THE CAUSES AND CONSEQUENCES OF REPRODUCTIVE INTERFERENCE IN THE LYGAEIDAE

Emily Rose Burdfield-Steel

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Declarations

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I Emily Burdfield-Steel hereby certify that this thesis, which is approximately 57,000 words in length, has been written by me, and that it is the record of work carried out by me, or principally by myself in collaboration with others as acknowledged, and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in September 2010 and as a candidate for the degree of PhD in August 2011; the higher study for which this is a record was carried out in the University of St Andrews between 2010 and 2014.

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Co-authorship statement

Chapter 1 is an independent interpretation of the current literature under the guidance of David Shuker. Mick Webb provided access to specimens in the Natural History Museum, London for the estimation of rates of aposematism in the Lygaeidae.

I conducted the experiments and data collection, performed the analysis and wrote the manuscripts for chapters 2, 3 and 6 under the guidance of David Shuker.

Chapter 4 was a collaboration between myself, Terry Smith, Ginny Greenway, Sam Auty and David Shuker. I collected and extracted the specimens. The analysis of the cuticular hydrocarbons was done in collaboration with Dr Terry Smith. The behavioural experiments were performed with the assistance of Ginny Greenway and Sam Auty. Paris Veltsos provided an R script for performing principle component analysis on CHC peaks which I then modified with the help of Darren Parker.

Experimental work and data collection for chapter 5 was carried out in collaboration with Liam Dougherty, Lynsey Smith and Laura Collins. I performed the analysis under the guidance of David Shuker. The data described in this chapter has been published as Burdfield-Steel, Dougherty, Smith, Collins and Shuker, Variation in social and sexual behaviour in four species of aposematic seed bugs (Hemiptera: Lygaeidae): The role of toxic and non-toxic food in *Behavioural Processes.* See Appendix 3.

The data collection for chapter 7 was done with the assistance of Ginny Greenway and Sam Auty. I performed the analysis under the guidance of David Shuker.

I wrote chapter 8 under the guidance of David Shuker.

Abstract

Reproductive interference occurs when individuals direct sexual behaviours towards other species and this causes a reduction in the fitness of one or both of the participants. While initially considered to be little more than an aberration, reproductive interference is increasingly recognised, not just as a factor in determining species co-existence and community dynamics, but also as an opportunity to further our understanding of sexual selection. In this thesis I investigate the causes and consequences of reproductive interference between several species of true bugs (Order: Hemiptera) in the family Lygaeidae. These species have a polgynandrous mating system, in which both males and females mate multiple times. I found that the interspecific mating attempts often witnessed in these bugs is likely a consequence of this mating system, as potential cues for species discrimination, in the form of cuticular hydrocarbons, are available and can be utilised by the bugs in some contexts. This is further supported by the finding that pre-copulatory selection on factors such as diet and chemical protection are weak in these insects. Furthermore, my work highlights the context-dependant nature of both the fitness costs associated with reproductive interference, and also of the bugs' intra-specific behaviour. When housed in groups, female *Lygaeus equestris* did not show consistent fitness losses in response to harassment by either conspecific or heterospecific males, despite previous evidence showing that such males inflict costs on both egg production and longevity when interacting one-to-one. This, combined with the finding that male *L. equestris* alter their mate-guarding behaviour in the presence of other males, highlights the potential of behavioural flexibility to influence the outcome of inter-species interactions, and the importance of context when attempting to measure phenomena such as reproductive interference.

Chapter 1

Introduction

1. An Introduction to Reproductive Interference

Reproductive interference (henceforth referred to as RI) occurs when individuals of one species display reproductive behaviours towards individuals of a different species and these behaviours result in a loss of fitness for one or both species (Gröning and Hochkirch, 2008, Burdfield-Steel and Shuker, 2011; see also Appendix 1). These reproductive behaviours can take many forms, ranging from overlap in sexual signalling (Amezquita et al., 2006), through to heterospecific courtship (Fea et al., 2013) and mating attempts (Andrews et al., 1982, de Bruyn et al., 2008, Cothran et al., 2013, Kyogoku and Nishida, 2013). While such interactions will automatically lead to wasted reproductive effort, the ultimate costs (in terms of a reduction in an individual's fitness) of RI are likely to vary based both on the form of RI occurring and the life history and mating system of the species involved. It is thought that heterospecific mating attempts are likely to carry the highest costs due to gamete wastage, energetic expenditure and, potentially, physical damage. In addition, in species where hybridisation can occur, this may come with its own fitness consequences (Rhymer and Simberloff, 1996).

In order to clarify the behaviours that constitute RI in my study species, and their potential evolutionary significance, I will first introduce the forms of RI, and their potential consequences, before discussing the role of RI in shaping species' ecology and evolution. Given that sexual conflict is known to occur in the Lygaeidae, and that two of my study species are capable of hybridisation, I will briefly cover the application of theory developed in the study of sexual conflict and hybridisation to RI. Finally, I will discuss the factors expected to influence the occurrence of reproductive interference in the wild as these may differ from those detected in laboratory studies such as mine.

1.1 Types of reproductive interference

As previously mentioned, one form of RI is misdirected courtship. As the name would suggest this is where an organism directs courtship behaviour towards an individual of a different species. In some cases this may lead into another form of reproductive interference: heterospecific mating attempts (Cothran et al., 2013). It should be noted, however, that the one form is not required for the other as many species lack noticeable pre-mating courtship behaviours (for example, see section 2 and subsequent chapters for the lack of courtship in lygaeid bugs). In the event that successful mating is possible between the two species in question, a further form of interference may occur: heterospecific mating and hybridization. Leaving aside the question of hybridisation for the time being, it is expected that, on average, such heterospecific matings will carry the highest immediate fitness costs. Not only will these matings produce no viable offspring, and hence pass no genes to the next generation, but they can also result in multiple indirect costs that will ultimately impact fitness. It has been well documented that mating behaviour can inflict penalties on female fecundity as well as survival in both sexes (Simmons, 2001, den Hollander and Gwynne, 2009, Shuker et al., 2006) even when undertaken with conspecific mates. Heterospecific matings carry not only these potential costs, but also the risk of physical damage from incompatible morphologies (Rönn et al., 2007) as well as threat of attack from the perceived "mate". One extreme example occurs in the mantid *Orthodera novaezealandiae* from New Zealand. Males of this species are attracted to the pheromone of the females of an invasive species, *Miomantis caffra*, and attempt to copulate with them. As *M. caffra* females show high levels of sexual cannibalism, such attempts frequently end in the male's death (Fea et al., 2013). A similar risk of cannibalism is experienced by the gift giving spider species *Paratrechalea ornate* (Costa-Schmidt and Machado, 2012). Even when mating does not occur, misdirected courtship can reduce both mating rate and future mating opportunities (Gröning et al., 2007). Male reptile ticks, *Amblyomma albolimbatum*, have been observed attempting to court females of the species *Aponomma hydrosauri*. Although unable to copulate with heterospecific females they re-attached to the host following courtship with their ventral surface against the female. This behaviour blocks access to her genital aperture and prevents conspecific males from mating with her for a period of more than 20 days (Andrews et al., 1982). Additionally, when courtship includes gifts, as in both *P.ornate* and its sibling species *P. azul*, misdirected courtship and mating attempts may also result in the loss of the nuptial gift (Costa-Schmidt and Machado, 2012), which often represents a significant expenditure for the male. While courtship gifts may increase the costs of RI for the sex that provides them, they may reduce the cost of inter-specific courtship or matings for the sex that receives the gift. Selection against RI is often thought to be stronger in females (Parker and Partridge, 1998: see section 1.3), however when females receive nutritional benefits from mating this may not be the case (Doherty and Howard, 1996).

Even when the fitness costs associated with mating itself are low, the ultimate consequences of such matings is expected to vary depending on the biology and reproductive strategy of the species involved. For example, in a species that mates only once such a mistake would be disastrous and reduce the fitness of the affected individual to zero. In contrast, in species that mate multiply, interspecific matings, while they may still waste both time and mating effort, are expected to enact a lesser cost on lifetime fitness. The species-specific nature of RI may explain why the fitness costs it inflicts often appear to be asymmetric (i.e. one species suffers more than another; Gröning et al., 2007).

In addition to this spectrum of misdirected inter-sexual behaviours, reproductive interference may take the form of heterospecific rivalry, also referred to as interspecific aggression. In this, individuals, often males, mistakenly perceive members of another species as potential rivals for mates and thus behave aggressively towards them. This is most commonly seen in territorial species (Brenowitz, 1982, Ord and Stamps, 2009) and is expected to be non-adaptive when territories are held solely for reproductive, rather than resource-based, purposes (Ord et al., 2011, Peiman and Robinson, 2010). Heterospecific rivalry has been found to drive character displacement in wing spots, a sexually selected trait, in the damselfly *Calopteryx splendens* (Tynkkynen et al., 2004). For a detailed review of how interspecies aggression can influence character displacement see (Grether et al., 2009).

Other recognized forms include erroneous (i.e. misdirected) female choice, where females prefer heterospecifics as mates, or potential mates, over conspecific males, as seen for example in pacific island geckos (Dame and Petren, 2006). When females show strong preferences for certain male traits, such as large body and territory size in cichlid fish, heterospecific males possessing these traits may be preferred over conspecific males that do not (Gerlai, 2007). Erroneous male choice has also been reported in a number of species including mantids (Fea et al., 2013) and crayfish (Butler and Stein, 1985). Both of these can exacerbate the effects of other forms of RI by increasing the frequency and intensity of intraspecific sexual interactions.

Another potentially common form of RI is so-called “signal jamming”, whereby the behaviour or ‘signals’ produced by one species in some way disrupt those necessary for successful reproduction in another species. (It should be noted that this is distinct from conspecific "signal-jamming" that can occur in response to intra-species mate competition and sexual conflict: Tobias and Seddon, 2009). An example of signal jamming would be the calls of one frog species interfering with the ability of a second species to locate mates. This could be the result of the conspecific calls simply masking those of heterospecifics, or it could be that the calls confuse individuals of the second species and cause them to approach conspecific callers rather than those of their own species. Evidence has been found in Amazonian frogs for decreased sensitivity in male *Allobates femoralis* to calls that fall within the range of *Epipedobates trivittatus* calls in areas where the two species co-occur. This sensory adaptation may represent a mechanism by which *A. femoralis* can avoid masking interference resulting from the overlapping frequency range of *E. trivittatus* calls (Amezquita et al., 2006). As these calls form the basis of male-male communication in these territorial frogs, responses to *E. trivittatus* calls by *A. femoralis* males would represent both ‘signal jamming’ and potentially also heterospecific rivalry. Signal jamming can also occur in other communication systems. Many chemical communication systems are known to be susceptible to environmental disturbance (Fisher et al., 2006) and the presence of heterospecific signals could conceivably lower signal efficiency. In the case of the reptile ticks described previously, male mate searching was reduced when heterospecific females were present on the same host, presumably due to interference in long-range pheromones (Andrews et al., 1982). Multiple forms of RI can often occur simultaneously. Indeed, phenomena such as signal jamming and erroneous mate preferences frequently result in heterospecific mating attempts (Andrews et al., 1982).

Finally, one particular case that should also be mentioned when considering RI is the occurrence of gynogenetic species. Gynogenesis is a form of parthenogenesis that requires sperm to trigger embryonic development. Despite this dependence on sperm, embryos produced in this manner contain only maternal chromosomes. Thus, gynogenetic species are almost exclusively female and require matings with males of close-related species in order to reproduce. As the males that mate with these females pass no genes to the offspring produced, gynogenetic species can be thought of as “sexual parasites”. One classic example is the Amazon Molly (*Poecilia formosa*) (Hubbs and Hubbs, 1932). The result of hybridisation between *Poecilia latipinna* and *Poecilia mexicana* (Lampert et al., 2005), this species can only occur sympatrically with one of its “host” species (Monaco et al., 1984). Given that the males of these host species do not gain paternity from these encounters we would expect rapid evolution of male preference for females of their own species in areas where *P. formosa* occurs. Indeed, there is evidence of reproductive character displacement in male *P. latipinna* mate preferences in areas where they co-occur with *P. formosa*. Males from sympatric populations show a stronger preference for conspecific females (Gabor and Ryam, 2001). Interestingly, it seems male *P. latipinna* may actually gain some benefits from these heterospecific matings. Schlupp et al (1994) found that males can increase their attractiveness to conspecific females by associating with *P. formosa*, although it is not clear if this is sufficient to outweigh the potential fitness costs of mating with them (Schlupp et al., 1994). Gynogenetic species are perhaps a special case however, as typically there are, by definition, no mating interactions between truly parthenogenetic species and so no reproductive interference (as defined above). With the exception of gynogenetic species, parthenogenetic species will only influence RI when they become a target for mis-directed mating interactions (for instance if there are closely related sexual and asexual species).

The above discussion considers examples of reproductive interference in the context of interactions between different animal species. However, RI is also known to occur in plants (and presumably also occurs in sexually reproducing fungi, where complementary mating types from different species may interfere with intra-specific mating competition; for an example of sexual selection in isogamous yeast see Rogers and Grieg 2009). Perhaps the most common form of RI in plants is pollen interference, whereby pollen from one species is transported to or lands on the stigma of another species. Heterospecific pollen may either prevent the deposition of con-specific pollen, displace existing pollen, or even block pollen tube formation by con-specific pollen (for a recent review see Ashman and Arceo-Gomez 2013). For the most part however, I will consider RI amongst animal species.

1.2 Ecological and evolutionary consequences of RI

Evidence for reproductive interference has now been found across a wide range of taxa (McLain and Shure, 1987) particularly in the context of invasive species (Dame and Petren, 2006, Liu et al., 2007) and hybridization (Mallet, 2005). A review of the field in 2008, by Gröning and Hochkirch, found reports of reproductive interference in 167 bi-species systems, excluding studies of hybridization. However the authors note that the ecological effects of reproductive interference are still poorly understood (Gröning and Hochkirch, 2008). While some reported cases may seem unlikely to have detectable effects on either the ecology or evolution of the species involved (de Bruyn et al., 2008), studies of invasive species have shown that reproductive interference may contribute to the displacement of native species (Dame and Petren, 2006). Evidence has also been found suggesting that reproductive interference between naturally co-occurring species may have sufficient fitness effects to influence their ecology, particularly habitat use (McLain and Pratt, 1999, McLain and Shure, 1987). Experiments with ground hoppers (Terigidae) suggest that RI can significantly reduce the fitness of both individuals and populations (Gröning et al., 2007). The authors suggest that RI can have comparable effects to competition and result in ‘sexual exclusion’ of one species from an area of habitat. They also observe that such effects are expected to be density dependent. Similarly, recent work on ladybirds suggests that asymmetric RI may select for specialisation, as specialised habitats may act as refugia (Noriyuki et al., 2012). The work of Pfennig and colleagues on the hybridization of spadefoot toads has found that selection to avoid hybridization can cause females to compromise on mate quality choice (Pfennig, 2000). The loss of the benefits associated with selecting high quality mates in this species may have consequences for the population as a whole as it weakens sexual selection and may lower its overall fitness. Thus RI may have complex effects on the biology and behaviour of the affected species. In particular, RI may influence mating strategy. For example, heterospecific rivalry may affect male mate-guarding behaviours or sperm allocation. In particular, this may cause over-investment in strategies to counteract apparent sperm competition. While evidence for such processes in males remains scarce, there is mounting evidence that the presence of heterospecifics can significantly alter female reproductive behaviour, particularly in the context of resource allocation (Miller et al., 2013) and sex allocation (Ivens et al., 2009). Thus RI has the potential to arise through more subtle processes as well as overt heterospecific mating attempts.

1.3 Relationship between RI and sexual conflict

Sexual conflict occurs when the evolutionary interests of the sexes differ. Despite much of the discussion around within- and among-population sexual conflict taking place in the context of reproductive isolation and speciation (e.g. Parker and Partridge, 1998, Gavrilets, 2000), much of the theory developed can also be applied to RI. Sexual conflict can occur in two ways, either conflicting selection on alleles at a single loci that influence a trait expressed in both males and females (intra-locus sexual conflict), or behavioural or physiological conflict between the sexes based on selection across many loci (inter-locus sexual conflict: Parker, 1979, Lessells, 1999, Chapman et al., 2003, Arnqvist and Rowe, 2005). Both forms can lead to sub-optimal outcomes for one or both sexes. The first form has been implicated in the reduced fitness often seen in hybrids. Such intra-locus conflict is often resolved via mechanisms that cause sex-limited expression and these may break down as a result of hybridisation (Parker and Partridge, 1998). It can also affect the chances of inter-population, and potentially interspecies, matings during periods of secondary contact. The second form can result in many different behavioural and physiological arms races between the sexes (Arnqvist and Rowe, 2005) but for the purposes of this discussion I will focus on one example to illustrate its effects on RI, conflict over mating frequency (Parker, 1979). This usually involves males coercing females to mate at a rate above (or in some cases below (Chapman et al., 1995)) the female optima (Shuker et al., 2006, Shuker and Day, 2001, Shuker and Day, 2002). Conflict over mating can take many forms and may even continue after fertilization (Chapman et al., 1995, Wigby et al., 2009, Perry et al., 2013). The co-evolution of male coercion and female resistance can result in males having greater mating success with females from different populations, as these will lack the co-evolved resistance present in females of their own population. Furthermore, if, as has been suggested (Parker and Partridge, 1998, Parker, 1979), females are likely to show robust species discrimination, processes that manipulate or circumvent female choice may increase the likelihood of RI. It should be noted, however, that such models assume that hybridisation is possible between the populations, and so current models only include true reproductive interference at the limits of their parameter space.

1.4 Hybridisation

As with sexual conflict, hybridisation has been heavily investigated in the context of speciation (Strasburg and Rieseberg, 2013). In a broad sense, we may expect the costs of RI to be lower when hybridisation is possible, as individuals do not suffer immediate gamete wastage. However, hybrids often show lower fitness than “pure-bred” offspring (but see Arnold, 1997). In addition to the breakdown of sex-limited expression mentioned above, this reduction in fitness can occur for several reasons including (but not limited to): the disruption of co-adapted gene complexes (Burke and Arnold, 2001), and chromosomal rearrangements (Rhode and Cruzan, 2005, Rieseberg, 2001). The extent of this fitness loss will, to a great extent, determine the outcome of hybridisation. If the hybrids are able to mate or backcross to the parentals then processes such as reinforcement may occur (Butlin, 1987, Liou and Price, 1994). This is the evolution of prezygotic reproductive isolation in zones where species, or populations, overlap, in response to selection against hybrids (Howard, 1993).

It should be noted that not all hybrids show this reduction in fitness. Within the plant literature successful hybridisation is a well-recognised phenomenon, and many plant species may even have a hybrid origin (Seehausen, 2004). While adaptive hybridisation is rarer in animals a few examples do exist (Veen et al., 2001). One particularly striking example takes place between two species of spadefoot toads. *Spea bombifrons* and *S. multiplicata* occur sympatrically. Hybridisation between the two species is usually costly to long term fitness as the resulting offspring show reduced breeding ability and, as previously mentioned*, S. multiplicata* females will compromise on mate quality to ensure they do not mate with *S. bombifrons* (Pfennig, 2000). However, hybrid tadpoles from *S. bombifrons* females and *S. multiplicata* males have faster development times than pure *S. bombifrons* tadpoles. As the toads breed in ephemeral pools that often dry out as the season progresses, this faster development can lead to higher survival of these hybrids in smaller pools. Thus, in some contexts, it may be beneficial *for S. bombifrons* females to mate with *S. multiplicata* males. This may explain the prevalence of these matings in this system as species recognition between the two is thought to be robust (Pfennig and Simovich, 2002).

1.5 Occurrence of RI

Much of the theory on the occurrence of hybridisation can be applied to the occurrence of RI more generally. For example, there is considerable evidence that hybridisation is more likely to occur in complex or disturbed environments. This is likely to be due to a combination of processes including impaired sexual communication, increased costs associated with mate-searching (for example greater energy expenditure or predation risk) (Willis et al., 2011, Willis et al., 2012) and scarcity of conspecifics (Rosenthal, 2013). Such observations, in combination with species discrimination theory, lead to several clear predictions about the occurrence of RI. Firstly, encounter rate should affect how animals behave towards heterospecifics. If encounters with a particular species are rare, the costs of investing in a recognition system (in the form of increased investment in neural mechanisms at the expense of reproduction, or simply a delay in mating that reduces lifetime fitness) may not outweigh the benefits. Secondly, closely-related species may be harder to distinguish due to similarity of cues used in discrimination, as well as insufficient evolutionary time to develop reliable discrimination mechanisms. Finally, the sex of the interacting individuals (Parker and Partridge, 1998), as well as the timing of the interaction, will affect the potential fitness costs and benefits of discrimination (Ord et al., 2011). Despite this, a meta-analysis by Ord et al in 2011 failed to find consistent effects of any of these factors on the response of individuals to heterospecifics. Instead, they concluded that the benefits of species discrimination appear to be highly species specific. Aspects of species biology that were implicated included, the spacing patterns of conspecifics, the intensity of sexual selection and predation pressure (Ord et al., 2011). Thus, if we extend this finding to reproductive interference, current evidence suggests that multiple aspects of species biology and ecology will influence not just the likelihood of RI occurring, but also its consequences. Given this, it is unsurprising that RI has often been found to be asymmetric (Gröning et al., 2007, Noriyuki et al., 2012, Liu et al., 2007).

The aim of my research is to use five species of seed bug in the family Lygaeidae to investigate the ecological, and possibly evolutionary, effects of reproductive interference at the population level. Reproductive interference between the seed bug species *Lygaeus equestris* and *Spilostethus pandurus* has already been shown to impose significant fitness costs on *L. equestris* females, in the form of reduce lifetime fecundity (Shuker et al, *in prep*). This previous work, combined with the ease of keeping large numbers of seed bugs in the laboratory, makes them an ideal model system for this type of study. In order to make best use of this system a good understanding of the life history of the species is required. The following is an introduction to general seed bug biology with particular attention paid to the species I will be working with, as well as those used in previous studies of reproductive interference.

2. Study system: The Lygaeidae

2.1 Introduction

Insects of the family Lygaeidae (Insecta: Hemiptera: Heteroptera), commonly known as seed bugs, ground bugs or milkweed bugs, are found on every continent except Antarctica and are one of the three largest families (loosely defined; see below) within the Heteroptera. The Heteroptera is itself one of the most successful exopterygote sub-orders with approximately 40 000 species. Several Lygaeidae species are of economic importance due to their status as pests (Sweet, 2000, Summers et al., 2010), and the ease with which some species can be maintained in the laboratory has meant that a number of species have been utilized as laboratory animals in a range of contexts (Feir, 1974), particularly for studies of insect physiology (Feir, 1974, Jin et al., 2010). As a result, a variety of information on their biology is available, albeit spread across a number of disciplines and journals. A great deal of this work is the result of research carried out by a small number of authors in the 1970s and 80s. However, a steady stream of literature on Lygaeidae has continued to be produced, particularly concerning their evolutionary ecology and sexual behaviour. Here I aim to bring this disparate literature together to make the family better known to evolutionary and behavioural ecologists, and also to highlight the potential to link reproductive physiology and ecology at both mechanistic and functional levels (e.g. Attisano et al., 2012). First I will place the Lygaeidae in its current (and admittedly poorly-resolved) phylogenetic context, before considering aspects of the family's basic biology. I will then explore in more detail the progress made in understanding the evolutionary ecology of this group of insects, considering the evolution of aposematism (warning colouration) and mimicry, population structure and patterns of migration, life history variation, mating system ecology, reproductive behaviour and sexual selection. Importantly, chemical ecology appears to play a key part in many aspects of lygaeid biology and, as a result, I will keep returning to the role of chemical signals and chemical defence throughout this chapter.

2.2 Phylogenetic status and basic biology

Recent studies have confirmed that the family Lygaeidae as traditionally defined is polyphyletic (Weirauch and Schuh, 2011), and here I consider “lygaeids” in terms of the super-family Lygaeoidea. The lineages that currently make up the Lygaeiodea exist within the infra-order Pentatomomorpha (with putative sister-taxa Coreoidea and Pyrrhocoroidea), with the Cimicomorpha as the most likely sister taxon (Figure 1). It should be noted however that further work on the families within the Lygaeoidea is required (Weirauch and Schuh, 2011) as the suggested number of families varies from five to 15 depending on the authority (Henry, 2009). Weirauch and Schuh recently proposed 11 families (including a family called Lygaeidae) (Weirauch and Schuh, 2011). For simplicity, here I use the terms “Lygaeidae” or “lygaeid(s)” when discussing the bugs, not least as most of the biology has been done on species placed within the family Lygaeidae *sensu stricto* (e.g. the genera *Oncopeltus*, *Spilostethus* and *Lygaeus*; see below). The poor resolution of the phylogeny across the Pentatomorpha is a clear constraint on comparative analyses that might wish to test evolutionary and ecological hypotheses, a constraint unfortunately shared with many other insect groups (e.g. Ross et al., 2012).

Lygaeids are typically small to medium sized insects, ranging in size from approximately 1 to 12mm. While aposematism is widespread within the family, particularly within the subfamily Lygaeinae (Aldrich et al., 1997), the majority of species are cryptically coloured (Schuh and Slater, 1995). They are oval in shape and generally slender although notably some species of the subfamilies Cyminae and Pachygronthinae resemble the shape of the seeds they feed on (Schuh and Slater, 1995). Most possess antennae made up of four segments, though some Lygaeidae have only three. They can be distinguished from their close relatives, the Coreidae, by the number of veins present on the forewing. Lygaeidae have five or fewer whilst coreids have six of more. The Lygaeidae also closely resemble the Miridae but, unlike the mirids, they lack a distinctive cuneus and possess ocelli. The identification of key morphological features that characterise the Lygaeidae is problematic due to the polyphyletic nature of the family.

2.2.1 Sexual systems

Chromosomal number in the Lygaeidae appears to vary considerably and previous studies have reported male diploid chromosomal complements within the family ranging from six to 30 (Souza et al., 2007, Bressa et al., 2002). However, despite this extensive variation, chromosomal numbers of 2n=14 and 2n=16 are thought to be the most common (Kaur and Suman, 2009). Sex determination within the Lygaeidae shows a similar diversity. While the majority (approximately 74% of Lygaeidae species studied as of 2007) have XY/XX sex determination, some species have X0/XX or multiple sex chromosomes (approximately 15% and 10% respectively)(Souza et al., 2007). The evolutionary biology of these changes is currently unknown. As discussed below, parthenogenetic populations of *Nysius groenlandicus* have also been recently discovered (Bocher and Nachman, 2011).

2.2.2 Oviposition

Female lygaeids generally lay eggs in clutches, which can range in size from 10 to over 100 eggs (Feir, 1974, McLain, 1991), and may lay many clutches in their lifetime. While parental care is widespread throughout the Heteroptera, with the most well-known example being the so-called “parent” bug *Elamucha grisea* (Tallamy and Schaefer, 1997), no evidence has yet been found for parental care within the Lygaeidae beyond oviposition preferences. For example, some *Nysius* species are reported to attach their eggs individually to the underside of leaves (Tallamy and Schaefer, 1997). While adults and nymphs of aposematic species can be found aggregating together, presumably to present a stronger aposematic signal (see below), such groupings are not necessarily formed of kin and therefore are not considered to be any form of parental care. Instead, eggs are laid in clutches, either in crevices in the ground or on host plants depending on the habitat of the species. Clutch size varies considerably within and between species and has been shown to be affected by temperature, food availability, photoperiod, population, female age and mating status (Table 1.).

Nymphs typically live in similar environments to their parents (Schuh and Slater, 1995) and are often gregarious (Aller and Caldwell, 1979). Importantly, sibling cannibalism is known to occur in Lygaeidae (Sweet, 2000), particularly during and shortly after hatching (*pers obs*.; Solbreck and Sillen-Tullberg, 1990). Indeed, the seemingly high frequencies of infertile eggs observed in laboratory populations of several lygaeid species suggests a potential role of provisioning for these eggs (so-called “trophic” or “nurse” eggs) as newly hatched nymphs will often eat un-hatched eggs (*pers obs*.; Root and Chaplin, 1976, Solbreck and Sillen-Tullberg, 1990). Indeed newly hatched *Spilostethus pandurus* nymphs prefer to attack un-fertilised eggs over unhatched fertilised eggs and consumption of a single egg by a newly hatched nymph doubled their survival time compared to a starved nymph (Anderson and Solbreck, 1992). This may be particularly important as *S. pandurus* frequently lay their eggs some distance from food sources (Anderson and Solbreck, 1992). In a recent review of trophic egg theory, Perry and Roitberg (2006) outline the key hypotheses and what experiments would be needed to test them. For instance, in many cases of apparent trophic egg production across insects, it is currently not clear whether unfertilised eggs are deliberately produced or are the result of other factors such as sperm-limitation (Perry and Roitberg, 2006). It is also not clear whether trophic eggs, if deliberate, are produced as a form of offspring provisioning or are instead produced to limit sibling cannibalism. In order for this to be the case, we would expect these “trophic” eggs to be less expensive to produce than a viable egg. However, to the authors’ knowledge, there are currently no studies that compare the structure and chemical composition of unhatched eggs and viable ones in the Lygaeidae. Interestingly, it is currently also unknown if the observed levels of sibling cannibalism reflect parent-offspring conflict. It has been suggested that females could manipulate hatching synchrony to reduce sibling cannibalism (Schausberger and Hoffmann, 2008) as early hatching nymphs will attack unhatched eggs. However, theory developed in birds suggests that females may also manipulate clutch size in order to regulate sibling conflict (Nilsson, 1995). The Lygaeidae may provide an interesting system for the study of parent-offspring conflicts in the form of sibling competition as their polgynandrous mating system may frequently result in clutches of mixed parentage (Economopoulos and Gordon, 1972, Sillen-Tullberg, 1981, Wang and Davis, 2006). If the average relatedness of a lygaeid nymph to its clutch mates is less than that of the mother to her offspring then the potential for such conflicts may be greater than in typically monogamous species. The scope for the study of parent-offspring and offspring-offspring conflict is clear, and numerous lygaeids may be suitable study systems.

Table 1. Recorded clutch sizes and lifetime egg production of several Lygaeidae species. Where these measures were unavailable other measures of egg production were substituted if available.

Species	Number of eggs	Factors that influence egg production	References
Lygaeus creticus	Mean clutch size 20.7 ± 1.76 for once-mated females (at 29 °C), lifetime egg production unknown	-	Burdfield-Steel (unpublished)
Lygaeus equestris	Clutch sizes range from 20 to 50 eggs. Lifetime egg production typically ranges from 300 to 500 eggs, however this can be exceed this with some females producing up to 1000 eggs.	Temperature, Population	Sillén-Tullberg (1981), Sillén-Tullberg and Solbreck (1990a, 1990b), Solbreck et al (1989), Burdfield-Steel (unpublished)
Lygaeus simulans	Larger clutches exceed 60 eggs. Mean number of eggs laid per female in the laboratory was 150.2 after one successful mating and mean number of clutches was 5	Mating status	Tadler (1999a and 1999b)
Neacoryphus bicrucis	Females lay clutches of approximately 20 eggs almost daily The mean number of eggs produced per female over a 6 week period is approximately 143. Older females produced more eggs that younger ones	Female age	McLain (1991), McLain and Pratt (1999)
Nysius huttoni	200-600 eggs laid over the course of the females' life. Dissected females contain 4-9 mature eggs	-	Yang and Wang (2004), Wang and Davis (2006)
Oncopeltus cingulifer	The average clutch size was 29 at 27 °C and 17.3 at 25 °C. Lifetime egg production was 222 at 25 °C	Diet	Phelan and Frumhoff (1991), Root and Chaplin (1976)
Oncopeltus fasciatus	Typical clutch size is approximately 30 eggs though this appears to be highly variable (recorded clutch sizes range from 5 to more than 50). Lifetime egg production is similarly variable with some studies reporting between 200 and 2000 eggs produced by a female over her lifespan	Temperature, Population, Photoperiod	Sauer and Feir (1973), Dingle (1974), Feir (1974), Baldwin and Dingle (1986), Groeters and Dingle (1987), Dingle, Evans and Palmer (1988), Groeters and Dingle (1988), Leslie (1990), Phelan and Frumhoff (1991), Attisano, Moore and Moore (2012) Burdfield-Steel (unpublished)

Oncopeltus unifasciatellus	Average clutch size 26.1 eggs (at 25 °C). Lifetime egg production approximately 749 eggs	Diet	Root and Chaplin (1976)
Ozophora baranowskii	Females had an oviposition rate of 4-6 eggs per day	-	Rodriguez (1998)
Spilostethus pandurus	Mean clutch size 41.9 ± 3.16 for one-mated females (at 29 °C)	-	Burdfield-Steel (unpublished)
Togo hemipterus	Females lay 137.6 ± 10.9 (mean ± SE) eggs throughout their lifetime at a rate of 3.4 ± 1.1 eggs per day for 45.8 ± 3.6 days	-	Himuro (2010)

2.2.3 Development

As with all hemimetabolous insects, the Lygaeidae do not undergo complete metamorphosis during their life cycle but instead typically have five wingless nymphal instars before becoming reproductive, and typically winged, adults (Cárdenas et al., 2001, Slater and Gagne, 1973). However, flexibility in the number of larval stages has been observed in the New Zealand species *Nysius huttoni*. In this species, the number of larval instars ranges from three to seven, though individuals with five instars still form the majority. In the laboratory, variation in the number of instars is affected by both temperature and photoperiod, with lower numbers of instars more frequent at lower temperatures (Wei, 2010). This suggests that flexibility in the number of larval instars may aid survival under changing environmental conditions (Wei, 2010). *N. huttoni* normally have three generations per year in their natural range and overwinter as adults, therefore a shorter nymphal stage could potentially be beneficial to enable individuals to reach adulthood during poor, shorter summers. So far this is the only species found to show this flexibility in nymphal instars within the Lygaeidae; however it is well documented in other insect groups (Esperk et al., 2007) and may well be far more common. Evidence for another form of developmental variation has been found in *Oncopeltus fasciatus* in which maternal effects influence size and development time, particularly with respect to the age of the mother (Phelan and Frumhoff, 1991). This has also been linked to the seasonal variation experienced by some populations, and has been suggested as a mechanism by which mothers can maximise the survival potential of their offspring as the offspring of older mothers develop faster (Groeters and Dingle, 1987). The link between nymph size and speed of development is strengthened by evidence that the timing of adult moult is determined by nymph weight, and delayed moulting under low-nutritional conditions may be a result of failure to reach the critical weight to trigger the required hormonal changes (Blakley and Goodner, 1978a).

The pattern of development can also have important consequences for the evolution of life histories more generally. For instance, tropical populations of *Oncopeltus fasciatus* and the con-gener *O.*

cingulifer experience naturally-occurring variation in host plant quality, which influences the growth and development of nymphs. On poorer quality food (i.e. vegetative plant parts rather than nutrient-rich seeds), nymphs take longer to reach a given size, leading to a lower probability of surviving to eclosion. On the other hand, insects that eclose into adults at a larger body size have a better chance of survival under conditions of food stress and perhaps are better able to disperse away to better quality food patches (for instance with flowering host plants; Blakley, 1981). This means that critical size at metamorphosis may be under conflicting selection pressures in juveniles and adults. Moreover, if these size-development-survival relationships differ between the sexes, we may also expect sexually antagonistic selection over development under nutritional stress.

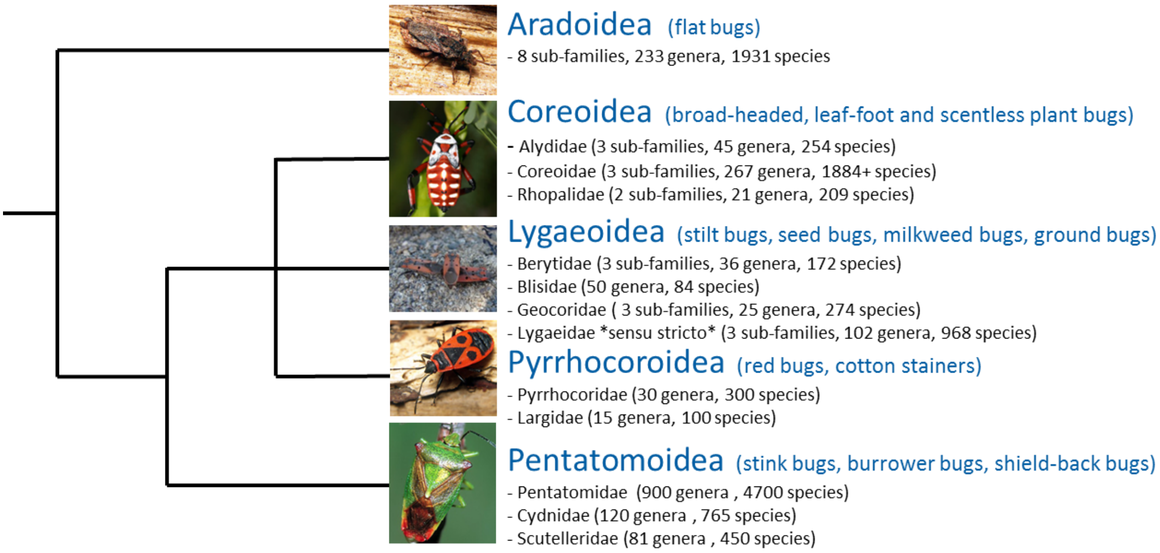

Figure 1. A schematic of the relationships among the infra-order Pentatomomorpha (after Li et al. 2005 and Henry 2009). Between 5 and 15 families are thought to comprise the Lygaeoidea, including the family Lygaeidae *sensu stricto*. Images taken from Tree of Life Web Project or DMS.

Figure 2. Left - An adult *Spilostethus pandurus* feeding on a *Lygaeus creticus* nymph. Sicily. Right – A *Lygaeus kalmii* feeding on an ant. Photo credits: (left to right) David Shuker, Mary Holland.

2.2.4 Diapause

A number of species in temperate areas, including *Lygaeus equestris* and the closely related species *L. simulans*, show reproductive diapause (i.e. diapause as adults) and migratory capabilities triggered by temperature and photoperiod (Dingle et al., 1980a, Solbreck and Sillen-Tullberg, 1981, Solbreck, 1979). These are thought to be adaptations to allow them to avoid or survive low temperatures during the winter months, as well as to migrate to follow seasonal patterns in host-plant abundance (Dingle et al., 1980a). Migration in *L. equestris* involves moving to overwintering sites, such as buildings or natural rock formations, rather than south to warmer climates (Solbreck et al., 1989, Sillen-Tullberg and Solbreck, 1990, Solbreck, 1976). Ovary development in the adult females does not start until after the spring migration flights from these overwintering areas to their breeding sites. As only adults can make the flights, typically the populations in northern Europe are univoltine, as only one generation can be produced per year. However occasionally, during particularly warm years, two generations have been recorded, and the degree of multi-voltinism increases with decreasing latitude. Additionally, in *L. equestris* copulation may determine the timing of diapause, as reproductively active females have been shown to be less likely to enter diapause (Sillen-Tullberg, 1984).

2.2.5 Feeding biology and economic impact

Turning to feeding, as one of the common names suggests many, though not all, species of Lygaeidae feed on seeds (Schuh and Slater, 1995). Like all Hemiptera, they have piercing-sucking mouth parts and feed through a flexible feeding tube called a proboscis. The proboscis, or rostrum, is made up of the mandibles and maxillae modified to form needle-like stylets lying within a grooved labium. Within this structure are two canals, one to deliver saliva and the other to take up food (Gullan and Cranston, 2005). Feeding methods can be divided into two “types” or manners of feeding: “stylet-sheath” feeders and “lacerate-flush” feeders (Schuh and Slater, 1995). In stylet-sheath feeders the salivary glands produce a so-called “feeding cone” that connects the apex of the labium to the feeding substrate. In addition, a salivary sheath may be produced that lines the puncture in the seed or plant through which the stylets are inserted (Sweet, 2000, Schuh and Slater, 1995). Lacerate-flush feeders on the other hand use the barbed apical portion of the proboscis to macerate the internal tissues of the host, be it plant or animal (Schuh and Slater, 1995). The tissues are then mixed with saliva, often containing digestive enzymes, and the resulting liquid is then sucked up through the food canal (Gullan and Cranston, 2005). Both feeding mechanisms require the production of saliva and at least in the laboratory access to water is crucial for successful rearing.

The majority of Lygaeidae are lacerate-flush feeders, the method commonly used by species that feed on portions of the plant that give a high nutrient return, such as seeds. However, an unnamed lygaeid species has been observed to secrete a feeding cone when using the lacerate-flush method and produce a stylet sheath when feeding on plant sap (Schuh and Slater, 1995). As sap feeding is widespread throughout the Lygaeidae, and many lygaeids do feed on sap in addition to seeds, it is likely that this behaviour may well apply to other species (Schuh and Slater, 1995). Moreover, the families Blissidae, Malcidae and Colobathristidae are predominantly sap feeders. The Blissidae, also known as chinch bugs, are arguably the most economically important group of lygaeids due to their status as pests (Samuels et al., 2002). For example, they are known to attack grasses including grain crops, sugar grasses and grasses used for lawns and playing fields (Sweet, 2000). Another lygaeid known to attack crops is the North American pest species the false chinch bug, *Nysius raphanus*. *N. raphanus* is multi-voltine, with adults overwintering on the ground underneath debris or rubbish (Demirel and Cranshaw, 2006). Populations can expand rapidly during favourable conditions and both adults and nymphs have been reported attacking a great variety of plants including tomatoes (Summers et al., 2010), cotton, tobacco, grapes and turf (Sweet, 2000), especially during the late spring when their preferred host plants begin to desiccate (Summers et al., 2010). The bugs aggregate in large groups on the plants and cause wilting, browning of the leaves, and deformation of any developing fruit (Sweet, 2000). *L. equestris* have also been reported as pests of sunflower seeds (Horvath and Frank, 2002, Horváth et al., 2004). Interestingly, species of the family *Geocoris* have been reported as predators of *Nysius spp*. (Sweet, 2000), suggesting they may be potential biological control agents of *Nysius raphanus.*

It is unclear what proportion of lygaeid species are food plant-specialists as opposed to generalists, as reliable data on habitat and feeding habits are available only for a few species (Solbreck et al., 1989, Ralph, 1976). Several species, such as *Oncopeltus fasciatus*, have been classified as “milkweed-specific” as they are restricted to milkweeds and other plants of the sub-family *Asclepiadaceae* (Wheeler, 1983). However, other species such as *Lygaeus equestris* (Kugelberg, 1974, Kugelberg, 1973)*, L. kalmii* (Wheeler, 1983)*, Spilostethus pandurus* (Elbanna et al., 2009, Sweet, 2000) and *Neacoryphus bicrucis* (Solbreck and Pehrson, 1979) have been found to feed on numerous plant families, even though asclepiads remain their preferred hosts (Wheeler, 1983). This host flexibility may account for many species’ sporadic role as pests. *S. pandurus* in particular often damages crops when its usual host plants are reduced by adverse weather or rendered insufficent by sudden population growth (see the next section for more on the pest status of *S. pandurus*). Laukkanen et al. (2013) recently looked for evidence forgenetic trade-offs between the ability to grow on one or severalhost plants that might favour the specialisation of herbivores, using *L. equestris* and four plant species, including the preferred host plant *Vincetoxicum hirundinaria*. They found no evidence for trade-offs in survival, development time or mass, suggesting that trade-offs do not affect the ability of *L. equestris* to adapt to novel food plants.

However, there is some evidence for a trade-off between survival and reproduction in *O. fasciatus* when feeding on novel food plants. Females show evidence of adaptive oosorption in response to a novel diet, thus conserving resources normally invested in eggs during periods of food stress (Moore and Attisano, 2011, Attisano et al., 2013). This ability to re-absorb eggs may represent an adaptation that allows the short term utilisation of a greater variety of host plants, allowing females to survive periods where their preferred food plants are scarce. During these periods females may invest less in reproduction so as to increase survival. Thus, lygaeids may offer an interesting set of model systems for the study of the evolution of life-histories under nutritional stress and in response to novel diet. Despite being primarily plant feeders, the Lygaeiodea contains one family, the Geocoridae, in which carnivory has become the most common feeding habit. Not surprisingly, some geocorids are therefore potentially important biocontrol agents (e.g. in the genus *Geocoris* (Carstens et al., 2008, Tillman, 2011, Lundgren, 2011) and their ecological interactions have received quite a lot of attention, particularly in the context of intra-guild predation (Müller and Brodeur, 2002, Polis et al., 1989). Perhaps more surprisingly, a small rhypirochromine lygaeid tribe, the Cleradini, has evolved to feed on the blood of small rodents (Harrington, 1983), although a study on *Clerada apicicornis* suggested that this species, whilst facultatively haematophagous, prefers to feed on other insects, including the blood-feeding reduviid bug *Rhodnius prolixus* (Torres et al., 2000). The observations of Torres et al. mean that the records of haematophagy in *C. apicicornis* obtained from museum specimens by Harrington (Harrington, 1990) might represent vertebrate blood meals obtained by feeing on other blood-feeders (so-called “cleptohaematophagy”) rather than from vertebrate hosts directly.

Generally, it appears that strict carnivory or phytophagy will be uncommon, with certain geocorids having been shown to be omnivorous, for example *Geocoris uliginosus* (Carstens et al., 2008). In addition, several otherwise phytophagous species having been observed engaging in opportunistic predation (e.g. Sweet, 1979, Sweet, 2000; Figure 2); indeed, this habit may well be common. Sweet (1979) observed that plants are typically low in protein and that secondary carnivory may well have evolved in a number of lygaeid species to provide an additional source of protein. Likewise, as we have already seen, egg cannibalism seems to be common among newly hatched nymphs, and may either represent offspring provisioning by the mother via the production of trophic eggs, or simple cannibalism of eggs that develop more slowly or fail to hatch (Perry and Roitberg, 2006). As previously discussed, such cannibalism could select for mothers to promote hatching synchrony, and for offspring to try and hatch first, leading to within- and between-generational conflict over time spent as an embryo. Eggs are also cannibalised by later-instar nymphs and also adults (Solbreck and Sillen-Tullberg, 1990) which is an important consideration for laboratory studies. Currently there are only limited data testing kin discrimination of eggs/nymphs in terms of cannibalism, and much remains to be discovered.

2.2.6 Endosymbionts

Interestingly, from across the insects there is the hint of an apparent syndrome of aposematism, sibling cannibalism, and the presence of male-killing endosymbionts (Majerus, 2003). To date, only one population of *Spilostethus hospes* has been identified as harbouring an (unidentified) male-killer bacterium (Groeters, 1996), with circumstantial evidence from a population of *Oncopeltus fasciatus* of a similar kind of sex ratio distorter (Leslie, 1984). However, we might predict that such sex ratio distorting endosymbionts will be more common in lygaeids than currently appreciated due to the habit of sibling cannibalism. In terms of symbiotic bacteria more generally, as with many of their Homoptera cousins (such as aphids, whiteflies and scale insects), many Heteroptera with restricted diets (such as blood-feeding cimicid bugs or sap feeding pentatomids) have bacterial symbionts, which are thought to facilitate production of otherwise limiting micro-nutrients. However, in Heteroptera these tend to be extra-cellular symbionts, housed in midgut sacs, crypts, or tubules, rather than within cells (see Kuechler et al., 2010, Matsuura et al., 2012). This extra-cellular habit requires an alternative suite of adaptations for cross-generational transfer of these useful bacteria (such as *Gammaproteobacteria*), such as mother-offspring transfer via so-called “egg smearing”, or gaining the bacteria from the environment (Kuechler et al., 2012). Recently, however, exceptions to this pattern have been found in a burst of exciting new papers.

First, the lygaeid *Kleidocerys resedae*, or birch catkin bug, has been found to have an obligate endosymbiotic proteobacterium which lives in a specialised endocellular mycetome structure (Kuechler et al., 2010). This species also has alpha-proteobacteria of the genera *Wolbachia* and *Rickettsia*. Second, the bulrush bug *Chilacis typhae* (Lygaeidae, sub-family Artheneinae) hosts an obligate intracellular gamma-proteobacterium in a set of enlarged midgut cells, arranged in a circular fashion around the mid-gut (called a "mycetocytic belt" by the authors: Kuechler et al., 2011). These authors suggest that this structure might be an intermediate structure between the mid-gut crypts or sacs of other Lygaeioids, Pentatomoids and Coreiods, and the more specialised bacteriome/mycetome of *Kleidocerys* (Kuechler et al., 2011).

Third, four species of *Nysius* (*Nysius plebeius*, *N*. *expressus* and two species not identified to species level) have been found to harbour the primary gamma-protobacterial endosymbiont *Schneideria nysicola* in a pair of bacteriome structures (Matsuura et al., 2012). These authors also identified *Wolbachia* and a novel alpha-proteobacterium in these species, finding the latter in five other species across the super-family as well. The gammaproteobacterium in *Nysius* does not form a clade with the bacterium found in *Kleidocerys*, suggesting an independent evolutionary origin of this symbiosis, with strict host-symbiont coevolution within the four *Nysius* species (Matsuura et al., 2012).

Fourth, five species from the Blissidae (*Ischnodemus sabuleti*) and Lygaeidae (*Arocatus longiceps*, *Belonochilus numenius*, *Orsillus depressus*, and *Ortholomus punctipennis*) have also been confirmed to host gamma-proteobacteria endosymbionts in paired bacteriome structures (Kuechler et al., 2012). The phylogenetically-diverse symbionts and the anatomical characteristics and location of the bacteriomes varied across the species, suggesting either independent evolutionary origins for the symbioses or (perhaps) some coevolution amongst hosts and symbionts. In addition to these findings, *Wolbachia* has also been recorded from two species of *Nysius* (not identified to species level: Weeks et al., 2003) and from a range of Heteroptera including 14 out of 24 species of Japanese Lygaeiodea (comprising species from the Lygaeidae, Malcidae, and Berytidae; (Kikuchi and Fukatsu, 2003). All told, these surveys suggest that more symbionts, including obligate endosymbionts with specialised host structures, probably await discovery. Given the possible ecological and evolutionary consequences of these symbionts (Moran et al., 2008), and perhaps the opportunity to study a range of colonisation events and a diversity of host specialisations (from simple crypts to dedicated host tissues) in the same super-family, there should be much interest in exploring this aspect of lygaeid biology further.

2.2.7 Social interactions

In addition to intra-guild predation, a variety of social behaviours and interactions are apparent across the Lygaeidae. Individuals of many species, including *O. fasciatus*, *S. pandurus* and several species of the genus *Lygaeus* aggregate as nymphs (Aller and Caldwell, 1979) as well as adults (Root and Chaplin, 1976). These aggregations may occur during nymph development, when adults may also be present, or during periods of hibernation (e.g. hibernating masses of *Lygaeus equestris*: (Solbreck and Sillen-Tullberg, 1990). Rather than try and characterise these interactions as "sub-social" or "communal", I follow Costa (2006) and consider all these behaviours as "social". Otherwise, adults are typically solitary barring sexual interactions, which are considered in more detail below, although food plants may often host several individuals of both sexes which may stay on the same plant for several days (e.g. *Spilostethus pandurus* on the milkweed *Gomphocarpus sinaicus*: (Elbanna, 2004, Elbanna et al., 2009). While nymphal aggregation may simply be the result of kin-aggregation in *Oncopeltus fasciatus*, this social behaviour has been found to be adaptive: feeding and ingestion rates are higher for bugs in aggregations versus solitary bugs, likely as a result of their feeding strategy (see above). Like many Lygaeidae, *O. fasciatus* must inject seeds with saliva before they are able to feed on them (Schuh and Slater, 1995). When many bugs are feeding on the same seed each individual is thought to "economise" on saliva (Root and Chaplin, 1976). This is likely to be true for other Lygaeids as other species of the *Oncopeltus* genus have been observed in aggregations containing both adults and nymphs (Root and Chaplin, 1976). I will also discuss aggregation in the Lygaeidae further below when considering the importance of pheromones. Whilst groups are often considered to comprise kin, the extent of non-kin aggregations has yet to be investigated. As mentioned above, in some temperate species adults also aggregate for hibernation. This has been most extensively studied in the species *Lygaeus equestris* where hibernating adults typically enter reproductive diapause before aggregating at overwintering sites.

In terms of inter-specific interactions, one particularly fascinating interaction concerns ant mimicry or ”myrmecomorphy” (McIver and Stonedahl, 1993), which is widespread in the Lygaeoidea and appears to have evolved multiple times (Schuh and Slater, 1995). This mimicry is thought to act as an anti-predator defence as many predators avoid ants (Durkee et al., 2011) and may be morphological (Figure 3) or behavioural in nature. For example, *Neopamera bilobata*, a Neotropical lygaeid, moves with jerky, ant-like movements when disturbed. This has been described as “action mimicry” and is thought to cause predators to hesitate in their attack, allowing the bugs time to escape (Schuh and Slater, 1995). While some species of Lygaeidae live in or associated with ants nests, it is currently unclear if any species mimic ants for this purpose though (Schuh and Slater, 1995).

Figure 3. An ant-mimicking Seed Bug - *Daerlac nigricans* – form the Family Rhyparochromidae. Photo courtesy of Peter Chew (http://www.brisbaneinsects.com).

2.2.8 Predators and parasites

Despite the interest in the aposematic species of Lygaeidae (see below), there have been few detailed studies of predation in the wild. Generally, it is thought that lygaeids are preyed upon by a variety of predators including birds (Gamberale-Stille and Sillen-Tullberg, 1999, Svadova et al., 2010), rodents (Aldrich, 1988), and invertebrate predators such as mantids (Berenbaum and Miliczky, 1984), whilst most studies on warning coloration and chemical defences have used model predators such as chicks (Gamberale-Stille and Sillen-Tullberg, 1999, but see Berenbaum and Miliczky, 1984). In addition, seed bugs have been found in the stomach contents of invasive *Anolis sagrei* in Taiwan and ants have been observed preying upon eggs of *L. equestris* (Kugelberg, 1977). However, it seems likely that parasites rather than predators pose the greatest threat to wild Lygaeidae (e.g. Tachinid flies: Lampman and Fashing, 1978, Müller and Fritsche, 1993, and Trypanosomes: Tieszen et al., 1986, Thorpe and Harrington, 1979) especially egg parasites (Root and Chaplin, 1976, Lampman and Fashing, 1978, Anderson and Solbreck, 1992). The impact of parasites appears to vary considerably between populations, for example Solbreck et al, (1989) suggest there is no predation or parasitism in Swedish populations of *L. equestris*, compared to populations in Sicily which fall prey to Tachinid flies (Solbreck et al., 1989).

2.3 Common study systems: a brief introduction

While there are more than 4000 species spread across approximately 102 genera within the Lygaeiodea (Henry, 2009), previous study has focused on a select few species (most notably those that have proven to be suitable organisms for study in the laboratory, Figure 4). As much of what we know about Lygaeidae is based around these species, a brief introduction to each is provided.

The most intensively studied species has been the large milkweed bug *Oncopeltus fasciatus*. This species' easy husbandry has made it a commonly-used organism for laboratory studies (Feir, 1974, Liu and Kaufman, 2009), including studies of development (Feir, 1974, Angelini and Kaufman, 2002, Angelini et al., 2005), the effects of pollution (Feir and Hale, 1983), endocrinology (Lorenz et al., 2009, Chung and Bowers, 1996), and gene function (Adler and Woodruff, 2000, Angelini et al., 2005, Chesebro et al., 2009). This has, in turn, led to an increased interest in the biology of the species. *O. fasciatus* ranges from the USA down through Central America and into the Caribbean. It feeds on seeds of milkweed plants, in particular *Asclepias*, though the species host plant varies across its range and includes *Nerium oleander* (Miller and Dingle, 1982, Klausner et al., 1980).

Several species from the genus *Lygaeus* have also received some attention, particularly the closely related species *Lygaeus equestris* and *Lygaeus simulans*. These are found throughout Northern and Central Europe and feed on the seeds of plants such as *Vincetoxicum hirundinaria* and *Nerium oleander*. As with its New World relative *Oncopeltus*, the host plant of *L. equestris* varies with locality (Solbreck et al., 1989). Most of the work on *Lygaeus* has focused on reproductive behaviour, aposematism and diapause.

The genus *Spilostethus* contains 24 species found throughout the Old World. The most studied of these is *Spilostethus pandurus*, which is widespread throughout the tropics, sub-tropics and up into the temperate zone. While this species has been shown to be a suitable lab animal, it is best known for its status as a pest species. *S. pandurus* is highly polyphagous, meaning it can switch from its preferred host plant, usually from the sub-family *Asclepidaceae,* to various crops. Thirty-three crops worldwide are reported to have been attacked by this species (Sweet, 2000). Despite being predominantly a seed eater, the presence of *S. pandurus* on crops has been shown to reduce the number of fruit developing as well as damage the body of the plant due to the sucking of sap from the leaves, stalks and flowers by both adults and nymphs. In severe cases, attacks by *S. pandurus* can cause plants to wither completely. In addition the species has been implicated in the spread of the fungus *Nematospora corgli* which causes yellow-spot disease, and is thought to transfer the fungus via its mouth parts (Sweet, 2000).

The final species I would like to highlight is *Neacoryphus bicrucis.* This North American species is found from southern Canada down to Brazil (Solbreck, 1979). It has been reported on a number of herbaceous plants, particularly ragworts (*Senecio* spp.), and as a result is sometimes known as the ragwort bug (McLain, 1991). *N. bicrucis* shares much of its range with *O. fasciatus* but is often found at higher altitudes (Solbreck, 1979). Below I will consider this species primarily in terms of its interactions with other lygaeids, in particular reproductive interactions and the phenomenon of reproductive interference.

Much of the research into the evolutionary ecology of the Lygaeidae in general, and the above mention species in particular, can be broadly classified into several topics. The first of these is research into the aposematic signals that characterise the best-known species of this group. A related topic is chemical communication, which is central too much of our understanding of lygaeid behaviour. I will then cover what is known so far about the population structure of the better-characterised lygaeid species and how this may impact their ecology and evolution. Finally I will consider reproductive behaviour, with an emphasis on mating systems and sexual selection in these species, and also the growing area of reproductive interference. Firstly however, I will discuss aposematism.

2.4 Aposematism, predator defence and life history effects

One of the most striking features of the Lygaeidae is that a number of species, in particular those in the sub-family Lygaeinae, exhibit distinctive black and red patterning (Figure 3). This is a form of aposematism, whereby the striking colours serve as a warning to predators that the organism possesses anti-predator defences, including toxic chemical defences (Ruxton et al., 2004, Mappes et al., 2005, Sillen-Tullberg, 1985a). A recent survey of museum specimens at the Natural History Museum in London estimated that more than 20% of the 1 951 species of Lygaeidae included have aposematic colouration (Burdfield-Steel, *unpublished data*). Additionally, 65 (approximately 14%) of the 452 genera surveyed contained at least one aposematic species. Of course, aposematic signals may take forms other than colour, such as odour or behaviour. Any cue that conveys to the predator that the prey is unprofitable may be considered aposematic. These signals are most effective if they are easily detected (i.e. conspicuous) and memorable, as this facilitates predator detection and learning (Mappes et al., 2005). In the case of the Lygaeidae, this defence is chemical (Aldrich, 1988, Aldrich et al., 1997, Zhang and Aldrich, 2003). These chemicals are typically cardiac glycosides, also referred to as cardenolides, which are distasteful to most predators (Duffey and Scudder, 1972, Voneuw et al., 1971). The prevalence of aposematism within the subfamily Lygaeinae has been linked to their feeding habits. While many lygaeids feed on fallen seeds, members of the Lygaeinae also feed on developing seeds and thus expose themselves to visually oriented predators such as birds (Aldrich, 1988). It is hypothesised that this has contributed to the evolution of the bright warning colours so often seen in members of this subfamily (Aldrich et al., 1997).

The availability of toxic compounds that can be sequestered in their food is also important (but also see below). Species such as *S. pandurus*, *O. fasciatus* and members of the genus *Lygaeus* feed on asclepiadaceous plants, such as milkweed, and both adult and larval stages sequester toxic cardiac glycosides obtained from these host plants (Aldrich, 1988, Aldrich et al., 1997, Elbanna et al., 2009). Cardiac glycosides inhibit the function of intracellular sodium pumps by binding to the alpha subunit of the sodium (Na+) and potassium (K+) ATPase. As the transportation of Na+ and K+ are crucial for many cellular functions, including muscle contraction, cardiac glycosides are highly toxic to most herbivores (Zhen et al., 2012). The Lygaeidae, in common with other insect species that feed on asclepiadaceous plants, possess amino acid changes in this ATPase that reduce the inhibitory effects of cardiac glycosides. In *O. fasciatus* these chemicals are sequestered in a double layered epidermis and can therefore be released through weak points in the cuticle if the bug is squeezed (Aldrich, 1988). The presence of these cardiac glycosides in the bodies of lygaeids makes them unpalatable to both vertebrate predators such as birds (Gamberale-Stille and Sillen-Tullberg, 1999, Sillen-Tullberg et al., 1982) and invertebrates. For instance, two species of praying mantis (*Tenodera ardifolia sinensis* and *Mantis religiosa*) have been shown to learn to avoid the bugs. The mantids frequently regurgitated after feeding on milkweed-fed *O. fasciatus* adults and learned to avoid the bugs after only a few encounters (Berenbaum and Miliczky, 1984). Additionally, secretions from the scent glands of adult *S. pandurus* contain cardiac glycosides and have been shown to repel birds, cats and even scorpions (Sweet, 2000).

In species such as *L. equestris*, *L. simulans*, *L. creticus*, *L. kalmii*, *O. fasciatus* and *S. pandurus*, both adults and nymphs are aposematic and often aggregate, possibly in order to amplify their warning colour signals (Sauer and Feir, 1973, see Figure 5). In an experimental study, the attack rate of domestic chicks on nymphs was found to decrease when nymphs were presented in groups compared to when presented alone (Sillen-Tullberg et al., 2000), suggesting that aggregation may help present a stronger aposematic signal. This suggests a possible link between the evolution of aposematism in Lygaeidae and various life-history traits, as aggregation frequency is variable across different species within the (super-) family (Sillen-Tullberg et al., 2000). This would be in keeping with the finding that aposematism is associated with the evolution of gregariousness in butterfly larvae (Sillen-Tullberg, 1988). However, see chapter 5 for data that suggests that while aggregation and aposematism may be linked, the specific level of defence a given individual has may not influence aggregation behaviour. Thus, processes such as automimicry, where individuals perform aggregation behaviour despite considerable variation in their own level of chemical defence (Speed et al., 2012), may play a key role, especially in species that can feed on more than one host plant such that levels of defence are likely to fluctuate both within and between individuals.

While much work on aposematism in Lygaeidae has focused on the learning ability of predators rather than the consequences for the bugs themselves, a series of studies by Sillén-Tullberg and colleagues have investigated the effect of diet on aposematic traits. In particular, they found that toxic defences are not always dependent on diet. While toxic defence was found to be food dependent in the polyphagous *L. equestris*, with individuals of the species showing, perhaps unsurprisingly, greater protection from predators (i.e. *Gallus gallus domesticus* chicks) when raised on the toxic *Vincetoxicum hirundinaria* than on the non-toxic *Helianthus annuus*, the naturally monophagous species *Tropidothorax leucopterus* showed no effect of host plant. Thus *T. leucopterus* was equally unappealing to predators when raised on *H. annuus* (sunflower) as it was when raised on *V. hirundinaria*. Interestingly however, both species were still relatively unappealing to predators compared with mealworms, suggesting the existence of further defences that are independent of diet. It has been hypothesised that these food-independent defences may be volatile substances or contact poisons produced in the stink glands and released when the animal is startled (Sillen-Tullberg et al., 2000). *O. fasciatus* raised on *H. annuus* have also been found to synthesise a histamine or a histamine-analog (Graham and Staddon, 1974), although predation studies using mantids found that sunflower-fed bugs were far less effective at inducing avoidance compared with those fed on milkweed (Berenbaum and Miliczky, 1984). This suggests that while some lygaeids suffer a cost of expanding their niche to include non-toxic host plants, in the form of increased predation, others many have developed alternative defences in addition to plant-sequestered toxins, although these appear to provide reduced levels of protection (Sillen-Tullberg et al., 2000). Additionally, as introduced above, lygaeids feeding on non-toxic host plants may benefit from their resemblance to their protected conspecifics feeding on other plant species through automimicry. It has been shown that, after attempting to eat toxic *O. fasciatus,* mantids of the species *Tenodera ardinfolia sinensi* avoided all *O. fasciatus*, even those raised on non-toxic hosts (Berenbaum and Miliczky, 1984). See chapter 5 for further discussion on the relationship between aggregation and chemical protection.

There is also evidence that chemical protection can be passed from parents to eggs. The eggs of many lygaeid species, such as *L. equestris*, *S. pandurus* and *O. fasciatus*, are brightly coloured, turning from pale yellow through to orange and red as they develop. Recent work in *O. fasciatus* has found that cardiac glycosides are transferred to eggs by both sexes though the maternal contribution is considerably bigger than the paternal (Newcombe, 2013). Eggs were found to contain a greater concentration of cardiac glycosides and showed greater protection from a predator (the larvae of the green lacewing, *Chrysopa carnea*) when the mother had been fed on milkweed compared to those where the mother had been feed on sunflower seeds (Newcombe, 2013). It is worth noting that these defences were only detected by the lacewing larvae after feeding on, and thus destroying, an egg. Therefore, these defences may benefit close kin by deterring predators from continuing to feed on a clutch.

In summary, the literature surrounding aposematism in insects makes it clear that a level of gregariousness is often associated with aposematic signalling, although much remains to be done to understand how individual levels of defence influence aggregation behaviour, including the extent to which animals in the wild are typically (well) defended. For instance, if sequestering and maintaining toxic compounds in the body requires considerable energy expenditure, is there the potential for “cheats” to evolve that avoid toxic foods but benefit from the mimicry ring of which they are part? It would be interesting to compare oligo- and polyphagous species in this regard, as there may be greater scope for cheating in the latter. As the aposematic lygaeids are often also part of wider inter-specific Müllerian mimicry rings (Svadova et al., 2010), there is the potential for complex, spatially variable relationships between intra- and inter-specific mimicry, combining Batesian, Müllerian and automimicry. There are clear opportunities to extend our understanding of mimicry in both the laboratory and more importantly in the field with these species. However, now I will move on to focus on the role of chemical communication beyond aposematism, in terms of both the maintenance of sociality in the Lygaeidae, and other aspects of their behaviour.

Figure 4. Four extensively studies species of Lygaeidae. Top right *Neacoryphus bicrucis* (permission pending), bottom right *Oncopeltus fasciatus* (photo courtesy of Alison Bockoven), bottom left *Spilostethus pandurus* (David Shuker) and top left *Lygaeus equestris* (Liam Dougherty).

Figure 5. Right – Laboratory raised second instar nymphs of the species *Spilostethus pandurus* showing characteristic black and red colouration. Left – an aggregation of late-instar *Lygaeus creticus* nymphs and adults. Sicily.

2.5 Pheromones in Lygaeidae – aggregation and communication.

One of the suggested mechanisms by which Lygaeidae aggregate is through the use of pheromones; the majority of Heteroptera possess scent glands and they have been found to produce a variety of pheromones (see table 2) (Aldrich, 1988) most of which appear to be for attracting conspecifics, although some may be for defence. For instance, it has been shown that aggregation in nymphs and young adults of *O. fasciatus* and *L. kalmia* is facilitated by the presence of a so-called aggregation pheromone found in acetone extracts taken from the insects (Aller and Caldwell, 1979).

Like other Heteroptera, lygaeids possess both metathoracic scent glands (MTG) and dorsal abdominal scent glands (DAG)(Aldrich, 1988). As discussed above, the secretions of both have been found to contain cardenolides sequestered from the plants they feed on. These odours are thought to act as an additional aposematic signal in their own right to deter potential predators (Aldrich et al., 1997) as many Insectivora, which are important predators of litter-dwelling Lygaeidae, are colour-blind (Aldrich, 1988). Distinctive scents could therefore act in the same way as distinctive markings and colouration to ward off potential visual predators such as birds.

In several species the MTG is reduced in size (Aldrich, 1988), however the chemistry of the MTG is complex and has been found to be sexually dimorphic in *Oncopeltus fasciatus* (Aldrich, 1988) and *Spilostethus rivularis* (Staddon et al., 1985b) among others. The presence of sexually dimorphic pheromones in adult lygaeids suggests they may play a role in mate choice. Males of *Neacoryphus bicrucis* and *Tropidothorax cruciger* have been found to produce compounds that attracted adults of both sexes in field bioassays (reviewed by Millar, 2005). Similarly, a study of *O. fasciatus* and *L. kalmii* by Aldrich et al (1999) found that pheromone traps baited with synthetic pheromone blends mimicking those extracted from the metathoracic scent glands of both species attracted females of the corresponding species. As both *O. fasciatus* and *L. kalmii* often have patchy distributions, it has been suggested that males which colonise new patches of host plants use long-range pheromones to guide potential mates to the patch. In addition, conspecific males and nymphs were also attracted to such pheromones (Aldrich et al., 1999). As such, these signals may indicate the presence of food, and hence, suitable habitat. Finally, females of the predatory species *Geocoris punctipes* produce pheromones that increase activity, specifically searching behaviour, in males (Millar, 2005). Clearly then, pheromonal signals must be considered when seeking to understand both migration dynamics and mate acquisition in these bugs.

We may expect signals such as pheromones to change over an individual's lifetime (e.g. virgin versus mated, diapause versus reproductively active). Indeed, the cotton seed bug *Oxycarenus hyalinipennis* changes the compounds synthesised by the MTG after the first day of adult life. It seems likely that the differences in scent glands often found between nymphs and adults (Schuh and Slater, 1995, Aller and Caldwell, 1979) may be reflective of the different roles pheromones play at these life stages, but data remain limited to date.

Another key chemical signalling system in insects is that associated with their cuticular hydrocarbon (CHC) repertoire. CHCs are long-chain fatty acids which are secreted onto the cuticle from specialised epidermal cells. These waxy compounds function primarily as protection from desiccation (i.e. water-proofing). However, the inter-specific diversity of CHCs presented on the cuticle is also used by a wide variety of species for species-discrimination (Kather and Martin, 2012), and sex-specific differences in CHC blends are also used for sex-discrimination and in processes such as mate choice (Everaerts et al., 2010, Thomas and Simmons, 2010). Finally, there is growing evidence that CHCs also allow discrimination of self from non-self (e.g. in the cricket *Gryllodes sigillatus*, females appear to recognise males they have already mated with by the presence of their own CHCs left on the male during previous copulations: (Weddle et al., 2012)). Despite this broad literature across a wide-range of insects, there is surprisingly little information about CHCs in the Lygaeidae (e.g. Jackson, 1983). This issue has been addressed in chapter 4.

Table 2. Compounds detected in the pheromones of several species of Lygaeidae.

Species	Compounds from Metathoracic scent gland	Defence substances	References
Lygaeus kalmia	(E)-2-Hexenyl acetate, (E,E)-2,4-Hexadienyl acetate, (E)-2,5-Hexadienyl acetate, (E)-2-Heptenyl acetate, (E)-2-Octenyl acetate, (E)-2,7-Octadienyl acetate, (E)-2-Hexenyl butyrate, (E,E)-2,4-Octadienyl acetate, (E)-2-Hexen-1-ol, (E)-2-Hexenal, (E)-2-Octenal, (E)-4-oxo-2-Hexenal, (E)-4-oxo-2-Octenal,		Aldrich et al. (1999)
Oncopeltus cingulifer	(E)-2-Hexenyl acetate, (E,E)-2,4-Hexadienyl acetate, (E)-2,5-Hexadienyl acetate, (E)-2-Heptenyl acetate, (E)-2-Octenyl acetate, (E,Z)-2,6-Octadienyl acetate, (E,E)-2,6-Octadienyl acetate,		(Aldrich et al., 1999)
Oncopeltus fasciatus	(E)-2-Hexenyl acetate, (E,E)-2,4-Hexadienyl acetate, (E)-2,5-Hexadienyl acetate, (E)-2-Heptenyl acetate, (E)-2-Octenyl acetate, (E)-2,7-Octadienyl acetate, (E,Z)-2,6-Octadienyl acetate, (E,E)-2,6-Octadienyl acetate, (E)-2-Hexenal, (E,E)-2,4-Hexadienal, (E)-2-Octenal, (E)-2,7-Octadienal, (E,Z)-2,6-Octadienal, (E,E)-2,6-Octadienal, 2-Octenal	2-Isobutyl-3-methoxypyrazine	Aldrich et al. (1999), Aldrich et al. (1997), Games and Staddon (1973)
Oncopeltus unifasciatellus	(E)-2-Hexenyl acetate, (E,E)-2,4-Hexadienyl acetate, (E)-2,5-Hexadienyl acetate, (E)-2-Heptenyl acetate, (E)-2-Octenyl acetate, (E)-2,7-Octadienyl acetate, (E,Z)-2,6-Octadienyl acetate, (E,E)-2,6-Octadienyl acetate, (E)-2-Hexenal, (E,E)-2,4-Hexadienal, (E)-2-Octenal, (E)-2,7-Octadienal, (E,Z)-2,6-Octadienal, (E,E)-2,6-Octadienal,		(Aldrich et al., 1999)
Spilostethus rivularis	(E)-2-Octenyl acetate, (E)-2-Hexenyl acetate, 3-Methylbutyl acetate, 3-Methyl-2-butenyl acetate, 2-		Staddon et al. (1985a)

	Phenylethanol acetate, (E,E)-2,4-Hexadienyl acetate,	
Geocoris punctipes	(E)-2-Octenyl acetate, (E)-2-Hexenyl acetate, (E)-2-Octenal, (E)-2-Hexenal, (E)-4-oxo-2-Hexenal, (E)-2-Decenal	Marques et al. (2000)
Geocoris varius	(E)-2-Hexenal, (E)-2-Decenal, Tridecane	Yamashita and Kanehisa (1979)
Neacoryphus bicrucis	(E,E)-2,4-Hexadienyl acetate, (E)-2-Octenyl acetate, 2-Phenylethanol acetate, (E)-2-Hexenal, (E)-2-Octenal, (E)-4-oxo-2-Hexenal, (E)-4-oxo-2-Octenal, (E,E)-2,4-Hexadienyl acetate, 2-Phenylethanol acetate,	Aldrich et al. (1999), Aldrich et al. (1997)
Oxycarenus hyalinipennis	(Z,E)-3,7,11-Trimethyl-1,3,6,10-dodecatetraene, (E)-2-Octenyl acetate, (E)-2-Octenal, 2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene, 1-Methyl-4-(1-methylethenyl)-cyclohexene, 2-Hexenal, 1,3,3-Trimethyl-2-oxabicyclo[2.2.2.]octane, (E)-2-Hexenyl acetate, 2-Octenal, (E)-4-oxo-2-Hexenal, 2-Octenyl acetate, (E)-4-oxo-2-Octenal,	Knight et al. (1984), Olagbemiro and Staddon (1983)
Tropidothorax cruciger	(E)-2,7-Octadienyl acetate, (E)-2-Octenyl acetate	Aldrich et al. (1997)

2.6 Population structure and ecology – Migration, diapause and genetic structuring

Despite the interest in terms of pest status and studies of migration, there have been very few genetic studies of the population structure of lygaeid species. To date, a study of allozyme frequencies among populations of *Lygaeus equestris* in Sweden has revealed evidence of genetic differentiation between the populations (Sillen-Tullberg, 1983), genetic differentiation that was echoed in differences in physiological response to photoperiod across the Swedish populations. A study of laboratory-reared specimens taken from four different locations in Sweden revealed a latitudinal cline in critical photoperiod, with longer critical photoperiods in the north and shorter photoperiods required to trigger reproductive diapause in southern populations (Solbreck and Sillen-Tullberg, 1981). Similarly, populations of *O. fasciatus* in different regions have also been found to be genetically distinct and show variation in reproductive diapause and migration tendency between populations (Dingle, 1972b, Dingle et al., 1980a, Dingle et al., 1980b). While *O. fasciatus* have not been found to overwinter in specific sites in the same way as *L. equestris*, some populations do show delayed reproduction and migration. Individuals from populations in variable, temperate climates can be triggered to enter diapause far more easily than those from more stable, tropical climates (Dingle et al., 1980a). This fits with the generally accepted theory that populations in the north of the species range are seasonal migrants that move north from subtropical overwintering sites during the spring (Chaplin and Chaplin, 1981). Further support for this comes from studies showing that bugs from these more northern populations have increased flying ability (Dingle et al., 1980b). *Spilostethus pandurus* also shows reproductive diapause triggered by falling temperatures. For instance, in northern India they pass the winter hibernating in leaf litter and fewer overlapping generations were found than in the south (Sweet, 2000). Whether the recorded differences in life history and host plant between populations in different geographic locations is reflected by genetic structuring in this species remains unknown though.

Another form within-and among-population variation can take is in terms of wing polymorphism, as lygaeids show a great deal of variation both in flying ability and wing morphology (Slater, 1977, Solbreck and Anderson, 1989). Currently, studies across many insects groups suggest that wing polymorphisms, such as brachyptery (described below), may be the result of trade-offs between flying ability and fecundity (Roff, 1986). There are many degrees of wing modifications within the Heteroptera, though only a few are typically found within the Lygaeidae. These fall into a four distinct categories (Schuh and Slater, 1995): (1) aptery, or the complete absence of wings; (2) sub-brachyptery where the forewings only reach to the end of the firth abdominal tergite; (3) brachyptery where the forewings are reduced and do not cover the sixth and seventh abdominal terga and the hind wings are reduced but usually not flap-like; (4) macroptery where the clavus and corium are distinct, the membrane is well-developed and the hind wings are elongate.

Natural populations of *Nysius huttoni* have been found to contain three wing forms. Field surveys over a four year period found that approximately 94.1% of the population were macropters, 5.5% were sub-brachypters and 0.4% were brachypters. Additionally, photoperiod was found to affect the production of the different wing morphs in the laboratory, with long photoperiods favouring the production of macropters. This was suggested to be a mechanism to enable the rapid dispersal of adults to new host plants during dry summers, which may reduce the availability of the bug's preferred weedy hosts (Wei, 2011). In the genus *Oncopeltus*, species in isolated habitats have been found to display both aptery and brachyptery, presumably as a result of selection for reduced dispersal. Additionally, flying ability within species can show considerable variation, even without obvious changes to wing morphology (Dingle et al., 1980b). When *Oncopeltus fasciatus* from both migratory and non-migratory populations were subjected to artificial selection for wing length in the laboratory, both showed a positive response, and flying ability also responded positively to selection (Dingle and Evans, 1987, Palmer and Dingle, 1989). It has been observed that within the Lygaeinae, wing reductions typically occur in ground-living species that feed upon fallen seeds while those that feed upon plants typically retain their flying ability. This may be the result of the increased habitat complexity experienced by these latter species, necessitating greater mobility (Solbreck et al., 1990). Wing polymorphisms in Lygaeidae have also been linked to habitat permanency though. Slater (1977) observed that ground-living Lygaeidae in old, stable areas such as southwest Australia and the Cape of South Africa have a greater degree of wing modification and are more likely to have flightless morphs compared to those in less stable areas (Slater, 1977). Interestingly, size and wing morphology seem to be correlated with wing dimorphisms, with aptery occurring more frequently in smaller species, although the reason for this pattern remains unknown (Solbreck et al., 1990).

As life history traits such as diapause and migratory capabilities have been shown to vary in several species, and that this variation between populations has been shown to remain despite laboratory culture (Solbreck and Sillen-Tullberg, 1981), it is reasonable to assume that many Lygaeidae species have structured populations and that this structure has allowed the evolution of climate-specific behavioural and physiological adaptations (i.e. local adaptation). This has been supported by work by Dingle and colleagues in *O fasciatus* which found variation in many traits including diapause (Dingle et al., 1980a), body size and flight ability (Dingle et al., 1980b) between tropical, sub-tropical and temperate populations. Additionally differences in body size and sexual harassment costs (in the form of reduced fecundity) have been found in lab populations of *L. equestris* collected from different locations in Europe (Shuker et al., 2006). The lack of recent genetic studies does, however, make it harder to predict how fine a scale this structuring extends. Many species, including *L. equestris* (Solbreck and Sillen-Tullberg, 1990) and *O. fasciatus* (Aldrich et al., 1999) are limited to a few plant species and as a result often have a patchy distribution across large parts of their ranges. For example, mark and recapture studies carried out in Sweden in the 1970s found that in areas of patchy food-plant distribution 82% of *L. equestris* individuals marked remained within the same area, no more than 50 meters from where they were originally captured, even after migration to overwintering sites (Sillen-Tullberg, 1983). While this seems initially like a high level of isolation, the inferred migration rate of 0.18 would still be sufficient to limit genetic differentiation and without studies using markers such as microsatellites or single-nucleotide polymorphisms (SNPs), capable of detecting relatively recent genetic differentiation, it is impossible to know if such spatial structuring of the bugs' habitat is reflected in patterns of gene flow. At least one closely-related species pair found in Europe, *L. equestris* and *L. simulans*, are hypothesised to have arisen via some form of local adaptation, possibly associated with pre- or post-glacial isolation (Hewitt, 2001, Hewitt, 1999).

More generally, one area that has received rather little attention in the Lygaeidae is that of speciation. As mentioned above, *L. simulans* and *L. equestris* may represent a relatively recent speciation event, perhaps associated with repeated range changes across Europe associated with glaciation events. Despite asymmetric reproductive isolation between the two, male *L. simulans* can mate successfully with *L. equestris* females, but *L. equestris* males are usually unable to fertilize *L. simulans* females (Evans, 2011), it is unknown whether introgression and/or reinforcement played any role in the evolution of reproductive isolation between these sibling species. Classic patterns of speciation, such as Haldane's Rule (Coyne and Orr, 2004), also remain to be tested. Hybridization has also been found in several species from the *Oncopeltus* genus (Leslie and Dingle, 1983, O'Rourke, 1979). Barriers to hybridization are frequently asymmetric within this genus as well and, despite the occasional observations of hybrid pairs in the wild, laboratory experiments confirmed strong conspecific mating preferences (Leslie and Dingle, 1983).

2.7 Mating systems – Promiscuity, sperm competition and harassment.

Mating systems describe where, when, how and how often individuals of the two sexes come together to mate (Emlen and Oring, 1977, Davies, 1991). Traditionally, mating systems terminology has served a mostly descriptive function, for instance defining how often males and females mate (monogyny or polygyny for once or multiply mating males, monandry or polyandry for once or multiply mating females; polgynandry for multiple mating amongst both males and females), with additional terms describing features of how access to mates of the opposite sex was achieved (e.g. resource-defence polygyny, scramble competition polygyny, and so forth: see Thornhill and Alcock, 1983 for examples of this kind of framework to describe mating systems across the insects). In addition to just describing mating systems however, various theoretical frameworks have been developed, primarily in order to help predict what mating systems should appear under a given ecological scenario, and to link mating systems to patterns of sexual selection. In terms of the former, the classic framework (e.g. Emlen and Oring, 1977, Davies, 1991) sought to predict mating systems based on the ecological factors that shaped the distribution of females in the environment (such as relevant resources or habitats), and the extent to which males were able to monopolise access to females or those resources (again influenced by local ecological factors). Two important additional components were the "operational sex ratio" (OSR), or the ratio of reproductively active males and females in the population at a given time (the common, and mostly valid assumption is that the OSR is more or less male-biased) and the so-called “Bateman gradient” (the relationship between fitness and number of mates, calculated separately for males and females, and which typically show a steeper gradient for males compared to females: Bateman 1948). This framework could then place species into harems or resource-based systems where males could economically defend females or the resources they needed, through to leks, swarms or scrambles, whereby females or resource territories were not defendable and alternative systems, including those relying on arbitrary "conventions" (such as leks, or hill-topping: Thornhill and Alcock 1983), arose instead.

Important early developments included the realisation that there was substantial variation in a population in terms of the "mating system" that different individuals would experience (e.g. the dunnock, *Prunella modularis*: Davies, 1992). However, more recently, the focus has been more on linking mating systems and sexual selection. For instance, Shuster and Wade (2003) defined mating systems in terms of the so-called "opportunity for sexual selection" in both males and females, based on the variance in mating success in males and females. Whilst they provided a more rigorous, quantitative approach for defining mating systems, the series of mating systems that resulted were nonetheless rather similar to the more traditional framework. The approach of Shuster and Wade has also been critiqued on theoretical grounds. Building on work of Parker and Simmons (1996), who explored the links between mating systems and sexual selection in terms of "time-in" and "time-out" of the mating pool (i.e. the OSR), in a series of papers Kokko and colleagues have clarified how OSR, variance in mating success, and patterns of sexual selection in males and females are linked across mating systems (Klug et al., 2010, Jennions et al,. 2012, Kokko et al., 2012, 2014). Key to this has been teasing apart the operational sex ratio and the Bateman gradient. In brief, both the OSR and the Bateman gradients for males and females are needed for a complete picture of how sexual selection is expected to act on males and females. A full discussion of these developments in mating systems theory is beyond the scope of this introductory chapter, but for an up-to-date review see Kokko et al. (2014). All that said, the basic descriptive function of "mating systems" is still informative, and from the species studied to date, lygaeids seem to be characterised by polgynandrous mating systems with both males and females mating multiple times (Wang and Davis, 2006). For instance, mating frequencies in both wild and laboratory populations of *Neacoryphus bicrucis* have been observed to average 0.8 copulations per day for females (McLain, 1989) and in laboratory populations of *O. fasciatus* 20-30% of individuals within a colony may be engaged in copulation at any one time (Economopoulos and Gordon, 1972). Preliminary courtship behaviours (or at least those that are easily observable) are very rare but exceptions do exist, for example in *N. huttoni* males have been observed to court females by antennating them prior to mounting (Yang and Wang, 2004), though how typical this behaviour is remains unknown. Fertilization is internal, and males produce free spermatozoa rather than spermatophores (Dallai and Afzelius, 1980). In the majority of species described, copulation is initiated by the male grasping the female with all three pairs of his legs. Males then orient themselves in the same direction as the female to initiate genital coupling (Figure 6). If genital coupling is successful, males then change orientation and the majority of the mating takes place with males and females facing opposite directions (i.e. back-to-back). This copulation behaviour appears to be the norm within the family and has been recorded in a number of species including *L. equestris* (Shuker et al., 2006, Sillen-Tullberg, 1981), *S. pandurus*, *O. fasciatus* (Walker, 1979) and *Nysius huttoni* (Yang and Wang, 2004).

Encounter polgynandry appears to be common within the group with little evidence of either resource-based (e.g. resource defence) or non-resource based territoriality (e.g. lekking). There is some evidence that male Lygaeidae attract potential mates via pheromonal cues (see above; Aldrich et al., 1999, Zhang and Aldrich, 2003) or exclude other insects from food patches (McLain and Shure, 1987), but whether these behaviours are sufficient to be considered different from encounter polgynandry is unclear. In particular, the exclusion of other individuals from food patches by males of species such as *N. bicrucis* appears to be a side effect of indiscriminate sexual harassment rather than directed behaviours towards potential rivals (but see Rodriguez S, 2000). This behaviour may also result in the exclusion of female conspecifics, reducing the future mating opportunities of the males in those patches (McLain and Pratt, 1999).

As with many insect species (Garcia-Barros, 2000, Salavert et al., 2011, Kanuch et al., 2013) increased female body size in the Lygaeidae is typically correlated with higher fecundity (but see Shuker et al., 2006). As a result we expect males to preferentially mate with larger females and indeed weak selection for larger females has been detected in laboratory populations (Dougherty and Shuker, 2014). Despite this, evidence of pre-copulatory selection in the Lygaeidae is generally rather scarce, although some patterns of non-random mating have been detected (*Neacoryphus bicrucis*, larger males gain more matings, McLain, 1992, in *Nysius huttoni*, males prefer females with broader abdomens, Yang and Wang, 2004). Within *L. equestris*, for example, there is suggestion that females prefer smaller, or intermediate-sized, males (this despite previous findings suggesting females gain fitness benefits from mating with heavier males, see below) (Dougherty and Shuker, 2014).

Females commonly mate with one or several males between oviposition events (Sillen-Tullberg, 1981, Economopoulos and Gordon, 1972, Wang and Davis, 2006). This creates the potential for intense sperm competition (e.g. Simmons, 2001) and studies *of L. equestris* have shown that the last male to mate with a female before oviposition fertilizes approximately 90% of the eggs she lays in that batch (Sillen-Tullberg, 1981). Similarly, studies in laboratory strains of *O. fasciatus* found that when two males were allowed to mate with a single female in succession, the mean number of progeny fathered by the first male is only 14% of the mean number of progeny of the second male (Economopoulos and Gordon, 1972), although additional research suggested this ratio can vary according to the photoperiod during which the second mating takes place (Walker, 1979). As such, changes in both male and female (physiological) state can perhaps significantly alter insemination and fertilization success. Overall, patterns of non-random fertilisation success may well be related to the complexities of lygaeid genitalia (see Figures 7 and 8). As in common within the Heteroptera, copulations are often prolonged (Sillen-Tullberg, 1981) (although insemination may not be) and depending on the species copulation duration can vary from less than an hour to over 15 hours. As successful insemination has been found to occur in matings less than an hour long, these extended copulations have been suggested to be a form of mate-guarding (Sillen-Tullberg, 1981, Wang et al., 2008). This hypothesis was supported by a study of *N. bicrucis* in 1989 which found that the mean fertilization success of the last male to mate with a female before oviposition was 78% despite a single insemination containing enough sperm to fertilize 10-20 clutches of eggs (McLain, 1989). This study also found that not only did females frequently re-mate before oviposition following matings of short (i.e. less than eight hours) duration, but that longer matings were more frequent when the sex ratio was male biased. The latter observation suggests plasticity of copulatory behaviour, both in terms of copulatory mate-guarding (Figure 6) and also perhaps in terms of strategic ejaculate allocation (i.e. longer copulations and greater sperm transfer with increasing sperm compertition intensity: Parker and Pizzari, 2010, Kelly and Jennions, 2011), and similar plasticity has been found in

N. huttoni (Wang et al., 2008). Interestingly however, longer copulations may not always result in greater sperm transfer as findings in *L. equestris* suggest that longer copulation durations produced no increase in the number of fertilised eggs, and the insemination rate was highest during the first hour of copulation (Sillen-Tullberg, 1981).

The genitalia of both male and female lygaeids are typically complex (Bonhag and Wick, 1953) and elongated (for details of the genetic patterning of these genitalia see Aspiras and Angelini, 2011, Aspiras et al., 2011). During mating males must thread their tube-like intromittent organ (or processus gonopori) through the female's spermathecal complex, in order to deliver their flagellated sperm to the receptaculum and thus successfully inseminate her (Tadler, 1999, Figures 7 and 8). Since not all matings result in insemination and fertilization, it is possible that cryptic female choice plays a role in the overall process of sexual selection in these species (Eberhard, 1996). However, this has yet to be confirmed and the exact mechanism of any possible form of cryptic choice is still uncertain. Gschwentner and Tadler (2000) suggested that a valve structure at the entrance to the female's spermatheca could allow females to deny entry to both the male organ and sperm, however a subsequent study utilizing three (albeit different) species, *O. fasciatus*, *Leptoglossus occidentalis* and *Leptocoris trivittatus* (Chiang, 2010b), has suggested that the male organ instead gains access to the spermatheca duct via a parallel duct named the insemination duct, thus bypassing the valve. As this was discovered in the box elder bug, *L. trivittatus*, it is uncertain whether it holds true for all Lygaeidae. Intriguingly, despite the male intromittent organ often exceeding half his body length (Higgins et al., 2009) there appears to be no positive selection for increased length. Indeed, studies in *L. equestris* and *L. simulans* have found evidence for stabilising or negative selection acting on male intromittent organ length (Tadler, 1999, Higgins, 2009) although this may vary depending upon the fitness measure used. It appears that in *L. equestris*, males with longer genitalia are less likely to gain matings. However, when fertilisation success is taken into account selection on genitalia is stabilising (Dougherty and Shuker, 2014). This suggests that different selective pressures may act on male genitalia at different stages of reproduction.

The lack of strong pre-copulatory selection, combined with the complex genitalia and high rate of variability in fertilisation success, suggest that post-copulatory choice plays a significant role in the sexual selection of the Lygaeidae. In their 2006 study, Shuker et al found that when female *L. equestris* were allowed to mate only once, those that mated with heavier males produced a greater number of eggs. This suggests that females may benefit from matings with some males more than others. It is possible then that females may have responded to the apparent male control of copulation by evolving mechanisms whereby they can bias paternity following the initiation of copulation. However, experimental evidence of post-copulatory selection in the Lygaeidae is scarce. While it has been hypothesised that behaviours such as the rocking and kicking that can occur during copulation may be attempts by one of both of the sexes to influence paternity, experimental studies have failed to detect a measurable effect of these behaviours on fertilisation success (Sillen-Tullberg, 1985b). This is despite kicking behaviour in females being found to reduce copulation duration (Rodriguez, 1998). Thus, the prevalence of post-copulatory selection within the Lygaeidae remains an interesting avenue for further study.

With so few courtship preliminaries, males effectively harass females as they try and engage genitalia. Studies have demonstrated a fitness cost to females of this repeated mating and harassment, confirming the existence of sexual conflict over mating frequency in Lygaeidae (Shuker et al., 2006). Sexual conflict, whereby the optimal strategies of males and females do not align during reproduction, has been recognised as a driving force in the evolution of reproductive morphologies and behaviour (Parker, 1984, Eberhard, 1996, Arnqvist and Rowe, 2005). A key source of sexual conflict within the Lygaeidae appears to be mating frequency, with the optimal number of matings for males far exceeding that of females. Females often appear to resist male attempts to mate (Rodriguez S, 2000) and males may continue to attempt to mate with 'unreceptive' females for several hours (*pers. obs*.). Females may well then have to balance the fitness costs of a sub-optimal mating rate with those of resisting male mating attempts. Studies in *L. equestris,* for example*,* have demonstrated a significant reduction in female longevity when females are housed with multiple males compared within one or no males (Shuker et al., 2006). This reduction was greatly reduced when males were rendered unable to mate, suggesting that while harassment by males may impose some penalties on the females, it is mating itself which is responsible for most of the survival and fitness costs observed (perhaps through damage imposed by the internal movements of the complex genitalia). Further evidence for a penalty of repeated mating in Lygaeidae comes from a study of the seed bug *Togo hemipterus*, which found that mating reduces lifespan and starvation tolerance in both males and females (Himuro and Fujisaki, 2010). Intriguingly, this species has also been found to produce seminal proteins that inhibit female remating (Himuro and Fujisaki, 2008), opening up the possibility for similar seminal-fluid mediated sexual conflicts over mating and reproduction as to those seen in *Drosophila* (e.g. Chapman et al., 1995).

Finally, sexual behaviour needs not always be present. Remarkably, a unisexual population of the seed bug *Nysius groenlandicus* has been found in Northern Greenland. In some areas of the Zackenberg Valley within the Northeast Greenland National Park, there exist populations in which males are rare or entirely absent. Due to the persistence of these unisexual populations it is assumed that females in these areas reproduce parthenogenetically. Unusually these asexual populations occur in close proximity to sexual populations (Bocher and Nachman, 2011). *N. groenlandicus* is widespread across northern Europe and is common throughout Greenland. It is a well-adapted arctic species and is univoltine, with all reproductive activity taking place during the brief arctic summer and only the eggs surviving the winter. The preferred habitat of the species is dry, sunny areas with sparse vegetation and a temperature of 30^{o}C. As a result it has a patchy distribution and its obligatory univoltinism makes it vulnerable to local extinction during particularly bad summers. It is thought that parthenogenetic reproduction may provide an advantage in such adverse conditions as it removes the need for females to find a mate prior to egg-laying, thus reducing the time needed to successfully complete an annual reproduction cycle. This is supported by the finding that differing sex ratios can be explained by climatic factors, with the female bias increasing with distance from the coast. As inland climatic conditions are more predictable than those along the coast it would seem that asexual reproduction provides a benefit in relatively stable environments while sexual reproduction remains the preferred mode of reproduction in the more changeable coastal regions (Bocher and Nachman, 2010). However, it is also possible that these asexual populations are the result of a male-killing parasite, like those described earlier, rather than an adaptation.

2.8 Reproductive interference – ecological and evolutionary consequences

As is assumed in many species, it is thought that male seed bugs can increase their fitness by mating with as many females as possible (Bateman, 1948). This selection for males to mate readily means that in areas where related species co-exist there is the potential for inter-specific matings to occur. Inter-specific matings and attempted matings have been observed in several species of Lygaeidae, both in the wild (Leslie and Dingle, 1983, McLain and Pratt, 1999) and in the lab (Shuker et al, *in prep*). These inter-specific mating interactions have been shown to have significant impacts both at an individual (Shuker et al, *in prep*) and an ecological scale (McLain and Pratt, 1999, McLain and Shure, 1987), and as such are examples of reproductive interference (Gröning and Hochkirch, 2008, Burdfield-Steel and Shuker, 2011). On an individual scale, harassment and mating by inter-specific males has been shown to impose a fitness cost on female *L. equestris* (Shuker et al, *in prep*). Large decreases in both longevity and lifetime fecundity, comparable with those experienced in the presence of a conspecific male, were recorded in females housed with male *S. pandurus*. No such reductions were recorded when *L. equestris* females were housed with females of either species. A similar pattern was found in *N. bicrucis* (McLain and Pratt, 1999). Females housed with males of either the same species or *Margus obscurator* following mating with a conspecific male had approximately half the fecundity of those housed with females of either species. On an ecological scale, this fitness cost of heterospecific courtship and harassment has been suggested as a reason why the two species are rarely found co-existing on the same plant despite having overlapping ranges (McLain and Pratt, 1999). In addition, a series of studies carried out on *N. bicrucis* in the field suggest that indiscriminate, and often aggressive, mating behaviour carried out by males of the species actively excludes other polyphagous insect species from habitats supporting high *N. bicrucis* densities (McLain and Shure, 1987). Thus there is emerging evidence that reproductive interference may play a significant role in determining habitat use in the Lygaeidae. More formal links between selection on (primarily) male mating frequency and the extent of reproductive interference remain to be made however. For instance, measuring Bateman gradients in the presence or absence of hetero-specifics remains to be done (in the laboratory, let alone in the wild). Incorporating such aspects of mating systems theory into the study of RI will help us predict when we might expect RI to have a major or minor role in mating system evolution.

3. Conclusion

The Lygaeidae offer a variety of opportunities for evolutionary and behavioural ecologists, including the study of sexual selection and reproductive interference. They are often easy to keep in the laboratory, and in the age of next-generation sequencing and sub-thousand dollar genomes, there is the potential for them to become a genetic model organism in the future(for an example of this see Zhen et al., 2012). In addition to their complex genitalia and varied inter-specific interactions several other areas that may ultimately influence their reproductive dynamics include: (1) the fascinating pattern of co-evolution between the bugs and their endosymbionts, including the evolution of specialised structures and the remarkable diversity *within genera*; (2) the individual variation in chemical defence and its potential to influence within- and among-population and species patterns of mimicry; (3) the patterns of cannibalism that remain poorly-studied, including egg- and sib-cannibalism, and the opportunities for testing kin selection theory; (4) the lack of pre-copulatory mate choice and the likely importance of post-copulatory sexual selection; (5) the ease with which these species can be used to study fundamental aspects of life-history evolution, including diet specialisation, wing polymorphisms, and diapause.

With this in mind my research has focused on several areas that contribute to reproductive interference in these insects. In chapter two I investigate the development time of my study species in the laboratory in order to inform further work on their inter-specific interactions. In chapter 3 I test the effects of both male and female heterospecifics and conspecifics on the fecundity of female *L. equestris*. Chapter four focuses on short range signalling between the bugs, in the form of cuticular hydrocarbons, and its implications for both species discrimination and mate choice. Chapter five looks at the effects of diet and levels of chemical protection on several life-history traits, including mate choice. Chapter six investigates the potential for reproductive interference to act via effects on male mate-guarding behaviours and male-male competition. In chapter seven I look for evidence of genetic compatibility driving the selection for multiple mating in *L. equestris* females, and how contact with heterospecifics may influence the benefits of polyandry. Finally, in chapter 8 I summarise my findings and lay out both the conclusions that can be drawn from my work and the areas in need of further study in order to fully understand the causes and consequences of reproductive interference in the Lygaeidae.

Figure 6. An assumed attempt at a copulation takeover in wild *Lygaeus creticus,* Sicily*.*

Figure 7. Photograph of the female genital tract of *Lygaeus simulans* with inserted male intromittent organ. Reproduced from Gschwentner and Tadler (2000) with permission.

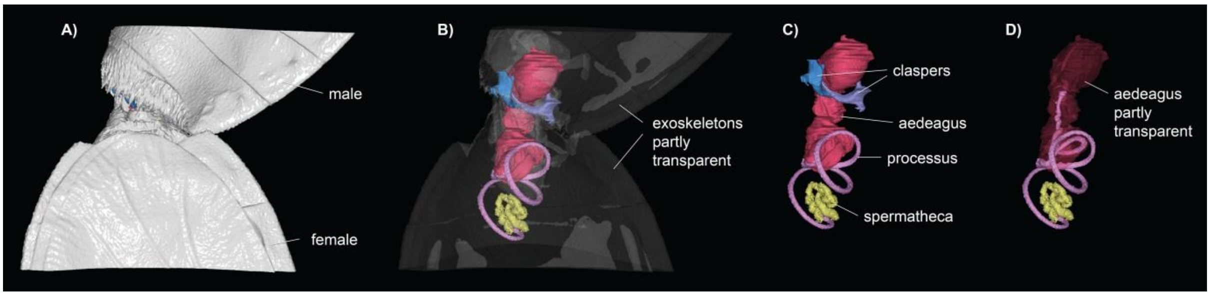

Figure 8. Reconstruction of micro-CT scans showing the male and female genitalia of *Lygaeus equestris* during copulation. Courtesy of Imran Rahman.

Chapter 2

General Husbandry and Development time in four species of Lygaeidae

1. General Husbandry

In this second chapter, I will briefly outline the general husbandry of the study species before discussing a preliminary experiment to compare patterns of development across four of these study species.

Five species of Lygaeidae, from six populations, were used during this work: *Lygaeus equestris* (two Pratomagno mountains in Saltino, Tuscany), *Lygaeus creticus* (from Sicily), *Spilostethus pandurus* (from Sicily), and *Oncopeltus fasciatus*. The latter species is a North American species that does not naturally co-occur with the other three species (Dingle et al 1977). Our population was provided by Dr Vernon French from the University of Edinburgh who maintained this species in long-term laboratory culture. The other three species were collected by David M. Shuker and colleagues between 2004 and 2009, with the exception of the Sicily population of *L. equestris*. This population was collected and then maintained in laboratory culture in Sweden before a new laboratory culture was founded at the University of Leeds by Professor Nina Wedell in 1996. The study population is descended from that Leeds population and has been studied previously alongside the Dolomites population (Shuker et al. 2006).

I maintained all five species in continuous culture in the laboratory subject to a fixed environmental regime of 22:2 hours light:dark cycle and a temperature of 29ºC. These conditions prevented the bugs from entering reproductive diapause (Shuker et al., 2006). I used 30x15x15 cm plastic boxes with ventilated lids as stock cages, and provided organic, dehusked sunflower seeds (*Goodness Direct*, UK) at a depth of five to six centimetres across the bottom of the cage. I also provided two tubes of demineralised water with cotton wool bungs as a water source and changed these tubes weekly. Finally, a piece of cotton wool was also provided as three-dimensional substrate for the bugs to sit on and hide in. Oviposition occurred both on this cotton wool and amongst the sunflower seeds. Stock cages were kept in continuous culture with overlapping generations and were replaced every six to eight weeks. New stock cages were founded from mixed age groups of upwards of 60 individuals removed at random from the original cage. Large-mouthed pooters were used to transfer the bugs between cages.

2. Development times in the laboratory

2.1 Introduction

When trying to assess the demographic outcomes of behavioural interactions it is vital to have a baseline to which any potential variation can be compared. Life history traits such as life cycle, development time and reproduction rate provide important context in which more nuanced information about variation in behaviour can be interpreted.

Species from the super family Lygaeiodea (Insecta: Hemiptera: Heteroptera, henceforth referred to as “lygaeids”) have frequently been utilized as laboratory model insects due to their ease of husbandry and short generation times (Feir, 1974, Solbreck et al., 1989). In addition, the polygamous mating system and complex, elongated genital structures that are typical of the group, have attracted increasing attention from researchers working on sexual selection and related phenomena such as sexual conflict and reproductive interference (McLain, 1991, McLain and Pratt, 1999, Shuker et al., 2006). Lygaeids are a common, and often familiar, group of bugs found worldwide with a variety of common names (e.g. seed bugs, ground bugs and milkweed bugs). They are often conspicuous, as some species are aposematic, displaying red and black warning colourations as part of putative Müllerian mimicry rings (Gamberale-Stille and Sillen-Tullberg, 1999, Sillen-Tullberg et al., 2000). Despite several species having economic importance as pest species (Sweet, 2000) the majority of what is known about lygaeids is a result of their use in laboratory experiments (Feir, 1974, Sillen-Tullberg, 1981, Jin et al., 2010). However, if species from this group are to be most usefully utilized in laboratory studies then more data on their basic biology is needed. For instance, studies of intra- or inter-specific mating interactions that utilise demographic, or “economic” costs and benefits of a given form of interaction, require us to understand the base-line developmental schedules of the focal species. This may be particularly relevant for inter-specific interactions, in which the effects of behavioural (interference) interactions need to be partitioned out from other forms of inter-specific resource competition. As such, studies using different species, or distinct populations of the same species, would benefit from information regarding variation in life-history traits, such as development time and reproductive rates, both within and between the species being studied.

Here I consider the patterns of egg-to-adult develop in four species of lygaeid under a common-garden experiment set-up. Of the species used in this experiment, three are old world species, *Spilostethus pandurus* (Scopoli)*, Lygaeus equestris* (Lucus.) and *Lygaeus creticus* (L.) and one, *Oncopeltus fasciatus* (Dallas), is found in the new world. All four species are seed eaters and undergo incomplete metamorphosis with five wingless nymphal instars before they reach the adult, winged, form.

I measured the developmental timings of four species of Lygaeidae kept in single species populations under identical lab conditions. In addition to recording development, I also considered clutch size and proportion of infertile clutches laid, as these may also impact the husbandry (and the interpretation of experiments) of these species in the lab.

2.2 Methods

I identified and isolated 5th instar nymphs from stock cages of each of the four species. As two separate strains of *Lygaeus equestris* are present in the lab, one descended from an experimental population originally collected in Sicily (and then maintained by Professor Nina Wedell at the University of Leeds: the “Leeds” population) and one descended from bugs collected in the Dolomites mountains in northern Italy in 2004, these were treated as separate populations for the purposes of this experiment. I kept the isolated nymphs in tubs and provided them with both organic sunflower seeds and water *ad libitum*. Virgin females were isolated from these tubs on a daily basis as they completed their final moult. Eight days after reaching adulthood, I paired each female with a male of the same species selected randomly from the stock colonies. These pairs were kept in petri dishes, again provided with seeds and water, and checked twice daily for mating over the course of ten days. If males died, I replaced them with new males from the colony cages. If the female died I discarded the pair. I isolated the first clutch laid and placed it in another tub with sunflower seeds and water again *ad libitum* (water tubes were monitored and replaced as necessary). The number of eggs in each clutch was counted and the date of collection was considered as the first day of the clutches' development. One clutch per female was included in the study. After ten days, I separated surviving females from the males and collected the first clutch laid after separation. The date of collection and age of mother was recorded for each clutch. I monitored clutches every day and the number of nymphs present at each instar was recorded. These were then plotted for inspection. The time, in days, taken for every individual in a clutch to reach adult form was recorded and analysed using PASW Statistics 18 by IBM. I tested for significant differences between the five populations in egg-to-adult development time using one-way ANOVA with post-hoc Fisher's LSD to determine which populations differed significantly from each other. The same method was then used to look for species differences in clutch size. Differences in fertility rate (measured as whether a clutch produced nymphs) were tested using logistic regression (i.e. a generalised linear model with a binomial error structure and logit-link function).

2.3 Results

Sample sizes across the five populations ranged from 36 to 50, with a total of 204 clutches followed (Table 1). The four species varied in their patterns of egg-to-adult development under a common-garden experiment situation (Figure 1). All individuals across all the replicates reached adulthood by 32 days, with adult eclosion commencing between 20-23 days post oviposition. Fourth instar nymphs and adults over-lapped across replicates for all the species except *L. creticus*. *O. fasciatus* was the fastest developer, followed by *L. creticus*, with *L. equestris* developing slowest (Table 1). The differences among the species were highly significant (ANOVA: $F_{4, 85} = 23.68$, $P < 0.0001$). Post-hoc analysis revealed that *O. fasciatus* and *L. creticus* did not differ significantly in development time, whilst *S. pandurus* developed significantly slower than *O. fasciatus* but not *L. creticus* (Table 2). However, these three species all developed faster than the two *L. equestris* populations (all $P < 0.001$; Table 2).

The five species also varied in terms of clutch size ($F_{4, 154} = 22.698$, $P < 0.0001$) and clutch fertility ($\chi^2 = 24.251$, *d.f* = 4, $P<0.0001$; Table 1). *S. pandurus* laid significantly more eggs per clutch (Table 3) and had a higher fertility rate than the other species (Table 1).

2.4 Discussion

Here I have considered patterns of egg-to-adult development in four species of lygaeid seed bug, plus clutch size and fertility. There is significant variation in the time taken for newly laid eggs to hatch and the nymphs to develop into adults between the four species. Despite this variation, the broad patterns of development are reasonably similar (Figure 1) such that no one instar stands out in terms of its duration across the five populations (i.e. the proportion of time spent at each instar, is similar across all four species). Once hatched the nymphs move relatively rapidly between instars until they reach the fifth instar. Nymphs of all four species spent more time at the fifth instar than they did at any other nymphal stage. This is in keeping with a previous finding in *O. fasciatus* that nymph size is related to the timing of the adult moult (Blakley, 1981). It is thought that nymphs must reach a certain weight in order to trigger this final moult (Blakley and Goodner, 1978b). As fifth instar nymphs are significantly bigger than those at earlier instars, it may take them longer to achieve a similar proportional increase in weight, thus causing a lag in development speed at the fifth instar as it takes longer to obtain the resources needed for the switch to adulthood than for switches between other instars.

Although found in previous studies to differ in a number of ways, including both body size and life expectancy in the lab (Shuker et al., 2006), the two populations of *L. equestris* do not differ significantly from each other with regards to development time. Both populations take significantly longer to reach adulthood than any of the other three species. The mean clutch size and fertility rates of these two populations were also similar. Therefore the two populations of *L. equestris* are considered together for the purposes of the rest of this discussion.

S. pandurus has the largest average clutch size and highest rate of fertility. This is in keeping with the observation that, in laboratory conditions, *S. pandurus* has the highest rate of population growth of all the four species (Burdfield-Steel, *pers. obs*.). This has both practical and theoretical implications. Firstly, this rapid growth may allow *S. pandurus* to competitively exclude the other lygaeid species studied in mixed populations. This must be considered when planning any experiments that require mixed species populations. Secondly, the large clutch sizes and rapid development makes the species susceptible to dramatic fluctuations in population size under laboratory culture. In a closed environment, such as a stock cage, *S. pandurus* can reach the maximum population size (i.e. carrying capacity) leading to population crashes if the cages are not monitored regularly and appropriately managed and maintained.

O. fasciatus was found to have the shortest development time from egg to adult. It also has the second largest average clutch size and percentage fertility after *S. pandurus*. Despite this, the differences between both the mean clutch sizes and fertility rates of the two species are considerable. This, in addition to its longer period of adaptation to laboratory conditions, could explain why *O. fasciatus* is observed to be less susceptible to very rapid population growth and subsequent crashes in the laboratory than *S. pandurus.* Interestingly, *O. fasciatus* also had the smallest mean body size of the four species (*pers. obs*.), suggesting that there may be a trade-off between development time and adult body size.

No significant differences were found in development time between *L. creticus* and the two species *S. pandurus* and *O. fasciatus* as it has an intermediate development time between the two. However, *L. creticus* has not been observed to suffer from rapid fluctuations in population size in the laboratory, presumably due to the lower average clutch size and fertility rate found in this species compared to *S. pandurus* and *O. fasciatus*. This suggests that, under laboratory conditions, it is clutch size and fertility rate that have the greatest effect on population growth, rather than development time.

It is not yet known if the variation in development time found in this study can translate into significant differences in generation time between the species. The species used in this study do not begin mating and producing eggs immediately after the adult moult. There is usually a period of several days before reproductive behaviour can be observed and it is not yet known how the length of this period varies between species. In addition, in Lygaeidae, generations typically overlap (Shuker et al., 2006) and females are expected to lay several clutches over the course of their lifespan. Thus, additional factors, such as the time period between oviposition events, may have a greater impact on generation time than the speed of development. In the wild the timings of mating and oviposition are largely determined by season. Populations from more northern latitudes are often uni-voltine, with adults undergoing reproductive diapause during the winter (Dingle, 1972a, Dingle et al., 1977, Sillen-Tullberg, 1984, Solbreck and Sillen-Tullberg, 1981). However, multi-voltine reproduction has been observed during long summers (Solbreck and Sillen-Tullberg, 1981, Solbreck and Sillen-Tullberg, 1990) and in more southern populations (Dingle, 1972a, Dingle et al., 1980a).

In conclusion, the four species differ considerably in development time and clutch size and fertility.

These differences have implications, both for future studies of competition and interference between the species, and also for the management of laboratory populations. In the next chapter I will describe the results of one such study, investigating the effects of RI on a group level.

Table 1. Summary of clutch size, fertility and mean time taken between collection of eggs to all individuals reaching adulthood across the five species across the five populations.

Species	**Number of clutches collected**	**Proportion of clutches fertile (%)**	**Mean clutch size (± s.e.)**	**Mean time to adult in days (± s.e.)**
Spilostethus pandurus	39	92	41.9 ± 3.16	26.66 ± 0.245
***Lygaeus equestris* (Dolomites)**	36	64	17.9 ± 1.62	29.92 ± 0.712
***Lygaeus equestris* (Leeds)**	50	52	15.2 ± 1.62	29.26 ± 0.314
Oncopeltus fasciatus	36	69	28.8 ± 3.95	25.23 ± 0.482
Lygaeus creticus	43	56	20.7 ± 1.76	26.00 ± 0.302

Table 2. Results of LSD post-hoc test on development times. I-J = mean differences, P = significance. Results significant to 95% confidence interval (P < 0.05) and marked with a *.

Species	***Spilostethus pandurus***	***Lygaeus equestris* (Dolomites)**	***Lygaeus equestris* (Leeds)**	***Oncopeltus fasciatus***	***Lygaeus creticus***
Spilostethus pandurus	-	I-J = 3.260*, P = 0.000	I-J = 2.606*, P = 0.000	I-J = -1.426*, P = 0.008	I-J = -0.657, P = 0.239
***Lygaeus equestris* (Dolomites)**	-	-	I-J = 0.654, P = 0.272	I-J = -4.686*, P = 0.000	I-J = -3.917*, P = 0.000
***Lygaeus equestris* (Leeds)**	-	-	-	I-J = -4.032*, P = 0.000	I-J = -3.263*, P = 0.000
Oncopeltus fasciatus	-	-	-	-	I-J = 0.769*, P = 0.245
Lygaeus creticus	-	-	-	-	-

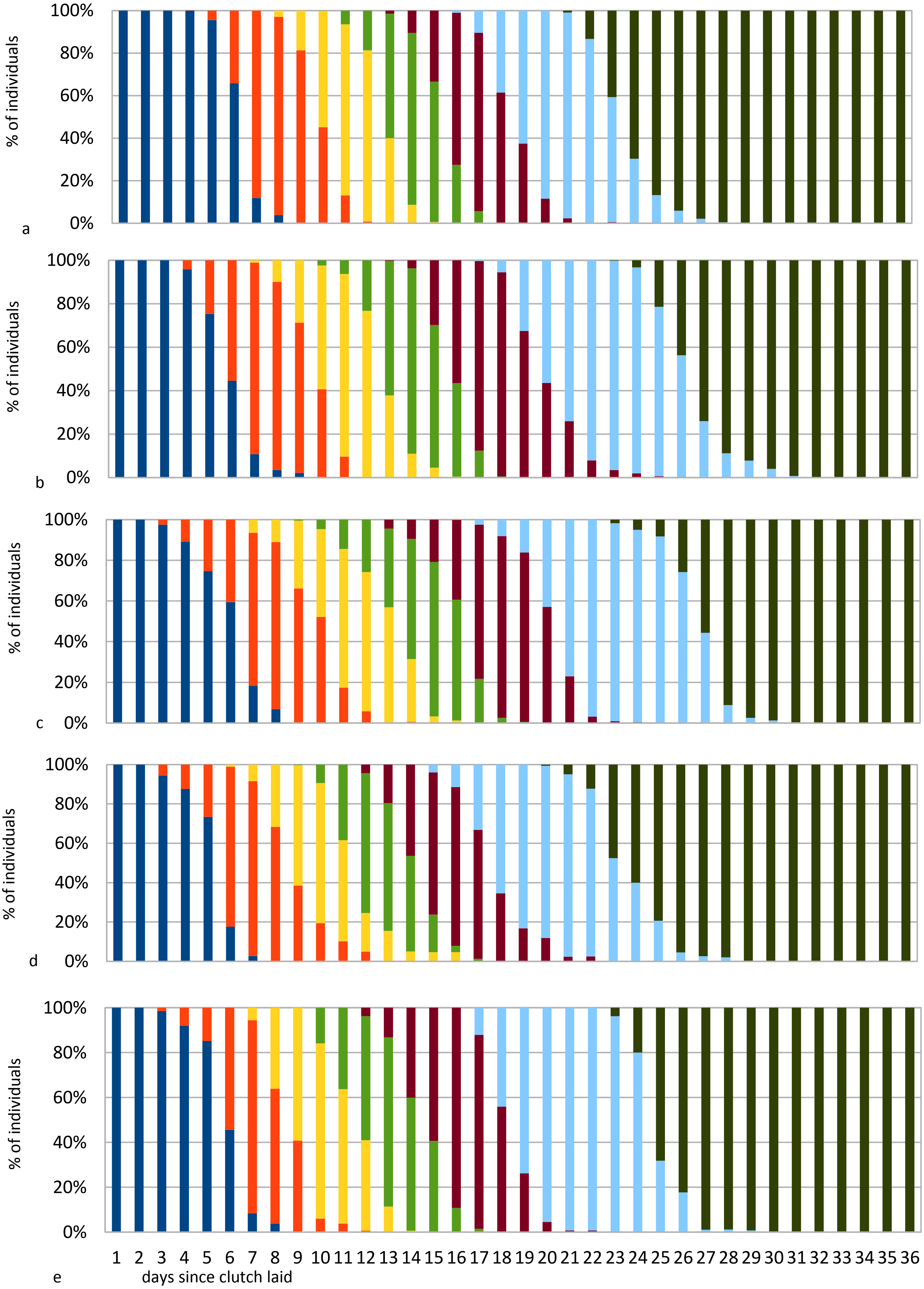

Figure 1a) *S. pandurus*, b) *L. equestris* (Dolomites), c) *L. equestris* (Leeds), d) *O. fasciatus*, e) *L. creticus*. The colours represent each stage of the bugs development. Dark blue - eggs, red – 1st instar, yellow - 2nd instar, light green - 3rd instar, purple - 4th instar, light blue - 5th instar, dark green – adult.

Table 3. Results of LSD post-hoc test on clutch sizes. I-J = mean differences, P = significance. Results significant to 95% confidence interval (P < 0.05) and marked with a *.

Species	***Spilostethus pandurus***	***Lygaeus equestris* (Dolomites)**	***Lygaeus equestris* (Leeds)**	***Oncopeltus fasciatus***	***Lygaeus creticus***
Spilostethus pandurus	-	I-J = -23.85*, P < 0.000	I-J = -25.25*, P < 0.000	I-J = -13.85*, P < 0.000	I-J = 21.17*, P < 0.000
***Lygaeus equestris* (Dolomites)**	-	-	I-J = 1.40, P = 0.641	I-J = -11.00*, P = 0.001	I-J = 2.674, P = 3.89
***Lygaeus equestris* (Leeds)**	-	-	-	I-J = -12.40*, P < 0.000	I-J = -4.07, P = 0.154
Oncopeltus fasciatus	-	-	-	-	I-J = 8.33*, P = 0.008
Lygaeus creticus	-	-	-	-	-

Chapter 3

Reproductive Interference between *Lygaeus equestris* and four Lygaeidae species

1. Introduction

Interspecies interactions can take many forms and play a vital role in shaping evolution. Perhaps the most commonly studied, and best understood, interactions are those that involve predation and competition. However, when different species are carrying out potentially complex behaviours (i.e. mating and reproduction) in the same area, seemingly non-adaptive behaviours, such as reproductive interference, can take place. Reproductive interference (RI) occurs when reproductive behaviours occur between organisms of different species and these behaviours result in a loss of fitness for one or both species. While inter-species sexual behaviours are well-documented (Andrews et al., 1982, Dame and Petren, 2006, de Bruyn et al., 2008), the ecological effects of such interactions are still poorly understood (Gröning and Hochkirch, 2008). RI has been implicated in both the exclusion of native species by invasive ones (Butler and Stein, 1985, Liu et al., 2007) and in determining the ecology and habitat use of naturally co-occurring species (Gröning et al., 2007, Noriyuki et al., 2012). The exact consequences of RI will vary depending on both the context and the form that the interference takes. For instance, signal jamming can reduce the efficiency of mate searching, as well as lead to the displacement of the signals themselves. For example, female spadefoot toads (*Spea multiplicata*) compromise on male song quality to ensure they mate with the correct species in areas where they co-occur with closely-related heterospecifics. As a result, females in these populations are unable to benefit from the increased fertilization success associated with high-quality males (Pfennig, 2000).

If the fitness consequences of RI are sufficient, then it may drive ecological or evolutionary changes to mitigate this cost. However, more empirical studies of the fitness costs of reproductive interference are needed in order to establish if this is the case (Gröning and Hochkirch, 2008). It should be noted at this point that while patterns such as reduced lifespan may be suggestive of potential fitness costs, changes in life history parameters such as number off eggs produced, or offspring survival, provide more convincing evidence of RI as they are generally considered to be closer approximations of an individual's true "fitness". Of the few published studies of the ecological effects of RI, several have used insect species (McLain and Shure, 1987, McLain and Pratt, 1999, Gröning et al., 2007). McLain and Pratt (1999) found that *N. bicrucis* females housed with males of either the same species or *Margus obscurator* following mating with a conspecific male had significantly reduced fecundity compared to those housed with females of either species. This cost of heterospecific courtship and harassment has been suggested as a reason the two species are rarely found on the same host plant in nature despite having overlapping ranges (McLain and Pratt, 1999). A series of studies carried out on *N. bicrucis* in the field suggest that indiscriminate, and characteristically aggressive, mating behaviour of the males of the species actively excludes other polyphagous insect species from habitats supporting high *N. bicrucis* densities, showing that RI can have significant ecological consequences (McLain and Shure, 1987). Similarly, Shuker et al (*in prep*) also used species from the super family Lygaeiodea (Insecta: Hemiptera: Heteroptera, referred to as "lygaeids") in order to investigate the consequences to female fitness of sexual harassment by conspecific males in a laboratory setting. Lygaeids are highly suitable for laboratory studies due to their easy husbandry and short generation time (Feir, 1974). In addition, previous research shows that in *Lygaeus equestris*, used as the focal species by Shuker et al, mating and sexual harassment by male conspecifics can reduced female longevity (Shuker et al., 2006). Although it is thought that, as in many species, male lygaeids can gain fitness benefits from mating with as many females as possible (Bateman, 1948), this willingness to mate may cause males to be less selective than females when assessing potential mates. Additionally, male *S. pandurus* have been shown to inflict similar fitness and survival penalties on female *L. equestris* as conspecific males (Figure 1, Shuker et al, *in prep*). The same experiments also found that *O. fasciatus* males did not inflict such penalties on *L. equestris*. Finally, harassment of *L. equestris* females by males of the species *Lygaeus creticus* has also been observed in the laboratory and in the field (*pers. obs.*), although its fitness consequences remain unknown.

This study was carried out to determine if the observed pattern of reduced fitness in individual *Lygaeus equestris* females in the presence of *Spilostethus pandurus* males (Shuker et al, *in prep*) extends to populations. In addition, the effects of both males and females of two other species, *O. fasciatus* and *L. creticus* on *L .equestris* females were also investigated. As I found evidence of egg predation occurring during the course of the experiment (see results), a second experiment was designed to limit the opportunity for such predation.

2. Methods

2.1 Experiment 1

The general husbandry of the species used was the same as that described in chapter 2. The focal species for this experiment was *Lygaeus equestris*. Small cages measuring 17x11x7 cm were set up with sunflower seeds and a source of water. Each cage contained ten reproductively mature *L. equestris* females which had been housed with a single male for three days prior to the start of the experiment to ensure mating. In addition, ten "companion" bugs were added to each cage. The sex and species of these varied as shown in Figure 2. There were eight possible treatments; four consisted of males of each species and four of females. Each treatment, as represented by a box in the schematic, was replicated five times bringing the total number of cages to 40. The additional females added to the cages were kept in single sex populations after eclosion to ensure they did not lay fertile eggs.

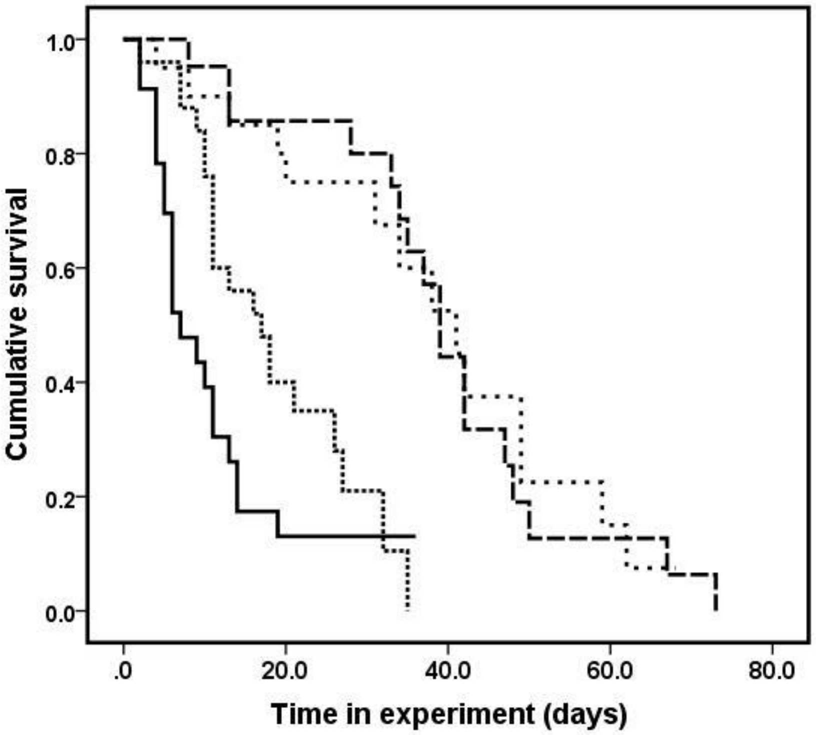

Figure 1. Interactions with conspecific males or male *Spilostethus pandurus* reduce the longevity of female *Lygaeus equestris*. Data are presented as Kaplan-Meier survival curves including censored individuals. Bold line: focal females kept with *S. pandurus* males; Dotted line: focal females kept with *L. equestris* males (conspecifics); Dashed line: focal females kept with *O. fasciatus* males; Exploded dotted line: focal females kept alone. Figure taken from Shuker et al. (*in prep*) with permission.

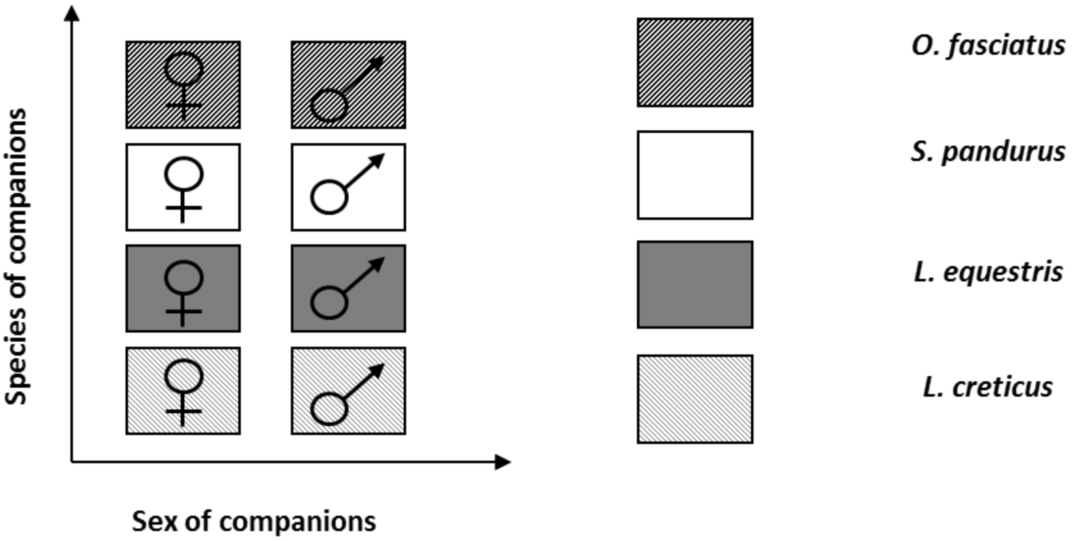

Figure 2. The 2x4 factorial design of the experiment. Colours represent species as indicated by the key.

The cages were maintained for seven days in a fixed environmental regime of 22:2 hours light:dark cycle and a temperature of 29ºC. During this time water tubes were replaced as needed. Any dead bugs were removed and their sex and species recorded. At the end of the seven days all adults were removed and the cages maintained for a further ten days to allow any eggs to hatch. At the end of this period all cages were frozen to kill the occupants and total number of nymphs at each instar in each cage was counted. The total number of nymphs present in the cage was taken as a measure of population "fitness". The number of focal *L. equestris* females found to have died during the course of the experiment was recorded. In the case of the *L. equestris* female treatment, it was not possible to tell if dead females were from the focal group or the treatment so all dead *L. equestris* females were recorded and the final number divided by two as it was assumed that focal and companion females contributed equally to the deaths recorded in this treatment.

A two-way ANOVA was carried out to determine if companion sex, companion species or the interaction between the two had an effect on the fitness of *L. equestris* females. The effect of these treatments on mortality of focal *L. equestris* females was modelled using a GLM with a poisson distribution with companion sex, companion species and the interaction between the two as the effects in the model. Analyses were carried out in PASW Statistics 18 by IBM.

2.2 Experiment 2

This experiment was designed to limit the occurrence of egg predation by adult bugs. As with the previous experiment, the focal species for this experiment was *L. equestris*. Small cages were set up with sunflower seeds and a source of water. Each cage contained ten reproductively mature *L. equestris* females which had been housed with a single male for three days prior to the start of the experiment to ensure mating. In addition ten "companion" bugs were added to each cage. The sex and species of these varied as previously shown in Figure 2 (above). There were eight possible treatments; four consisted of males of each species and four of females. Each treatment, as represented by a box in the schematic, was replicated five times. The additional females added to the cages were kept in single sex populations after eclosion to ensure they did not lay fertile eggs.

During course of the experiment, which lasted for seven days, the adult bugs were transferred daily to a new cage with an identical set up. Once the adult bugs were removed from a cage it was maintained for ten days to allow any eggs laid to hatch and nymphs to develop. At the end of the ten day period the cages were frozen to kill the occupants and total number of nymphs at each instar in each cage was counted. During all stages of this experiment cages were kept in the same environmental conditions as before. During this time water tubes were replaced as needed and any dead adult bugs removed and their sex and species recorded. After seven days the adult bugs were removed from the final cage. A total of seven cages were produced per replicate and the combined number of nymphs present in these cages was again taken as a measure of the population “fitness”. The number of focal *L. equestris* females found to have died during the course of the experiment was recorded. In the case of the *L. equestris* female treatment it was not possible to tell if dead females were from the focal group or the treatment so all dead *L. equestris* females were recorded and the final number divided by two as it was assumed that focal and companion females contributed equally to the deaths recorded in this treatment. Data analysis was carried out as described for experiment 1.

3. Results

3.1 Experiment 1

The total number of nymphs was collected for 37 cages with N = 4 or 5 for each treatment. One cage was discarded due to contamination with *S. pandurus* nymphs and two cages produced no nymphs, which was attributed to a failure of the focal females to mate prior to the start of the experiment. The average number of nymphs produced by *L. equestris* females in each of the eight treatments is displayed in Figure 3a. The results of the two-way ANOVA showed that neither sex ($F_{1, 35}$ = 2.69, P = 0.11), species ($F_{3, 33}$ = 0.50, P = 068), nor the interaction between the two ($F_{3, 33}$ = 0.76, P = 0.53) had any significant effect on the number of nymphs produced by the focal *L. equestris* females.

A total of 60 *L. equestris* females died during the course of the experiment. A breakdown of these deaths by treatment is shown in Figure 4a. The sex of the companion bugs was found to affect the mortality of the focal females. The average number of focal *L. equestris females* that died when housed with male companion bugs was 2.2 while the average number that died when housed with female companion bugs was 1.2. This difference is significant (Poisson GLM: LR = 7.51, d.f. = 1, P = 0.006). There was no effect of the species of companion bug (LR = 4.47, d.f. = 3, P = 0.22) and no interaction between sex and species of companion bug (LR = 5.27, d.f. = 3, P = 0.15).

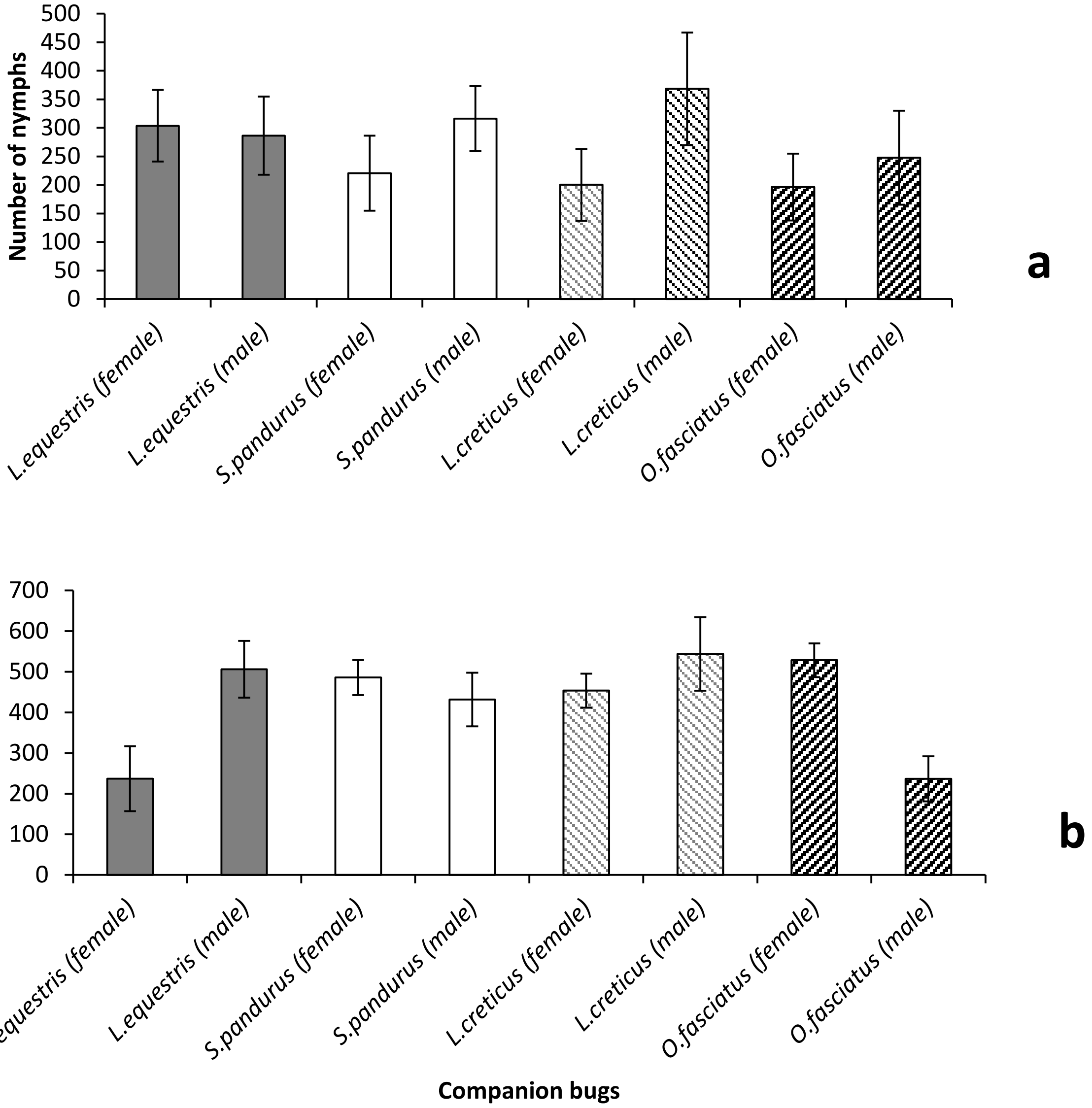

Figure 3. Plot showing the average number of *Lygaeus equestris* nymphs produced by 10 mated females in the presence of 10 companion bugs, a) data from experiment 1 b) data from experiment 2. The species of companion bug is denoted by colour.

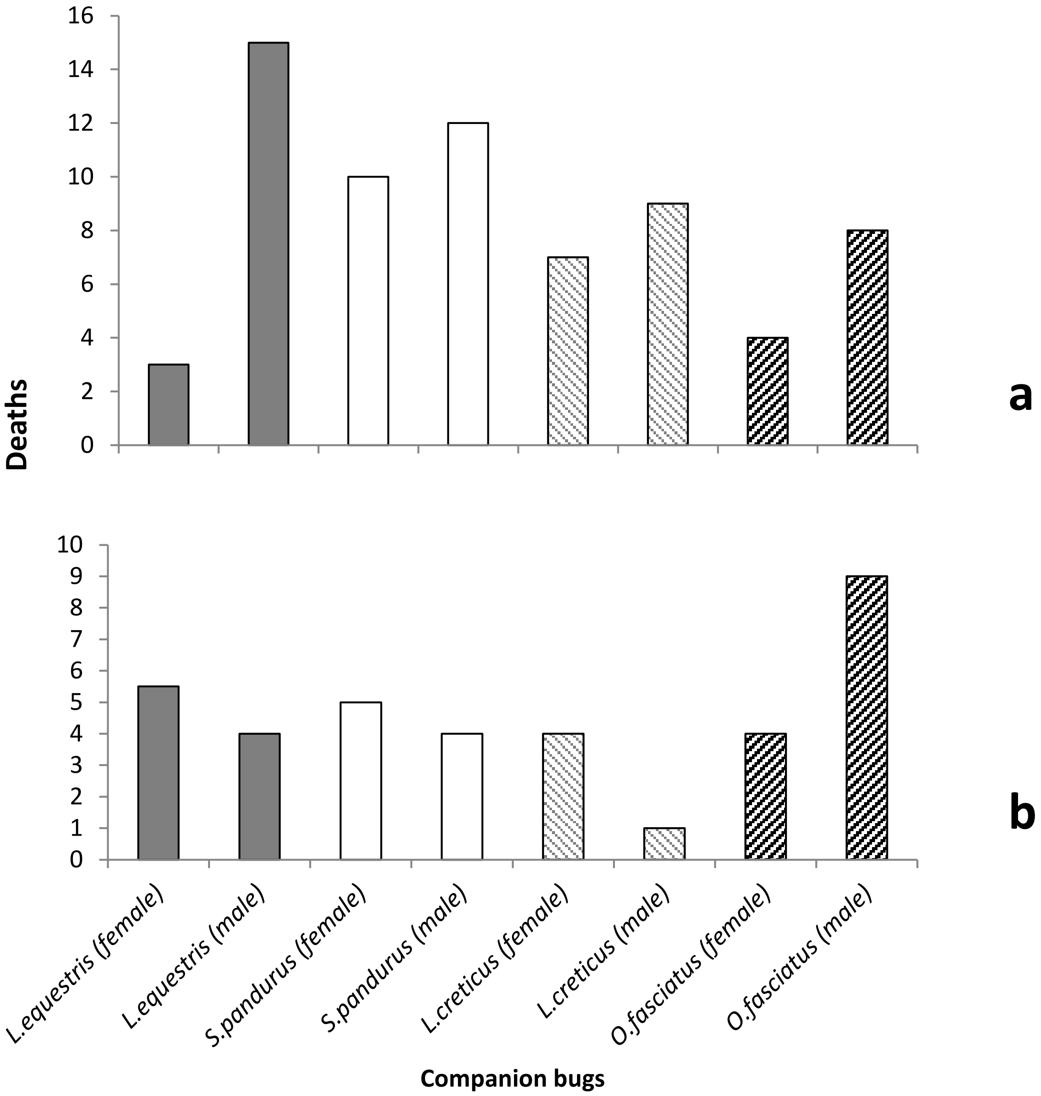

Figure 4. Plot showing the total number of focal *Lygaeus equestris* females that died during the course of the experiment, a) data from experiment 1, b) data from experiment 2. The species of companion bug is denoted by colour.

3.2 Experiment 2

The total number of nymphs was collected for 40 cages with N = 5 for each treatment. The average number of nymphs produced by *L. equestris* females in each of the eight treatments is displayed in Figure 3b. The results of the two-way ANOVA showed that while sex and species had no significant effect ($F_{1, 39}$ = 0.26, P = 0.620 and $F_{3, 36}$ = 1.22, P = 0.320 respectively), nymph production was influenced by an interaction between sex of the companion bugs and their species identity (ANOVA: $F_{3, 36}$ = 5.04, P = 0.006). This is the result of opposing sex effects in the *O. fasciatus* and *L. equestris* treatments. *L. equestris* females had lower nymph production in the presence of females of their own species compared with males; however the pattern was reversed when the treatment bugs were *O. fasciatus*. Fewer nymphs were produced in the presence of *O. fasciatus* males than females. (Figure 3b).

In this run, there were however no differences between the number of focal females that died in relation to the sex or species identify of the bugs (LR = 0.62, d.f. = 1, P = 0.430 and LR = 3.92, d.f. = 3, P = 0.27 respectively) and no significant interaction term (LR = 4.39, d.f. = 3, P = 0.220).

4. Discussion

There was high variability in the number of nymphs produced within the treatments, which may have contributed to the lack of significant effects detected. However, the pattern of reduced fitness in the presence of male *L. equestris* and *S. pandurus* predicted from previous studies was not found (Shuker et al *in prep*). Sexual harassment of female *L. equestris* by male *S. pandurus* certainly took place as cross-species matings were observed on several occasions over the course of the experiment (Figure 5) and the results of the first experiment suggested some penalties to harassment, though not ones that affected fecundity. Whether, then, the reductions in fecundity caused by these interactions were diluted in a larger and more complex environment, or other interactions, such as resource competition or egg predation, are masking the effects of these reductions remains unknown. Egg predation has been previously recorded in some lygaeid species (Solbreck and Sillen-Tullberg, 1990, Costa, 2006) and *S. pandurus,* particularly females, have been observed preying on *L. creticus* nymphs in the wild (*pers obs*.). It is therefore possible that increased egg predation, or even predation of early instar nymphs, by the female companion bugs (who are on average larger than the males of their species) could be imposing fitness costs on the focal females. More generally, there is growing evidence that intra-guild predation may play a greater role in ecological interactions than previously assumed (Polis et al., 1989, Müller and Brodeur, 2002, Noriyuki et al., 2012).

The male *L. equestris* treatment had the highest mortality among the focal females, and this is in keeping with previous work that found repeated matings by conspecific males reduced longevity in *L. equestris* females, and that these repeated matings had greater consequences than sexual harassment alone (Shuker et al., 2006). For three of the four species, male treatment appeared to result in higher mortality, although the small samples size means these results must be interpreted cautiously. In the *S. pandurus* treatments, however, this pattern appears to be reversed, thus providing a further indication that there may be other ecological and behavioural interactions at work. It is especially interesting that increased mortality does not seem to correlate with reduced "fitness" in this study. One possible explanation might be that the period of time for which the reproductive output of the focal females was measured during this experiment was insufficient to capture the fitness costs that sexual harassment and reproductive interference can impose on a female over the course of her lifespan.

Experiment two was designed to limit opportunities for egg predation. The higher average numbers of nymphs produced by the focal females during the course of this experiment (see Figure 4) indicates this was at least partially successful, however some egg predation was still observed. While the interaction between sex and species was found to have a significant effect on the number of nymphs produced the pattern observed did not correspond with previous findings (Shuker et al, *in prep*). The lack of significant trends in mortality may be a result of the reduced number of deaths overall during the course of this experiment compared to experiment one.

Previous work has shown that ecological and demographic variation may influence both the likelihood and effects of RI (Gröning et al., 2007, Ord et al., 2011, Noriyuki et al., 2012). Given that this experiment allowed for interactions between conspecifics as well as heterospecifics, it is perhaps unsurprising that I did not see the clear patterns of RI apparent in previous, simpler, experiments (Shuker et al, *in prep*). While the current data suggests that interactions between *L. equestris* and heterospecific Lygaeidae may be more complex than previously anticipated, further investigation is required before any conclusions can be drawn. Clearly experimental design must be carefully considered when trying to identify, and partition out, the effects of specific behavioural interactions.

In the next chapter I will investigate the role of cuticular hydrocarbons in both species recognition, and sexual signally within and between these species.

Figure 5. Photo showing a mating between a male *Spilostethus pandurus* (right) and a female *Lygaeus equestris* (left).

Chapter 4

Cuticular hydrocarbons and the potential for sex- and species-discrimination in five species of Lygaeidae (hemiptera: heteroptera)

1. Introduction

Animals use a variety of signals and cues to identify and select appropriate mates including visual (Petrie and Williams, 1993, Booksmythe et al., 2011), auditory (Shaw and Herlihy, 2000, Rantala and Kortet, 2003) and olfactory cues (Thomas and Simmons, 2010). Insects frequently use both volatile (Aldrich et al., 1999, Zhang and Aldrich, 2003) and contact (Everaerts et al., 2010, Weddle et al., 2012) pheromones to communicate information on location, sex and status. These pheromones can take the form of long-chain fatty acids and their derivatives in the insects' waxy cuticle, and are known as cuticular hydrocarbons (henceforth referred to as CHCs) (Blomquist et al., 1987). CHCs are produced by specialised epidermal cells and function primarily as a form of water-proofing to protect the insect from desiccation. However, they have also been found to play a significant role in mate choice in many species, allowing them to communicate a wealth of information including sex (Fukaya et al., 1996), mate quality (Thomas and Simmons, 2010), mating status (Everaerts et al., 2010), parasite load (Wagoner et al., 2013), nutritional status (Fedina et al., 2012) and immuno-compatibility (Ali and Tallamy, 2010). The role of CHCs in sexual selection, as well as their genetic basis, has been extensively studied in *Drosophila* (see Ferveur 2005, for a review). CHCs can also allow discrimination of self from non-self. For example, in the cricket *Gryllodes sigillatus*, females appear to recognise males they have already mated with by the presence of their own CHCs left on the male during previous copulations (Weddle et al., 2013). Finally, the composition of these CHCs is often species-specific and can be used for species discrimination (Kather and Martin, 2012). Indeed CHCs have been used as tools to differentiate cryptic species in a number of insect groups (Antonialli et al., 2008).

As a result of their potential for chemical communication, CHCs may well play a role in determining the outcome of heterospecific encounters in closely related species. Long range pheromones have long been known to attract heterospecifics as well as conspecifics, with one striking example coming from New Zealand, where the invasive praying mantis species *Miomantis caffra* has female pheromones that are highly attractive to males of the native species *Orthodera novaezealandiae*, which can result in heterospecific sexual cannibalism (Fea et al., 2013). For species that often mate opportunistically, and with little or no pre-copulatory courtship, CHCs may provide a reliable source of information for determining the compatibility of a suitable mate. This may be of particular significance when other morphological characteristics are constrained, for example in mimicry rings, where there is the potential for a trade-off between accurate mimicry and species recognition (Kikuchi and Pfennig, 2013).

Species of the family Lygaeidae, commonly known as seed bugs, ground bugs or milkweed bugs, are seed-feeding true bugs found almost worldwide, the most conspicuous and well-known of which are aposematic (Burdfield-Steel and Shuker, 2014). They typically have a polgynandrous mating system in which both males and females often mate with several different individuals during their lifetime. Despite some studies into mate preferences in the Lygaeidae (e.g. McLain, 1991, McLain, 1992, Burdfield-Steel et al., 2013), mate choice in this family remains poorly understood. However, several species are known to use chemical communication, in the form of pheromones, throughout their life-cycle (Aldrich, 1988, Aldrich et al., 1997). For example, previous studies have found evidence for an “aggregation pheromone” used by nymphs to facilitate aggregation for predator defence (Aller and Caldwell, 1979). There is also evidence that adults may use long-distance pheromones to attract conspecifics (Aldrich et al., 1999, Zhang and Aldrich, 2003), an ability that may be of great importance given the often patchy habitat and food sources many species encounter in the wild (Root and Chaplin, 1976, Doak, 2000). However, less is known about their close-range communication and mate choice. Several studies have found a male preference for larger females (presumable due to increased fecundity)(Dougherty and Shuker, 2014) but the mechanisms by which bugs of both sexes assess potential mates is unclear.

Despite the interest in their volatile pheromones, to date the composition and function of CHCs in the Lygaeidae remains poorly described. A single study carried out in 1983 showed evidence of age and sex variation in *Oncopeltus fasciatus*, suggesting that they may be a possible source of information about potential mates (Jackson, 1983).The study found distinct quantitative changes in the CHC profile of the bugs as they moved from the third to the fifth nymphal instar and that the ratio of the various hydrocarbons detected changed throughout the bugs adult lifetime. Jackson also found differences in the ratio of certain hydrocarbon between adult males and females 12 hours after eclosion (Jackson, 1983). These results, in combination with the finding that CHCs and other close-range pheromones may be instrumental in eliciting male courtship behaviour in a related bug group, the Miridae (Drijfhout and Groot, 2001), suggests that CHCs may play a role in mate choice in the Lygaeidae.

Here I analysed the CHCs of five species of Lygaeidae to investigate both sex and age differences and variation within species. In particular I compared the CHC profiles of males and females, looking for any sex-specific variation that would indicate CHCs may be involved in mate choice within this group. I then experimentally manipulated the CHC profiles of *Lygaeus equestris* and *L. simulans* females to investigate the potential role of CHCs in mate choice and species discrimination by *L. equestris* males.

2. Methods

2.1 CHC characterisation

Individuals from five species of Lygaeidae, *L. equestris*, *L. simulans*, *L. creticus*, *Spilostethus pandurus* and *O. fasciatus* were maintained in continuous culture in the laboratory as described in chapter 2.

I identified and isolated 5th instar nymphs, sexed them, and housed males and females of each species separately in plastic tubs. The tubs were checked daily and newly eclosed adults were removed and housed separately. Bugs were frozen pending extraction at three ages: 5th instar, one day after eclosion and seven days after eclosion. I placed bugs in individual tubes prior to freezing and too care not to touch the bugs with bare hands.

The bugs remained in a -25°C incubator for 24 hours to ensure they were dead. They were then removed and allowed to thaw. The thawed insects were placed in borosilicate tubes and 1 ml of hexane added along with 100 pmoles of pentacosane as internal standard. After vigorous vortexing for approximately one minute, the organic extract was filtered through glass wool directly into a 2 ml glass screw-top vial. A further 1 ml of chloroform was then added to the pre-extracted insect and vortexed vigorously for approximately one minute, after which the organic extract was filtered through glass wool directly into the same 2 ml glass screw-top vial. The contents of the 2 ml vial was evaporated to dryness on a nitrogen gas line, sealed and stored at -80 °C until analysed by GC-MS. The sample was dissolved in hexane (20 µl) and 1 µl analysed by GC-MS on a Agilent Technologies (GC-6890N, MS detector-5973) with a DB-1 column, (12 M x 0.20 mm x 0.33 µm) with a temperature program of 100 °C for ten minutes followed by a gradient to 290 °C at 5 °C /min and held at 290 °C for a further ten minutes. Mass spectra were acquired from 50-500 amu. Two separate runs were carried out. The first contained sexually mature (i.e. seven day post-eclosion) adults of all five species. The second contained bugs from three different developmental stages (see above). Due to changes in the column the retention times of the peaks were different between the two runs. As a result, I analysed them separately.

2.2 Species and sex differences

The first run contained 65 mature adult bugs from the five species. Five female *S. pandurus*, three male *S. pandurus*, seven *L. equestris* males, five *L. equestris* females, seven *L. creticus* females, seven *L. creticus* males, nine *L. simulans* females, five *L. simulans* males, 11 *O. fasciatus* females and six *O. fasciatus* males.

Peaks were identified for each species and the abundance of each was recorded. All abundances were standardised using the internal standard (100 pmoles pentacosane). The abundance at each peak was then recorded for each individual and any peaks detected in fewer than two individuals were excluded from the analysis. As overall abundances were higher in larger individuals, each peak was divided by the abundance of the highest peak detected from that individual to create a measure of relative abundance. Subsequent analyses were carried out in R 2.14. Principle component analysis was carried out on the abundances of each of the included peaks across all individuals in the run. Eigen values were calculated in order to determine which principle components to include in the analysis. GLMs were then run to test for species and sex differences among the selected principle components.

The identification of CHCs was carried out by comparisons of relative retention times and fragmentation patterns, including diagnostic ions for CHC species as described in literature (Kroiss et al., 2011, Yocum et al., 2011, Thomas et al., 2011, Mullen et al., 2008). Phthalate, a common contaminant found in plastics, was detected in some of the samples and was found to co-migrate with the 2-methyl-C25 peak. In order to correct for this, I calculated the percentage of phthalate verses 2-methyl-C25 and adjusted the peak abundance accordingly.

2.3 Species and age differences

The second run contained 111 individuals. Sample sizes for each species, sex and developmental stage varied from one to six. Notably no mature *O. fasciatus* adults were included as none were available at the time, and age differences in *O. fasciatus* are already well-characterised (Jackson, 1983). Individual CHCs were not identified for this experiment but all other aspects of the analysis were carried out as described above. GLMs were run to test for species, sex and age differences in the selected principle components.

2.4 CHC removal

I isolated virgin males and females from stock populations of *L. equestris* and *L. simulans* at 5th instar and separated them into single sex tubs following eclosion. Experimental bugs were aged seven to ten days post eclosion. Females were divided into two treatments: 'washed' and control. Females in the 'washed' treatment were washed using two strokes of a fine paintbrush dipped in hexane along the dorsal surface. Control females were isolated and held, but no hexane was applied. In both treatments *L. equestris* and *L. simulans* females were distinguished by painting with a dot of white paint on either the right or left side of the thorax. Females were then randomly assigned to petri dishes in heterospecific pairs within each treatment. An *L. equestris* male was placed in each fresh petri dish with a pair of females and any mating attempts (classified as mounting a female and attempting genital contact) or matings (back-to-back positioning) were recorded during the first hour. Petri dishes were then checked every 45 minutes and any mating pairs were recorded. I did not use the number of copulations for analysis due to possible confounding effects of female resistance to mating. 37 males were observed for each treatment giving a total of 74. Differences in mating activity between the two treatments were tested using a chi-squared test. Using a binomial test, the proportion of mating attempts by a *L. equestris* male directed towards a *L. equestris* female as opposed to *L. simulans* females under both control and 'washed' treatments was compared to a null expectation of 0.5 representing no male preference for either species.

3. Results

3.1 CHC characterisation and species and sex differences

A total of 43 peaks were included in the analysis (see supplementary Figures). Of these 13 contributed to one or more of the first three principal components (i.e. had a loading greater than

0.1, see Table 1). With the help of my collaborator, 11 of these peaks were identified (Table 1). The identified peaks were dominated by alkenes, alkanes and methyl-branched alkanes.

As all of the eigenvalues were less than one, the first three principle components were selected as they cumulatively explained over 72% of the variance in the dataset. The low eigenvalues may be a result of many of the peaks being species-specific.

The five species showed species-specific and sex-specific CHC profiles. The first principal component varied significantly among the species ($F_{4, 60}$=222.19, P < 0.001) and also between the sexes ($F_{1, 63}$=9.23, P = 0.004) (see Figure 1). Similarly, both the second and third principal components differed among the species ($F_{4, 60}$= 43.11, P < 0.001 and $F_{4, 60}$= 2.96, P= 0.028 respectively) and between the sexes ($F_{1, 63}$ = 6.21, P=0.016 and $F_{1, 63}$= 11.47, P= 0.001 respectively). For the third principal component, the sex-specific difference varied between the species (interaction: $F_{4, 60}$ = 2.73, P =0.038) although this difference is not strongly significant. All other sex by species interactions were not significant (all P > 0.05).

3.2 Species, sex and age differences

A total of 46 peaks were included in the analysis. As all of the eigenvalues were less than one, the first three principle components were selected as they cumulatively explained over 58% of the variance in the dataset. As with the previous run, the low eigenvalues may be a result of many of the peaks being species-specific.

CHC profiles varied with species, sex (as above) but also with developmental stage. The first principal component showed a significant effect of species ($F_{4, 96}$= 172.69, P < 0.001), and age ($F_{2, 108}$= 9.59, P< 0.005) as well as an interaction between age and species ($F_{4, 106}$ = 10.24, P < 0.001)(see Figure 3). The second principal component significantly varied with respect to species ($F_{4, 106}$=28.10, P < 0.005), sex

($F_{1, 109}$ =9.83, $P < 0.001$) and age ($F_{2, 108}$ =8.31, $P < 0.005$). The third component also varied significantly in terms of species ($F_{4, 106}$= 10.69, $P < 0.001$), age ($F_{2, 108}$= 30.56, $P< 0.001$) and the interaction between the two ($F_{4, 106}$ = 2.47, $P < 0.05$).

3.3 CHC removal

Manipulating the CHC profiles of female bugs significantly influenced con- versus hetero-specific mating attempts. In the control treatment, *L. equestris* males directed a significantly higher proportion of mating attempts towards conspecific *L. equestris* females compared to heterospecific *L. simulans* females (34 attempts towards *L. equestris* females vs. 10 towards *L. simulans* females, Binomial test: $P <0.001$). In contrast, *L. equestris* males showed no significant preference for conspecific females when both con-specific and hetero-specific females were washed with hexane (14 attempts towards *L. equestris* females vs. 24 towards *L. simulans*, Binomial test: $P = 0.188$; Figure 3).

CHC removal did not significantly influence overall mating activity. From a total of 82 mating attempts recorded, 44 were in the control group and 38 in the 'washed' group ($\chi^2 = 0.065$, d.f. = 1, $P = 0.798$). The total number of males who displayed mating attempts also did not differ between the treatments ($\chi^2 = 0.138$, d.f. = 1, $P = 0.711$). Of these mating attempts, 30 resulted in stable copulations in the control treatment (21 with *L. equestris* females and nine with *L. simulans* females) and 22 did so in the 'washed' treatment (six with *L. equestris* and 16 with *L. simulans*).

Table 1. Loadings of the peaks onto the first three principal components. Those loadings under 0.1 were omitted

Retention time	Loading onto Comp.1	Loading onto Comp.2	Loading onto Comp.3	Diagnostic ions (m/z)	Carbon number	Hydrocarbon
26.11	0.132	-0.133		282, 201, 57	5- Me-C19	Tricosane
31.4	0.327	0.688		280, 264, 57	7- Me-C25	7-Methyl-pentacosane
31.5	-0.137	0.212	0.896	366, 351, 309, 244, 71, 55	5- Me-C25	5-Methyl-pentacosane
31.6	0.122	-0.118	-0.169	284, 241, 65	2- Me-C25	2-Methyl-pentacosane
31.97			0.170			Unknown
33.02			-0.153	350,209, 69	5- Me-C26	5-Methyl-hexacosane
34.1	0.269	-0.164		374, 281, 207, 71, 57	C27:3	Heptacosatriene
34.57	0.641	0.223		378, 283, 269, 83, 57	C27:1	Heptacosene
34.79	-0.542	0.438	-0.169	408, 341, 281,69	C27:0	Heptacosane
39.68		0.344	-0.118			Unknown
41.11			0.123	460, 408, 127, 113, 71, 57	C33:2	Tritriacontadiene
46.66	0.123			379, 351, 281, 71, 57	7- Me-C33	7-Methyl-tritriacontane
50.3			0.111	490, 365, 57	C35:1	Pentatriacontene

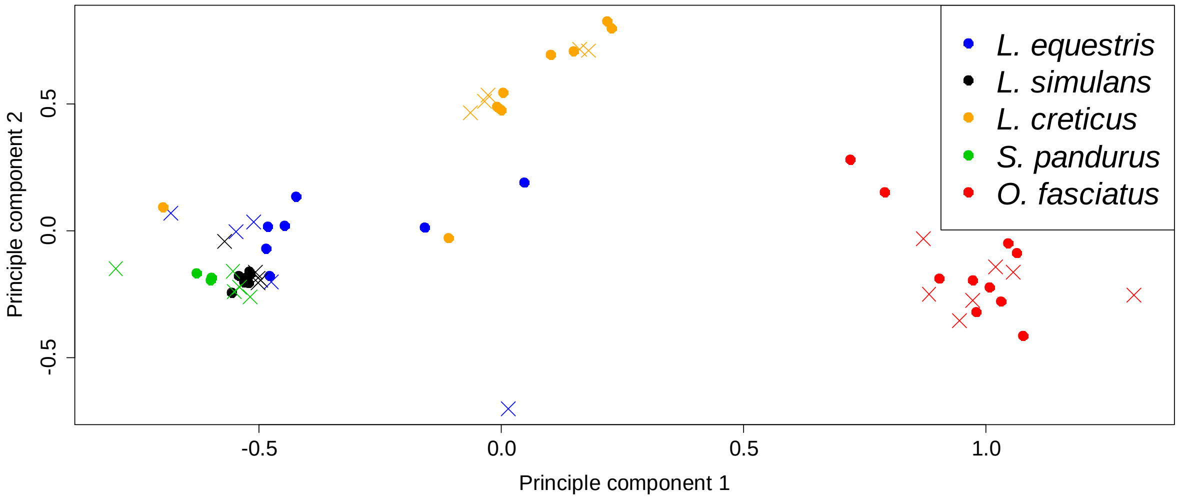

Figure 1. Sex and species differences between seven day adults for the first and second principle components. Crosses denote males, circles denote females.

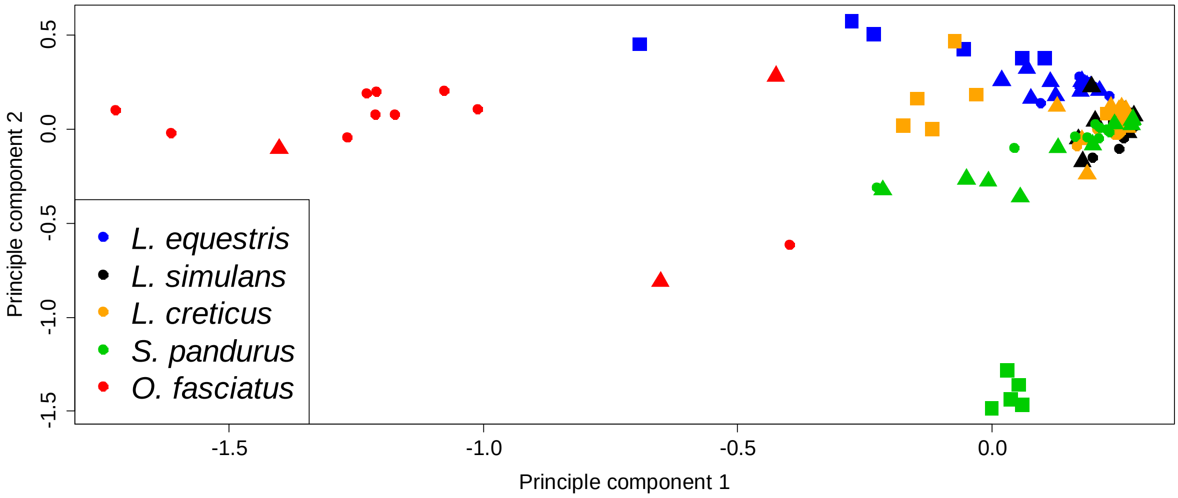

Figure 2. Age and species differences in the first and second principle components for the mixed age run. Circles denote 5th instar nymphs, triangles adults 1 day post-eclosion and squares adults seven days post-eclosion.

4. Discussion

In keeping with the findings of Jackson (1983), the CHC composition of the five species of Lygaeidae considered here was broadly similar to those of other insects. The CHC profiles of all five species were dominated by linear alkanes, linear alkenes and methyl-branched alkanes (Blomquist et al., 1987, Blomquist, 2010). As these are all classes of hydrocarbon commonly found in insects, previous studies have broadly described the biosynthetic pathways thought to be involved in their production. Much of what is known is summarised in (Blomquist, 2010).

As expected, the CHC profiles of the five species differed significantly. The new world species, *O. fasciatus* had the most distinct CHC profile from the other species (see Figures 1 and 2 and Appendix 2), as its profile was dominated by the alkene heptacosene, while all other species have heptacosane as their most abundant hydrocarbon. This is in contrast with the findings of Jackson who found heptasosane as the most abundant hydrocarbon in adult *O. fasciatus* (Jackson, 1983). As the two compounds have very similar carbon numbers and retention times in the column (see Table 1) this may have been due to an inability to distinguish them. Other compounds found only in *O. fasciatus* include 7-methyl-tritriacontane and heptacosatriene (see Appendix 2, tables 2A and 2B). The distinctness of *O. fasciatus* to the other four species fits with its presumed phylogenetic position relative to them. However, even the closely related species *L. equestris* and *L. simulans* showed some consistent differences. While their overall profile was similar (see Appendix 2, Figure 3) *L. equestris* females produced 2-methyl-pentacosane (peak at 31.6 minutes) while *L. simulans* females did not. Indeed, 2-methyl-pentacosane only occurred in *L. equestris* and *O. fasciatus* and only showed sex-biased abundance in *L .equestris*. As displayed in Appendix 2, Figures 2 and 3, *S. pandurus* showed the greatest similarity with *L. simulans*. Pentatriancontene was found in abundance only in *S. pandurus* and some *L. simulans* males and tritriacontadiene also occurred predominantly in those two species.

Despite *O. fasciatus* forming the most district group, patterns of CHCs variation did not appear to be explained by phylogeny alone. While the phylogeny of Lygaeidae may not yet be fully resolved (Schuh and Slater, 1995, Weirauch and Schuh, 2011), the first and second principle components of variance show that *S. pandurus* is clustered closely with the *Lygaeus* species, in particular *L. simulans*. Given that *S. pandurus* was originally placed in the genus Lygaeus when it was first described it is likely to be closely related to both species, although subsequent re-classification suggests less so than *L. creticus*. There are, however, clear differences between the species, although these are not as great as the differences with *O. fasciatus. S. pandurus*, *L. simulans*, *L. equestris* and *L. creticus* all share overlapping ranges (Lis et al., 2013, Lis, 1998, Solbreck et al., 1989, Gusev and Tatarnikov, 1992, Sweet, 2000), though not necessarily habitats (*pers. coms*.), and the extent to which character displacement or other processes (such as sexual selection, or selection for desiccation resistance across the species ranges) has shaped these differences remains uncertain. However, the greater variation in CHCs between *L. equestris* and *L. simulans* in comparison to more distantly related species, such as *S. pandurus*, may be a result of character displacement and reflect a role in their reproductive isolation (the two species are known to co-occur micro-sympatrically, i.e. in the same micro-habitats, at sites in central Europe (e.g. Lis et al., 2013, see also Deckert, 1985)). These two species show asymmetric behavioural reproductive isolation: male *L. simulans* are able to copulate with *L. equestris* females, but *L. equestris* males are rarely able to mate successfully with *L. simulans* females (Evans, 2011). When heterospecific pairings do occur they typically result in significantly reduced fitness (see chapter 7) so selection favouring species discrimination is thought to be strong. Indeed male *L. equestris* appear to be able to distinguish between conspecific and *L. simulans* male rivals in the context of mate guarding (Burdfield-Steel and Shuker, *in press*) and the differences in CHC profiles between the species provide a potential mechanism for this discrimination.

Age and developmental stage also influenced CHC composition (Figure 2). In particular, with the exception of *O. fasciatus*, the CHCs of adults appeared to show greater species differences. The overlap between the species was much greater in 5th instar nymphs. In keeping with the results of the adults-only run, *O. fasciatus* was the most distinct species as both nymphs and adults overlapped little with the other species. There also appears to be differences between the CHCs of newly eclosed verses sexually mature adult bugs. This fits with the hypothesis that CHCs may be involved in adult sexual signalling, as the changes may allow bugs to determine the receptivity of potential mates. Interestingly, given these clear differences between nymphs and sexually mature adults, adult males of *L. creticus* have been observed attempting to copulate with nymphs in the wild, and adult-nymph mating attempts also occur in all five species in the laboratory (*pers. obs*: see Figure 4). Additionally, there appears to be greater variation amongst the adults, mature adults in particular, although larger sample sizes would be required to confirm this. While the causes of this variation remain unknown in these bugs, it may be indicative some aspect of quality or genotype and thus a source of information during mate choice. While CHCs are known to vary as a result of environment (Weddle et al., 2012), the identical rearing condition of these bugs in the laboratory means any variation detected is unlikely to be environmental in origin. These changes, both between nymphs and adults and over the course of the adult life-stage, are in keeping with previous work on *O. fasciatus* (Jackson, 1983). There is also evidence in *O. fasciatus*, and in a related bug species, *Oxycarenus hyalinipennis,* that individuals may alter their volatile pheromones as they age (Aller and Caldwell, 1979, Olagbemiro and Staddon, 1983, Schuh and Slater, 1995).

When the CHCs of female *L. equestris* and *L. simulans* were experimentally reduced, male *L. equestris* no-longer showed a preference for *L. equestris* females (Figure 2). There was no significant change in overall mating activity between the treatments. It should be noted, however, that when the CHCs were experimentally reduced, *L. equestris* males started to show a hint of a preference for *L. simulans* females (Figure 2). Whilst not significant, it is possible that the (reduced) effects of CHCs are interacting with the known male preference for larger females, as the average body size of both sexes in *L. simulans* is significantly larger than that of *L. equestris* (Burdfield-Steel et al., 2013). The interactions between different CHC and morphological traits in terms of mate choice remain to be fully characterised however.

Despite clear differences between the species, there was also considerable within-species variation. This may account for the observation of heterospecific mating attempts between *S. pandurus*, *L. equestris*, *L. simulans* and *L. creticus* as the CHC profiles of these species overlap (Figure 1; Shuker et al, *in review*). One of the main components of within species variation is, of course, sex-specific variation. Males and females varied significantly in terms of their CHC composition, even when all five species were analysed together. As expected, sex differences appeared to be species-specific and more subtle than the differences between species. Some sex-specific peaks were found. For instance, 5-methyl-hexacosane was present in male *L. creticus* but not females, and a similar pattern was found for 5-methyl-pentacosane in *L. equestris*. In contrast, 2-methyl-pentacosane was female-specific in *L. equestris*, and completely absent in the other *Lygaeus* species.

Of the hydrocarbons identified, several (including tricosane, 7-methyl-pentacosane, 5-methyl-pentacosane, heptacosatriene , heptacosene and hritriacontadiene) have been found to show sex- and species-specific variation in other insect species . 7-Methyl-pentacosane and tricosane show species-specific variation in necrophagous flies (Ye et al., 2007) and sex and caste-specific variation in the stingless bee *Melipona scutellaris* (Kerr et al., 2004), while 5-methyl-pentacosane abundance has been found to vary between CHC phenotypes of *Reticulitermes* termites in southern California (Nelson et al., 2008) and also termites of the genus *Zootermopsis* across the southern USA (Haverty et al., 1988). Tricosane and Heptacosatriene have also been implicated in sexual attraction in the house fly (Howard and Blomquist, 1982), *Drosophila melanogaster* (Yew et al., 2009), and the yellow peach moth (Xiao et al., 2012), while Tritriacontadiene is thought to play a similar role in *Drosophila* *pallidosa* (Nemoto et al., 1994). Tricosane also shows male-specific abundance in the trap-jaw ant, *Odontomachus brunneus* (Smith et al., 2014). Additionally, heptacosane has been found in the cockroaches *Leucophaea maderae* and *Blatta orientalis* (Tartivita and Jackson, 1970) as well as several species of fly in the family Sarcophagidae (Braga et al., 2013). Perhaps more interestingly, it has been implicated in queen-worker chemical communication in ants (Endler et al., 2004).

Although some species-specific peaks were identified (for example heptacosatriene and 7-methyl-tritriacontane in *O. fasciatus*), much of the between-species variation was in the abundance ratio of the shared hydrocarbons. There is an increasing call for chemical ecologists to consider the full CHC profile of an organism rather than specific peaks (Hefetz et al., 2010). While some specific compounds may have a disproportionate role in chemical signalling (for example, those identified as sex pheromones) it is likely that insects will evaluate CHC profiles as a whole, and thus subtle variation within these profiles may have biological significance. Even when variation is dominated by a few peaks, this variation may be due to differences in the ratio of various compounds as opposed to simple presence/absence (Neems and Butlin, 1994, Buckley et al., 2003). This seems to be the case in several social insects; however it may also be true for other groups such as the Lygaeidae. Clearly, the CHCs of these species are a potential source of information on species, developmental stage and sex. This information is of great importance during mate-choice, and, at least in *L. equestris*, males do appear to use CHC cues when choosing mates. Despite this however, interspecies, male-male and adult-nymph copulation attempts are frequently observed in these species. Nor is species discrimination perfect even when CHC cues are intact (Figure 3). Thus, it seems that males may not be utilizing this information in all contexts, or that environmental variation is reducing the clarity of CHC signals in some circumstances.

In summary, my results suggest that CHCs play a role in seed bug species discrimination and mate choice. Not only is there sufficient within- and among-species variation for discrimination to act upon, but manipulating CHCs in female *L. equestris* and *L. simulans* removed male preference for conspecific females. The extent to which CHCs have influenced the evolution of reproductive isolation between these sister species remains to be explored.

In the next chapter I will explore the effect of environmental factors, such as diet, on sexual selection and other life-history traits of the Lygaeidae.

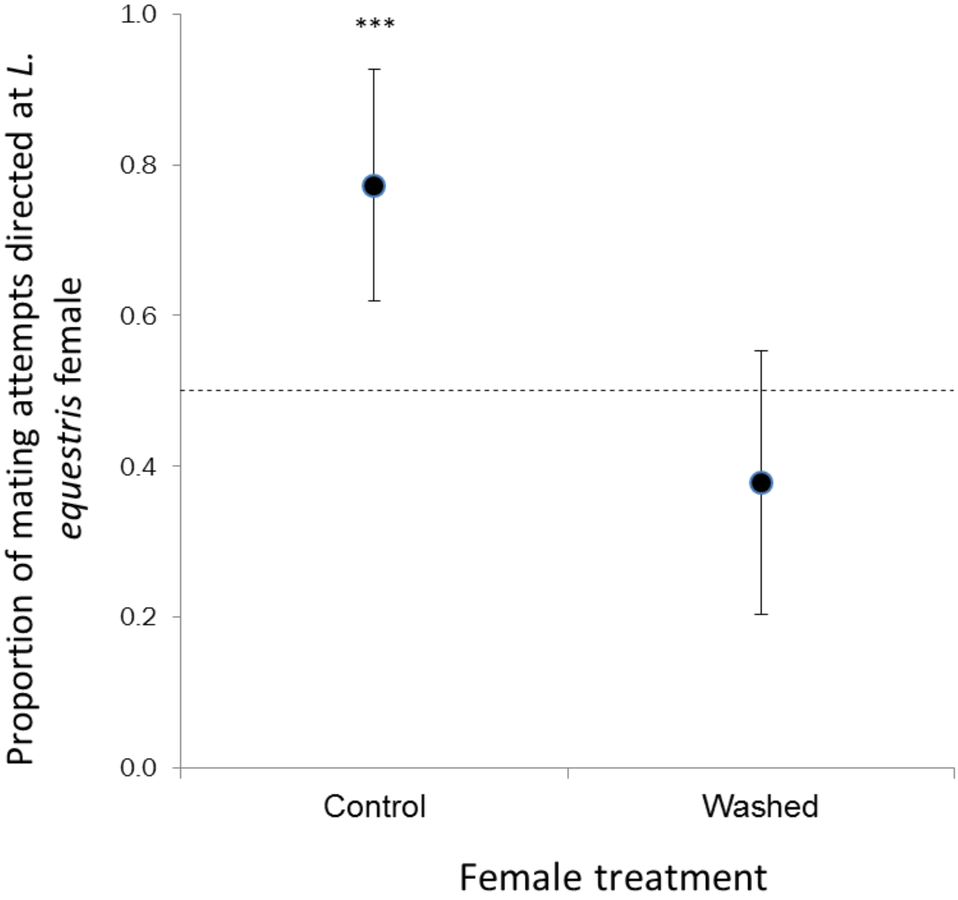

Figure 3. CHC profile influences species discrimination by male *Lygaeus equestris*. Mating attempts directed at *L. equestris* females by males in the two treatments. Error bars represent 95% confidence intervals. *** P >0.001 significance from null hypothesis of no species discrimination.

Figure 4. Adult male *Lygaeus creticus* attempting to mate with a 5th instar *L. creticus* nymph in Sicily. Photo credit: David Shuker.

Chapter 5

Variation in social and sexual behaviour in four species of aposematic seed bugs (Hemiptera: Lygaeidae): the role of toxic and non-toxic food

1. Introduction

Social interactions are key components of fitness. These range from potentially brief interactions between males and females associated with mating, through to animals that aggregate either temporarily or in the form of long term groups (including over-wintering assemblages and colonies) (Bleakley et al., 2010). Such interactions often vary over the course of an animal's life, but the factors that cause variation in social behaviours, both within and between species, are not yet fully understood (Ebensperger et al., 2012). For instance, there has been a lot of attention given to variation in mate choice (reviewed by Jennions and Petrie, 1997, Bateson and Healy, 2005), including the extent to which mating outcomes are repeatable (e.g. Shuker and Day, 2002). Despite this attention, it is still unclear whether such variation is adaptive, and the extent to which the variation we see is explained by factors such as learning, local ecology, or chance. One problem is that much of the theoretical machinery we have for predicting the outcomes of social behaviour seeks to provide an optimal behaviour for a given set of circumstances (e.g. the classic behavioural ecological approach: Davies et al., 2012). Whilst this approach has been enormously successful, such models are often not geared towards predicting (and explaining) among-individual variation in behaviour (Sih et al., 2012). This has led to a renewed focus on understanding individual variation in behaviour more generally (including investigation of the related concepts of animal personalities and behavioural syndromes: (e.g. Pruitt and Ferrari, 2011, Pruitt et al., 2012, Bell, 2012, Sih et al., 2012, Sih and Del Giudice, 2012)).

As discussed in chapter 1, aposematism is widespread throughout the Lygaeidae and all the species studied in this thesis derive chemical protection from their food. However, the majority of laboratory populations used in the study of these insects, including our own, are raised on sunflower seeds (*Helianthus anuus*), which offer no chemical protection. Thus, in order to generalise the behaviours observed in laboratory populations to those exiting in the wild it is important to understand what, if any, behaviours are altered in response to this non-natural diet. In this chapter I consider the effects of diet on these insects and explore how variation in diet influences variation in behaviour, in particular mating preferences. Aposematic species display their chemical defences using warning colours, such as red and black, or yellow and black (Ruxton et al., 2004). Additionally other signals may be used, such as chemical cues (Aldrich, 1988), sounds or distinctive behaviours (De Wert et al., 2012). These cues signal to potential predators that the organism is toxic or distasteful. In order for such signals to be successful, predators must learn to associate them with unpalatability or toxicity, and so there is selection favouring members of the same population that exhibit the same signals. That way, once a predator has encountered one individual of an aposematic species it will, theoretically at least, avoid all members of that species (Ruxton et al., 2004). However, not all aposematically coloured animals are defended, as non-defended species may mimic the markings of defended ones in order to take advantage of predator’s reluctance to attack potentially toxic prey (i.e. Batesian mimicry: (Ruxton et al., 2004). This contrasts with Müllerian mimicry, when two or more protected species evolve similar markings, sharing the costs of predator education and benefiting from predators generalising from one toxic prey species to another (Sillen-Tullberg et al., 2000). However, even among truly distasteful species, not all individuals may be chemically defended or the levels of chemical protection may not be uniform across all individuals in a species, or even a population (Speed et al., 2012). This is especially true for animals that sequester their protective chemicals from their food (Bowers, 1992). This “automimicry” may arise from individuals being unable to find toxic defensive compounds to sequester, or as a “cheating” strategy by which undefended individuals do not pay the (presumed) cost of being chemically defended (Speed et al., 2012, Holen and Svennungsen, 2012). Here, I consider the extent to which social and sexual behaviour in four species of aposematic seed bug varies with access to a diet that either does or does not provide compounds used for chemical defence.

I focused on two behaviours. First, aposematic animals often appear to behave in ways that promote the effectiveness of their warning coloration, for instance by aggregating together as nymphs or larvae (see chapter 1). For example, nymphs of the seed bug *Lygaeus equestris* suffered fewer attacks from domestic chicks (*Gallus gallus domesticus*) when presented in groups than when presented alone (Sillen-Tullberg et al., 2000). This behaviour is presumed to present a stronger "signal" to warn off potential predators or help predators associate aposematically-coloured prey with toxins/bad-taste (Sillen-Tullberg, 1988, Gamberale and Sillen-Tullberg, 1998). Additionally, group size has been found to positively affect the survival in the wild of nymphs of another aposematic seed bug, *Oncopeltus fasciatus* (Ralph, 1976). However, recent work has shown that even undefended, non-aposematic individuals can benefit from lowered predation rates by aggregating with aposematic individuals (De Wert et al., 2012). Despite this, it remains unclear though how chemical defence influences aggregation behaviour.

Second, mate choice remains an important component of organismal fitness (Andersson, 1994, Kokko et al., 2003, Kotiaho and Puurtinen, 2007). There has been recent interest in the role of nutrition in sexual selection and mate choice, from the perspectives of both the chooser and the chosen, across a range of organisms (e.g. ground crickets, Hall et al., 2008, house mice, Costello et al., 2009, wolf spiders, Lomborg and Toft, 2009, fruit flies, Fricke et al., 2010, Nile tilapia, Giaquinto et al., 2010, bluefin killifish, McGhee and Travis, 2011, and cockroaches, South et al., 2011). Nutrition is important in terms of understanding condition-dependence of ornaments, the role of life-history trade-offs in sexual selection, and the nature of the benefits that may accrue to the choosy sex. Choosiness may also vary depending on the condition of the choosing sex (Cotton et al., 2006). As well as the potential for indirect benefits arising from mate choice (i.e. good genes or sexy sons: reviewed by Andersson, 1994, Koella, 1989, Bateson, 1983a, Prokop et al., 2012) many species also receive direct benefits from matings (Arnqvist and Nilsson, 2000). These benefits can take the form of ejaculates that act as food (DiRienzo and Marshall, 2013) or as a source of water (Edvardsson, 2007), to the female or her offspring (Gwynne, 2008).

However, females may receive other benefits from ejaculates or nuptial gifts such as increased chemical protection against predation (Cothran et al., 2012). For example, it may be adaptive for individuals to mate with chemically well-defended partners, if those partners can transfer those chemical defences to their offspring (either via eggs or via the ejaculate). One of the most elegant examples concerns the moth *Utethesia ornatrix*. This species sequesters pyrrolizidine alkaloids from food as larvae. Females can transfer this chemical protection to their offspring; however males can also transfer additional pyrrolizidine alkaloids to the female as part of his sperm package. This has been shown to not only increase the amount of protection a female can pass on to her offspring, but also to increase the female's own protection against predators following mating (Gonzalez et al., 1999). Perhaps unsurprisingly, females show a marked preference for males with high pyrrolizidine alkaloid levels (Dussourd et al., 1991). A similar phenomenon has also been found in the beetle *Neopyrochroa flabellate* (Eisner et al., 1996a, Eisner et al., 1996b). With this in mind, we might expect environmental factors, such as diet or chemical protection, to affect the potential benefits females can gain from mating, and thus, female mating decisions.

The Lygaeidae (Figure 1) typically sequester protective toxic compounds such as cardiac glycosides from the plants they feed on and both adults and nymphs advertise this protection with bright red or orange markings (Aldrich, 1988, see chapter 1). In this study I considered four species. First, *O. fasciatus*, the 'milkweed bug' is considered to be a milkweed specialist as it is typically found on the common milkweed *Asclepias syriaca* and other plants of the sub-family Asclepiadaceae (Dingle et al., 1980a, Dingle et al., 1980b). Despite this however, it has been reported feeding on *Nerium oleander* which suggests its diet is not as restricted as previously though (Dingle et al., 1980a, Dingle et al., 1980b). The cardiac glycosides that individuals of this species sequester from their food provide them with protection from predators (Sillen-Tullberg et al., 1982, Berenbaum and Miliczky, 1984, Scudder et al., 1986). Adult *O. fasciatus* exhibit several morphological and physiological adaptations to allow for the efficient sequestering of cardiac glycosides, including a double layered epidermis in which the inner layer is specialized for glycoside storage, and weak points in the cuticle which rupture easily, releasing glycoside-rich fluid (Scudder *et al*., 1986). *O. fasciatus* fed on milkweed seeds have been shown to be distasteful to mantids (Berenbaum and Miliczky, 1984) and lizards (Feir and Suen, 1971). *L. equestris,* its sister species *Lyageus simulans,* and *Spilostethus pandurus* typically feed on asclepiads (Wheeler, 1983) and show evidence of chemical protection from predators (Sillen-Tullberg et al., 1982, Gamberale and Sillen-Tullberg, 1996, Svadova et al., 2010, Sillen-Tullberg et al., 2000) (although they too have been reported to feed on numerous plant species when their preffer host is not avilable). Predation experiments have shown that chicks, *Gallus gallus domesticus,* and great tits, *Parus major*, quickly learn to avoid *L. equestris* nymphs and adults in the laboratory, with bugs frequently surviving being seized and immediately dropped by the birds (Sillen-Tullberg *et al*., 1982; 2000). This protection has been shown to be diet-dependent (Sillen-Tullberg et al., 2000). Additionally, there is evidence of avoidance by some bird species in the wild (Hotova Svadova *et al*., 2010). *S. pandurus* also sequesteres cardiac glycosides, which have been shown to be incorporated into defensive secretions produced in the scent glands (Abushama and Ahmed, 1976) and naïve chicks, *Gallus gallus domesticus,* show learned avoidance to 5th instar nymphs (Gamberale & Sillen-Tullberg, 1996). While all this suggests that chemical defence is in these species is reliant on compunds sequestered from their diet, there is evidence that related species, such as *Tropidothorax leucopterus,* can manufacture at least some defences de novo (Sillen-Tullberg et al., 2000), and *O. fasciatus* may release endogenously-produced chemicals from its scent glands on initial contact (Games & Staddon, 1973). However, it appears that individuals feeding on plants other than asclepiads may sacrifice some measure of chemical protection in exhange for niche expansion. Despite this, these unprotected (or less protected) individuals may still benefit from their resembalance to protected conspecifics (Berenbaum and Miliczky, 1984). For example predators such as mantids have been found to avoid all *O. fasciatus* after attempting to eat a single toxic individual (Berenbaum and Miliczky, 1984). This automimicry may well be very common (Speed et al., 2012), especially in groups such as lygaeids where variation in food sources may strongly influence levels of chemical protection (Sillen-Tullberg et al., 2000).

Here I explore how exposure to toxic versus non-toxic seeds influences social and sexual behaviour in these four Lygaeid species, in a series of four experiments. First I consider the extent to which nymphs preferentially select toxic compound-bearing food. I allowed nymphs to feed on either non-toxic sunflower seeds (*Helianthus anuus*) or a mixture of sunflower seeds and toxic milkweed seeds (*Asclepias syriaca*) from hatching. I predict that given a choice between toxic and non-toxic seeds nymphs of all four species will prefer the toxic food. In the second experiment, I compared the aggregation behaviour of nymphs fed on the two different diets. I expect to see a greater tendency to aggregate in nymphs fed on a toxic diet as these are likely to have greater chemical defence and reinforce the aposematic signal for potential predators. The third experiment examines the effect of the two different diets on body size in both sexes of adult bugs to look for any evidence of life-history trade-offs associated with consuming toxic versus non-toxic foods. My final experiment will consider mating preferences of both males and females fed on either toxic or non-toxic diets. I predict that female bugs will prefer to mate with males possessing greater chemical defences; alternatively there may be a preference in both sexes for bugs that were fed on the same food. Importantly our design allows us to test whether any variation in mate preference is associated with the diet of the focal individual. By comparing four separate species with similar ecologies I hope to identify more general effects of diet on patterns of variation in social behaviour.

2. Methods

The natural ranges of all four species are described in chapter 1. The preferred host of *L. equestris* and its sister-species *L. simulans* is *Vincetoxicum hirundinaria*. *S. pandurus* has a wide range and feeds on many different plants while *O. fasciatus* is a new world species that feeds on the common milkweed plant *Asclepias syriaca.* Thus, *O. fasciatus* is the only species in this study that uses milkweed seeds in its natural diet. However, all species used in the study are able to sequester cardiac glycosides to some extent. All populations were collected and maintained as described in chapter 2.

2.1 Experiment 1 - Nymph feeding preferences

Mated females of each species were isolated from stock cages (whilst in copula, with the male removed once mating was complete) and housed individually in small plastic tubs containing sunflower seeds *ad libitum*, demineralised water and cotton wool for oviposition. Females were allowed to lay eggs which were left to hatch. Second instar offspring were collected and separated into two groups of approximately equal numbers, for each female. The two groups were then placed in 17 x 11 x 7 cm tubs containing either: (1) one (60mm diameter) open plastic dish containing sunflower seeds (Dish S) and one plastic dish containing milkweed seeds (Dish M), subsequently referred to as the choice box; or (2) two plastic dishes containing sunflower seeds, referred to as the control box. Thus nymphs from a single mother were split between the two treatments. Water tubes were provided and regularly changed. Food was provided *ad libitum* across the developmental period. The mean number of nymphs per box was 19 at the beginning of the trial and 14 at the end (due to some nymphal mortality).

The boxes were surveyed each day between 10 and 11am and the number of nymphs observed in each dish was recorded. This continued until the majority of the clutch reached 5th instar. This took approximately 11 days, but varied slightly between species. I also counted the total number of bugs surviving in each box every seven days. Nymphs from 56 females were included in the paired design resulting in a total of 112 boxes, half control and half choice for each species. The breakdown by species was as follows: 32 boxes contained *L. equestris*, 32 contained *L. simulans*, 20 *O. fasciatus* and 28 boxes contained *S. pandurus*. I tested for differences among the four species in feeding preferences in two ways. First, I analysed feeding preferences using counts of the number of days nymphs were observed in each dish (nymphs observed in a food dish were assumed to be feeding) across the experiment (giving one count for each replicate). I used these counts to generate the proportion of days nymphs were observed feeding from one or both food dishes for each replicate (with number of days each replicate was surveyed as the denominator). I compared the proportion of days nymphs were observed feeding from sunflower and milkweed feeding dishes in the choice condition for the four species using a generalised linear model with binomial errors and a logit-link function (using IBM SPSS Statistics 19). Second, I compared the proportion of nymphs observed feeding from the S and M dishes in the choice condition for the four species using a generalised linear model with binomial errors and a logit-link function (again using SPSS). To again avoid pseudoreplication, each replicate provided only one data point representing the number of nymphs observed for each replicate divided by the total number of nymphs in the box. The average proportion across the surveys for each replication was then used as the data point for each replicate. Finally, I also checked the overall feeding rates in the presence of only sunflower or sunflower plus milkweed by comparing the feeding rates between choice and control boxes. I did this by comparing the proportion of nymphs observed in both dishes in both kinds of box (i.e. combining sunflower + sunflower for control boxes and sunflower + milkweed for choice boxes), again averaged across the experimental surveys for each replicate.

2.2 Experiment 2 - Nymph aggregation

Once the nymphs from experiment 1 reached 5th instar, ten nymphs from each treatment (where possible) were isolated and tested for aggregation behaviour. I set up 85mm plastic Petri dishes on paper circles that were divided into four equal quadrants numbered 1-4, on the bench (24-25°C). Nymphs were placed in an empty 30ml Universal tube which was then inverted over the centre of the Petri dish, thus introducing the nymphs into the centre of the arena together. At 30 and 60 minutes after introduction of the nymphs to the Petri dish I recorded the following: (1) how many nymphs were in each quadrant (if nymphs were aggregated together and over a section line I counted all nymphs in the aggregation as in the section that contained the majority of the group); (2) the number of aggregations in each dish (an aggregation was classed as two or more nymphs less than a body-length away from each other and not moving); (3) the number of nymphs in each aggregation; (4) the furthest distance between two nymphs in the dish. The effect of species, diet and their interaction on the number of nymphs in aggregations was analysed using a generalised linear model with a negative binomial (again in SPSS). Due to the non-normality of the data, the effect of species on the furthest distance between nymphs was tested using a Kruskal-Wallis test and the effect of diet on furthest distance was tested separately for each species with Mann-Whitney U tests (again in SPSS). I excluded dishes containing fewer than 10 nymphs, leaving us with a total of 50 trials, comprising: 18 *S. pandurus* replicates, six *O. fasciatus* replicates, 12 *L. equestris* replicates and 14 *L. simulans* replicates split between the two treatments.

2.3 Experiment 3 - Body length

Following mating trials (see below), bugs were frozen and stored at -20°C. To test for any effects of diet on adult body size, I measured body length of all individuals after thawing using a dissecting microscope fitted with a measuring graticule. 614 individuals were measured: 188 *L. equestris*, 190 *L. simulans*, 50 *O. fasciatus* and 186 *S. pandurus*. I measured body length as the tip of the snout to the end of the underside of the abdomen, with the bug lying on its back (Figure 2). The effect of diet, sex and species on body length was tested using a general linear model with LSD post-hoc tests to compare between the species.

2.4 Experiment 4 - Mating trials

Mating trials were performed over two weeks. Seven days after adult eclosion, males and females of the same species were paired up according to four combinations: (i) milkweed-fed male with milkweed-fed female; (ii) milkweed-fed male with sunflower-fed female; (iii) sunflower-fed male with milkweed-fed female; and (iv) sunflower-fed male with sunflower-fed female. Each individual was used in a single trial only. The pairs were introduced into plastic dishes 60mm in diameter and observed continuously for a period of two hours, on the bench (24-25°C). The order in which the two sexes were put into the dishes was randomised. I recorded the number of male mating attempts observed, as well as the initiation and duration of any copulations. In all four species, copulation is typically initiated by the male grasping the female with his legs and attempting genital coupling, with no obvious courtship phase (for more details of copulation see chapter 1). Pairs were classed as being in copula once they have moved into the end-to-end mating position typical of lygaeids (Micholitsch et al., 2000, Shuker et al., 2006).

The effect of treatment on mating success was analysed using logistic regression (i.e. a generalised linear model with a binomial error structure and logit-link function) in R 2.15.1, with male diet and female diet modelled as separate factors and species included as a main effect. A total of 318 pairs were included in this experiment: 36 *O. fasciatus,* 93 *S. pandurus,* 94 *L. equestris and* 95 *L. simulans.* Wald tests were used to test the significance of the main effects. The ratio of the residual deviance and the residual degrees of freedom was 1.2, so over-dispersion was not considered to be a problem.

3. Results

3.1 Experiment 1 - Nymph feeding preferences

I recorded two measures of feeding preference in the choice treatment: the average proportion of days bugs were observed feeding in either food dish, and the average proportion of nymphs observed in each food dish over all days. There was a significant preference for milkweed seeds over sunflower seeds in the milkweed boxes, for both the proportion of days nymphs were observed in the different dishes (likelihood ratio test of the main effect of food type: LR= 209.36, *d.f.* = 1, P < 0.001; Figure 3) and the proportion of nymphs found in each dish (main effect of food type: LR = 5541.03, *d.f.* = 1, P < 0.001; Figure 4). However this pattern was not seen in all species: there was a significant species x food type interaction, for both the presence in dishes (LR =158.24, *d.f.* = 3, P < 0.001) and the proportion of total nymphs in each dish (LR =3368.28, *d.f.* = 3, P < 0.001). *S. pandurus* nymphs did not show a significant preference for milkweed seeds while nymphs of the other three species did (Figures 3 & 4). Moreover, *O. fasciatus* nymphs appeared to show the greatest preference for milkweed over sunflower seeds (Figure 4). The four species also differed in their levels of feeding within the choice treatment, both in terms of the proportion of days nymphs were observed in both dishes (main effect of species: LR =131.72, *d.f.* = 3, P < 0.001) and in terms of the proportion of nymphs observed in both dishes (main effect of species: LR = 14587.35, *d.f.* = 3 P < 0.001; Figure 4).

Comparing across control (Sunflower only) and choice (Sunflower & Milkweed) treatments there were differences between the species in the overall level of feeding by nymphs (LR = 938.1, *d.f.* = 3, P < 0.001): *S. pandurus* and *O. fasciatus* nymphs had a higher level of feeding than *L. equestris* and *L. simulans* nymphs (P < 0.001 for all pairwise LSD contrasts) (Figure 4). There was also a significant interaction effect between species and treatment (LR = 36.36, *d.f.* = 3, P < 0.001), which is probably due to a higher level of feeding in the milkweed boxes by *S. pandurus*. Within the other three species the feeding rate was similar regardless of the presence of milkweed seeds, suggesting that overall nutritional requirements were being satisfied (or not satisfied) to the same extent in both treatments. Indeed, the main effect for treatment was not significant (L.R. = 0.44, *d.f.* = 1, P = 0.510). The average proportion of nymphs feeding in each dish for each treatment is displayed in Figure 5. My method of recording feeding preferences (counts once a day) means I am likely to have missed the majority of feeding events. Accordingly, the proportion of nymphs was never above 0.3 for any dish, across all species.

3.2 Experiment 2 - Nymph aggregation

Nymph aggregation behaviour after 30 or 60 minutes in dishes together did not differ between the species or in terms of diet ($P > 0.05$ in all cases including interactions).When I consider the furthest distance between two nymphs per dish however, some effects of species emerge. There were significant species differences in spacing after both 30 (result of Kruskal-Wallis test: P = 0.04, *d. f.* = 3, 47) and 60 minutes (P = 0.007, *d. f.* = 3, 47). The furthest distance between two nymphs in a dish was on average smaller in *O. fasciatus* and *S. pandurus* than in *L. equestris* and *L. simulans* (Figure 6). Diet had no effect on the furthest distance between nymphs at either 30 or 60 minutes ($P > 0.05$ for all tests).

3.3 Experiment 3 - Body length

The four species used in the experiment differed significantly in body size (main effect of species: $F_{3, 611} = 511.22$, $P < 0.001$). *O. fasciatus* was the smallest species with a mean body size of 10.7mm, followed by *L. equestris* at 11.1mm, then *L. simulans* at 11.9mm. *S. pandurus* was the largest species with a mean body size of 12.8mm. Females were significantly larger than males in all species (main effect of sex: $F_{1, 613} = 677.85$, $P < 0.001$). The extent of sexual dimorphism varies between the species (interaction between species and sex: $F_{3, 611} = 15.33$, $P < 0.001$) with *O. fasciatus* showing less sexual dimorphism than the other three species. The average body sizes of each species according to sex and species are displayed in Figure 7.

Individuals fed on milkweed seeds were found to be significantly larger than those fed only on sunflower seeds (main effect of diet: $F_{1, 613} = 18.29$, $P < 0.001$; Figure 8). This is true in all cases except for *O. fasciatus* (Figure 7). The effect of diet depended on sex as females showed a larger size increase when fed on milkweed than males (interaction between diet and sex: $F_{1, 613} = 7.82$, $P = 0.005$). However, diet did not change overall patterns of size variation among the four species (interaction between diet and species: $F_{3, 611} = 1.407$, $P = 0.240$), nor did the interaction between species, diet and sex significantly affect body size ($F_{3, 611} = 2.02$, $P = 0.109$).

3.4 Experiment 4 - Mating trials

Across all the trials, approximately 57% of pairs copulated during the 2 hour observation period. Mating frequency varied between species, independently of diet ($\chi^2 = 39.88$, *d.f* = 3, $P<0.001$). *L. equestris* and *O. fasciatus* both had higher rates of mating (75% and 81% of pairs respectively) than *L. simulans* and *S. pandurus* (48% and 38% of pairs respectively) (Figure 8). Contrary to our predictions, milkweed-fed males had significantly lower mating success than sunflower-fed males ($\chi^2= 7.90$, *d.f* = 1, $P=0.005$). This pattern was most strongly influenced by the decreased mating frequency observed in *L. equestris* males when fed on milkweed and the effect becomes non-significant when *L. equestris* is removed from the analysis ($\chi^2= 1.60$, *d.f* = 1, $P=0.205$). There was no difference in mating success for females fed either milkweed or sunflower seeds ($\chi^2= 2.07$, *d.f* = 1, $P=0.150$). There were also no significant interactions between any of the factors ($P> 0.05$ in all cases), such that the attractiveness of one sex was not influenced by the diet of the other.

Figure 1. Photos of the four Lygaeidae species used in this study. Clock wise from top left: *Lygaeus simulans*, *Lygaeus equestris*, *Oncopeltus fasciatus* (photo courtesy of Alison Bockoven) and *Spilostethus pandurus*.

Figure 2. The ventral view of a male *Lygaeus simulans*. The lines indicate the portion of the bug that was used for our measure of body length, i.e. from the tip of the snout to the end of the underside of the abdomen.

4. Discussion

I tested the feeding preferences and aggregation behaviour of nymphs of four species of aposematic Lygaeidae, as well as their adult mating preferences, to look at the effect of early diet on social behaviour in these insects. I found only a limited effect of diet on key social interactions. Contrary to our predictions, our results showed no influence of diet on the aggregation behaviour of nymphs. While the species clearly differed considerably in their propensity to aggregate, no significant effect of diet on aggregation behaviour was found. *O. fasciatus* and *S. pandurus* both readily formed aggregations regardless of diet while *L. equestris* and *L. simulans* were less gregarious in nature. The effect of diet on mating behaviour also ran contrary to our predictions. Despite both sexes showing larger body sizes when fed milkweed, only in one species did diet affect mating success: in *L. equestris*, milkweed-fed males had lower mating success than those fed on sunflower seeds. Diet had no effect on female mating success in all species tested.

While aggregation has been shown to increase the protection associated with aposematic signals (Sillen-Tullberg et al., 2000), it may be that aggregation in these species is not state-dependent but rather an evolved response regardless of the level of individual chemical protection. As even nymphs living in close proximity may vary in their chemical defence due to food availability, it could be the case that a relatively unprotected individual may be surrounded by well-protected conspecifics and thus still benefit from the increased aposematic signal presented by aggregation. Research has shown that predators with experience of protected prey items will avoid other individuals of that species regardless of their level of chemical protection (Berenbaum and Miliczky, 1984). For example, praying mantids with experience of milkweed-fed *O. fasciatus* adults would then avoid bugs fed on sunflower seeds, despite these being palatable to naive mantids (Berenbaum and Miliczky, 1984). If group size increases the level of protection from predators for all members of the group then it may be advantageous for protected nymphs to associate with any conspecifics in the area, regardless of their level of protection.

In terms of the life-history consequences of nymph diet, I found that for all species (except *O. fasciatus*) adult individuals were larger when fed on milkweed seeds. The lack of a response in *O. fasciatus* is more puzzling as milkweed is its natural food plant and thus we might expect to see greater increases in body size of milkweed-fed individuals. A recent study has suggested that milkweed-fed *O. fasciatus* males invest more of their resources in mating and reproduction at the expense of longevity (Attisano et al., 2012), although this study only manipulated adult diet. Previous evidence suggests that adult body size is strongly affected by nutrition during development (Blakley and Goodner, 1978a, Solbreck et al., 1989, Blakley, 1981), especially in 5th instar nymphs, suggesting dietary effects may affect nymphs and adults differently (Blakley, 1981). Attisano and colleagues suggested that the differences in resource allocation between the two diets in their study were the result of a shift in life-history trade-offs that accompanied the adaptation of *O. fasciatus* to sunflower seeds. Adaptation of most seed bug species to sunflower seeds takes place very quickly in a laboratory environment, and all the populations used in this experiment have been raised on sunflower seeds for many generations prior to their use in this experiment. Thus the lack of change in body size in *O. fasciatus* may be reflective of different life history trade-offs operating in this species verses *S. pandurus*, *L. equestris* and *L. simulans*. However, we must be cautious in interpreting this finding as it may be an artefact of the smaller sample size for *O. fasciatus* compared with the other species.

No increase in mating success was detected in the milkweed-fed individuals, as would be expected if sequestered chemical protection increased attractiveness to the opposite sex. Indeed while male diet did appear to affect the rate of successful mating, it was in the opposite direction to that predicted. This was especially pronounced in *L. equestris* where males fed only on sunflower seeds had a much higher mating rate than those fed on milkweed seeds. However, it must be noted that sunflower-fed males only enjoyed greater mating success in *L. equestris*. If this species is removed from the analysis, the effect of male diet on mating success becomes non-significant. It could be that the variation in effects observed across the species is not caused by variation in the potential levels of chemical defence but by some other aspect of nutrition. The reason for this is unclear however; it may be related to the finding that milkweed-fed males had a greater body size as there is suggestion that female *L. equestris* prefer smaller males (Dougherty & Shuker, 2014). In contrast, we might expect increased body size to correlate with improved nutrition and it has been suggested that in other organisms females may prefer well-fed males (Giaquinto et al., 2010). For example, improved nutrition has been found to correlate with increased courtship behaviour and mating success in male wolf spiders (*Pardosa prativaga*: Lomborg and Toft, 2009) and increased reproductive success in spruce budworms (*Choristoneura fumiferana*: Delisle and Hardy, 1997) The lack of a significant male preference for females fed on milkweed (and thus assumed to possess greater levels of cardiac glycosides) is also unexpected as very recent work has found that female *O. fasciatus* may pass defensive chemicals to their eggs, giving them some protection from predators (Newcombe 2013 unpublished thesis).

Mate choice outcomes were tested using a no-choice paradigm, in which each bug was tested once with a single potential mate. No-choice paradigms can test for absolute mating preferences, but are probably less likely to detect small differences in preference between stimuli (Wagner Jr, 1998, MacLaren and Rowland, 2006). When compared to choice tests, rejection of a mate in a no-choice test may be more risky due to the potential lost mating opportunities (Barry and Kokko, 2010, Booksmythe et al., 2011). However, it has recently been shown that there is little variation in mating outcomes between choice and no-choice paradigms in *L. equestris*, based on male and female morphology (Dougherty & Shuker, 2014), so perhaps this criticism is less relevant here. What may be more important is that Dougherty and colleagues showed rather little pre-copulatory choice in *L. equestris*, across all experimental paradigms. To date, studies of pre-copulatory sexual selection in lygaeids have shown some patterns of non-random mating in terms of aspects of morphology in both males and females (Yang and Wang, 2004, McLain, 1992, see chapter 1). While mate choice may occur either before, during or after copulation, our experiment only tested pre-copulatory preferences. There is growing evidence that post-copulatory sexual selection may play a greater role in lygaeids, as indicated by the extreme genital morphology in these species (Gschwentner and Tadler, 2000, Micholitsch et al., 2000, Bonhag and Wick, 1953), and evidence for post-copulatory selection on aspects of male and female genitalia (Tadler et al., 1999, Yang and Wang, 2004, Higgins et al., 2009). As discussed in chapter 1, all four species exhibit very long copulations (Walker, 1979, Sillen-Tullberg, 1981, Micholitsch et al., 2000), which likely function as a form of post-copulatory mate-guarding (Tadler et al., 1999, Sillen-Tullberg, 1981, see chapter 6 for further investigation of mate-guarding), as well as a high frequency of mating failures, even after successful intromission by the male (Tadler et al., 1999, Micholitsch et al., 2000, see chapter 7). This suggests that sperm competition and potentially cryptic female choice may play a significant role in sexual selection in these species. Future experiments that test cryptic female choice for males in terms of nutrition or chemical defence may be more fruitful (see also Newcombe 2013 unpublished thesis). Additionally, given the sex and species-variation demonstrated in chapter 4, it would be useful to look for any diet-dependent effects on cuticular hydrocarbon composition that may provide information on either chemical defence or nutrition.

Despite the apparent lack of strong effects on behaviour, *L. equestris*, *L. simulans* and *O. fasciatus* nymphs all showed a preference for feeding on milkweed seeds over sunflower seeds when provided with both. The only species in this trial not to show this preference was *S. pandurus.* However, *S. pandurus* was observed in the food dishes more frequently than any of the other species and this may have masked any weaker diet preferences. It is also possible that as *S. pandurus* is one of the larger species used in this experiment (see experiment 4) and often had higher numbers within a single clutch (see chapter 2) there may have been greater competition for food within the *S. pandurus* boxes.

Milkweed was clearly preferred as a food source by the nymphs of all but one of the species used in this study, however, diet did not play a significant role in determining the social behaviour of the bugs. While there was variation among the different species, neither the propensity to aggregate as nymphs, nor mate choice as adults, was much associated with diet. Although our study did not measure chemical defence directly, these findings suggest that social behaviour in the Lygaeidae may depend less on nutritional or chemical-defence state than previously thought. Rather than adjusting their behaviour in response to either their own internal states, or that of others, lygaeids may instead base their social interactions on behavioural “rules of thumb” which are advantageous in the majority of circumstances. The next step will be to explore patterns of social behaviour in terms of nutrition and chemical defence in the wild. Our data here suggest that processes like automimicry, where individuals vary in their chemical defence, and perhaps are completely undefended yet still perform aggregation behaviour, may be more common (Speed et al., 2012).

The lack of mating preferences based on diet does suggest that the interspecific mating attempts observed in the laboratory are not the result of a breakdown of host-plant mediated signals. If mate choice in these species was heavily influenced by diet-derived signals then we would expect to see changes in mating success as a result of different diets, especially in *O. fasciatus* as milkweed is its natural diet. Instead it seems mating behaviour is diet-independent, at least when the diets do not vary greatly in terms of nutritional content, and observations, including those in chapters 3, 4, 6 and 7, made using sunflower-fed individuals are unlikely to differ from those feeding on more natural diets.

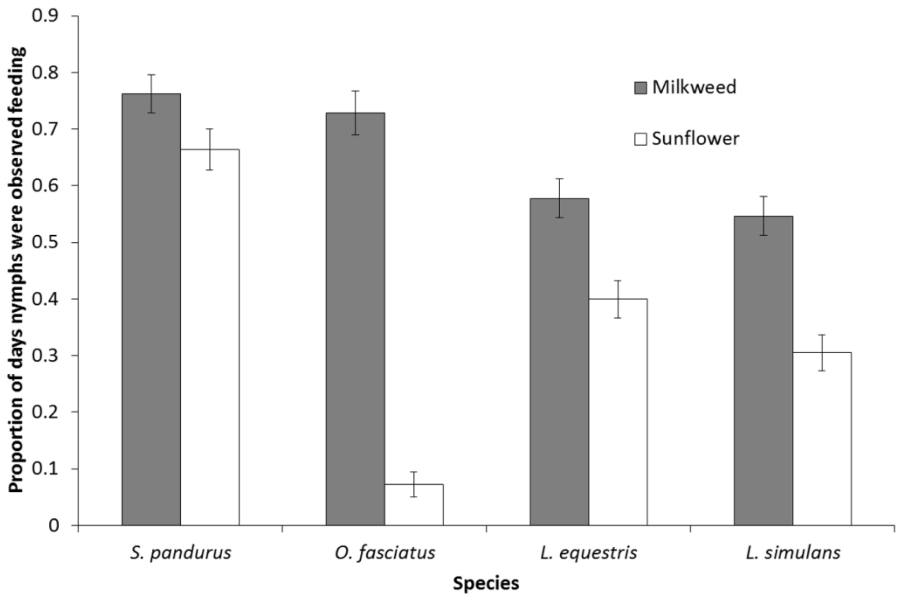

Figure 3. Proportion of days that nymphs were observed in the different food dishes for each species in the choice (Sunflower & Milkweed) treatment. Bars indicate ± 1 standard error.

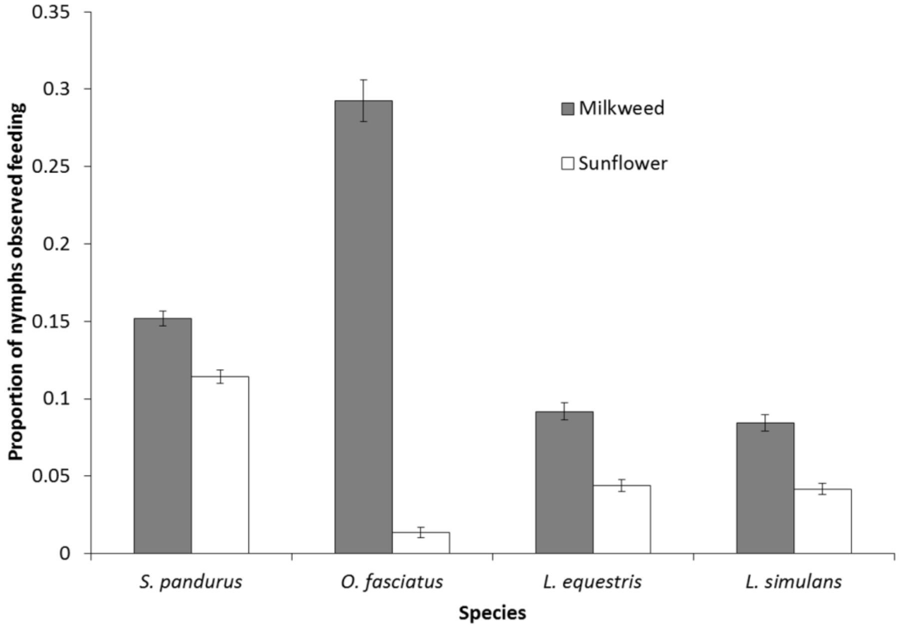

Figure 4. Proportion of nymphs that were observed in the different food dishes for each species in the choice (Sunflower & Milkweed) treatment. Bars indicate ± 1 standard error.

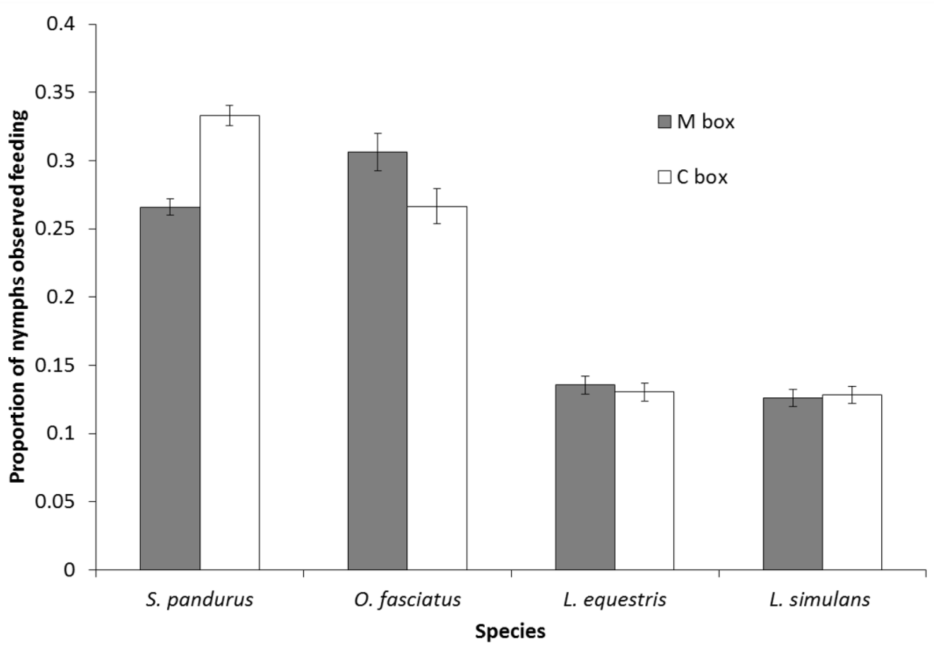

Figure 5**.** Overall proportions of nymphs feeding in the milkweed (M) and control (C) boxes. Bars indicate the number of nymphs present in both dishes as a proportion of the total nymphs present in each box. Error bars indicate ± 1 standard error.

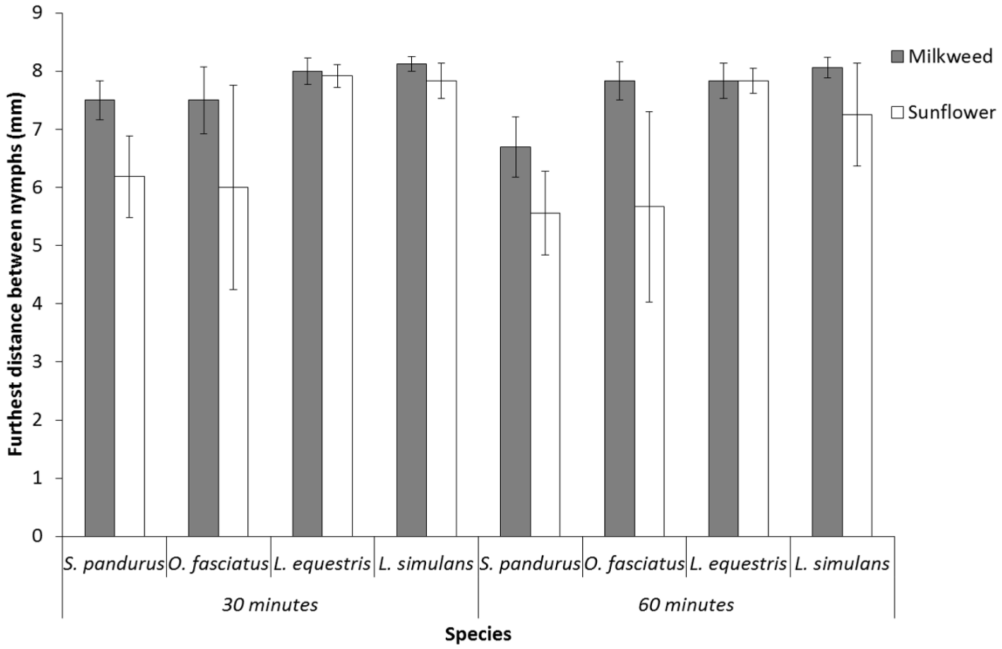

Figure 6. The mean furthest distance between two nymphs at 30 and 60 minutes (when dishes with fewer than 10 nymphs were excluded), bars indicate ± 1 standard error.

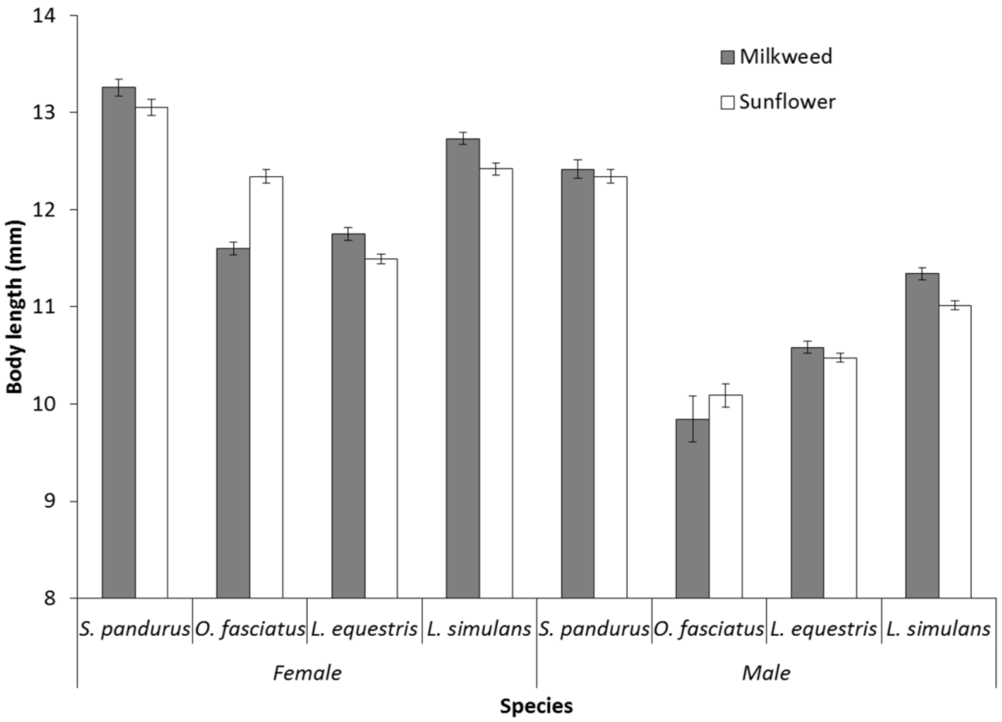

Figure 7. The mean body length of each species for females and males according to diet and species. Bars indicate ± 1 standard error.

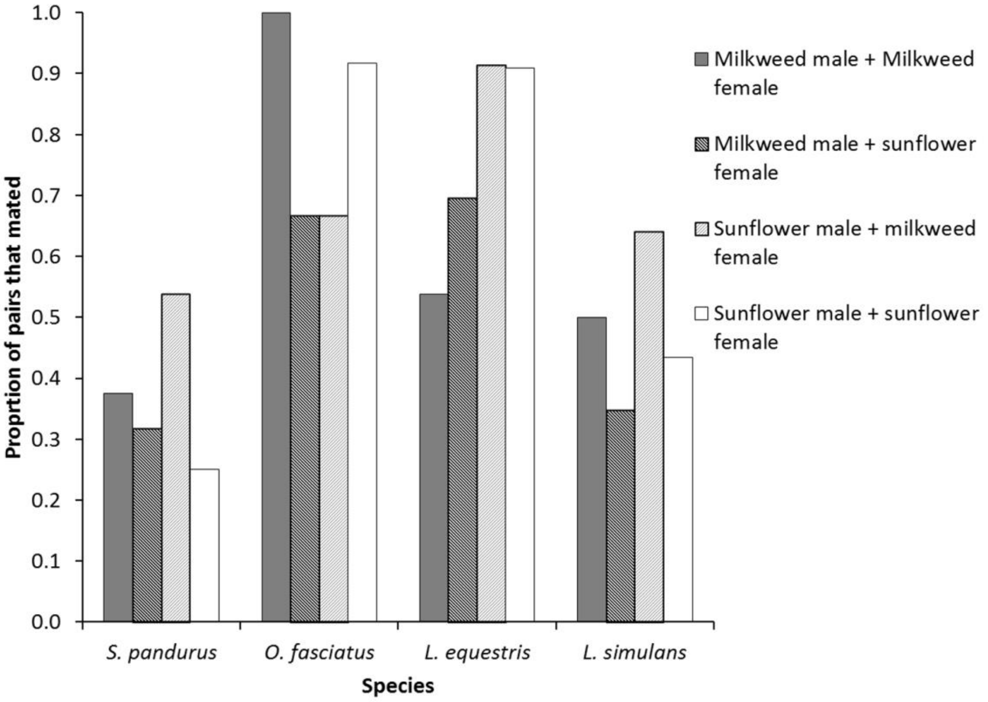

Figure 8. The proportion of pairs which mated during the course of the trial for each species and treatment.

Chapter 6

Mate-guarding in a polygamous insect: species discrimination influences reproductive interference in seed bugs

1. Introduction

The ability to distinguish between conspecifics and heterospecifics is an important component of successful mate choice. The failure or breakdown of this species discrimination can have many effects, both on an individual and population level. One particular outcome that has received increasing attention in recent years is reproductive interference (RI). RI occurs when organisms of one species attempt reproductive behaviours with another species, leading to a reduction in fitness for one or both species (Gröning and Hochkirch, 2008, Burdfield-Steel and Shuker, 2011). Examples of RI can range from straightforward heterospecific copulation attempts, (i.e. de Bruyn et al., 2008) to more subtle behavioural changes such as forgoing normal mate-quality assessment in favour of reliable species discrimination (Pfennig and Pfennig, 2009). The presence of heterospecific signals in the environment can interfere with sexual signalling e.g. in the frog, *Allobates femoralis*, and Neotropical singing mice, *Scotinomys sps*. (Amezquita et al., 2006, Pasch et al., 2013), influencing both mate-attraction and mate-searching. Additionally, males may, erroneously, exhibit aggressive behaviour towards heterospecifics whom they perceive as rivals (Grether et al., 2009). While much of the literature on the effects of RI has focused on responses to heterospecific copulation attempts, including for example avoidance behaviours leading to changes in habitat use (McLain and Shure, 1987), or erroneous mate choice (Butler and Stein, 1985, Dame and Petren, 2006), heterospecific matings do not actually have to occur for RI to be present. If individuals are unable to differentiate between heterospecifics and conspecifics it may influence their reproductive behaviour in more than one way (Miller et al., 2013). For example, studies in *Drosophila melanogaster* have shown that heterospecific song can influence female gene expression and that these changes are broadly comparable to those caused by conspecific song (Immonen and Ritchie, 2012) . Thus RI can potentially arise through a variety of different processes.

Context-dependent male mating strategies may be particularly vulnerable to influence from RI. Sperm production and mating both pose non-trivial investments in males (Simmons, 2001) and numerous studies have illustrated the interaction between social environment and male sexual behaviour. Facultative changes in behaviour, especially in the context of sperm competition and post-copulatory sexual selection, have been demonstrated in a huge variety of taxa, from insects (Bretman et al., 2009a, Wigby et al., 2009, Bailey, 2011, Billeter et al., 2012, Bailey et al., 2013, Han and Brooks, 2013) to vertebrates (recently reviewed by Kelly and Jennions, 2011, Firman et al., 2013). Regarding sperm competition, two main factors have been identified that should change a male's optimal mating strategy: risk and intensity. Sperm competition risk refers to the risk that a female the male mates with will mate with a second mate. It is the risk that the male's sperm will experience competition to fertilise the female's gametes. In contrast, sperm competition intensity refers to the number of males a male's sperm may be in competition with. Theory predicts that male investment in traits and behaviours that increase the competitiveness of his sperm should increase as sperm competition risk increases, but decrease with local increased sperm competition intensity. While males may employ a variety of strategies in response to sperm competition (including, but not restricted too: changes in sperm number (Wedell et al., 2002), ejaculation size (Gage, 1991, Garcia-Gonzalez and Gomendio, 2004), seminal protein composition (Wigby et al., 2009, Perry et al., 2013) and sperm morphology (Gage, 1994)), behavioural changes have the benefit of being "cheaper" and may allow for more rapid responses to fluctuations in local environment (Bretman et al., 2011). However, failure to discriminate between con- and hetero-specifics may result in males adopting sub-optimal mating strategies, ultimately reducing their fitness.

One other behaviour that has been shown to vary considerably according to social environment is mate guarding. Mate guarding is an umbrella terms for prolonged periods of association between males and females beyond that required for fertilization (Simmons, 2001). While these interactions may have other benefits to both sexes (Alcock, 1994), the term mate guarding refers specifically to behaviours designed to reduce sperm competition (Parker and Vahed, 2010). This can be achieved either by reducing female polyandry or securing a last male advantage (Adler and Adler, 1991). As such it is predicted that mate-guarding will be favoured when the risk of sperm competition is high. In order to enact these context-dependent behavioural 'strategies' however, males must first acquire information on their social environment. Several variables have been predicted to influence mate-guarding, including the operational sex ratio (OSR), population density (both of which will contribute to search time, mate encounter rate and the risk and intensity of sperm competition) as well as the time required to achieve insemination and the time required for mate guarding to be beneficial (Carroll, 1993). Of these, OSR and population density are most likely to vary over time and space. Equally, perception of these factors may be influenced by the presence of heterospecifics, however there appears to be little in the literature exploring how RI may arise in the context of mate-guarding. Here I examine the influence of closely related heterospecifics on the mating strategy of male seed bugs (Hemiptera: Heteroptera: Lygaeidae) to explore both copulation strategies and how males of this group discriminate among competitors.

Lygaeus equestris and *Lygaeus simulans* are closely related species found across mainland Europe. They can be distinguished from each other by the morphology of their antennae (Gusev and Tatarnikov, 1992). While the two species can hybridize (Evans, 2011), there is evidence for asymmetric pre-zygotic isolation: male *L. simulans* can copulate with *L. equestris* females, but although *L. equestris* males attempt to mate with *L. simulans* females they are usually unable to do so due to high levels of female resistance (Evans, 2011). Additionally, fertilization success is considerably lower in heterospecific crosses; although the exact mechanism behind this remains unknown (see chapter 7). *Spilostethus pandurus* also shares part of its range with *L. equestris* but is far more diverged from *L. equestris* than *L. simulans* and thus completely reproductively isolated. *Oncopeltus fasciatus* is a new world species and would never naturally co-exist with *L. equestris*. While the phylogeny of this group remains largely unclear (Weirauch and Schuh, 2011) it is probable that *O. fasciatus* is more distantly related to *L. equestris* than *S. pandurus*. Previous laboratory studies have found evidence of sexual conflict and RI between *L. equestris* and *S. pandurus*. Shuker et al (*in prep*) showed that *L. equestris* females housed with male *S. pandurus* suffered comparable fitness costs, in the form of lifetime egg production, to those housed with male conspecifics. However, those housed with *O. fasciatus* did not show any such fitness costs. All four species belong to the family Lygaeidae and share a similar, polygamous, mating system (Sillen-Tullberg, 1981). No pre-copulatory courtship has been recorded in these species; instead males rush or jump towards females and grasp them with their legs before initiating genital coupling. If coupling is successful, the pair then moves so they are facing away from each other, end to end (see Figure 1). Bugs can continue to move and feed in this position, with females typically directing movement and males walking backwards or being dragged along (Walker, 1979, Sillen-Tullberg, 1981, Shuker et al., 2006); pairs have been observed to remain coupled in this manner for over 15 hours (Sillen-Tullberg, 1981). As successful insemination has been known to occur from copulations lasting between one and two hours, this prolonged copulation is thought to be a form of mate guarding (McLain, 1989, Alcock, 1994, Wang et al., 2008). This is further supported by the finding that sperm displacement during second matings may be as high as 90% and average copulation duration is longer when the local sex-ratio is male-biased (Sillen-Tullberg, 1981).

By measuring the response of male *L. equestris* to heterospecifics that differ both in their evolutionary relatedness but also in their likelihood of being encountered by *L. equestris* in the wild we can test various predictions about species recognition and reproductive interference. Firstly, I would predict that as male *L. simulans* are both closely related to *L. equestris* males and capable of fertilizing female *L. equestris*, male *L. equestris* may respond to them as if they were conspecific rivals. Secondly, it has been hypothesised that species that naturally co-occur are more likely to have effective species recognition mechanisms than those that would never naturally encounter each other (Gröning and Hochkirch, 2008). Thus we might expect male *L. equestris* to respond differently to *S. pandurus* and *O. fasciatus* males, viewing *O. fasciatus* males as potential rivals, but not *S. pandurus*.

The original paper describing the influence of sex ratio on copulation duration in *L. equestris* (Sillén-Tullberg, 1981) used only two treatments, one male with four females, and one female with four males. Thus, it is hard to distinguish the effects of sperm competition verses mate availability on male behaviour in this study. I therefore added a control treatment of one male and one female in order to distinguish between these two mechanisms. If male *L. equestris* increase copulation duration only in response to perceived sperm competition then we expect to see no difference in copulation duration between the control and the female-biased treatments. By extension, if male *L. equestris* do alter copulation duration in response to the availability of potential mates then the presence of heterospecific females may also influence their behaviour.

Here I investigate the effect of sex ratio on the duration of copulation in *L. equestris*, taking into account any effects of heterospecifics of both sexes, to determine if *L. equestris* males differentiate between conspecifics and heterospecifics when making context-dependent mating decisions. If prolonged copulation serves as a form of mate-guarding, we would expect to see longer copulations occurring when the sex ratio is male-biased, due to increased levels of perceived sperm competition. Similarly, if *L. equestris* males react to heterospecifics as though they were conspecifics, we can expect to see a similar pattern when the sex ratio of *L. equestris* is equal, but the overall sex ratio including heterospecific individuals is biased.

2. Methods

The focal species for this experiment was *Lygaeus equestris.* A sister species, *Lygaeus simulans*, and two other Lygaeid species, *Spilostethus pandurus* and *Oncopeltus fasciatus*, were used as treatment bugs. I maintained all four species in continuous culture as described in chapter 2.

For each species I isolated 5th instar nymphs and housed them in tubs with organic sunflower seeds and water *ad libitum*. I checked the tubs daily and any adults that had eclosed were removed and housed individually. At day five of adulthood (48 hours prior to the trials) males were marked with enamel paint to allow easy identification and re-housed with their experimental group. Females were marked but kept separate.

2.1 Experiment 1

All bugs were assigned to one of five treatments (see table 1.) Groups in the female biased (*L. simulans*) treatment contained three *L. simulans* females, one *L. equestris* females and one *L. equestris male*. Groups in the female biased (*L. equestris*) treatment contained four *L. equestris* females and one *L. equestris* male. Groups in the control treatment contained one *L. equestris* female and one *L. equestris* male. Groups in the male biased (*L. equestris*) treatment contained *one L. equestris* female and four male *L. equestris* and groups in the male biased (*L. simulans*) treatment contained one *L. equestris* female, one *L. equestris* male and three *L. simulans* males. All groups contained five bugs, except treatment the control groups which contained only two. All groups contained at least one male and one female *L. equestris*. A total of 98 groups were observed, 19 of the female biased (*L. simulans*) treatment, 20 of the female biased (*L. equestris*) treatment, 20 of the control treatment, 19 of the male biased (*L. equestris*) treatment and 20 of the male biased (*L. simulans*) treatment.

On the day of the behavioural trial, the male experimental groups were placed in a large petri dish 85mm in diameter. Females were then added at the start of the trial. I recorded the presence/ absence of mated pairs after 15 minutes and subsequently every 30 minutes for nine hours. At the end of the experiment all pairs still copulating were separated and bugs were frozen at -20°C. The trials were started at 9am in the morning and finished at 6pm in the evening.

Mating time was calculated by adding up the time between the observations. For example, a pair observed copulating at 30 and 60 minutes were considered to have been copulating for 30 minutes as this is the minimum time they could have been coupled. Matings that were observed only once (i.e. for only one observation period) were not included. The effect of treatment on average length of both the first observed copulation, and the longest copulation observed during the nine hour period was tested with a Univariate Analysis of Variance. The difference between the treatments was assessed with a LSD post-hoc test. All analyses were carried out in PASW Statistics 18 by IBM.

2.2 Experiment 2

Nymphs of *L. equestris*, *S. pandurus* and *O. fasciatus* were isolated as described above. As these species are easily distinguished from each other, bugs were not marked with enamel paint. All other methods remained the same. Four treatments were carried out, as detailed in table 2 . A total of 68 pairs were observed: 19 of the female biased (*O. fasciatus*) treatment, 20 of the male biased (*O. fasciatus*) treatment, 11 of the female biased (*S. pandurus*) treatment and 18 of the male biased (*S. pandurus*) treatment.

3. Results

3.1 Experiment 1

The copulation behaviour of *L. equestris* was context-dependent. The length of both the first and the longest copulation recorded varied significantly between the treatments ($F_{4, 94}$ = 3.448, P = 0.011 and $F_{4, 94}$ = 3.502, P = 0.010 respectively). However, only the male biased (one female, four conspecific males) treatment containing *L. equestris* males produced significantly longer copulations (mean time of approximately 380 and 409 minutes for first and longest copulation respectively) than any of the other treatments (mean copulation time ranged between 180 - 230 and 230 - 270 minutes respectively, see Figure 2 and table 3). Copulation duration in the *L. simulans* male biased treatment was not significantly different from the control treatment. Thus, there was therefore no evidence of reproductive interference in terms of mate-guarding. Additionally, copulation duration did not differ from the control in either of the female biased treatments, suggesting that male *L. equestris* do not alter their copulation duration in response to multiple females, regardless of female species.

3.2 Experiment 2

There was likewise no effect of the species or sex of the heterospecifics present on copulation duration in Experiment 2. Treatment had no effect on the length of first, or longest, copulation ($F_{3, 64}$ = 25.183, P = 0.189 and $F_{3, 64 =}$ 35.928, P = 0.120 respectively; Figure 3). Male *L. equestris* did not therefore behave differently in response the presence of *S. pandurus* or *O. fasciatus* males or females. Although Figure 3 appears to show longer copulations in the presence of male *O. fasciatus* compared with female, this effect was not significant.

Finally, no heterospecific pairings were observed during the course of the experiment. All recorded copulations involved only individuals of *L. equestris*.

Figure 1. Photos of two *Lygaeus equestris*. Left: male initiating copulation, right: pair *in copula.* Photo credit Liam Dougherty.

4. Discussion

These results suggest that male *L. equestris* are able to correctly distinguish between conspecific rivals and heterospecifics and adjust their behaviour accordingly. In keeping with previous studies (Sillen-Tullberg, 1981), male *L. equestris* responded to the presence of multiple male conspecifics by extending the duration of copulation. This supports the hypotheses that extended copulations in *L. equestris* can act as a form of mate guarding (McLain, 1989, Alcock, 1994). However, no such changes in copulation duration were observed in the presence of multiple male *L. simulans, O. fasciatus* or *S. pandurus*, despite morphological similarities between *L. equestris* and *L. simulans*, and previous observations that male *S. pandurus* frequently attempt to mate with *L. equestris* females. Additionally, male *L. equestris* did not adjust copulation duration in response to increased numbers of hetero- or conspecific females. Nor did *L. equestris* males ever successfully pair with *L. simulans* females, although it is possible that heterospecific mating attempts may have occurred during the experiment as not all unsuccessful attempts were recorded.

These findings suggest that heterospecific harassment and mating attempts in this group are likely to be the result of male mating strategy, rather than a breakdown of species discrimination *per se*. As *L. equestris* is a multiply-mating species without parental care, the main limit to male fitness is the number of successful matings they can acquire (Bateman, 1948). Thus males are expected to mate opportunistically which may lead them to attempt to mate with females perceived to be of low "quality", such as heterospecifics, in the absence of other mating opportunities. However, clearly males do discriminate between species in some contexts, as shown here. This conclusion is further supported by recent work showing that all four species have distinctive cuticular hydrocarbon (CHC) profiles (chapter 4). Therefore, in keeping with many other insect species (Everaerts et al., 2010, Thomas and Simmons, 2010, Weddle et al., 2012), CHCs may provide information on an individual's species identity that can be utilized by male bugs in the context of mating decisions. Additionally, previous studies in *L. equestris* have found not only sexual conflict over mating frequency, but also weak pre-copulatory sexual selection (Burdfield-Steel et al., 2013, Dougherty and Shuker, 2014). Thus, it seems that the mating system of encounter polgynandry, rather than species discrimination, may be the driving force behind reproductive interference in this group.

While male *L. equestris* increased mating duration in the presence of conspecific rivals, they did not show any reduction in copulation duration in the presence of multiple females. Although we might expect to see copulation duration reduced when multiple potential mates are available, there are several possible explanations for the lack of such a pattern in this species. One explanation is that male seed bugs may trade off mating with more females against increased fertilisation rates with each female. Previous studies have found that insemination rates are highest during the first hour of copulation, and matings lasting between one and two hours are sufficient to achieve fertilization in the majority of cases (Sillen-Tullberg, 1981, Sillen-Tullberg, 1985b). However, the genitalia of many Lygaeidae, including *L. equestris*, are elongate and complex (Bonhag and Wick, 1953, Gschwentner and Tadler, 2000, Micholitsch et al., 2000, Higgins et al., 2009, Aspiras and Angelini, 2011) and the delay in insemination may be a result of time take for the male processus to traverse the female reproductive tract to the spermatheca. This, combined with the high levels of mating failure observed in this group (Tadler et al., 1999, Gschwentner and Tadler, 2000, Micholitsch et al., 2000), suggests that males may make significant investments on copulation duration due to processes of post-copulatory sexual selection (or “cryptic female choice”: Eberhard 1996). As such, even if other females are in the environment, minimum copulation duration may still represent a significant time investment for males. However, given that the average length of the first and longest copulations (approximately 200 minutes) was considerably greater than the two hours suggested to be necessary for insemination.

Alternatively, this lack of response to female-biased OSRs may simply reflect the limits of behavioural plasticity in this species. In the wild, *L. equestris* habitats are often patchy, with spatial and temporal variation in the availability of seeds causing fluctuations in population density (Sillen-Tullberg and Solbreck, 1990, Solbreck, 1994), but it is not known if these fluctuations also result in significant changes to the OSR. Previous studies have shown that considerable selective pressure is needed to maintain behavioural plasticity in populations (Carroll, 1993, Carroll and Corneli, 1995), and this may not be the case in *L. equestris*. However, this seems unlikely given the presence of behavioural plasticity in response to the presence of rival males.

Finally, if males are selected to mate opportunistically, and given that pre-copulatory selection appears to be weak, males may be adapted to assess females sequentially. Thus, the number of females present would perhaps not influence male copulation duration as each mating interaction is assessed independently. This is supported by work by Dougherty and Shuker (2014) on mate choice in *L. equestris* which suggests that the bugs are unable to simultaneously assess mates. They found no differences in the strength of mate choice between no-choice and choice tests, even when both males and females had access to more than one potential mate simultaneously (Dougherty and Shuker, 2014).

In conclusion, male *L. equestris* can correctly distinguish between rival and heterospecific males in the context of phenotypically plastic copulation behaviour. This, combined with the observations that males will often attempt copulation with female heterospecifics in the absence of conspecific females, suggests that while males are able to distinguish even closely-related heterospecifics from their own species, they may use this information differently in different contexts. I hope this work will encourage evolutionary ecologists to explore the potential for reproductive interference in a broad range of mating contexts, where social information informs behaviour.

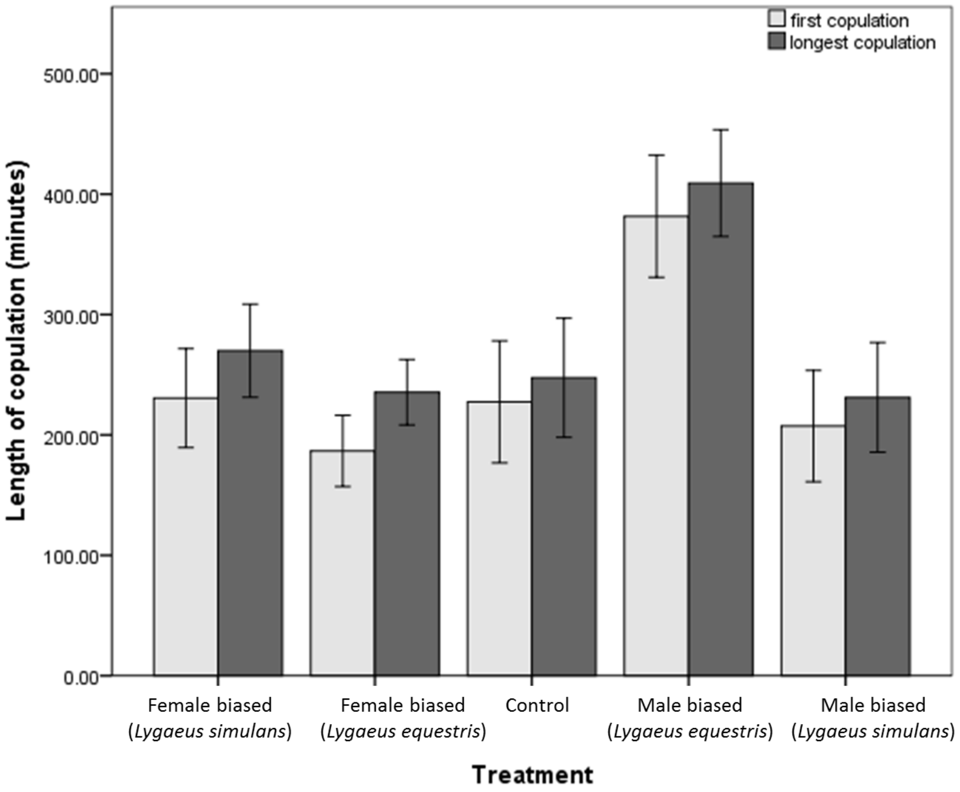

Figure 2. The mean length of both the first recorded copulations and the longest recorded copulations from each treatment from experiment 1. Error bars indicate ±1 standard error.

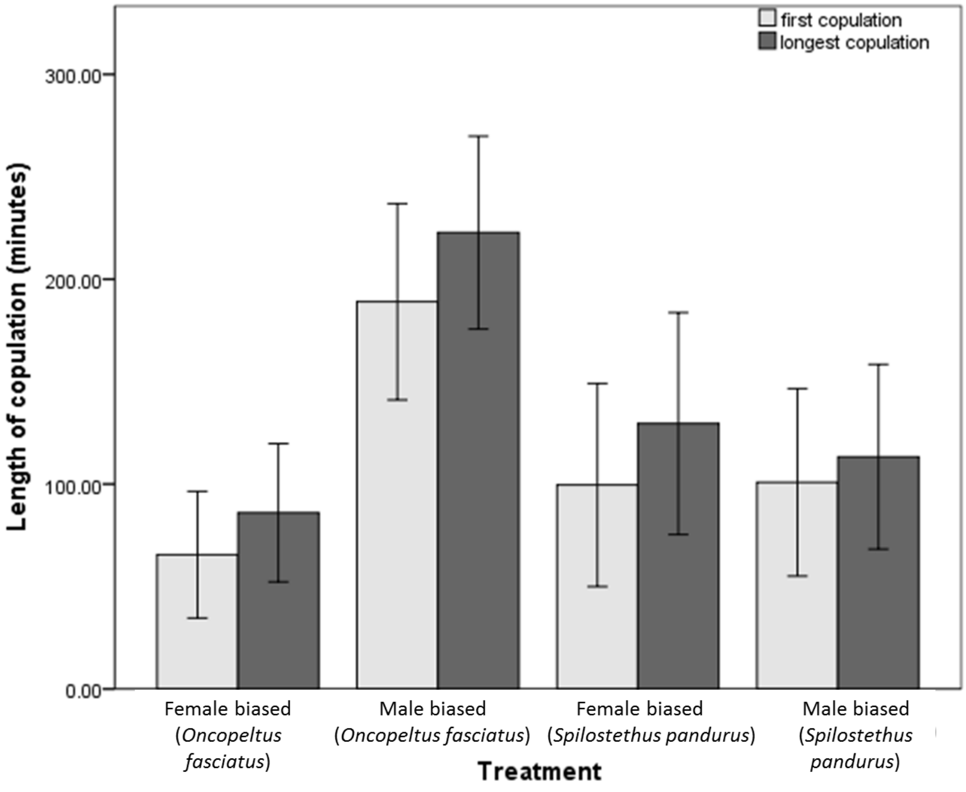

Figure 3.The mean length of both the first recorded copulations and the longest recorded copulations from each treatment in experiment 2. Error bars indicate ± 1 standard error.

Table 1. Numbers of male and female *L. equestris* and *L. simulans* bugs in each treatment.

Treatment	**Females (*L. simulans*)**	**Females (*L. equestris*)**	**Males (*L. equestris*)**	**Males (*L. simulans*)**	**Total bugs**
Female biased (*L. simulans*)	3	1	1	0	5
Female biased (*L. equestris*)	0	4	1	0	5
Control	0	1	1	0	2
Male biased (*L. equestris*)	0	1	4	0	5
Male biased (*L. simulans*)	0	1	1	3	5

Table 2. Numbers of male and female *Spilostethus pandurus* and *Oncopeltus fasciatus* bugs in each treatment. All treatments contained one male and one female *Lygaeus equestris*.

Treatment	**Females (*O. fasciatus*)**	**Males (*O. fasciatus*)**	**Females (*S. pandurus*)**	**Males (*S. pandurus*)**	**Total bugs**
Female biased (*O. fasciatus*)	3	0	0	0	5
Male biased (*O. fasciatus*)	0	3	0	0	5
Female biased (*S. pandurus*)	0	0	3	0	5
Male biased (*S. pandurus*)	0	0	0	3	5

Table 3. Results of a LSD post-hoc test on the effect of treatment on the length of first copulation (above the diagonal) and length of longest copulation (below the diagonal). I-J refers to the mean difference between the groups. * indicates the mean difference is significant at the 0.05 level.

Treatment	Female biased (*L. simulans*)	Female biased (*L. equestris*)	Control	Male biased (*L. equestris*)	Male biased (*L. simulans*)
Female biased (*L. simulans*)	-	I-J = 43.78 P = 0.469	I-J = 9.28 P = 0.878	I-J = -158.68 P = 0.011*	I-J = 8.53 P = 0.888
Female biased (*L. equestris*)	I-J = 34.50 P = 0.541	-	I-J = -34.50 P = 0.563	I-J = -202.46 P = 0.001*	I-J = -32.25 P = 0.555
Control	I-J = 30.75 P = 0.586	I-J = -3.75 P = 0.946	-	I-J = -167.96 P = 0.006*	I-J = -0.75 P = 0.99
Male biased (*L. equestris*)	I-J = -145.26 P = 0.012*	I-J = -179.76 P = 0.002*	I-J =-176.01 P = 0.002*	-	I-J = 167.21 P = 0.007*
Male biased (*L. simulans*)	I-J = 12.00 P = 0.831	I-J = -22.5 P = 0.686	I-J = -18.75 P = 0.736	I-J = 157.26 P = 0.006*	-

Chapter 7

Do the benefits of polyandry scale with inbreeding?

1. Introduction

Mating is known to carry non-trivial penalties for females, yet female polyandry is widespread in insects (Simmons, 2005, Arnqvist and Nilsson, 2000, Pizzari and Wedell, 2013). Potential explanations for this can be broadly categorized under several headings (Boulton and Shuker, 2013). Firstly, female mating rate may be the result of conflict between the sexes shifting mating rate away from the (presumably low) female optima. One example of this is convenience polyandry, where females mate to mitigate the costs of harassment by males (Thornhill and Alcock, 1983, good examples include water striders: Rowe et al., 1994, and seaweed flies: Shuker and Day, 2001). Secondly, females may receive benefits from mating more than once. These benefits can be direct or indirect. Direct benefits range from females simply requiring sperm from more than one mating to fertilize all her eggs, to nutritional benefits, such as food gifts during courtship (Gwynne, 2008), and increased male parental care or protection (Yasuo Ihara, 2002). Indirect benefits are those that increase offspring fitness through genetic mechanisms (Boulton and Shuker, 2013). These include the benefits of “good genes” or “sexy sons” (Jennions and Petrie, 2000) as well as genetic compatibility. One mechanism by which female polyandry could be favoured is the acquisition of such indirect (i.e. genetic) benefits. For instance, mating with multiple males might allow females to avoid the costs of genetically incompatible sperm (Tregenza and Wedell, 2002, Tregenza and Wedell, 2000).

Females of the seed bug *Lygaeus equestris* show high rates of infertility, presumably as a result of mating failure. While mating failure has been documented in many insect species, it has only recently been receiving theoretical attention (Rhainds, 2010). Mating failure is often viewed in the context of females failing to mate, presumably due to lack of opportunity. Less is known about situations in which females mate but these matings do not lead to the production of offspring. I hypothesise that the polygamous mating system of *Lygaeus equestris*, whereby both males and females mate with multiple individuals, could have evolved in response to this high risk of mating failure.

Post-mating infertility has been demonstrated in several species of Lygaeidae (McLain, 1991, Tadler et al., 1999). The exact mechanism behind these mating failures is unknown but there are a number of potential explanations. Firstly, it may be the result of failure by the male to successfully inseminate the female during copulation (all species reported to show mating failure have complex genitalia, and a minimum copulation duration of one hour is necessary for sperm transfer (Micholitsch et al., 2000, Higgins et al., 2009, Dougherty and Shuker, 2014)). Additionally, as many laboratory pairings are done with virgins, there could be an effect of first matings, as males may be less efficient at achieving insemination. It has been demonstrated that the mating preferences of both sexes are often weaker in virgin insects (Bateman et al., 2001) and this may alter the patterns of investment, resulting in different levels of fecundity from virgin pairs. Other hypotheses include genetic incompatibility, possibly exaggerated by inbreeding and population bottle-necks in laboratory populations (Tregenza and Wedell, 2000). This is a common problem as laboratory populations of insects are often founded from relatively small groups of founders of unknown pedigree. In addition, species such as *Lygaeus equestris* and *Lygaeus simulans* are often kept on non-natural foods which can cause selective events that further reduce genetic diversity (Attisano et al., 2012, Laukkanen, 2014).

The polgynandrous mating system of the Lygaeidae could be a response to this risk of mating failure, if it is a result of genetic incompatibility. The negative effects of homozygosity in the presence of deleterious recessive alleles mean that close relatives are more likely to lead incompatible matings and reduced offspring production or viability (Tregenza and Wedell, 2002). If this is indeed the case, then we would expect to see an interaction between the effect of mate relatedness and the effect of number of mates on egg fertility. In the polyandrous cricket *Gryllus bimaculatus*, the eggs of females that mated with two siblings had significantly lower hatching success than those of females that had mated to at least one non-sibling (Tregenza and Wedell, 2002). The fact that only one non-sibling mating was sufficient to confer equal hatching success to females who had two non-sibling mates suggests that female crickets are able to bias sperm storage, and hence sperm use, in favor of compatible sperm (Bretman et al., 2009b). Follow up studies in *G. bimaculatus* support the conclusion that polyandry in this species is driven by genetic incompatibilities arising from inbreeding (Tregenza and Wedell, 2000, Bretman et al., 2004, Rodríguez-Muñoz et al., 2008). Thus, by mating with multiple males females can select the most compatible sperm and hence reduce their chances of producing offspring of low fitness. A similar pattern of females limiting the production of inbred offspring has been found in *Drosophila mojavensis* (Markow, 1997). Females of this species mated to siblings fail to oviposit, despite the presence of both motile sperm and mature ovarian oocytes. Interestingly, oviposition could be "rescued" by the injection of sperm–free seminal fluids from non-related males, suggesting that sperm use may be determined by non-sperm seminal compounds (Markow, 1997).

Genetic incompatibility can also be viewed as a spectrum, arising from inbreeding depression at one end, through to genetic incompatibility between divergent gene pools following reproductive isolation at the other (often viewed in the framework of Dobzhansky-Muller incompatabilities: Orr and Turelli, 2001, see Shuker et al., 2005 for an empirical example). Polyandry could therefore evolve to avoid incompatibilities not just by avoiding inbreeding, but by helping to promote optimal outbreeding (Bateson, 1982, Bateson, 1983b), whereby too little or too much outbreeding (i.e. across a species barrier) is to be avoided. There is evidence in several systems for homogamy, a bias for conspecific sperm (Fricke and Arnqvist, 2004, Kyogoku and Nishida, 2013), which could allow polyandrous females to avoid the costs associated with mating with a heterospecific.

In this experiment I aim to explore the role of polyandry in *L. equestris* female fitness in the light of variation in inbreeding, from full-sibs through to heterospecific matings. The experimental design used non-virgin males and gave all females two mating opportunities in order to reduce the chances that the level of mating failure recorded was due to male or female sexual inexperience. I take advantage of the multiple levels of genetic distance available in the laboratory to test the effect of different levels of in-and out-breeding of the benefits of polyandry in *L. equestris* females. I expect to see greater benefits at the two extremes of the genetic range, i.e. in the full-sibling treatment, and when females are mated to *L. simulans* males, as this is where genetic incompatibilities are most likely to occur.

2. Methods

For this study I utilized the two populations of *L. equestris*, and the population of *L. simulans*, described in chapter 2. I isolated 5th instar *L. equestris* nymphs (from the Dolomites population) and housed them in tubes with food and water provided. Nymphs were checked daily and newly eclosed adults were removed and housed in single sex tubs with no more than ten individuals. Seven days after eclosion, virgin females were assigned to a male and housed with him for 24 hours to allow mating. Males were then removed and females left to oviposit. A single clutch was collected from each female in order to create full-sib cohorts. When the nymphs from these cohorts reached 5th instar they were checked daily and newly-eclosed adults removed and house in single-sex groups of siblings. Males from the Leeds population of *L. equestris* and *L. simulans* males were isolated from continuous culture as 5th instar nymphs and, after eclosion, were kept in single sex tubs of no more than ten individuals. At seven days virgin males from each cohort, as well as those from the other population and species, were assigned to a single (non-sibling) female from the same population and housed with her for two hours to allow mating. They were then kept in male only groups, with either siblings or males from the same population, for 24 hours prior to the start of the experiment. The females were discarded.

At the start of the experiment females from the same clutch were assigned to one of eight treatments. In treatment one, females were given the opportunity to mate twice with a (once mated) male from the same clutch. In treatment two, females were mated twice to a male from the same population but a different clutch. In treatment three, females were mated twice to a male from the other population of *L. equestris* and in treatment four, females were mated twice to a *L. simulans* male. Treatments five to eight follow the same pattern but rather than being mated twice to the same male, females were instead mated to two different males. In all cases females were housed for 24 hours with a male, and then isolated for 24 hours before being housed either with the same or a different male for a further 24 hours. Thus males had 24 hours between matings to replenish sperm reserves. All males in the experiment mated twice during the course of the experiment in addition to their one mating prior, those that were in treatments five to eight mated with two different females while those in treatments one to four mated with same female twice.

After mating, females were housed individually in tubs until death. The number of eggs laid was checked daily and if eggs were present the female was moved to a new tub. All eggs were then returned to the incubator for a further ten days after which the number of nymphs present was recorded as a measure of hatching success. Eggs were collected from a total of 306 females. The breakdown of females per treatment is shown in table 1. A total of 91935 eggs and 41511 nymphs were recorded during the course of the experiment.

The effect of number of mates and level of inbreeding on both nymph and egg production was analysed in R version 2.15.1. Liner mixed models were fitted with family as a random factor. Age at death was also included in the model as lifespan and egg and nymph production were correlated. The influence of both factors on the rate of mating failure (defined as a female producing no nymphs in the course of her life) was modelled with a binary logistic generalised linear model in PASW Statistics 18 by IBM.

3. Results

Female fitness was influenced by outbreeding level, but not by the number of mates she had. Mate number did not significantly affect the number of nymphs ($F_{1, 305} = 2.24$, $P = 0.140$) or eggs ($F_{1, 305} = 0.89$, $P = 0.350$) a female produced. However, both measures were affected by inbreeding level ($F_{3, 303} = 22.27$, $P <0.001$ and $F_{3, 303} = 11.21$, $P < 0.001$, for nymph and egg number respectively) and this seems to be a result of lowered egg and nymph production in females mated to *L. simulans* males (Figures 1 and 2). Unsurprisingly, there was a significant effect of age ($F_{1, 305} = 37.45$, $P <0.001$ and $F_{1, 305} = 163.85$, $P < 0.001$ for nymph and egg number respectively) as, on average, females who lived longer produced more eggs. There was also a significant interaction effect between age and inbreeding level ($F_{3, 303} = 7.90$, $P <0.001$ and $F_{3, 303} = 5.55$, $P < 0.01$ for nymph and egg number respectively), as this relationship broke down in the *L. simulans* treatment due to the lower number of eggs and nymphs produced by females mated to heterospecifics. All other interactions were non-significant ($P > 0.05$).

If the *L. simulans* treatment was excluded from the analysis of nymph number then inbreeding level no-longer significantly influenced number of nymphs produced ($F_{2, 234} = 0.10$, $P = 0.904$), nor did number of mates ($F_{1, 235} = 2.744$, $P = 0.098$). Age, and the interaction between age and both remained significant ($F_{1, 235} = 37.94$, $P < 0.001$ and $F_{2, 234} = 4.36$, $P = 0.014$ respectively). All other interactions were non-significant.

Level had a significant effect on the probability of mating failure ($\chi2 = 56.94$, *d.f.* = 3, $P < 0.001$), however number of mates did not ($\chi^2= 0.98$, *d.f.* = 1, $P = 0.324$). Nor did the interaction between the two (χ^2= 4.71, *d.f.* = 3, P = 0.195). As with nymph number, this effect was driven by the high levels of infertility in the *L. simulans* treatment.

Table 1. Sample sizes per treatment

Treatment	One male	Two males
Inbred (full-sibling)	50	45
Same population (non-sibling)	40	38
Different population	31	33
Lygaeus simulans	34	35

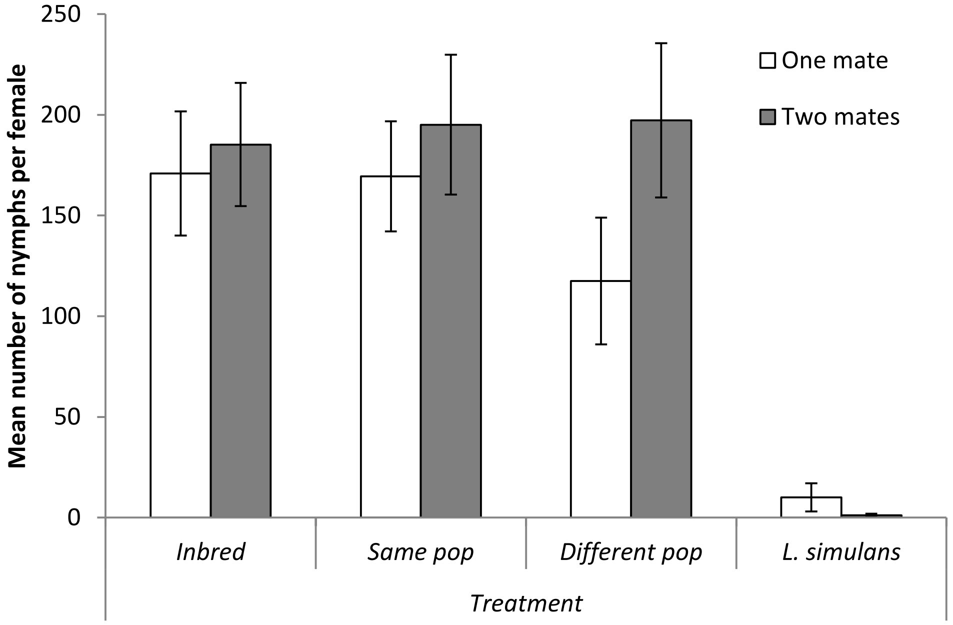

Figure 1. The mean number of nymphs produced per female across the treatments. Error bars indicate ± 1 standard error.

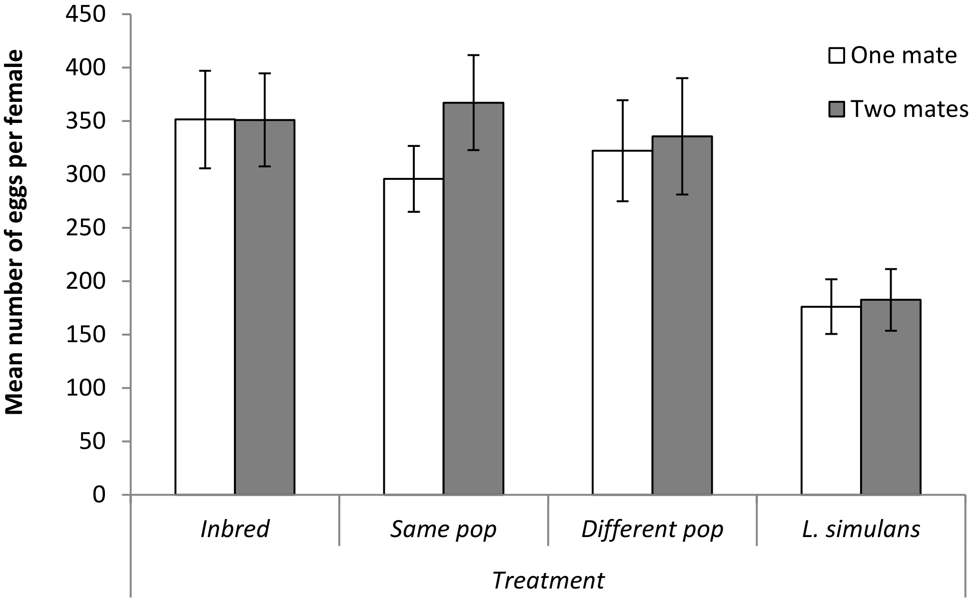

Figure 2. The mean number of eggs produced per female across the treatments. Error bars indicate ± 1 standard error.

4. Discussion

The level of inbreeding within *L. equestris* has no effect on either number of eggs or nymphs produced. Nor was there any effect of polyandry. There was an effect of male relatedness; however this was driven by the low fitness of the *L. equestris* x *L. simulans* crosses. This is surprising given that inbreeding depression, in the form of a reduction in fertile eggs, has been described in other populations of *L. equestris* (Laukkanen, 2014). It is possible that prolonged inbreeding in the laboratory has purged these populations of deleterious recessive alleles that contribute to inbreeding depression (Tregenza and Wedell, 2000). Alternatively, the costs of inbreeding may be expressed later in development in this population, as our design measured only the number of eggs laid, and hatching success. Furthermore, I found no benefit to females of mating with more than one male, regardless of her level of relatedness to her mates. While this is in contrast to the findings in *G. bimaculatus* (Tregenza and Wedell, 2002), a review of the polyandry literature in 2005 found that, while there was an positive effect of polyandry on hatching success, the effect size was small, and that pattern did not hold true for all systems (Simmons, 2005).Thus, with the exception of heterospecific crosses, which I will talk about in more detail below, female polyandry in these populations is unlikely to be driven by increased fertilization success resulting from the avoidance of incompatible sperm. Instead, female multiple-mating in this system may be driven by a number of alternative processes, including the risk of sperm depletion (Wang and Davis, 2006) and male harassment.

The first of these is convenience polyandry. This is particularly plausible in this system given that harassment for matings has been shown to impose fitness costs on *L. equestris* females (Shuker et al., 2006). While repeated mating itself has also been shown to impose fitness costs on females (Sillen-Tullberg, 1985b, Shuker et al., 2006), during copulation females typically lead movement and can continue to feed, thus mating may be less energetically costly than repeatedly resisting male mating attempts. As mentioned in the introduction, convenience polyandry has often been evoked in various insects with apparent mating struggles (i.e. seaweed flies: Shuker and Day, 2001).

Secondly, female mating resistance to unwanted matings may be balanced against the potential fitness costs of dying a virgin (Kokko and Mappes, 2013). Given that females cannot predict their future encounter rate with potential mates, selection may act to remove females that frequently reject mates due to the increased risk of those females dying before mating and hence leaving no offspring. Females that frequently reject matings may also spend a greater proportion of their life as virgins and have a reduced window for reproduction, leading to fewer offspring. Such selection may lead to a scenario in which females mate above their otherwise optimal level (Kokko and Mappes, 2013).

Thirdly, indirect selection on males may be influencing female behaviour and increasing female mating rate (Halliday and Arnold, 1987). While typically described in the context of extrapair matings (e.g. positive genetic correlation between males and females for levels of extrapair mating in zebra finches: Forstmeier et al., 2011, reviewed by Arnqvist and Kirkpatrick, 2005), this form of sexually antagonistic selection can also be applied to promiscuous mating systems. It suggests that male and female extrapair (promiscuous) behaviours are influenced by the same alleles. Thus, female polyandry could evolve in the absence of fitness benefits and even when such behaviours are costly to the females themselves (Lande and Arnold, 1983).

As mentioned above, crosses with *L. simulans* showed significantly reduced hatching success and egg number. This suggests there may be a similar mechanism operating as in *Drosophila*, where females can reduce egg laying when mated to incompatible or poor quality mates (Markow, 1997). While hybridization is clearly possible between the two species, little is known about the viability or fitness of these hybrids, although F1s can be produced (Evans 2011). Most notably perhaps, we currently have no information about Haldane's Rule (which would predict loss of viability or fertility in male hybrids in this cross, as males are the heterogametic sex: Coyne and Orr, 2004). We cannot tell from these results if reduced hatching is a result of females not using heterospecific sperm, as seen in flour beetles (Fricke and Arnqvist, 2004)and *Drosophila* (Markow, 1997), or simply a result of incomplete post-mating reproductive isolation. It is possible that this is the result of decreased mating activity in these treatments as matings were not directly recorded. Previous work by Evans has shown that mating frequency between *L. simulans* males and *L. equestris* females is sometimes reduced compared with *L. equestris* only pairs, although this effect is population-dependent (Evans, 2011). However, mixed species pairings were frequently observed throughout the duration of the experiment and even when infertile females were excluded from the analysis, mean nymph production was lower in the *L. simulans* treatments, suggesting that this effect is not merely due to a failure to mate. A similar pattern was found by Evans in that, while the proportion of females producing fertile eggs did not differ between conspecific and heterospecific crosses, the number of nymphs produced did, with matings between *L. simulans* and *L. equestris* producing far fewer nymphs. Thus, it may be the case that, while sperm transfer is possible in these pairings, it is less efficient than in conspecific matings, or some early embryonic mortality is taking place that this design cannot detect. One aspect of heterospecific matings that may be of great importance in determining the extent of sperm transfer is copulation duration. In both *L. equestris* and *L. simulans*, copulations must typically exceed one hour for sperm transfer to take place (Tadler, 1999, Tadler et al., 1999). As previously discussed, copulation duration is assumed to be largely under male control (but see Sillen-Tullberg, 1985b for potential mechanisms by which females may influence copulation duration), and male seed bugs have claspers with which they grasp females during matings. Variation in these claspers is one of the key features by which *L. equestris* and *L. simulans* can be distinguished from one another (Deckert, 1985), and this variation may also affect the ability of males to remain in copula with females for long periods of time. If this is indeed the case then it may result in smaller amounts of sperm being transferred during heterospecific matings, leading to the lower number of fertilized eggs observed. Other mechanisms that may be influencing female fecundity in this treatment include physical damage caused by mismatched genital morphology during mating (Rönn et al., 2007) and the presence of male seminal proteins. Many species have been found to have non-sperm components of the seminal fluid or package that can influence female physiology and behavior (Perry et al., 2013, Wigby et al., 2009, Chapman et al., 1995). While little is known about the components of seminal fluid in the Lygaeidae, males of one species (*Togo hemipterus*) have been found to influence female refractory period via accessory glad substances (Himuro and Fujisaki, 2008). Given that such male adaptations usually coevolve alongside female resistance to the effects of these compounds within populations (Rönn et al., 2007, Andrés and Arnqvist, 2001, Holland and Rice, 1999), there is the potential for heterospecific male seminal compounds to negatively affect *L. equestris* females. Additionally, any compounds that have evolved to reduce the success of rival sperm, due to the risk of sperm competition in these species, may also impede the survival or fertilization success of heterospecific sperm within the female tract.

One way of distinguishing between the many mechanisms that may cause the lowered fitness seen in heterospecific crosses is to see a single con-specific mating can rescue fitness in the case of extreme outbreeding (i.e. *L. equestris- L. simulans* hybrid matings). A recent extension of this work did just that and found that, similar to some other systems (Tregenza and Wedell, 2002, Kyogoku and Nishida, 2013, Nakano, 1985), a single mating with a conspecific male, either before or after a heterospecific mating is for the most part sufficient to completely rescue female fitness (Burdfield-Steel, Auty, Shuker, *unpublished data*). Thus it seems genital damage, and seminal components are unlikely to be a factor in this system.

The level of mating failure was consistent across all *L. equestris* x *L. equestris* crosses. This suggests that mating failure is not the result of inbreeding or first-mating effects, and the overall phenomenon of mating failure in these bugs is currently a focus of research. As previously described

(Evans, 2011) *L. equestris* females show considerably reduced fecundity when mated to *L. simulans* males. However, no benefits of polyandry were detected across any of the levels of in- and out-breeding. Thus, in conclusion, polyandry in this species seems unlikely to be the result of indirect benefits to females.

Chapter 8

Discussion

1. General discussion

From the work I have presented in this thesis we can conclude that, in *Lygaeus equestris*, the fitness costs of reproductive interference (RI) to females are context dependent, and that inter-species interactions and other forms of competition may have a greater role in determining female fitness than RI (chapter 3). I found clear evidence that species discrimination mechanisms, in the form of species-specific cuticular hydrocarbon (CHC) profiles, do exist between the five lygaeid species considered in this thesis (chapter 4) and that these play a role in mate choice and species discrimination. Thus, heterospecific harassment and mating are unlikely to be a result of a failure of species discrimination alone in this system. However, as I also found no evidence of pre-copulatory selection on chemical defence, an attribute predicted to have considerable fitness consequences (chapter 5). It appears that there is little by way of pre-copulatory choice in these insects, which may explain the low levels of discrimination between females shown by males. This is despite their ability to reliably identify, and respond to, conspecific male rivals in the context of mate guarding (chapter 6). Additionally, I did not detect any significant fecundity benefits to females from polyandry, suggesting that there may be other processes driving multiple mating in female Lygaeidae (chapter 7). I will now discuss the implications of these findings and place them in the context of other recent, and on-going, work on this system, as well as in the field of RI more generally.

2. The causes and consequences of RI

In their 2008 review, Gröning and Hochkirch defined RI as “any kind of interspecific interaction during the process of mate acquisition that adversely affects the fitness of at least one of the species involved and that is caused by incomplete species recognition”. In this thesis, however, I have chosen only to use the first part of this definition, as I believe the second part, “caused by incomplete species recognition”, is perhaps less useful. Indeed, as my research suggests, determining if incomplete species recognition really is the cause of the behaviours we see between species is not always straightforward. While we can generally assume that failures in species discrimination play a role in RI, they may not be the driving force shaping the behavioural outcomes. If, for example, selection for greater mate discrimination, that would prevent interspecies mating attempts, is less than that for ensuring mating then we would see a lack of species discrimination. However, this does not mean that the organisms in question are unable to develop such capabilities; merely that selection does not sufficiently favour them in this instance. In cases like this, describing the cause of RI as a failure of species recognition would overlook the, potentially more interesting, underlying causes. With this in mind I have outlined some of the factors I consider to be most likely to shape the occurrence of RI, and its consequences, in figure 1. As the figure shows, I predict that multiple factors are likely to influence both the occurrence and outcome of RI. While I have grouped these factors under two distinct headings, mating systems and sexual selection and ecology, the two clearly overlap in many ways. Nor is it easy to predict the outcome when multiple factors are acting together. For example, while cross-species aggregations may increase the opportunity for RI, the presence of a large number of potential mates in one area may also reduce the potential fitness costs of both choosiness and mate-searching, leading to the evolution of stronger species discrimination mechanisms, and hence a reduction in the occurrence of RI. Additionally, some factors, like learning, have the potential to both reduce and increase the incidence of RI depending on the circumstances in which they occur. Learned mate preferences have now been displayed in multiple species (Magurran and Ramnarine, 2004, Kozak and Boughman, 2009) including insects (Svensson et al., 2010). While acquiring a mate preference based on experience may allow for greater species discrimination, in situations where the focal species is locally rare, or simply outnumbered by the “interfering” species, individuals may actually acquire preferences for the “wrong” mate, thus increasing RI. However, an example of learning in the butterfly *Bicyclus anynana* provides a potential solution to this problem. In this species males have varying numbers of eye-spots on their wings, which are thought to play a role in mate-choice. A recent study found that naïve females have an innate preference for males with two eyespots (the most common phenotype found in nature). When exposed to males with increased ornamentation (i.e. four eyespots) shortly

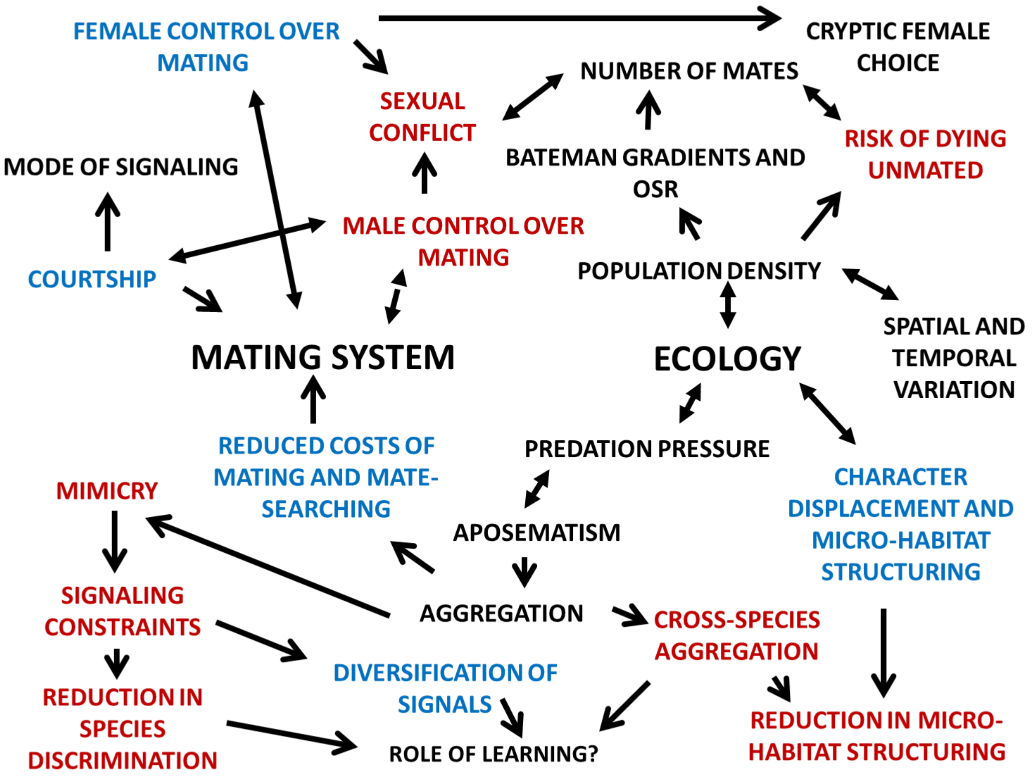

Figure 1. A mind map showing the factors predicted to influence the occurrence of RI. Those in red are expected to increase the likelihood of RI, while those in blue are expected to reduce it.

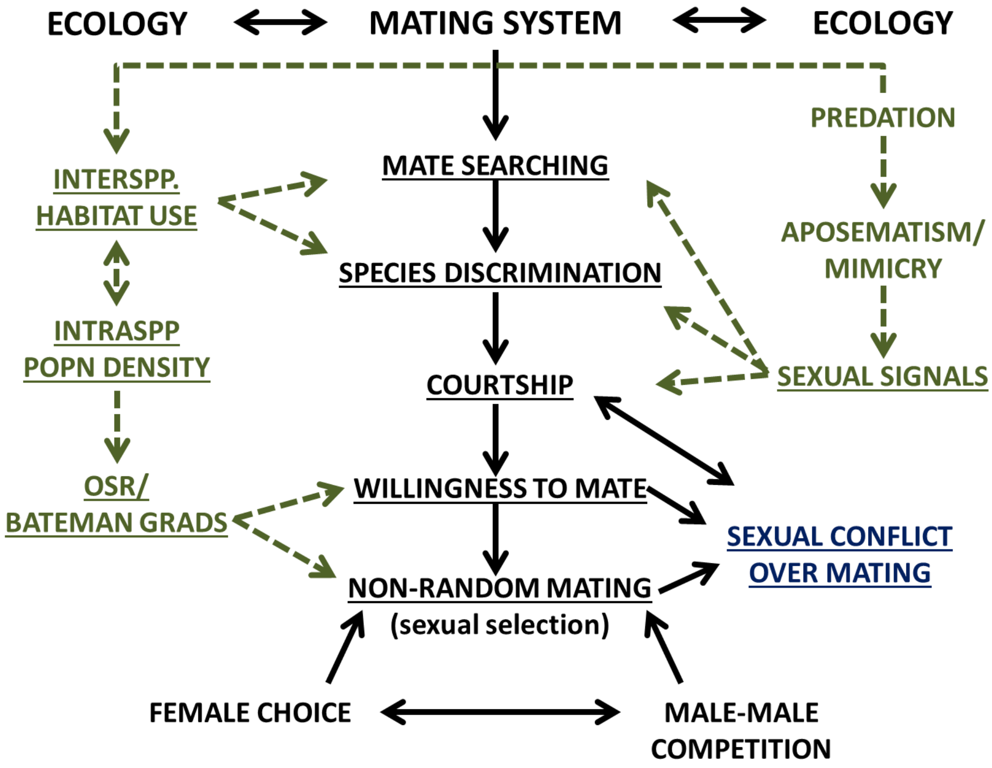

Figure 2. A flow diagram showing the interaction between ecology and mating system. Those aspects that are predicted to be influenced by RI are underlined.

after emergence, the females developed a preference for this phenotype, however they did not show any change in preference if exposed to males with no eyespots. This suggests that mate-preference learning is biased in this species. While the exact cause of this bias is still under investigation, the presence of closely-related species in sympatry with *B. anynana* which possess fewer eyespots does raise the possibility that this bias has arisen in order to prevent females from acquiring preferences for heterospecific males (Westerman et al., 2012).

Rather than simply considering how existing ecologies and mating systems will affect the incidence of RI, it is also interesting to consider the way in which RI may help to shape these attributes in a species. Figure 2 shows a simplified view of the interactions between ecology and mating system, highlighting the processes that may be influenced by RI. As with all studies of evolutionary systems, separating cause and consequence of RI in natural systems is challenging, as the history that lead to any particular dynamic observed in the present day can only be inferred. Therefore, rather than trying to discuss the two separately, I will instead discuss the way RI may interact with various aspects of both mating systems and ecology, with a particular emphasis on those likely to be involved in the RI observed within the Lygaeidae.

2.1 The role of mating systems and sexual selection in RI

As mentioned in chapter 1, sexual selection (and, by extension, mating system) within a species is predicted be an important factor in determining the occurrence of RI (Ord et al., 2011). First, the operational sex ratio could influence the extent of RI. For instance, high skew in OSR (with one sex being rare for whatever reason) may make hetero-specific interactions, and hence RI, more likely, as the common sex searches for possible mates. Another key component of mating systems theory, the Bateman gradient (see Chapter 1), may also influence the scope for RI. If Bateman gradients are steep (i.e. fitness increases sharply with increased numbers of matings), then selection for less selective mating behaviour may lead to higher RI. However, increased RI may then feed back into the system, as high RI may eventually reduce the slope of the Bateman gradient (as selection favours individuals that mate less, but more selectively). This means that reproductive interference may be both a consequence of the mating system, and also a contributing cause of mating system structure. As yet, a formal theoretical consideration of OSR and Bateman gradients in the context of the ecological and evolutionary consequences of reproductive interference is lacking though (see also Chapter 1 for a discussion of existing models relevant to RI). Additionally, if the same traits are used for both mate choice and species discrimination (Bath et al., 2012) strong sexual selection may impede reproductive character displacement, and hence allow for RI.

Another potential interaction between RI and mating systems and sexual selection is courtship. Courtship behaviours may play a number of roles, including mate choice (Hoikkala et al., 1998, Rantala and Kortet, 2003), manipulation of female (and, potentially male) state (Immonen and Ritchie, 2012), and even avoidance of sexual cannibalism (Herberstein et al., 2002). It can be reasonably expected that courtship may also play a major role in species discrimination (Ritchie et al., 1999), and that sequential courtship behaviours in particular, may promote the evolution of species discrimination by separating the signals used to identify potential mates, and assess mate quality. However, evidence that species with pre-copulatory courtship are less susceptible to RI is not as abundant as might be expected (Gray, 2005). Despite this, it seems likely that courtship will reduce the chances of an individual actually mating with other species, even if it may make them vulnerable to other forms of RI, such as misplaced courtship or signal jamming, and potentially even increase the fitness costs associated with RI through time and energy expenditure (for an example see the loss of courtship gifts discussed in chapter 1). Thus it would be premature to say that the risk, or even occurrence, of RI will influence the evolution of courtship in any easily-predictable way. One factor I have previously identified as having close ties to both courtship and RI is sexual conflict. However, I have already discussed the relationship between sexual conflict and RI in chapter 1 I will not repeat it here.

One of the main conclusions that can be drawn from my work is that understanding inter-specific interactions between lygaeid species will only be possible once we have a better grasp of the intra-specific ones. In particular, my results highlight two main aspects of the lygaeid mating system that warrant further investigation: cryptic female choice and male mating strategy.

2.1.1 Cryptic female choice

I found that heterospecific mating attempts appear to be a result of male mating strategy and “weak” pre-copulatory mating selection, rather than a lack of species discrimination *per se*. Both my work (Burdfield-Steel et al., 2013), and that of others (Dougherty and Shuker, 2014) suggests that mate choice on morphology or dietary cues is unlikely to play a strong role in the mating system of these insects. This is unexpected given the long duration of copulations (Sillen-Tullberg, 1981, McLain, 1989), and the penalties they inflict on females (Shuker et al., 2006). Therefore, one aspect of lygaeid reproductive behaviour clearly warrants further attention: cryptic female choice. In his 2011 paper, Eberhard identified 23 possible mechanisms by which cryptic female choice can occur, ranging from abortion of zygotes, to removal of mating plugs to differential investment in parental care (Eberhard, 2011). Given our current knowledge of the Lygaeidae mating system, three mechanisms stand out as promising avenues of further research.

Firstly, females may be able to influence the depth of penetration of the male processus, and thus his ability to deposit sperm in the optimal site for fertilization or storage. As the high levels of mating failure, discussed in chapter 7, appear to be independent of inbreeding effects, they may instead arise from processes affecting sperm use within the female reproductive tract, rather than some measure of sperm “quality”. However, it seems unlikely that a phenomena that results in so many females dying without offspring could be adaptive. Instead, it would be sensible to ask what patterns of selection could have led to so complicated an insemination process, with such a high apparent risk of failure. As discussed in chapter 1, the genitalia of the Lygaeidae are highly complex (Tadler, 1999, Higgins, 2009), and this complexity may contribute to the long copulation duration and mating failures so often observed in the laboratory. It has been shown that in *L. equestris* male intromittent organ length is heritable (Higgins et al., 2009) and appears to be under differing selective pressures at different stages of the mating process. Males with relatively shorter organs show greater success at achieving matings, although the mechanism behind this remains unknown. However, when fertilization success was considered, the selection appeared to be stabilizing, favouring males with intermediate length processes (Tadler, 1999, Dougherty and Shuker, 2014, but see Higgins, 2009). As discussed in chapter 1, there is conflicting evidence as to whether females can control access to the spermatheca (Gschwentner and Tadler, 2000, Chiang, 2010a, Chiang, 2010b). In order to fully understand the selective processes acting on genitalia in these species, more study of the female, as well as the male, genitalia is needed. Additionally, we need a greater understanding of how male and female morphologies interact*. L. equestris,* and its sister species *L. simulans,* provide a promising system for studying the evolution of genital morphologies, as variation in male genital morphology has been found both within- and between these species (Deckert, 1985, Higgins, 2009, Evans, 2011). Given ongoing debate about the relevance of processes like the lock and key hypothesis (Simmons, 2014) to the evolution of genitalia, information about the variation in female genitalia between these two species, and how such variation may influence reproductive isolation, would be of particular interest. With this in mind, I am currently assisting with on-going work that uses micro-CT scanning to visualise the genitalia of *L. equestris* that have been flash frozen in copula (see chapter 1, figure 8). This work, in combination with experimental manipulation of male processus length, may help understand both selection on male genitalia, and potential mechanisms by which cryptic female choice may be operating. Additionally, if this study proves successful, it could be extended to look at inter-specific pairings in order to better understand the mechanisms behind the reduced female fitness seen in these pairings.

Secondly, females may bias paternity by either terminating copulation early, or by refraining from copulating at all. Mating in the Lygaeidae appears to be under male control as males initiate copulations, often aggressively (McLain and Pratt, 1999). Despite this, females have been observed resisting, and indeed can do so effectively, albeit at a cost (Shuker et al., 2006). Previous studies have found that female kicking and rocking behaviour can shorten copulation duration, however it is unclear if this affects male fertilization success (Walker, 1979, Sillen-Tullberg, 1985b). Males possess specialised claspers with which they grasp the female during mating. These claspers show clear species differences (Deckert, 1985). Given that a minimum mating duration of one hour is typically required for fertilization, both between and within-species variation in the structure of male claspers may be of great importance in determining male mating success. Furthermore, as species with male control of mating are predicted to be more susceptible to RI (chapter 1), confirmation that that is indeed the case in these species would contribute greatly to our understanding of the factors driving the heterospecific matings observed.

Finally, females may invest differentially in eggs depending on the “quality” of the father. Both the number of eggs laid, and the size of the eggs, can vary greatly between females of the same species (Feir, 1974, McLain, 1991, Newcombe, 2013, *pers. obs*.). However it remains unknown if this variation has any relation to mate quality, or if it is simply a maternal or dietary effect (Newcombe, 2013). *O. fasciatus* females also pass chemical protection, in the form of cardiac glycosides, to their eggs (Newcombe et al., 2013). Thus, female investment in eggs may have considerable effects on the survival of their offspring. As discussed in chapter 1, further provisioning of offspring via the production of trophic eggs may be possible in lygaeids, and if this is the case it may provide another avenue for females to bias investment in favour of offspring sired by their preferred mate or mates.

2.1.2 Male mating strategy

The male mating strategy of the five lygaeid species included in this work remains unclear. Current evidence, both of the aggressive nature of male mating attempts (McLain, 1991, McLain and Pratt, 1999), and the weak pre-copulatory choice shown by males (Burdfield-Steel et al., 2013, Dougherty and Shuker, 2014), suggests an opportunistic mating strategy. Despite this, males are clearly responding to the threat of sperm competition as they both mate-guard, in the form of extended copulations, and also vary the duration of this guarding in different social contexts (Sillen-Tullberg, 1981, McLain, 1989, Wang et al., 2008, chapter 6). However, it is not known whether males show any context, or condition-dependent, differential investment. Work presented in chapter 6 suggests that, while males alter copulation duration in response to male rivals, they do not appear to do so based on the availability of females. Additionally, little is known about male control over ejaculation. Context-dependent variation in sperm number, and in other components of the seminal fluid, has been shown in species, including *Drosophila* (Wigby et al., 2009, Perry et al., 2013). However it remains unknown whether such mechanisms are operating in the Lygaeidae.

Another aspect of male behaviour that may influence both sexual selection and RI is male-male competition and aggression. Such behaviour is rarely observed in the laboratory (*pers obs*), but has been seen in some wild populations. McLain (1984) found that male *Neacoryphus bicrucis* excluded other males from their host plants, but that this territoriality broke down at high population densities when males were frequently observed sharing host plants (McLain, 1984). Thus the lack of territoriality in laboratory populations may be a result of the high densities at which they are kept. Male-male sexual harassment may also cause RI (Bath et al., 2012). Male-male mating attempts have been reported in the Lygaeidae (*pers. obs*., Sillen-Tullberg et al., 1982) and it remains unknown whether these represent a form of male aggression, or simply a case of mistaken identity. Given the aggressive nature of male mating attempts (McLain, 1984, McLain and Pratt, 1999), distinguishing between territoriality and mating attempts can be difficult. For example, McLain (1984) found that territorial males also chased unreceptive females from their host plants, but it is not clear if this was intentional or simply a female response to harassment (McLain, 1984). Similar exclusion of other species by *N. bicrucis* males is also difficult to classify for the same reason (McLain and Shure, 1987). If species such as *L. equestris* do indeed show male territoriality in the wild, then this could significantly alter both their interspecies sexual selection, and the nature of their interactions with heterospecifics. In the territorial disputes described in *N. bicrucis* larger males almost always excluded smaller ones, however females choice in *Lygaeus* species seems to favour small or intermediate body sizes in males (Dougherty and Shuker, 2014), thus the patterns of selection may vary greatly in the presence or absence of male territoriality.

2.2 Ecology and RI

The other crucial aspect of the study of RI is ecological context. My results in chapter 3 and 6 only serve to emphasise the context-dependent nature both of inter-species and intra-species interactions. Given that sexual behaviour itself shows significant flexibility in these species (see chapter 6), it is unsurprising that RI should also be highly dependent on the context in which it is measured. This provides an interesting challenge when estimating both the occurrence of RI and its ecological and evolutionary consequences. If RI is context-dependent then what is the correct context in which to measure it? Given the obvious differences between the laboratory and the field, behaviours in the laboratory may not accurately reflect the situation in wild populations. However, as I will discuss, conditions in the “field” are neither spatially or temporally stable.

2.2.1 Population density and context dependence

Preliminary field work suggests that population density is likely to vary considerable from year to year for Lygaeidae. *L. equestris*, *L. simulans, L. creticus* and *S. pandurus* are all highly seasonal across most of Europe. As seeds are required for successful development of nymphs, the distribution of these species is spatially structured around patchy food plants (McLain, 1984, Solbreck and Sillen-Tullberg, 1990). Thus, density is also likely to fluctuate over the course of a single season (Sillen-

Tullberg and Solbreck, 1990, Solbreck and Sillen-Tullberg, 1990). The mating system appears to be opportunistic (i.e. encounter polgynandry; see Chapter 1), but the extent and costs of mate-searching behaviour in natural populations is unknown. Given that both density and mate-searching costs are expected to influence RI, the levels of reproductive interference, and indeed more traditional forms of intra-specific competition (McLain, 1984), may also vary across time. Thus, before we can ask “in what context should we measure RI?” we must first estimate the range of densities likely to be experienced by the population in question, as well as establish how both selection and behaviour can vary within this range. From this perspective, laboratory experiments can be useful in determining the effect of variation in specific factors, such as population density, on sexual behaviour. This knowledge can then be applied in field studies, where different ecological factors are likely to co-vary.

2.2.2 The role of predation and aposematism

Another factor that is predicted to influence mate-searching, and by extension, RI is predation. Mating is thought to increase predation risk in most insects (Bath et al., 2012) but this may not be the case in the Lygaeidae due to prevalence of aposematism in this group. Indeed, given that a nymphs of species, in particular *L. equestris,* have been shown to benefit from greater protection from predators when aggregated (Sillen-Tullberg et al., 2000), it may even be the case that copulating pairs also benefit from a stronger aposematic signal. One barrier to testing this hypothesis is that little is known about the natural predators of lygaeids. Their chemical protectants mean that both vertebrate and invertebrate predators have been shown to rapid learn to avoid them in laboratory experiments (Berenbaum and Miliczky, 1984, Gamberale-Stille and Sillen-Tullberg, 1999, Gamberale-Stille et al., 2009, Sillen-Tullberg et al., 2000, Svadova et al., 2010) and the level of predation by invertebrate predators in the wild appears to be low (McLain and Shure, 1987). Given the bright colours used as warning signals by these species, it seems sensible to assume that visual predators, such as birds, played an important role in shaping their evolution. However, their signalling is not only visual, as *S. pandurus* have been show to produce secretions that repel both vertebrates and invertebrates (Sweet, 2000), and other species may well produce similar scents. It is worth noting that, despite the current research focus on aposematic species, many of the Lygaeidae are cryptic (see chapter 1). Thus, comparative work with cryptic species may allow us to determine if aposematism is playing a role in weakening the influence of predation on the evolution of mating behaviour in their aposematic relatives. Finally, in addition to more classical forms of predation, intra-guild predation and cannibalism may also exert an influence, both on community dynamics in general (Ohlberger et al., 2012), and RI in particular. Given that both these phenomena have been observed in wild populations (see chapter 1) it seems prudent to regard them as a potentially significant component of the overall predation risk, and thus mate-searching costs. However, intra-guild predation in particular, is likely to be dependent both on population density and on food availability, but the dynamics of such behaviours in the wild remain unknown.

3. Conclusion

Heterospecific mating attempts frequently occur between laboratory populations of five species of Lygaeidae (chapter 1). While clear fitness costs of this harassment to female *Lygaeus equestris* occur when females are kept singly with heterospecific males, this effect is not found when the experimental design includes multiple males and females of each species (chapter 3). Furthermore, such interactions are not solely the result of failures in mate recognition. Clear differences in the cuticular hydrocarbon (CHC) profiles of the five species were detected, and the experimental reduction of these signals resulted in a reduction in preference of male *L. equestris* for females of their own species (chapter 4). Male *L. equestris* can both identify and respond to heterospecific and conspecific males appropriately in the context of mate-guarding (chapter 6), and this, in combination with a lack of evidence for strong pre-copulatory selection (chapter 5), suggests that heterospecific mating attempts may be the result of male mating strategy. Thus, further investigation of intersexual selection in these insects, and how this is influenced by variation in their population and ecological dynamics, will be crucial in determining the extent of RI in natural populations.

Furthermore, studies on RI clearly have a role in play in the study of mating system evolution more generally, as they provide a window into the underlying pressures that shape the evolution of sexual behaviour, both at an individual and a population level. In addition, RI itself may be playing a previously-overlooked role in shaping these sexual behaviours. While it appears there is considerable potential for RI to interact with numerous aspects of species biology and ecology, we currently have very little data, experimental or otherwise, with which to test these hypotheses. Areas I would suggest are of particular interest for further study are the role of courtship in species discrimination, and the effect of heterospecific reproductive interference on a the “optimal” mating strategy of an individual, as predicted by concepts such as the OSR and the Bateman gradient.

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Appendix 1

Quick Guide to Reproductive Interference. Current Biology, 21, R450-R451.

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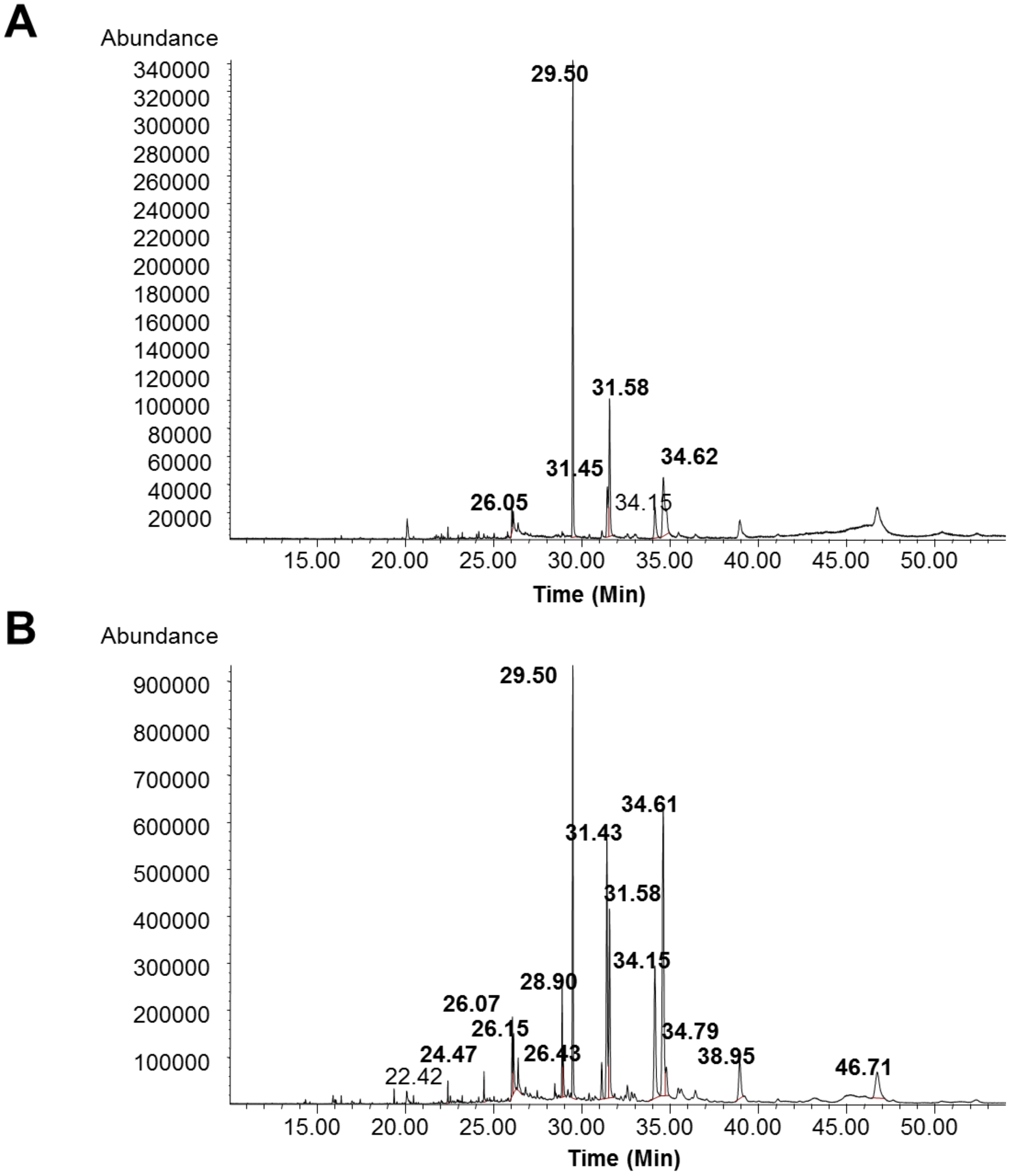
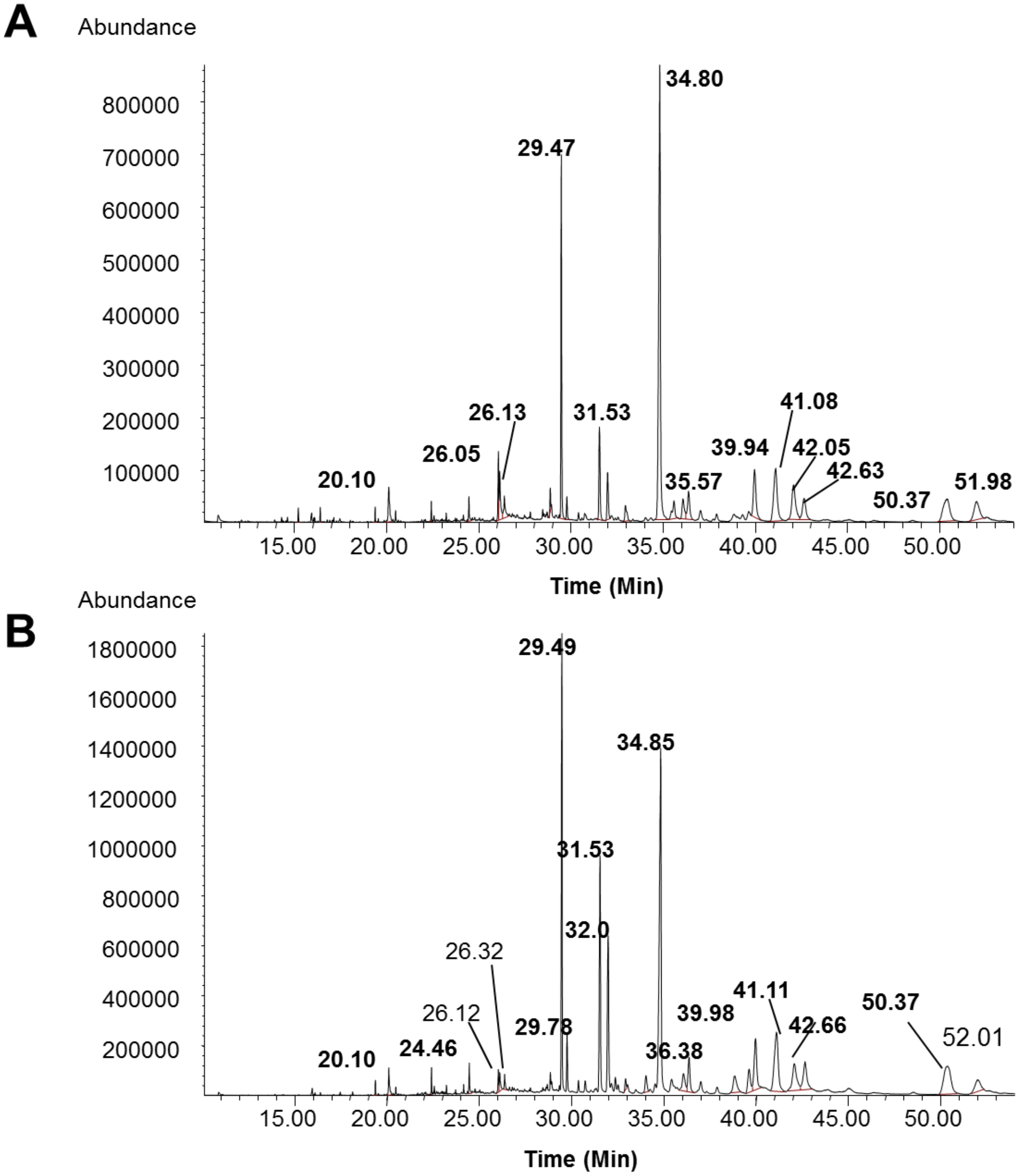
Appendix 2

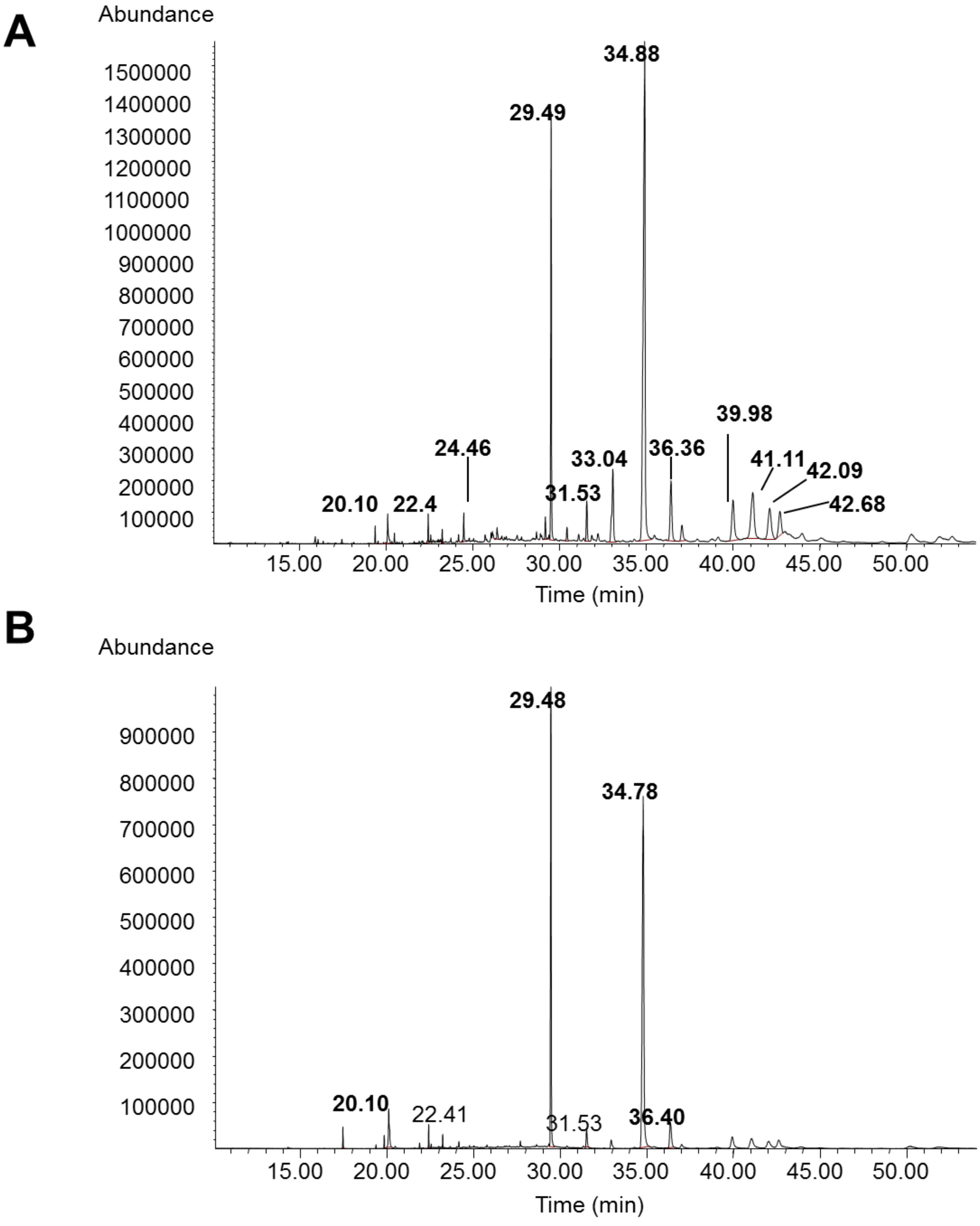
Supplementary tables and figures for Chapter 4



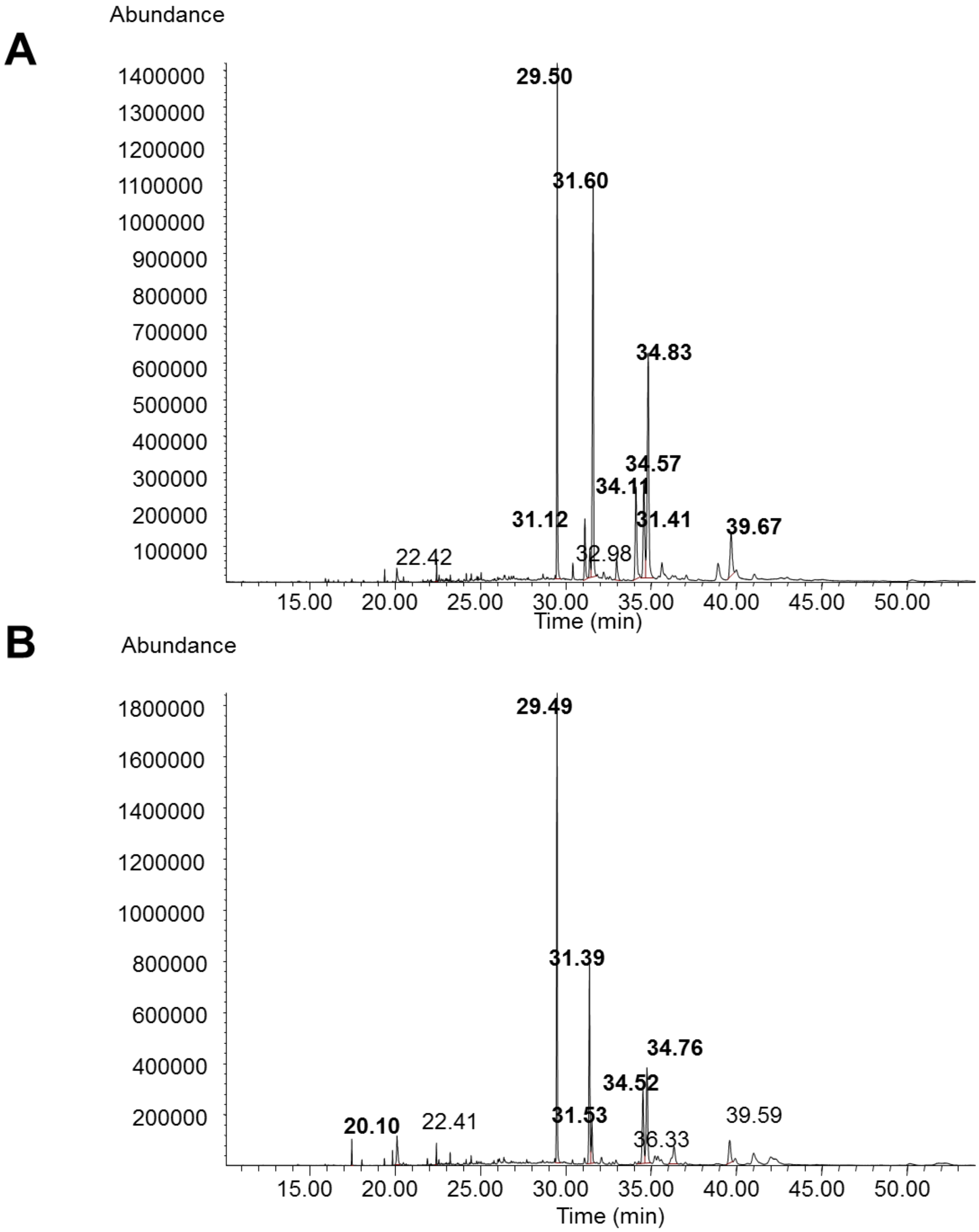
Appendix Figure 1. Typical GC-MS, total ion current chromatogram of cuticular hydrocarbons from adult *Oncopeltus fasciatus* (A) female, (B) male.



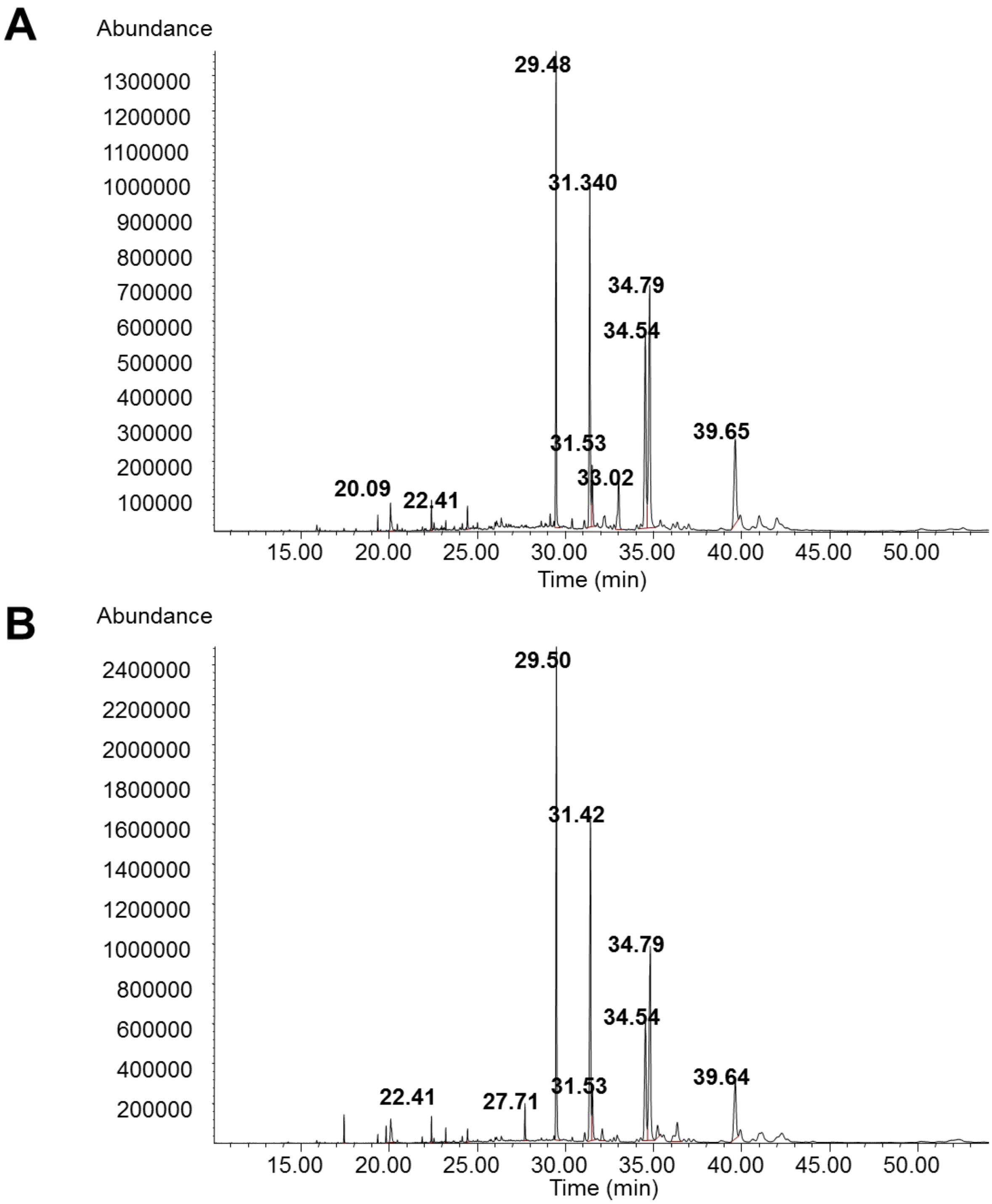
Appendix Figure 2. Typical GC-MS, total ion current chromatogram of cuticular hydrocarbons from adult *Spilostethus pandurus* (A) female, (B) male.



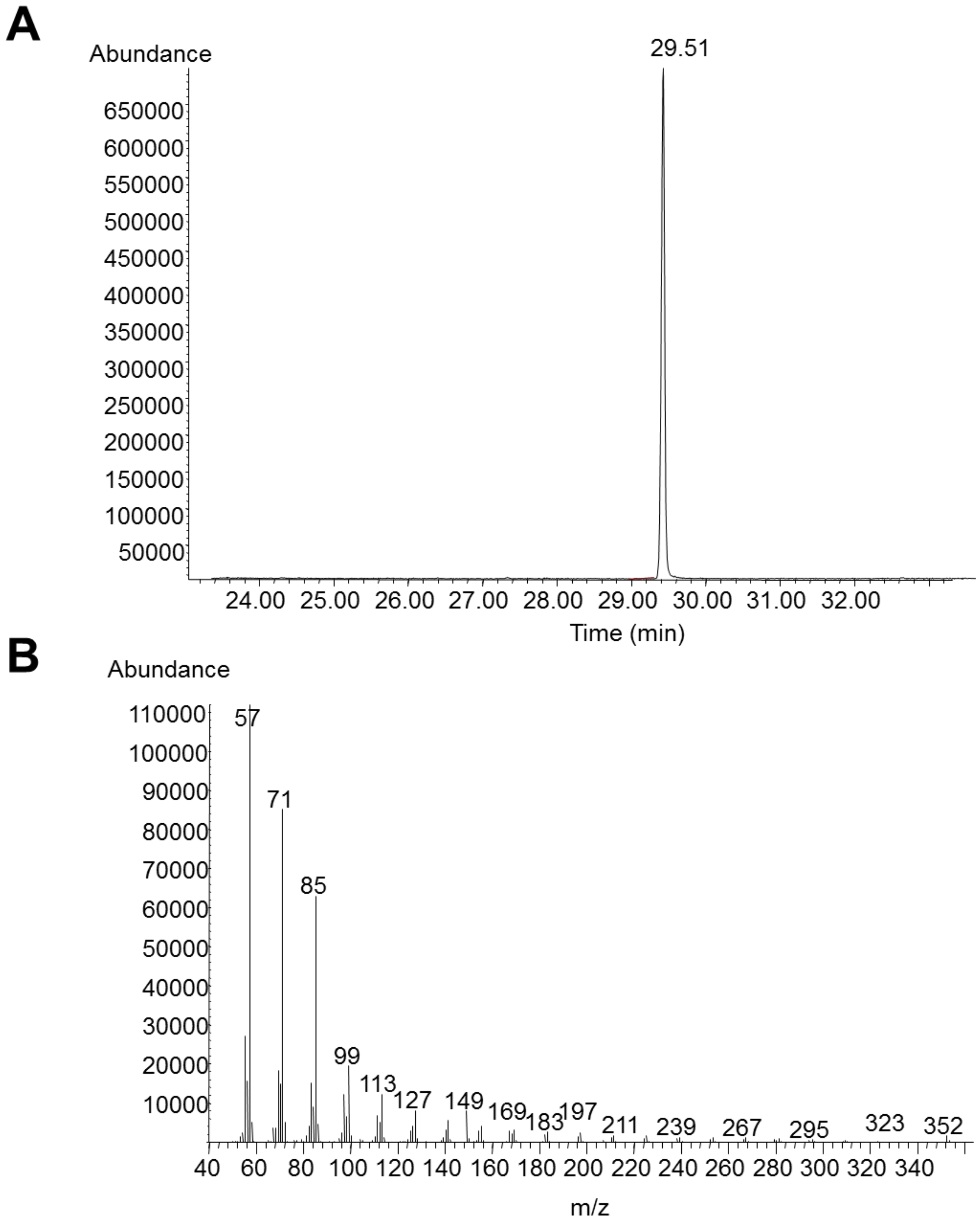
Appendix Figure 3. Typical GC-MS, total ion current chromatogram of cuticular hydrocarbons from adult *Lygaeus simulans* (A) female, (B) male.



Appendix Figure 4. Typical GC-MS, total ion current chromatogram of cuticular hydrocarbons from adult *Lygaeus equestris* (A) female, (B) male.



Appendix Figure 5. Typical GC-MS, total ion current chromatogram of cuticular hydrocarbons from adult *Lygaeus creticus* (A) female, (B) male.



Appendix Figure 6. Typical GC-MS, total ion current chromatogram (A), and spectrum (B) of pentacosane, used as internal standard.

Appendix table 1 (A) *Oncopeltus fasciatus* Female.

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
26.05	282, 207, 194, 91	C23:1	Tricosene
31.45	280, 112, 69, 55	7-Me-C25	7-Methyl-pentacosane
31.58	350,323, 85, 57	2-Me-C25	2-Methyl-pentacosane
34.15	376, 293, 281, 71, 57	C27:2	Heptacosdiene
34.62	378,295, 281, 71, 57	C27:1	Heptacosene

Appendix table 1 (B) *Oncopeltus fasciatus* Male.

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
22.42	282, 201, 57	3-Me-C19	3-Methyl-nonadecane
24.47	284, 249, 55	C21:0	Heneicosane
26.07	282, 207, 194, 91	C23:1	Tricosene
26.15	280,223, 55	C23:0	Tricosane
26.43	284, 241, 57, 55	3-Me-C23	3-Methyl-tricosane
28.90	262, 117, 91	C25:2	Pentacosadiene
31.43	280,112, 69, 55	7- Me-C25	7-Methyl-pentacosane
31.58	350, 85, 57	2- Me-C25	2-Methyl-pentacosane
34.15	376,, 281, 71, 57	C27:2	Heptacosadiene
34.61	378, 295, 281, 71, 57	C27:1	Heptacosene
34.79	380, 207, 99, 85, 71	C27:0	Heptacosane
38.95	422, 355, 341,, 99	5- Me-C29	5-Methyl-nonacosane
46.71	379, 351, 281, 71, 57	7- Me-C33	7-Methyl-tritriacontane

Appendix table 2 (A) *Spilostethus pandurus* Female

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
20.10	256, 213, 129	C19:0	Nonadecane
26.05	282, 207, 194, 91	C23:1	Tricosene
26.13	284, 241, 71, 55	C23:0	Tricosane
31.53	350, 252, 71, 55	9- Me-C25	9-Methyl-pentacosane
34.80	380, 99, 85, 71, 51	C27:0	Heptacosane
41.08	460, 408, 127, 113, 71, 57	C33:2	Tritriacontadiene
42.05	458, 435, 309, 168, 57	C33:3	Tritriacontatriene
42.63	462, 449, 168, 57	C33:0	Tritriacontane
50.37	490, 365, 57	C35:1	Pentatriacontene
51.98	518, 429, 313, 57	C37:1	Heptatriacontene

Appendix table 2 (B) *Spilostethus pandurus* Male.

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
20.10	256, 213, 129	C19:0	Nonadecane
24.46	296,227, 73, 57	C21:0	Heneicosane
26.12	284, 241, 71, 55	C23:0	Tricosane
26.32	284, 241, 55	5- Me-C23	5-Methyl-tricosane
29.78	366, 312, 207, 117, 91	9- Me-C25	9-Methyl-pentacosane
31.53	366, 351, 309, 244, 71, 55	5- Me-C25	5-Methyl-pentacosane
32.00	362, 308, 71, 57	C26:1	Hexacosene
34.84	364, 85, 71, 51	C27:0	Heptacosane
39.98	355, 341, 313,281, 252, 71, 57	C31:0	Hentriacontane
41.11	460, 408, 127, 113, 71, 57	C33:2	Tritriacontdiene
42.66	464, 449, 168, 57	C33:0	Tritriacontane
50.37	490, 365, 57	C35:1	Pentatriacontene
52.01	518, 429, 313, 57	C37:1	Heptatriacontene

Appendix table 3 (A) *Lygaeus simulans* Female.

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
20.10	256, 213, 129, 73	C19:0	Nonadecane
22.40	282, 201, 57	5- Me-C19	5-Methyl-nonadecane
31.53	366, 351, 309, 244, 71, 55	5- Me-C25	5-Methyl-pentacosane
34.79	408, 341,281,69	C27:0	Heptacosane
36.36	394, 365, 323, 285, 57	3- Me-C27	3-Methyl-heptacosane
39.98	432, 341, 313,281, 252, 71, 57	C31:2	Hentriacontadiene
41.12	460, 127, 113, 71, 57	C33:2	Tritriacontadiene

Appendix table 3 (B) *Lygaeus simulans* Male.

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
20.22	256, 213, 129, 73	C19:0	Nonadecane
31.54	366, 351, 309, 244, 71, 55	5- Me-C25	5-Methyl-pentacosane
34.78	408, 341, 281,69	C27:0	Heptacosane
36.40	394, 365, 323, 285, 57	3- Me-C27	3-Methyl-heptacosane

Appendix table 4 (A) *Lygaeus equestris* Female.

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
22.42	282, 201, 57	5- Me-C19	5-Methyl-nonadecane
31.12	280, 262, 67	9- Me-C19	9-Methyl-nonadecane
31.41	280, 264, 57	7- Me-C25	7-Methyl-pentacosane
31.60	284, 241, 65	2- Me-C25	2-Methyl-pentacosane
32.98	380,85, 71, 57	3- Me-C26	3-Methyl-hexacosane
34.11	374, 281, 207, 71, 57	C27:3	Heptacosatriene
34.57	378, 283, 269, 83, 57	C27:1	Heptacosene
34.83	408, 57	C27:0	Heptacosane
39.67	428, 313,, 252, 71, 57	C31:4	Hentriacontatetraene

Appendix table 4 (B) Lygaeus equestris Male.

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
20.12	280, 213, 129, 73	C19:0	Nonadecane
22.41	282, 201, 57	5- Me-C19	5-Methyl-nonadecane
31.39	280, 264, 57	7- Me-C25	7-Methyl-pentacosane
31.53	366, 351, 309, 244, 71, 55	5- Me-C25	5-Methyl-pentacosane
34.52	378, 283, 269, 83, 57	C27:1	Heptacosene
34.76	408, 57	C27:0	Heptacosane
36.33	394, 309, 295, 171,	7- Me-C27	7-Methyl-heptacosane
39.59	434, 323, 281, 207	C31:1	Hentriacontene

Appendix table 5 (A) *Lygaeus creticus* Female

Retention Time (Min)	**Diagnostic ions(m/z)**	**Carbon number**	**Hydrocarbon**
22.41	282, 201, 57	5- Me-C19	5-Methyl-nonadecane
31.40	280, 264, 57	7- Me-C25	7-Methyl-pentacosane
31.53	366, 351, 309, 244, 71, 55	5- Me-C25	3-Methyl-pentacosane
33.02	350,209, 69	5- Me-C26	5-Methyl-hexacosane
34.54	378, 283, 269, 83, 57	C27:1	Heptacosene
34.79	408, 57	C27:0	Heptacosane
39.65	430, 313, 289,207, 57	C31:3	Hentriacontriene

Appendix table 5 (B) *Lygaeus creticus* Male

Retention Time (Min)	**Diagnostic ions (m/z)**	**Carbon number**	**Hydrocarbon**
22.41	282, 201, 57	5- Me-C19	5-Methyl-nonadecane
31.38	280, 264, 57	7- Me-C25	7-Methyl-pentacosane
31.53	351, 244, 71, 55	5- Me-C25	5-Methyl-pentacosane
33.02	350,209, 69	5- Me-C26	5-Methyl-hexacosane
34.54	378, 283, 269, 83, 57	C27:1	Heptacosene
34.79	408, 57	C27:0	Heptacosane
39.64	430, 313, 289,207, 57	C31:3	Hentriacontrine

Appendix 3

Variation in social and sexual behaviour in four species of aposematic seed bugs (Hemiptera: Lygaeidae): The role of toxic and non-toxic food, Behavioural Processes, 99, 52-61

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Appendix 4

Sexual Stereotypes: The case of sexual cannibalism, Animal Behaviour, 85, 313-322

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