Patterns of reproductive isolation within- and between- species characterised by a sexual conflict over mating

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ABSTRACT

Theory suggests that under some circumstances sexual conflict over mating can lead to divergent sexually antagonistic coevolution (SAC) among populations for traits associated with mating, and that this can promote reproductive isolation and hence speciation. However, sexual conflict over mating may also select for traits, such as male willingness to mate, that enhance gene flow between populations, limiting population divergence. Here we compare pre- and post-mating isolation within and between two species characterised by male-female conflict over mating rate. We quantify sexual isolation among five populations of the seed bug Lygaeus equestris collected from Italy and Sweden, and two replicates of a population of the sister-species Lygaeus simulans, also collected from Italy. We find no evidence of reproductive isolation amongst populations of L. equestris, suggesting that sexual conflict over mating has not led to population divergence in relevant mating traits in L. equestris. However, there was strong asymmetric pre-mating isolation between L. equestris and L. simulans: male L. simulans were able to mate successfully with female L. equestris, but male L. equestris were largely unable to mate with female L. simulans. We found little evidence for strong post-mating isolation between the two species however, with hybrid F₂ offspring being produced. Our results suggest that sexual conflict over mating has not led to population divergence, and indeed perhaps supports the contrary theoretical prediction that male willingness to mate may retard speciation by promoting gene flow.

Key words:
Lygaeus – population divergence – sexually antagonistic co-evolution – sexual conflict – sexual isolation – speciation
INTRODUCTION

The evolution of reproductive isolation is the key event in speciation, and understanding how and why reproductive isolation arises remains a central topic in evolutionary biology (Coyne & Orr, 2004; Butlin et al., 2012). Over the last decade or so, interest has turned to the role that sexual conflict, and in particular sexual conflict over mating, might play in reproductive isolation (Holland & Rice, 1998; Parker & Partridge, 1998; Gavrilets, 2000; Arnvist et al., 2000; Martin & Hosken, 2003; Arnvist & Rowe, 2005). Sexual conflict arises when different evolutionary optima exist for males and females for a given trait (Parker, 1979; Chapman et al., 2003; Arnvist & Rowe, 2005). In terms of mating, sexual conflict may arise over whether mating between two individuals takes place at all (for instance if male and female mate preferences do not coincide) or over the frequency of mating. Generally speaking, males are typically thought to be selected to mate more often than is optimal for females (Bateman, 1948; Trivers, 1972; Arnvist & Nilsson, 2000) and to be less discriminating in terms of their mating partners, leading to sexual conflict between males and females over mating rate (reviewed by Arnvist & Rowe, 2005).

Opposing selection on males and females may be an important driver of evolutionary change, especially if it results in sexually antagonistic co-evolution (SAC). Within populations, SAC from inter-locus sexual conflict may be rapid, taking the form of irresolvable “arms-races” between the sexes, or cyclical dynamics as males and females constantly co-evolve in response to adaptations and manipulation of the other sex (Parker, 1979; Rice, 2000; Arnvist & Rowe, 2005; Lessells, 2006). As such, SAC has received increasing attention due to its potential to drive populations along divergent co-evolutionary trajectories, facilitating population divergence, sexual isolation, and finally speciation. Laboratory studies have demonstrated that sexual conflict over mating rate can lead to SAC, with the evolution of increased (or decreased) female resistance to mating under situations of increased (or decreased) conflict over mating (Holland & Rice, 1999; Wigby & Chapman, 2004; Stewart et al., 2005; Rice et al., 2006). Some studies have also confirmed that SAC over mating rate can result in population divergence (Hosken et al., 2002; Martin & Hosken, 2003), although divergence has not always
The role of sexual conflict in terms of reproductive isolation is not therefore clear-cut. First, SAC may be less commonly expressed in the field than under laboratory conditions (Chapman, 2006). Indeed, studies conclusively showing past operation of SAC remain limited in number (Arnqvist & Rowe, 2002a,b; Koene & Schulenburg, 2005; Bergsten & Miller, 2007; Anthes et al., 2008). Fewer studies still demonstrate active SAC within species to be driving current population divergence in the field (but see Hebets & Maddison, 2005; Sugano & Akimoto, 2007; Gagnon & Turgeon, 2011). However, continued cycles of antagonistic co-evolution are not the only outcome of opposing selection, and may not even be the most likely (Parker, 1979; Lessells, 2006). Second, SAC over mating is expected to select for generally persistent, or exploitative, males and concomitant female resistance to male mating attempts (Parker & Partridge, 1998). This means that among-population or even hetero-specific matings may occur more readily than conspecific matings, if males are selected to be highly coercive and if females are less able to resist males that they have not co-evolved with (Jennions & Petrie, 1997). Sexual conflict, therefore, may actually retard population divergence rather than promoting it, by promoting or maintaining gene-flow across populations (Holland & Rice, 1998; Markow & Hocutt, 1998; Parker & Partridge, 1998; Gavrilets et al., 2001). Indeed more recent theoretical studies suggest that population divergence from sexual conflict may be much less likely than first thought, even when SAC is apparent, with only two of six possible SAC dynamics resulting in population divergence (Gavrilets & Hayashi, 2005); this may account for the lack of population divergence observed amongst some laboratory evolution studies (see above). Moreover, the renewed appreciation of phenomena such as same-sex matings (Bailey & Zuk, 2009) and reproductive interference (Burdfield-Steel & Shuker, 2011) reminds us that mate discrimination is sometimes far from perfect, both in the laboratory and the field.

Measuring the degree of sexual isolation among allopatric populations that show variation in sexual conflict over mating is one way to gain insight into the role of sexual
conflict in population divergence and speciation in the field (Gay et al., 2009; Gagnon & Turgeon, 2011). Population crosses are an important first step towards identifying current episodes of population divergence, and in exploring the involvement of sexual conflict and SAC in such diversification episodes (e.g. Hebets & Maddison, 2005; Long et al., 2006; Panhuis et al., 2006; Sugano & Akimoto, 2007), although interpreting patterns of sexual conflict themselves from population crosses requires caution (Long et al., 2006).

Here, we explore patterns of pre- and post-mating reproductive isolation among populations of two closely related species of seed bug characterised by sexual conflict over mating, *Lygaeus equestris* (LE) and *Lygaeus simulan* (LS). These species have similar ecologies, including aposematic warning colouration, and both have promiscuous mating systems characterised by sexual conflict such that multiple mating is extremely costly to females in terms of reduced longevity and fecundity (Shuker et al., 2006). Males therefore benefit more from extra copulations than females do. Two LE populations were derived from Sweden, three from Northern Italy, and a LS population was twice sampled from Central Italy. In terms of predictions, given that the two species are presumed sister species, first we expect pre-zygotic isolation to be more developed than the post-zygotic isolation (Coyne & Orr, 2004). Second, if sexual conflict is associated with increased population divergence for mating traits, then we should expect variation in the extent to which populations of LE mate with each other, and perhaps also in the fitness of hybrid offspring. If this isolation is in part associated with ecological factors then we might also expect LE individuals from the Swedish or Italian populations to be more likely to mate with individuals from the same region as themselves. We performed two sets of within- and between-species no-choice mating experiments to test these predictions. The first assayed mating over a short period for reproductively mature individuals, allowing investigation of the latency of individuals to mate among four populations (three LE and one LS). The second experiment expanded the number of populations studied to seven populations (five LE and two LS), and assayed mating over a longer period of adult life, as well as the production of an F₁ and F₂ offspring generation.

MATERIALS AND METHODS
The study species

*Lygaeus equestris* (Linnaeus; Hemiptera: Lygaeidae) is an aposematic seed feeding insect with a wide geographic distribution, ranging from Spain to Russia (Deckert, 1985; Solbreck *et al.*, 1989; Péricart, 1998; for a general review of lygaeid biology see Burdfield-Steel & Shuker 2014a). It is a generalist seed predator of various composites (Solbreck & Kugelberg, 1972; Kugelberg, 1973; Solbreck *et al.*, 1989) however its preferred host plant through much of Europe is *Vincetoxicum hirundinaria* (Gentianales, Asclepiadaceae: Kugelberg, 1974, 1977; Solbreck *et al.*, 1989). It is largely univoltine, producing one offspring generation per year. However, *L. equestris* retains the potential for multi-voltinism across its distribution (Solbreck & Sillén-Tullberg, 1981; Solbreck, 1991) which is more likely to occur in southern regions (Solbreck *et al.*, 1989; Shuker *et al.*, 2006). Relatively recently, studies concluded the presence of a sister species, *Lygaeus simulans* (Deckert, 1985), in central and southern Europe (Deckert, 1985; Péricart, 1998; Tadler *et al.*, 1999). *Lygaeus simulans* differs to *L. equestris* in the morphology of the base of the antennae and male parameres (genital claspers: Deckert, 1985; Péricart, 1998), but clear discrimination with the naked eye is difficult (Tadler *et al.*, 1999). Much of the ecology of the two species is thought to be similar, and both display polygamous mating systems characterised by sexual conflict over mating (Sillén-Tullberg, 1981; Deckert, 1985; Tadler, 1999; Tadler *et al.*, 1999; Micholitsch *et al.*, 2000; Shuker *et al.*, 2006). The two species are parapatric across parts of their range and to date no hybrids have been found in the field (Maschler, 2002).

**Experiment 1: Four populations**

In terms of general husbandry, both species were maintained in stock cages in an incubator at 29˚C with a 22:2 hour light: dark cycle in order to prevent the initiation of reproductive diapause. Stock cages (plastic sandwich boxes measuring 30x15x15cm with fine mesh over part of the lids) were provisioned with organic sunflower seeds (Goodness Direct, UK) to a depth of about 3cm (Shuker *et al.*, 2006). A piece of cotton wool was added to the cages for bugs to cling on to and hide in. Oviposition was either amongst the sunflower seeds or on this piece of cotton wool. Each week two universal tubes were
filled with distilled water and stoppered with cotton wool were provided as a water
source. Populations comprised two to three stock cages and were maintained in
continuous culture with overlapping generations, with stock cages being replaced
approximately every 6-8 weeks. A sample of around 60 individuals of all age classes was
haphazardly removed from the population cages to initiate a new one. Egg-to-adult
development under these conditions is broadly similar for both species, taking
approximately 23-28 days.

To ensure a continual supply of bugs for the experiments, we transferred late larval
instars periodically (every 4-5 days) from continuous culture cages into smaller nymph
development cages for adult eclosion. In our first experiment, mating interactions were
examined for sexually mature individuals from four populations, in a 4x4 reciprocal
design (i.e. 16 replicated combinations). The populations were two Swedish LE
populations (from Morga and Geta), a LE population from the Dolomites region of
northern Italy, and a population of LS sampled from the Tuscany region of central Italy.

From each population, we removed newly eclosed adults from nymph cages every two
days, and placed them in same sex pots (within their respective populations) with others
of the same age to develop, ensuring virginity. Densities were restricted to six bugs per
pot (measuring 8 x 8 x 5.5cm, transparent with perforated lids). Each pot contained a
30mm diameter Petri dish lid and base containing water soaked cotton wool and organic
sunflower seeds respectively. Seeds and water were replaced every three days ensuring
an ad libitum supply of both. All bugs were retained in these conditions for at least seven
days prior to experimentation ensuring sexual maturity.

Adult bugs aged between seven and 12 days post-adult eclosion were then used in ‘no
choice’ mating trials. We randomly assigned these sexually mature males and females to
partners of the opposite sex from each of the four populations (LE: Morga, Geta,
Dolomites; LS: Tuscany) in a fully-factorial reciprocal design. Pairs were placed in
transparent pots, without any seeds or water, and were randomly distributed in trays,
before returning to the incubator. We scored the pairs every 30 minutes, for eight hours,
for copulation (stable end to end position: Sillén-Tullberg, 1981; Tadler et al., 1999).

Each hour, pots were rotated in their position within the incubator to minimise potential position effects. Pairing treatments were performed in blocks, with at least two replicates of each combination attained per block. Pairs where one or both individuals died during the observational period were discarded, and not included in the analysis. From the eight hour observation period, we recorded whether mating occurred and, for each pair that mated, the time taken for mating to occur. We obtained 25 replicates for each reciprocal cross (total $N = 400$ trials).

Experiment 2: Seven populations

In this experiment, we recorded the incidence of mating over a prolonged period of adult development (up to approximately three weeks). In addition, we used individuals from this second experiment to further explore aspects of pre- and post-mating sexual isolation. We reared any $F_1$ generation offspring produced, and allowed them to reproduce, thus enabling assessment of hybrid $F_1$ fertility.

We included two further LE populations from Northern Italy (Ledro and Predazzo), and a replicate LS population from the same area in Tuscany, thus creating a 7x7 reciprocal population design (i.e. 49 replicated combinations). All populations were maintained as before. For this experiment, males and females were paired from 0-2 days post adult eclosion and retained in small pots (8 x 8 x 5.5cm) containing a layer of organic, de-husked, sunflower seeds, and a 7ml plastic tube containing carbon filtered water capped with a cotton wool bung, for a maximum of 20 days. Water tubes were replaced every 10 days, or when necessary, to ensure a constant supply of water. From days 3 to 12 of being paired (bugs aged ~ 5 to 14 days post eclosion, allowing for the necessary development time required for sexual maturity), we scored pairs twice daily for mating. Males that died during this time, without mating being observed, were replaced by another virgin male of similar age from the appropriate population. Where males died and mating was observed the female was isolated in a similar pot and left to oviposit. If a female died without any observed matings or eggs the males were likewise re-used with new females. However if a female died after producing eggs, the eggs (within the pot) were retained
and left to develop, and the male discarded. After day 12, adults were left to continue mating (without scanning for mating) and females left to oviposit until day 20, whereupon both adults were removed and discarded.

Following removal of the adults on day 20, eggs were left to develop in their respective pots without their parents. After a further 7 days (when all viable eggs have hatched), we scored pots for the presence and number of offspring (hatched eggs). We then transferred up to 25 nymphs to a fresh pot with seeds and water (as above) and allowed them to develop to adulthood. Pots were then checked for adult eclosion every 5 days and a maximum density of 5 males and 5 females were retained together in fresh pots upon eclosion (with a minimum of at least 3 F1 males and 3 F1 females per pot). When either no males or no females eclosed (i.e. one sex absent), virgin bugs from other replicated pots (of the appropriate cross) were used to allow for the mating and fertility of this F1 generation to be assayed. Once again, we replaced the water every 10 days or earlier to allow for a constant supply of water. We retained all pots for up to 20 days and scored them for the presence/absence of F2 offspring (hatched eggs).

We observed between 8 and 14 replicate pairs for each of the 49 treatment combinations (median \(N = 10\)). Out of the overall 491 replicates, 12 replicates produced an F1 generation without mating being directly observed between the two daily scans. All combinations of crosses that produced F1 offspring were observed mating for at least a subset of the replicates however, indicating that twice daily observational scans were adequate for assessing mating (as is likely given the prolonged copulation durations exhibited within these species: Shuker et al., 2006). Of the 223 replicates that successfully produced F1 offspring, 22 had insufficient numbers of adults emerging (i.e. below our threshold of three males and three females) to assay for the production of F2 offspring within these respective replicate families.

Analysis

Experiment 1
We analysed the incidence of mating using binary logistic regression with a logit link function. First, we compared the within-population trials to examine if there were population differences in their baseline mating frequencies. Second, male and female identities (their population origin) were used as factors, along with the interaction term between them to test for differences in likelihood of mating across all population combinations. Sexual isolation is indicated by significant interaction effects, and was assessed in each case using likelihood ratio (LR) tests. These analyses were performed using R (R version 2.11.1).

We further analysed sexual isolation among the populations and species using an overall sexual isolation index (IPSI, Rolan-Alvarez & Caballero, 2000; Perez-Figueroa et al., 2005; see the latter paper for a detailed comparison of different indices). This was performed as a global analysis (IPSItotal) as well as for each pairwise comparison (IPSIA,b), and estimates of asymmetry among these crosses (IAPSIA,b) were also tested (Carvajal-Rodriguez & Rolan-Alvarez, 2006). “Asymmetry” describes the extent to which reproductive isolation in one direction of cross differs from the reciprocal cross. Significance of these sexual isolation indices was tested using bootstrap resampling with 10,000 iterations. Where no mating was observed among pairs, zeros were replaced with 0.5 to allow for bootstrap resampling. Tests of sexual isolation were performed using the programme JMATING (Carvajal-Rodriguez & Rolan-Alvarez, 2006).

In terms of the latency to mating, we analysed the time taken to mate using a generalised linear model with a quasi-poisson error distribution and log link to account for overdispersion of the data.

Experiment 2

Mating propensity and sexual isolation among the populations and species was assessed as above. We also tested F1 progeny production (from those pairs that were observed in copula), and the number of progeny produced (of those that produced offspring) by each
mating pair. We analysed the incidence of F₁ progeny production among pairs with logistic regression, firstly for conspecific pairs (within populations), and subsequently using all crosses to examine the effects of male and female identity and their interaction on mating success respectively. This was then repeated using only the subset of pairs that mated to investigate potential post mating reproductive barriers, firstly for all pairs and, secondly, among L. equestris populations only. We analysed variation in the number of progeny produced (by pairs that produced offspring) again firstly for conspecific pairs (within populations), and subsequently using all crosses to examine effects of male and female identity and their interaction on fecundity respectively, using ANOVA. Lastly, L. equestris populations were further analysed to test for differential mating success in terms of the number of hatched offspring produced by reproductive pairs. Where the interaction term was not significant ($p > 0.05$), it was removed and the model refitted using only the main effects. Again, these analyses were performed using R (R version 2.11.1). The data presented in Figures 1-3 are also given as raw proportions in the Supplementary Information file.

RESULTS

Experiment 1

Mating frequencies for within-population mate trials were similar across all the four populations (mean proportion mating = 0.89, $N = 100$; Likelihood ratio test: LR = 3.00, $df = 3$, $p = 0.39$; Figure 1). Across all 16 combinations however there was significant behavioural reproductive isolation, generally associated with the inter-specific pairings of L. equestris and L. simulans (Figure 1). As such, there was a highly significant interaction effect between male and female population on the incidence of mating (interaction effect; LR = 231.12, $df = 9$, $p < 0.0001$) as well as differences among the populations in overall male willingness to mate (LR = 40.55, $df = 3$, $p < 0.0001$) and female willingness to mate (LR = 71.22, $df = 3$, $p < 0.0001$). Within just LE, Morga males mated less overall than males from other populations (LR = 9.17, $df = 2$, $p = 0.01$; see Figure 1), but for LE females there were no population differences in the incidence of mating (LR = 0.35, $df =
2, \( p = 0.84 \) and no interaction between male identity and female identity on the incidence of mating (LR = 4.73, \( df = 4, p = 0.32 \)).

Using the isolation statistics, we confirmed strong pre-mating sexual isolation between LE (Morga, Geta, and Dolomites) and LS (Total \( I_{psi} = 0.573, p < 0.001 \); Table 1), but no sexual isolation effects among LE populations (see Table 1). Furthermore, the sexual isolation effect between LE and LS was asymmetric for the Dolomites*Tuscany cross (\( I_{apsi} = 4.065, p = 0.005 \)) with LS males able to mate with LE females whilst the opposite was not found (Figure 1 and Table 1).

Overall, when mating did occur there was little difference either within or between species in latency to mating. The time to mating did not differ among the populations or species when paired with conspecific partners (mean = 1.43 hours, SE = 0.15, \( F_{3,85} = 0.16, p = 0.92 \)). Additionally, including heterospecific pairings revealed no interaction effect between male and female identity on the time to mating (\( F_{5,219} = 1.42, p = 0.22 \)), and no overall difference among any of the populations or species in the time taken for males or females to mate (male population: \( F_{3,224} = 1.48, p = 0.22 \); female population: \( F_{3,224} = 0.25, p = 0.86 \)). Analysing only LE populations gave the same qualitative results of no differences in time to mating (data not shown).

*Experiment 2*

As with the first experiment, there was no difference between the populations, or species, in their mating propensities when paired with conspecific partners (mean proportion mating = 0.813, \( N = 64 \); LR = 7.32, \( d.f. = 6, p = 0.29 \)). However, there was again evidence of inter-specific mating isolation. Including hetero-specific pairings revealed significant heterogeneity among the populations in the likelihood of mating associated with female population and the interaction between male and female population (female population: LR = 126.44, \( d.f. = 6, p <0.0001 \); interaction effect: LR = 225.11, \( d.f. = 36, p <0.0001 \); Figure 2), but not associated with the main effect of male population (LR = 1.85, \( d.f. = 6, p = 0.93 \)). These effects are associated with the hetero-specific trials, as
within LE populations there was no significant interaction between male and female population \((N = 255, \text{interaction effect: LR} = 22.94, d.f. = 16, p = 0.13)\). The LE populations did differ in the overall mating propensity of females \((LR = 11.175, d.f. = 4, p = 0.025)\), suggesting that the LE populations differ in their baseline level of female receptivity to mating (Figure 2), but there were no among-population differences for the overall mating propensity of males \((LR = 3.14, d.f. = 4, p = 0.54)\).

Again, closer examination using Ipsi statistics revealed significant pre-mating sexual isolation between the two species, but not within species \((\text{Total Ipsi} = 0.255, p = 0.006; \text{Table 1})\). Moreover, as with the four population experiment, sexual isolation between the species was significantly asymmetric when LE from Ledro, Morga, and Predazzo, were paired with LS (Table 1 and Figure 2).

**F₁ offspring production**

There was a significant interaction effect between male and female identity on the incidence of hatched offspring \((LR = 167.74, d.f. = 36, p << 0.0001, \text{male population}; \text{LR} = 16.32, d.f. = 6, p = 0.012, \text{female population}; \text{LR} = 94.30, d.f. = 6, p << 0.001)\), indicating strong heterogeneity amongst the populations in the likelihood of successfully interbreeding (Figure 3). As expected, the incidence of hatched offspring production did not differ between populations when individuals were paired with conspecifics \((\text{mean} = 0.64, N = 64; \text{LR} = 5.82, d.f. = 6, p = 0.44)\); it is worth highlighting that this means that there was an overall “mating failure” rate of 36\%). However, reducing the data set to contain only those pairs that did mate, the likelihood of a male from a particular population producing hatched offspring with a female did not depend on the identity of the female partner and vice versa \((N = 305, \text{interaction effect; LR} = 36.46, d.f. = 27, p = 0.11)\) suggesting limited post-mating reproductive isolation between the species. Refitting the model (using mated pairs) without this interaction effect still revealed population differences for both males and females in the likelihood of producing hatched offspring following successful mating \((\text{male population; LR} = 34.35, d.f. = 6, p < 0.001, \text{female population; LR} = 25.99, d.f. = 6, p < 0.001)\). Contrary to the result of no population differences in the likelihood of producing hatched offspring in conspecific pairings, this
suggests that there is some variability among the populations in the likelihood of producing hatched offspring (Figure 3). Indeed analysis of LE populations only showed a similar story to mating propensity, with a significant effect of female population identity on the incidence of hatched offspring among mated individuals, but no effect of male identity and no interaction between the two ($N = 218$: female identity LR = 17.13, $d.f. = 4$, $p = 0.002$; male identity LR = 6.02, $d.f. = 4$, $p = 0.20$; interaction term LR = 17.97, $d.f. = 16$, $p = 0.37$).

There was no difference among the populations in the number of hatched offspring produced (by reproductive pairs) when paired with conspecific partners (mean = 59.88, se = 5.67, $F_{6,35} = 1.067$, $p = 0.40$). Analysing all population crosses, there were highly significant population differences for males and females in the number of hatched offspring produced overall, (male population: $F_{6,183} = 9.29$, $p < 0.0001$; female population: $F_{6,183} = 7.49$, $p < 0.0001$), which is driven largely by the low numbers of offspring produced among LE by LS population crosses (Figure 3). However, the interaction was found to be non-significant ($F_{25,183} = 0.85$, $p = 0.67$), suggesting that there was little difference in the numbers of hybrid versus pure-bred offspring, although this might be in part due to low statistical power from low sample sizes.

Analysing LE populations in isolation, again there was no significant interaction effect between male and female identity on the number of offspring produced ($F_{16,151} = 1.167$, $p = 0.30$), however, male and female identity were significant as main effects (male: $F_{4,167} = 3.12$, $p = 0.017$; female: $F_{4,167} = 2.73$, $p = 0.031$), suggesting there is variance in fertility across populations.

**$F_2$ offspring production**

Intra-specific crosses showed no restrictions in the ability of populations to interbreed and produce fertile hybrids as $F_2$ generations were produced in each case (Figure 4). However, there was some evidence for partial post-mating reproductive isolation between LE and LS as crosses between LE males and LS females did not produce any $F_1$ offspring (perhaps not surprising as only one of these pairings mated in the first instance: Figure 2).
However, there were more matings in the reciprocal direction (i.e. between male LS and female LE; Figures 1 and 3) and they did produce both an F1 generation (Figure 3) and a subsequent F2 generation in some cases (Figure 4). Although low numbers of F1 offspring were produced from these crosses, sexual isolation between LE and LS would appear to be largely in terms of pre-mating isolation rather than post-mating isolation therefore, and largely in one direction.

DISCUSSION

The importance of sexual conflict in facilitating reproductive divergence among populations remains unclear. Here we have investigated the extent of reproductive isolation within and between two sister species of seed bug (Lygaeus equestris and L. simulans), species that are characterised by sexual conflict (Shuker et al., 2006). No sexual isolation was apparent among different LE populations, as might be expected if the sexual conflict over mating had selected for diverging reproductive traits. Instead, our two experiments revealed pre-mating isolation between LE and LS, although this isolation was asymmetric: male LS were able to mate with female LE, but male LE were largely unable to mate with female LS. The results were similar whether we allowed pairs to interact for a few hours or for a few days, and were largely consistent across multiple LE populations and two replicate LS populations. In our first experiment, we found no differences in time to mating for those crosses where mating occurred, again suggesting little divergence in behaviour or key mating signals. We found little evidence for strong post-mating isolation between the two species either however, with hybrid F1 and F2 offspring sometimes being produced if matings occurred.

Pre-zygotic sexual isolation may act over many systems involving behavioural, physiological and/or morphological characters (Coyne & Orr 1989, 1997). Although no overt courtship occurs in the two species studied here (Solbreck, 1972; Sillén-Tullberg, 1981; Tadler et al., 1999; Dougherty & Shuker, 2014), morphological differences between the species could explain the pre-mating isolation observed to some extent. Indeed, one of the few characters that distinguish the species is the morphology of the
male parameres (genital claspers; Deckert, 1985; Péricart, 1998), which are used by
males to secure position during mating, facilitating successful copulation (Tadler, 1999;
Tadler et al., 1999). We are currently exploring the role that claspers, and also the
internal male genitalia, may play in reproductive isolation.

In addition to morphological differences, we have recently identified differences in
cuticular hydrocarbon (CHC) profiles between five species of Lygaeidae, including L.
equestris and L. simulans (Burdfield-Steel et al., unpublished data). Our data suggest
differences in CHC profiles between these latter two species, including sex-specific and
ontogenetic differences. These differences will provide a starting point for experimental
tests of species-discrimination mechanisms. Hetero-specific mating attempts are not
infrequent across four species of lygaeid more generally however (L. equestris, L.
creticus, Spilostethus pandurus, and Oncopeltus fasciatus: Shuker et al., 2015; see also
McLain & Shure, 1987; McLain & Pratt, 1999 for another example from the Lygaeidae),
and such hetero-specific mating interactions can be as costly as intra-specific mating
harassment for female LE (i.e. there is reproductive interference: Shuker et al., 2015).
Given the highly polygamous mating systems of these species, and the occurrence of
reproductive interference, our current inference is that despite the availability of cues,
such as CHCs, to facilitate species discrimination, males have been selected to be highly
opportunistic when it comes to possible mating partners, and this selection has weakened
selection for strong species discrimination (Parker & Partridge, 1998; Burdfield-Steel &
Shuker 2014b; Shuker et al 2015).

This means that sexual conflict over mating, although possible in the field, may not be
driving divergence and speciation among LE populations. Indeed, our interpretation
supports the contrary theoretical proposition, that male willingness to mate may impede
speciation through maintaining gene flow across populations (Parker & Partridge, 1998;
Gavrilets & Hayashi, 2005), and would concur with other studies. For example, Gagnon
& Turgeon (2011) found that despite significant correlations between morphological
traits of males and females associated with sexual conflict over mating in populations of
Gerris gillettei, there were no mating asymmetries among allopatric populations,
suggesting that sexual conflict was not driving population divergence. And as highlighted above, even in laboratory evolution studies, support for SAC over mating promoting allopatric population divergence is mixed.

In terms of the reproductive isolation between LE and LS, there was a clear pattern of asymmetric pre-mating isolation. Asymmetric sexual isolation is very common, both in terms of pre-mating, behavioural isolation (e.g. Kaneshiro, 1980; Arnold et al., 1996) and also in terms of post-mating isolation (e.g. Turelli & Moyle, 2007). Two such examples of the former include isolation between populations of the grasshopper, Podisma sapporensis (Sugano & Akimoto, 2007), and between species of Sonoran desert Drosophila (Markow & Hocutt, 1998). In both of these cases, divergent sexual antagonistic co-evolution among the populations or species (with differential levels of male vigour and female resistance) was attributed as a likely explanation for the patterns observed, but our failure to link between-species isolation with any among-population, within-species isolation suggests that SAC has not generated important divergence in behaviour between LE and LS. We note here that our analysis of pre-mating isolation was based on a no-choice experimental paradigm. A recent meta-analysis has confirmed that the strength of mate preferences can vary with experimental paradigm, i.e. between choice and no-choice paradigms (Dougherty & Shuker 2015; but see Dougherty & Shuker 2014 for no effect of experimental design on intra-specific mate preferences in L. equestris). Mate preferences are generally stronger when measured via choice experiments (typically the presentation of two options), although the meta-analysis suggested that experimental paradigm had no effect on the strength of preference for con-over hetero-specifics, as studied here. However, the number of studies available for that comparison was small (Dougherty & Shuker 2015).

In terms of post-mating isolation, no LE populations (from Sweden or Italy) showed any restriction in their ability to interbreed, producing F₂ offspring and thus demonstrating fertile F₁ hybrids. No breeding restrictions were found between the two L. simulans replicate populations either. That is not to say that all matings involve successful sperm transfer, as “mating failures” appear to be rather common, even among con-specifics.
Mating failure is probably more common than often realised (Eberhard, 1996; García-González, 2004) and interest in this phenomenon is growing (Rhaiinds, 2010; Greenway et al., 2015).

In terms of the inter-specific crosses, only a subset of LE female and LS male replicate crosses produced sufficient numbers of F1 generation individuals that developed to adults (and could reliably test for an F2 generation), thus we could not determine with certainty whether all types of crosses are equally (in)compatible, but there appears to be variation in the successful production of F1 and F2 offspring. As such, the data we have suggest there might be segregating variation in the genetic basis of any post-zygotic incompatibilities, an increasingly common finding (e.g. Shuker et al., 2005; Cutter, 2012). Nonetheless, the production of some hybrid F2 offspring clearly demonstrates that, given the no-choice mating experiments performed here, the two species can interbreed and produce viable offspring under laboratory conditions.

There remain a number of outstanding questions. For instance, more needs to be done unravelling how and under what circumstances males and females discriminate between species (e.g. Burdfield-Steel & Shuker, 2014b; Shuker et al., 2015), including further exploration of the role of CHCs (and variation in CHC profiles within and among populations). Second, the work presented here only begins to touch upon the nature and extent of post-mating isolation. Our data show that a proportion of F1 hybrids are viable and also fertile. However, the extent of variation in hybrid viability or fertility remains to be fully explored, including the extent of any reproductive incompatibilities that fit the pattern of Haldane’s rule (Coyne & Orr, 2004). Lygaeus have an XO chromosomal sex determination system, therefore we would predict that if failure to produce offspring (or variation in the failure to produce offspring) is associated with only one sex, it should be associated with the heterogametic males. Such experiments are currently underway.

Finally, our results suggest that this species-pair may be a useful system for exploring the genetic basis of reproductive isolation, as we have behavioural variation across the two species, an asymmetry in the direction of reproductive isolation, and the ability to form
viable F2 hybrids, which would facilitate genetic mapping. As the study of reproductive isolation in insects is still somewhat dominated by species of *Drosophila* and a number of Orthoptera (e.g. Coyne & Orr, 2004), this system may provide a welcome new study organism for exploring the evolutionary genetics of reproductive isolation in the wild.

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Hosken DJ, Blanckenhorn WU, Garner TWJ. 2002. Heteropopulation males have a fertilization advantage during sperm competition in the yellow dung fly


Figure legends

Figure 1. Proportion of pairs mating within and between populations and species \((N = 25\) each, total \(N = 400\)) in the first, four population experiment. Grey Bars denote pairings within and between populations of *Lygaeus equestris*: Morga, Geta, and Dolomites. White Bars denote pairings involving the sister species *L. simulans*: Tuscany. The data are also presented in tabular form in Supplementary Information.

Figure 2. Proportion of pairs mating within and between populations and species \((\text{total } N = 491)\) in the second, seven population experiment. Grey Bars denote pairings within and between populations of *Lygaeus equestris*: Morga, Geta, Dolomites, Ledro and Predazzo. White Bars denote pairings involving the sister species *L. simulans*: Tuscany 1 and Tuscany 2. The data are also presented in tabular form in Supplementary Information.

Figure 3. Proportion of pairs producing offspring within and between populations and species \((\text{total } N = 491)\) in the second, seven population experiment. Grey Bars again denote pairings within and between populations of *Lygaeus equestris*: Morga, Geta, Dolomites, Ledro and Predazzo. White Bars again denote pairings involving the sister species *L. simulans*: Tuscany 1 and Tuscany 2. The data are also presented in tabular form in Supplementary Information.

Figure 4. F2 progeny production for each reciprocal combination. Bugs denote successful production of F2 progeny. Dark coloured cells represent those combinations where no F1 generation was produced. Light shaded cells represent those combinations where F1 progeny were produced but insufficient numbers survived to test reliably for F2 offspring production. Only one combination, denoted as “*”, produced no F2 generation from sufficient numbers of F1 offspring to test this (3 males and 3 females).
Table 1. Global analysis of sexual isolation (\(I_{psi}\)), and estimates of asymmetry (\(IA_{psi}\)) in mating among populations using \(F_0\) crosses for the four and seven population experiments respectively. SD is the standard deviation and \(p\) is the two tail probability of rejecting the null hypothesis being true (isolation/asymmetry = 0) in the bootstrap resampling distribution (derived from 10,000 iterations). Crosses displaying significant pre-mating isolation are shown in bold. Morga, Geta, Dolomites, Ledro and Predazzo are *Lygaeus equestris*, and the Tuscany populations are *L. simulans* (see text for details).

<table>
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<tr>
<th>Population Cross</th>
<th>(I_{psi})</th>
<th>SD</th>
<th>(p)</th>
<th>(IA_{psi})</th>
<th>SD</th>
<th>(p)</th>
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<td></td>
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<td>Morga * Geta</td>
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<td>0.9922</td>
<td>1.003</td>
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<td>1.006</td>
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<tr>
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