.115	0.02
8.967	**0.8**	**-0.496**	-0.115	0.107	0.17	0.099	0.029
9.016	**0.819**	**-0.461**	-0.139	0.09	0.157	0.114	0.037
9.070	**0.79**	**-0.504**	-0.18	0.083	0.155	0.104	0.013
9.212	**0.562**	-0.139	**-0.672**	0.074	-0.159	0.076	-0.082
9.278	**0.7**	**-0.335**	-0.187	0.13	0.048	-0.197	0.038
9.733	**0.639**	-0.036	0.148	-0.028	0.084	0.079	0.128
9.844	**0.803**	**-0.419**	-0.07	-0.015	0.057	-0.076	-0.026
10.839	**0.628**	**-0.461**	-0.177	0.196	**0.26**	0.047	-0.012
10.912	**0.662**	-0.206	**-0.513**	-0.04	-0.016	0	-0.13
11.201	**0.743**	**-0.396**	-0.034	0.071	0.076	-0.116	-0.02
11.987	**0.789**	-0.191	0.175	0.001	-0.084	-0.045	0.112
12.074	**0.699**	**-0.438**	-0.042	0.095	**0.269**	0.041	0.022
12.569	**0.665**	-0.215	**0.319**	-0.072	-0.246	0.146	-0.014
12.668	**0.317**	**0.292**	**0.309**	**0.478**	**-0.281**	-0.023	0.048
12.8	0.205	-0.057	0.103	**0.286**	**-0.517**	**0.34**	0.078
13.143	**0.445**	0.114	**0.365**	**0.258**	**-0.418**	0.081	-0.003
13.594	**0.753**	**-0.262**	0.148	-0.016	-0.107	-0.124	-0.084
13.755	**0.717**	-0.241	0.222	0.063	-0.214	0.029	0.114
14.207	**0.556**	0.159	-0.104	0.036	0.102	0.054	-0.054
14.276	**0.713**	-0.103	0.141	0.083	**-0.291**	-0.029	0.146
14.405	**0.776**	-0.083	0.016	-0.063	**-0.313**	-0.059	-0.148
14.638	**0.829**	0.068	-0.098	-0.221	-0.249	-0.129	-0.224
14.729	**0.771**	0.03	0.225	-0.117	-0.093	**-0.281**	**-0.254**
14.968	**0.81**	0.065	0.2	-0.05	-0.052	**-0.384**	-0.093
15.271	0.072	-0.226	**0.597**	**-0.274**	0.238	**0.281**	-0.06
15.462	**0.728**	**0.31**	**0.428**	-0.146	0.028	0.051	0.121
16.165	**0.48**	**0.261**	**0.324**	0.009	-0.069	0.029	**0.411**
16.333	**0.398**	**0.441**	**0.392**	0.144	0.133	0.178	**-0.403**
16.439	**0.465**	**0.548**	-0.173	**-0.264**	-0.007	0.195	**-0.375**
16.834	**0.651**	**0.347**	**0.308**	-0.199	0.036	-0.11	**-0.297**
17.064	**0.621**	**0.552**	**0.424**	0.193	0.081	-0.017	0.052
17.246	**0.462**	**0.516**	**-0.425**	0.048	**-0.252**	-0.125	-0.087
17.689	**0.447**	**0.35**	-0.118	**-0.426**	0.055	**0.346**	0.097
17.885	**0.524**	**0.379**	**0.341**	**-0.323**	0.16	0.052	0.126
18.594	**0.534**	0.219	0.17	**-0.558**	0.021	0.061	**0.378**
18.823	**0.427**	**0.45**	**-0.396**	**-0.424**	0.004	0.024	-0.097
19.166	**0.529**	**0.309**	**-0.349**	**-0.294**	-0.175	**-0.29**	-0.04
19.357	**0.397**	**0.528**	**-0.266**	0.139	0.024	**-0.305**	**0.255**
19.592	**0.285**	**0.523**	-0.056	0.214	**0.259**	**-0.286**	0.227

20.025	0.033	**-0.258**	**0.53**	**-0.253**	**0.251**	**-0.385**	0.017
20.300	**0.333**	**0.368**	-0.2	0.067	-0.05	**0.526**	0.055
21.303	**0.266**	**0.435**	**-0.303**	**-0.266**	0.055	0.101	0.166
21.466	0.244	**0.592**	0.079	**0.408**	0.141	0.07	**-0.375**
22.11	**0.524**	**0.36**	0.233	-0.021	**0.289**	0.188	-0.21
22.321	0.135	**0.578**	-0.016	**0.51**	0.248	-0.06	-0.07
22.435	**0.385**	**0.569**	**-0.426**	-0.017	0.165	-0.002	0.009
22.716	**0.316**	**0.632**	-0.093	**0.266**	0.204	-0.045	**0.27**
22.922	**0.386**	**0.3**	0.031	**0.458**	0.09	0.043	0.117
26.653	**0.552**	**0.299**	-0.234	-0.049	-0.098	0.052	**0.327**

Table C.4. Continued

Retention time	**PC8**	**PC9**	**PC10**	**PC11**
8.696	0.078	-0.17	0.016	0
8.908	0.067	-0.159	-0.025	-0.017
8.967	0.082	-0.156	-0.004	-0.023
9.016	0.075	-0.151	-0.024	-0.033
9.070	0.083	-0.155	-0.014	-0.045
9.212	0.218	-0.051	-0.084	-0.13
9.278	-0.205	0.05	-0.096	-0.109
9.733	-0.24	0.103	-0.119	**-0.252**
9.844	-0.114	0.075	0.066	-0.02
10.839	0.039	0.134	0.12	0.108
10.912	0.07	**0.26**	**0.275**	0.008
11.201	-0.118	**0.294**	0.118	0.145
11.987	0.068	0.132	-0.168	**0.27**
12.074	0.036	**-0.285**	0.031	-0.098
12.569	-0.018	0.085	-0.062	-0.171
12.668	0.052	-0.144	**0.309**	0.043
12.8	0.1	0.215	0.196	-0.001
13.143	0.038	-0.243	**0.299**	-0.143
13.594	-0.094	0.07	-0.032	0.077
13.755	-0.025	0.142	-0.118	0.076
14.207	**-0.253**	-0.004	0.21	0.121
14.276	0.105	0.038	**-0.316**	0.193
14.405	0.041	-0.047	-0.084	**0.28**
14.638	0.089	0.021	0.084	-0.055
14.729	-0.085	-0.071	-0.033	0.002
14.968	-0.088	-0.077	0.063	-0.045
15.271	0.104	**0.254**	0.243	0.049
15.462	-0.111	-0.072	-0.026	-0.092
16.165	0.119	-0.144	0.083	-0.004
16.333	0.094	-0.146	-0.155	0.053

16.439	0.078	-0.039	0.066	-0.07
16.834	**-0.293**	0.045	0.012	-0.125
17.064	0.099	-0.049	-0.041	0.039
17.246	0.196	-0.021	**-0.259**	0.074
17.689	**0.321**	0.168	-0.115	-0.058
17.885	0.078	-0.13	-0.024	-0.238
18.594	0.072	-0.08	0.01	0.175
18.823	0.167	-0.089	**0.26**	-0.195
19.166	0.04	0.127	0.206	-0.036
19.357	-0.221	0.036	-0.008	0.063
19.592	-0.157	-0.205	0.149	-0.043
20.025	**0.404**	0.123	0.002	0.09
20.300	**-0.462**	-0.057	0.031	0.159
21.303	0.001	**-0.302**	0.096	**0.491**
21.466	0.064	-0.109	**-0.285**	-0.01
22.11	-0.247	**0.298**	-0.024	0.145
22.321	**0.356**	0.06	0.12	0.07
22.435	-0.006	0.15	-0.073	0.095
22.716	0.084	**0.255**	0.053	-0.035
22.922	0.182	**0.331**	0.029	-0.135
26.653	-0.091	0.116	-0.25	**-0.345**

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