1

2 Running Title: Sexual conflict and speciation

3

4 **Patterns of reproductive isolation within- and between- species**

5 **characterised by a sexual conflict over mating**

6

7 Gethin M.V. Evans[1], Toby Nowlan[1] and David M. Shuker*[2]

8

9 1. Institute of Evolutionary Biology, University of Edinburgh, West Mains Road,

10 Edinburgh, EH9 3JT, U.K.

11 2. School of Biology, University of St Andrews, Harold Mitchell Building, St Andrews,

12 KY16 9TH, U.K.

13

14 * author for correspondence: david.shuker@st-andrews.ac.uk

15

16

17

18   ABSTRACT

19

20   Theory suggests that under some circumstances sexual conflict over mating can lead to

21   divergent sexually antagonistic coevolution (SAC) among populations for traits

22   associated with mating, and that this can promote reproductive isolation and hence

23   speciation. However, sexual conflict over mating may also select for traits, such as male

24   willingness to mate, that enhance gene flow between populations, limiting population

25   divergence. Here we compare pre- and post-mating isolation within and between two

26   species characterised by male-female conflict over mating rate. We quantify sexual

27   isolation among five populations of the seed bug *Lygaeus equestris* collected from Italy

28   and Sweden, and two replicates of a population of the sister-species *Lygaeus simulans*,

29   also collected from Italy. We find no evidence of reproductive isolation amongst

30   populations of *L. equestris*, suggesting that sexual conflict over mating has not led to

31   population divergence in relevant mating traits in *L. equestris*. However, there was strong

32   asymmetric pre-mating isolation between *L. equestris* and *L. simulans*: male *L. simulans*

33   were able to mate successfully with female *L. equestris*, but male *L. equestris* were

34   largely unable to mate with female *L. simulans*. We found little evidence for strong post-

35   mating isolation between the two species however, with hybrid F_2 offspring being

36   produced. Our results suggest that sexual conflict over mating has not led to population

37   divergence, and indeed perhaps supports the contrary theoretical prediction that male

38   willingness to mate may retard speciation by promoting gene flow.

39

40

41   Key words:

42   *Lygaeus* – population divergence – sexually antagonistic co-evolution – sexual conflict –

43   sexual isolation – speciation

44

45 INTRODUCTION

46

47 The evolution of reproductive isolation is the key event in speciation, and understanding

48 how and why reproductive isolation arises remains a central topic in evolutionary biology

49 (Coyne & Orr, 2004; Butlin *et al*., 2012). Over the last decade or so, interest has turned to

50 the role that sexual conflict, and in particular sexual conflict over mating, might play in

51 reproductive isolation (Holland & Rice, 1998; Parker & Partridge, 1998; Gavrilets, 2000;

52 Arnqvist *et al*., 2000; Martin & Hosken, 2003; Arnqvist & Rowe, 2005). Sexual conflict

53 arises when different evolutionary optima exist for males and females for a given trait

54 (Parker, 1979; Chapman *et al.,* 2003; Arnqvist & Rowe, 2005). In terms of mating, sexual

55 conflict may arise over whether mating between two individuals takes place at all (for

56 instance if male and female mate preferences do not coincide) or over the frequency of

57 mating. Generally speaking, males are typically thought to be selected to mate more often

58 than is optimal for females (Bateman, 1948; Trivers, 1972; Arnqvist & Nilsson, 2000)

59 and to be less discriminating in terms of their mating partners, leading to sexual conflict

60 between males and females over mating rate (reviewed by Arnqvist & Rowe, 2005).

61

62 Opposing selection on males and females may be an important driver of evolutionary

63 change, especially if it results in sexually antagonistic co-evolution (SAC). Within

64 populations, SAC from inter-locus sexual conflict may be rapid, taking the form of

65 irresolvable "arms-races" between the sexes, or cyclical dynamics as males and females

66 constantly co-evolve in response to adaptations and manipulation of the other sex

67 (Parker, 1979; Rice, 2000; Arnqvist & Rowe, 2005; Lessells, 2006). As such, SAC has

68 received increasing attention due to its potential to drive populations along divergent co-

69 evolutionary trajectories, facilitating population divergence, sexual isolation, and finally

70 speciation. Laboratory studies have demonstrated that sexual conflict over mating rate

71 can lead to SAC, with the evolution of increased (or decreased) female resistance to

72 mating under situations of increased (or decreased) conflict over mating (Holland & Rice,

73 1999; Wigby & Chapman, 2004; Stewart *et al*., 2005; Rice *et al*., 2006). Some studies

74 have also confirmed that SAC over mating rate can result in population divergence

75 (Hosken *et al*., 2002; Martin & Hosken, 2003), although divergence has not always

76 resulted (Wigby & Chapman, 2006; Bacigalupe *et al*., 2007; Gay *et al*., 2009; Maklakov
77 *et al*., 2010).

78

79 The role of sexual conflict in terms of reproductive isolation is not therefore clear-cut.
80 First, SAC may be less commonly expressed in the field than under laboratory conditions
81 (Chapman, 2006). Indeed, studies conclusively showing past operation of SAC remain
82 limited in number (Arnqvist & Rowe, 2002a,b; Koene & Schulenburg, 2005; Bergsten &
83 Miller, 2007; Anthes *et al*., 2008). Fewer studies still demonstrate active SAC within
84 species to be driving current population divergence in the field (but see Hebets &
85 Maddison, 2005; Sugano & Akimoto, 2007; Gagnon & Turgeon, 2011). However,
86 continued cycles of antagonistic co-evolution are not the only outcome of opposing
87 selection, and may not even be the most likely (Parker, 1979; Lessells, 2006). Second,
88 SAC over mating is expected to select for generally persistent, or exploitative, males and
89 concomitant female resistance to male mating attempts (Parker & Partridge, 1998). This
90 means that among-population or even hetero-specific matings may occur *more* readily
91 than conspecific matings, if males are selected to be highly coercive and if females are
92 less able to resist males that they have not co-evolved with (Jennions & Petrie, 1997).
93 Sexual conflict, therefore, may actually retard population divergence rather than
94 promoting it, by promoting or maintaining gene-flow across populations (Holland &
95 Rice, 1998; Markow & Hocutt, 1998; Parker & Partridge, 1998; Gavrilets *et al*., 2001).
96 Indeed more recent theoretical studies suggest that population divergence from sexual
97 conflict may be much less likely than first thought, even when SAC is apparent, with
98 only two of six possible SAC dynamics resulting in population divergence (Gavrilets &
99 Hayashi, 2005); this may account for the lack of population divergence observed amongst
100 some laboratory evolution studies (see above). Moreover, the renewed appreciation of
101 phenomena such as same-sex matings (Bailey & Zuk, 2009) and reproductive
102 interference (Burdfield-Steel & Shuker, 2011) reminds us that mate discrimination is
103 sometimes far from perfect, both in the laboratory and the field.

104

105 Measuring the degree of sexual isolation among allopatric populations that show
106 variation in sexual conflict over mating is one way to gain insight into the role of sexual

107   conflict in population divergence and speciation in the field (Gay *et al*., 2009; Gagnon &

108   Turgeon, 2011). Population crosses are an important first step towards identifying current

109   episodes of population divergence, and in exploring the involvement of sexual conflict

110   and SAC in such diversification episodes (e.g. Hebets & Maddison, 2005; Long *et al*.,

111   2006; Panhuis *et al*., 2006; Sugano & Akimoto, 2007), although interpreting patterns of

112   sexual conflict themselves from population crosses requires caution (Long *et al*., 2006).

113

114   Here, we explore patterns of pre- and post-mating reproductive isolation among

115   populations of two closely related species of seed bug characterised by sexual conflict

116   over mating, *Lygaeus equestris* (LE) and *Lygaeus simulans* (LS). These species have

117   similar ecologies, including aposematic warning colouration, and both have promiscuous

118   mating systems characterised by sexual conflict such that multiple mating is extremely

119   costly to females in terms of reduced longevity and fecundity (Shuker *et al*., 2006). Males

120   therefore benefit more from extra copulations than females do. Two LE populations were

121   derived from Sweden, three from Northern Italy, and a LS population was twice sampled

122   from Central Italy. In terms of predictions, given that the two species are presumed sister

123   species, first we expect pre-zygotic isolation to be more developed than the post-zygotic

124   isolation (Coyne & Orr, 2004). Second, if sexual conflict is associated with increased

125   population divergence for mating traits, then we should expect variation in the extent to

126   which populations of LE mate with each other, and perhaps also in the fitness of hybrid

127   offspring. If this isolation is in part associated with ecological factors then we might also

128   expect LE individuals from the Swedish or Italian populations to be more likely to mate

129   with individuals from the same region as themselves. We performed two sets of within-

130   and between-species no-choice mating experiments to test these predictions. The first

131   assayed mating over a short period for reproductively mature individuals, allowing

132   investigation of the latency of individuals to mate among four populations (three LE and

133   one LS). The second experiment expanded the number of populations studied to seven

134   populations (five LE and two LS), and assayed mating over a longer period of adult life,

135   as well as the production of an F_1 and F_2 offspring generation.

136

137   MATERIALS AND METHODS

138

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, Asclepiadacaea: 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

169   filled with distilled water and stoppered with cotton wool were provided as a water

170   source. Populations comprised two to three stock cages and were maintained in

171   continuous culture with overlapping generations, with stock cages being replaced

172   approximately every 6-8 weeks. A sample of around 60 individuals of all age classes was

173   haphazardly removed from the population cages to initiate a new one. Egg-to-adult

174   development under these conditions is broadly similar for both species, taking

175   approximately 23-28 days.

176

177   To ensure a continual supply of bugs for the experiments, we transferred late larval

178   instars periodically (every 4-5 days) from continuous culture cages into smaller nymph

179   development cages for adult eclosion. In our first experiment, mating interactions were

180   examined for sexually mature individuals from four populations, in a 4x4 reciprocal

181   design (i.e. 16 replicated combinations). The populations were two Swedish LE

182   populations (from Morga and Geta), a LE population from the Dolomites region of

183   northern Italy, and a population of LS sampled from the Tuscany region of central Italy.

184

185   From each population, we removed newly eclosed adults from nymph cages every two

186   days, and placed them in same sex pots (within their respective populations) with others

187   of the same age to develop, ensuring virginity. Densities were restricted to six bugs per

188   pot (measuring 8 x 8 x 5.5cm, transparent with perforated lids). Each pot contained a

189   30mm diameter Petri dish lid and base containing water soaked cotton wool and organic

190   sunflower seeds respectively. Seeds and water were replaced every three days ensuring

191   an *ad libitum* supply of both. All bugs were retained in these conditions for at least seven

192   days prior to experimentation ensuring sexual maturity.

193

194   Adult bugs aged between seven and 12 days post-adult eclosion were then used in 'no

195   choice' mating trials. We randomly assigned these sexually mature males and females to

196   partners of the opposite sex from each of the four populations (LE: Morga, Geta,

197   Dolomites; LS: Tuscany) in a fully-factorial reciprocal design. Pairs were placed in

198   transparent pots, without any seeds or water, and were randomly distributed in trays,

199   before returning to the incubator. We scored the pairs every 30 minutes, for eight hours,

200   for copulation (stable end to end position: Sillén-Tullberg, 1981; Tadler *et al*., 1999).

201   Each hour, pots were rotated in their position within the incubator to minimise potential

202   position effects. Pairing treatments were performed in blocks, with at least two replicates

203   of each combination attained per block. Pairs where one or both individuals died during

204   the observational period were discarded, and not included in the analysis. From the eight

205   hour observation period, we recorded whether mating occurred and, for each pair that

206   mated, the time taken for mating to occur. We obtained 25 replicates for each reciprocal

207   cross (total $N = 400$ trials).

208

209   *Experiment 2: Seven populations*

210   In this experiment, we recorded the incidence of mating over a prolonged period of adult

211   development (up to approximately three weeks). In addition, we used individuals from

212   this second experiment to further explore aspects of pre- and post-mating sexual

213   isolation. We reared any F_1 generation offspring produced, and allowed them to

214   reproduce, thus enabling assessment of hybrid F_1 fertility.

215

216   We included two further LE populations from Northern Italy (Ledro and Predazzo), and a

217   replicate LS population from the same area in Tuscany, thus creating a 7x7 reciprocal

218   population design (i.e. 49 replicated combinations). All populations were maintained as

219   before. For this experiment, males and females were paired from 0-2 days post adult

220   eclosion and retained in small pots (8 x 8 x 5.5cm) containing a layer of organic, de-

221   husked, sunflower seeds, and a 7ml plastic tube containing carbon filtered water capped

222   with a cotton wool bung, for a maximum of 20 days. Water tubes were replaced every 10

223   days, or when necessary, to ensure a constant supply of water. From days 3 to 12 of being

224   paired (bugs aged ~ 5 to 14 days post eclosion, allowing for the necessary development

225   time required for sexual maturity), we scored pairs twice daily for mating. Males that

226   died during this time, without mating being observed, were replaced by another virgin

227   male of similar age from the appropriate population. Where males died and mating was

228   observed the female was isolated in a similar pot and left to oviposit. If a female died

229   without any observed matings or eggs the males were likewise re-used with new females.

230   However if a female died after producing eggs, the eggs (within the pot) were retained

231   and left to develop, and the male discarded. After day 12, adults were left to continue

232   mating (without scanning for mating) and females left to oviposit until day 20,

233   whereupon both adults were removed and discarded.

234

235   Following removal of the adults on day 20, eggs were left to develop in their respective

236   pots without their parents. After a further 7 days (when all viable eggs have hatched), we

237   scored pots for the presence and number of offspring (hatched eggs). We then transferred

238   up to 25 nymphs to a fresh pot with seeds and water (as above) and allowed them to

239   develop to adulthood. Pots were then checked for adult eclosion every 5 days and a

240   maximum density of 5 males and 5 females were retained together in fresh pots upon

241   eclosion (with a minimum of at least 3 F1 males and 3 F1 females per pot). When either

242   no males or no females eclosed (i.e. one sex absent), virgin bugs from other replicated

243   pots (of the appropriate cross) were used to allow for the mating and fertility of this F_1

244   generation to be assayed. Once again, we replaced the water every 10 days or earlier to

245   allow for a constant supply of water. We retained all pots for up to 20 days and scored

246   them for the presence/absence of F_2 offspring (hatched eggs).

247

248   We observed between 8 and 14 replicate pairs for each of the 49 treatment combinations

249   (median $N = 10$). Out of the overall 491 replicates, 12 replicates produced an F_1

250   generation without mating being directly observed between the two daily scans. All

251   combinations of crosses that produced F_1 offspring were observed mating for at least a

252   subset of the replicates however, indicating that twice daily observational scans were

253   adequate for assessing mating (as is likely given the prolonged copulation durations

254   exhibited within these species: Shuker *et al.*, 2006). Of the 223 replicates that

255   successfully produced F_1 offspring, 22 had insufficient numbers of adults emerging (i.e.

256   below our threshold of three males and three females) to assay for the production of F_2

257   offspring within these respective replicate families.

258

259   *Analysis*

260

261   *Experiment 1*

262

263   We analysed the incidence of mating using binary logistic regression with a logit link

264   function. First, we compared the within-population trials to examine if there were

265   population differences in their baseline mating frequencies. Second, male and female

266   identities (their population origin) were used as factors, along with the interaction term

267   between them to test for differences in likelihood of mating across all population

268   combinations. Sexual isolation is indicated by significant interaction effects, and was

269   assessed in each case using likelihood ratio (LR) tests. These analyses were performed

270   using R (R version 2.11.1).

271

272   We further analysed sexual isolation among the populations and species using an overall

273   sexual isolation index (IPSI, Rolan-Alvarez & Caballero, 2000; Perez-Figueroa *et al*.,

274   2005; see the latter paper for a detailed comparison of different indices). This was

275   performed as a global analysis (I_{PSItotal}) as well as for each pairwise comparison ($I_{\text{PSI}a,b}$),

276   and estimates of asymmetry among these crosses ($I_{\text{APSI}a,b}$) were also tested (Carvajal-

277   Rodriguez & Rolan-Alvarez, 2006). "Asymmetry" describes the extent to which

278   reproductive isolation in one direction of cross differs from the reciprocal cross.

279   Significance of these sexual isolation indices was tested using bootstrap resampling with

280   10,000 iterations. Where no mating was observed among pairs, zeros were replaced with

281   0.5 to allow for bootstrap resampling. Tests of sexual isolation were performed using the

282   programme JMATING (Carvajal-Rodriguez & Rolan-Alvarez, 2006).

283

284   In terms of the latency to mating, we analysed the time taken to mate using a generalised

285   linear model with a quasi-poisson error distribution and log link to account for

286   overdispersion of the data.

287

288   *Experiment 2*

289

290   Mating propensity and sexual isolation among the populations and species was assessed

291   as above. We also tested F_1 progeny production (from those pairs that were observed *in*

292   *copula*), and the number of progeny produced (of those that produced offspring) by each

10

293   mating pair. We analysed the incidence of F_1 progeny production among pairs with

294   logistic regression, firstly for conspecific pairs (within populations), and subsequently

295   using all crosses to examine the effects of male and female identity and their interaction

296   on mating success respectively. This was then repeated using only the subset of pairs that

297   mated to investigate potential post mating reproductive barriers, firstly for all pairs and,

298   secondly, among *L. equestris* populations only. We analysed variation in the number of

299   progeny produced (by pairs that produced offspring) again firstly for conspecific pairs

300   (within populations), and subsequently using all crosses to examine effects of male and

301   female identity and their interaction on fecundity respectively, using ANOVA. Lastly, *L.*

302   *equestris* populations were further analysed to test for differential mating success in terms

303   of the number of hatched offspring produced by reproductive pairs. Where the interaction

304   term was not significant ($p > 0.05$), it was removed and the model refitted using only the

305   main effects. Again, these analyses were performed using R (R version 2.11.1). The data

306   presented in Figures 1-3 are also given as raw proportions in the Supplementary

307   Information file.

308

309   RESULTS

310

311   *Experiment 1*

312

313   Mating frequencies for within-population mate trials were similar across all the four

314   populations (mean proportion mating = 0.89, $N = 100$; Likelihood ratio test: LR = 3.00, *df*

315   = 3, $p = 0.39$; Figure 1). Across all 16 combinations however there was significant

316   behavioural reproductive isolation, generally associated with the inter-specific pairings of

317   *L. equestris* and *L. simulans* (Figure 1). As such, there was a highly significant interaction

318   effect between male and female population on the incidence of mating (interaction effect;

319   LR = 231.12, *df* = 9, $p < 0.0001$) as well as differences among the populations in overall

320   male willingness to mate (LR = 40.55, *df* = 3, $p < 0.0001$) and female willingness to mate

321   (LR = 71.22, *df* = 3, $p < 0.0001$). Within just LE, Morga males mated less overall than

322   males from other populations (LR = 9.17, *df* = 2, $p = 0.01$; see Figure 1), but for LE

323   females there were no population differences in the incidence of mating (LR = 0.35, *df* =

324   2, $p = 0.84$) and no interaction between male identity and female identity on the incidence

325   of mating (LR = 4.73, $df = 4$, $p = 0.32$).

326

327   Using the isolation statistics, we confirmed strong pre-mating sexual isolation between

328   LE (Morga, Geta, and Dolomites) and LS (Total $I_{psi} = 0.573$, $p < 0.001$; Table 1), but no

329   sexual isolation effects among LE populations (see Table 1). Furthermore, the sexual

330   isolation effect between LE and LS was asymmetric for the Dolomites*Tuscany cross

331   ($I_{Apsi} = 4.065$, $p = 0.005$) with LS males able to mate with LE females whilst the opposite

332   was not found (Figure 1 and Table 1).

333

334   Overall, when mating did occur there was little difference either within or between

335   species in latency to mating. The time to mating did not differ among the populations or

336   species when paired with conspecific partners (mean = 1.43 hours, SE = 0.15, $F_{3,85} =$

337   0.16, $p = 0.92$). Additionally, including heterospecific pairings revealed no interaction

338   effect between male and female identity on the time to mating ($F_{5,219} = 1.42$, $p = 0.22$),

339   and no overall difference among any of the populations or species in the time taken for

340   males or females to mate (male population: $F_{3,224} = 1.48$, $p = 0.22$; female population:

341    $F_{3,224} = 0.25$, $p = 0.86$). Analysing only LE populations gave the same qualitative results

342   of no differences in time to mating (data not shown).

343

344   *Experiment 2*

345

346   As with the first experiment, there was no difference between the populations, or species,

347   in their mating propensities when paired with conspecific partners (mean proportion

348   mating = 0.813, $N = 64$; LR = 7.32, $d.f. = 6$, $p = 0.29$). However, there was again

349   evidence of inter-specific mating isolation. Including hetero-specific pairings revealed

350   significant heterogeneity among the populations in the likelihood of mating associated

351   with female population and the interaction between male and female population (female

352   population: LR = 126.44, $d.f. = 6$, $p < 0.0001$; interaction effect: LR = 225.11, $d.f. = 36$, p

353   < 0.0001; Figure 2), but not associated with the main effect of male population (LR =

354   1.85, $d.f. = 6$, $p = 0.93$). These effects are associated with the hetero-specific trials, as

355   within LE populations there was no significant interaction between male and female

356   population ($N = 255$, interaction effect: LR = 22.94, $d.f. = 16$, $p = 0.13$). The LE

357   populations did differ in the overall mating propensity of females (LR = 11.175, $d.f. = 4$,

358    $p = 0.025$), suggesting that the LE populations differ in their baseline level of female

359   receptivity to mating (Figure 2), but there were no among-population differences for the

360   overall mating propensity of males (LR = 3.14, $d.f. = 4$, $p = 0.54$).

361

362   Again, closer examination using *Ipsi* statistics revealed significant pre-mating sexual

363   isolation between the two species, but not within species (Total *Ipsi* = 0.255, $p = 0.006$;

364   Table 1). Moreover, as with the four population experiment, sexual isolation between the

365   species was significantly asymmetric when LE from Ledro, Morga, and Predazzo, were

366   paired with LS (Table 1 and Figure 2).

367

368   *F₁ offspring production*

369   There was a significant interaction effect between male and female identity on the

370   incidence of hatched offspring (LR = 167.74, $d.f. = 36$, $p \ll 0.0001$, male population; LR

371   = 16.32, $d.f. = 6$, $p = 0.012$, female population; LR = 94.30, $d.f. = 6$, $p \ll 0.001$),

372   indicating strong heterogeneity amongst the populations in the likelihood of successfully

373   interbreeding (Figure 3). As expected, the incidence of hatched offspring production did

374   not differ between populations when individuals were paired with conspecifics (mean =

375   0.64, $N = 64$; LR = 5.82, $d.f. = 6$, $p = 0.44$; it is worth highlighting that this means that

376   there was an overall "mating failure" rate of 36%). However, reducing the data set to

377   contain only those pairs that did mate, the likelihood of a male from a particular

378   population producing hatched offspring with a female did not depend on the identity of

379   the female partner and vice versa ($N = 305$, interaction effect; LR = 36.46, $d.f. = 27$, $p =$

380   0.11) suggesting limited post-mating reproductive isolation between the species. Refitting

381   the model (using mated pairs) without this interaction effect still revealed population

382   differences for both males and females in the likelihood of producing hatched offspring

383   following successful mating (male population; LR = 34.35, $d.f. = 6$, $p < 0.001$, female

384   population; LR = 25.99, $d.f. = 6$, $p < 0.001$). Contrary to the result of no population

385   differences in the likelihood of producing hatched offspring in conspecific pairings, this

386 suggests that there is some variability among the populations in the likelihood of

387 producing hatched offspring (Figure 3). Indeed analysis of LE populations only showed a

388 similar story to mating propensity, with a significant effect of female population identity

389 on the incidence of hatched offspring among mated individuals, but no effect of male

390 identity and no interaction between the two ($N = 218$: female identity LR = 17.13, $d.f.$ =

391 4, $p = 0.002$; male identity LR = 6.02, $d.f.$ = 4, $p = 0.20$; interaction term LR = 17.97, $d.f.$

392 = 16, $p = 0.37$).

393

394 There was no difference among the populations in the number of hatched offspring

395 produced (by reproductive pairs) when paired with conspecific partners (mean = 59.88, se

396 = 5.67, $F_{6,35} = 1.067$, $p = 0.40$). Analysing all population crosses, there were highly

397 significant population differences for males and females in the number of hatched

398 offspring produced overall, (male population: $F_{6,183} = 9.29$, $p < 0.0001$; female

399 population: $F_{6,183} = 7.49$, $p < 0.0001$), which is driven largely by the low numbers of

400 offspring produced among LE by LS population crosses (Figure 3). However, the

401 interaction was found to be non-significant ($F_{25,183} = 0.85$, $p = 0.67$), suggesting that there

402 was little difference in the numbers of hybrid versus pure-bred offspring, although this

403 might be in part due to low statistical power from low sample sizes.

404

405 Analysing LE populations in isolation, again there was no significant interaction effect

406 between male and female identity on the number of offspring produced ($F_{16,151} = 1.167$, p

407 = 0.30), however, male and female identity were significant as main effects (male: $F_{4,167}$

408 = 3.12, $p = 0.017$; female: $F_{4,167} = 2.73$, $p = 0.031$), suggesting there is variance in

409 fertility across populations.

410

411 *F_2 offspring production*

412 Intra-specific crosses showed no restrictions in the ability of populations to interbreed

413 and produce fertile hybrids as F_2 generations were produced in each case (Figure 4).

414 However, there was some evidence for partial post-mating reproductive isolation between

415 LE and LS as crosses between LE males and LS females did not produce any F_1 offspring

416 (perhaps not surprising as only one of these pairings mated in the first instance: Figure 2).

417   However, there were more matings in the reciprocal direction (i.e. between male LS and

418   female LE; Figures 1 and 3) and they did produce both an F_1 generation (Figure 3) and a

419   subsequent F_2 generation in some cases (Figure 4). Although low numbers of F_1 offspring

420   were produced from these crosses, sexual isolation between LE and LS would appear to

421   be largely in terms of pre-mating isolation rather than post-mating isolation therefore, and

422   largely in one direction.

423

424   DISCUSSION

425

426   The importance of sexual conflict in facilitating reproductive divergence among

427   populations remains unclear. Here we have investigated the extent of reproductive

428   isolation within and between two sister species of seed bug (*Lygaeus equestris* and *L.

429   simulans*), species that are characterised by sexual conflict (Shuker *et al*., 2006). No

430   sexual isolation was apparent among different LE populations, as might be expected if

431   the sexual conflict over mating had selected for diverging reproductive traits. Instead, our

432   two experiments revealed pre-mating isolation between LE and LS, although this

433   isolation was asymmetric: male LS were able to mate with female LE, but male LE were

434   largely unable to mate with female LS. The results were similar whether we allowed pairs

435   to interact for a few hours or for a few days, and were largely consistent across multiple

436   LE populations and two replicate LS populations. In our first experiment, we found no

437   differences in time to mating for those crosses where mating occurred, again suggesting

438   little divergence in behaviour or key mating signals. We found little evidence for strong

439   post-mating isolation between the two species either however, with hybrid F1 and F2

440   offspring sometimes being produced if matings occurred.

441

442   Pre-zygotic sexual isolation may act over many systems involving behavioural,

443   physiological and/or morphological characters (Coyne & Orr 1989, 1997). Although no

444   overt courtship occurs in the two species studied here (Solbreck, 1972; Sillén-Tullberg,

445   1981; Tadler *et al*., 1999; Dougherty & Shuker, 2014), morphological differences

446   between the species could explain the pre-mating isolation observed to some extent.

447   Indeed, one of the few characters that distinguish the species is the morphology of the

15

448   male parameres (genital claspers; Deckert, 1985; Péricart, 1998), which are used by

449   males to secure position during mating, facilitating successful copulation (Tadler, 1999;

450   Tadler *et al*., 1999). We are currently exploring the role that claspers, and also the

451   internal male genitalia, may play in reproductive isolation.

452

453   In addition to morphological differences, we have recently identified differences in

454   cuticular hydrocarbon (CHC) profiles between five species of Lygaeidae, including *L.*

455   *equestris* and *L. simulans* (Burdfield-Steel *et al*., unpublished data). Our data suggest

456   differences in CHC profiles between these latter two species, including sex-specific and

457   ontogenetic differences. These differences will provide a starting point for experimental

458   tests of species-discrimination mechanisms. Hetero-specific mating attempts are not

459   infrequent across four species of lygaeid more generally however (*L. equestris*, *L.*

460   *creticus*, *Spilostethus pandurus*, and *Oncopeltus fasciatus*: Shuker *et al*,. 2015; see also

461   McLain & Shure, 1987; McLain & Pratt, 1999 for another example from the Lygaeidae),

462   and such hetero-specific mating interactions can be as costly as intra-specific mating

463   harassment for female LE (i.e. there is reproductive interference: Shuker *et al*., 2015).

464   Given the highly polygamous mating systems of these species, and the occurrence of

465   reproductive interference, our current inference is that despite the availability of cues,

466   such as CHCs, to facilitate species discrimination, males have been selected to be highly

467   opportunistic when it comes to possible mating partners, and this selection has weakened

468   selection for strong species discrimination (Parker & Partridge, 1998; Burdfield-Steel &

469   Shuker 2014b; Shuker et al 2015).

470

471   This means that sexual conflict over mating, although possible in the field, may not be

472   driving divergence and speciation among LE populations. Indeed, our interpretation

473   supports the contrary theoretical proposition, that male willingness to mate may impede

474   speciation through maintaining gene flow across populations (Parker & Partridge, 1998;

475   Gavrilets & Hayashi, 2005), and would concur with other studies. For example, Gagnon

476   & Turgeon (2011) found that despite significant correlations between morphological

477   traits of males and females associated with sexual conflict over mating in populations of

478   *Gerris gillettei*, there were no mating asymmetries among allopatric populations,

16

479 suggesting that sexual conflict was not driving population divergence. And as highlighted

480 above, even in laboratory evolution studies, support for SAC over mating promoting

481 allopatric population divergence is mixed.

482

483 In terms of the reproductive isolation between LE and LS, there was a clear pattern of

484 asymmetric pre-mating isolation. Asymmetric sexual isolation is very common, both in

485 terms of pre-mating, behavioural isolation (e.g. Kaneshiro, 1980; Arnold *et al.*, 1996) and

486 also in terms of post-mating isolation (e.g. Turelli & Moyle, 2007). Two such examples

487 of the former include isolation between populations of the grasshopper, *Podisma*

488 *sapporensis* (Sugano & Akimoto, 2007), and between species of Sonoran desert

489 *Drosophila* (Markow & Hocutt, 1998). In both of these cases, divergent sexual

490 antagonistic co-evolution among the populations or species (with differential levels of

491 male vigour and female resistance) was attributed as a likely explanation for the patterns

492 observed, but our failure to link between-species isolation with any among-population,

493 within-species isolation suggests that SAC has not generated important divergence in

494 behaviour between LE and LS. We note here that our analysis of pre-mating isolation

495 was based on a no-choice experimental paradigm. A recent meta-analysis has confirmed

496 that the strength of mate preferences can vary with experimental paradigm, i.e. between

497 choice and no-choice paradigms (Dougherty & Shuker 2015; but see Dougherty &

498 Shuker 2014 for no effect of experimental design on intra-specific mate preferences in *L.*

499 *equestris*). Mate preferences are generally stronger when measured via choice

500 experiments (typically the presentation of two options), although the meta-analysis

501 suggested that experimental paradigm had no effect on the strength of preference for con-

502 over hetero-specifics, as studied here. However, the number of studies available for that

503 comparison was small (Dougherty & Shuker 2015).

504

505 In terms of post-mating isolation, no LE populations (from Sweden or Italy) showed any

506 restriction in their ability to interbreed, producing F_2 offspring and thus demonstrating

507 fertile F_1 hybrids. No breeding restrictions were found between the two *L. simulans*

508 replicate populations either. That is not to say that all matings involve successful sperm

509 transfer, as "mating failures" appear to be rather common, even among con-specifics.

17

510 Mating failure is probably more common than often realised (Eberhard, 1996; García-

511 González, 2004) and interest in this phenomenon is growing (Rhainds, 2010; Greenway

512 *et al.*, 2015).

513

514 In terms of the inter-specific crosses, only a subset of LE female and LS male replicate

515 crosses produced sufficient numbers of F_1 generation individuals that developed to adults

516 (and could reliably test for an F_2 generation), thus we could not determine with certainty

517 whether all types of crosses are equally (in)compatible, but there appears to be variation

518 in the successful production of F1 and F2 offspring. As such, the data we have suggest

519 there might be segregating variation in the genetic basis of any post-zygotic

520 incompatibilities, an increasingly common finding (e.g. Shuker *et al.*, 2005; Cutter,

521 2012). Nonetheless, the production of some hybrid F_2 offspring clearly demonstrates that,

522 given the no-choice mating experiments performed here, the two species can interbreed

523 and produce viable offspring under laboratory conditions.

524

525 There remain a number of outstanding questions. For instance, more needs to be done

526 unravelling how and under what circumstances males and females discriminate between

527 species (e.g. Burdfield-Steel & Shuker, 2014b; Shuker *et al.*, 2015), including further

528 exploration of the role of CHCs (and variation in CHC profiles within and among

529 populations). Second, the work presented here only begins to touch upon the nature and

530 extent of post-mating isolation. Our data show that a proportion of F1 hybrids are viable

531 and also fertile. However, the extent of variation in hybrid viability or fertility remains to

532 be fully explored, including the extent of any reproductive incompatibilities that fit the

533 pattern of Haldane's rule (Coyne & Orr, 2004). *Lygaeus* have an XO chromosomal sex

534 determination system, therefore we would predict that if failure to produce offspring (or

535 variation in the failure to produce offspring) is associated with only one sex, it should be

536 associated with the heterogametic males. Such experiments are currently underway.

537

538 Finally, our results suggest that this species-pair may be a useful system for exploring the

539 genetic basis of reproductive isolation, as we have behavioural variation across the two

540 species, an asymmetry in the direction of reproductive isolation, and the ability to form

541   viable F2 hybrids, which would facilitate genetic mapping. As the study of reproductive

542   isolation in insects is still somewhat dominated by species of *Drosophila* and a number of

543   Orthoptera (e.g. Coyne & Orr, 2004), this system may provide a welcome new study

544   organism for exploring the evolutionary genetics of reproductive isolation in the wild.

545

546

547

548

549

556

REFERENCES

Anthes N, Schulenburg H, Michiels NK. 2008. Evolutionary links between reproductive morphology, ecology and mating behavior in opisthobranch gastropods. *Evolution* **62**: 900-916.

Arnold SJ, Verrell PA, Tilley SG. 1996. The evolution of asymmetry in sexual isolation: a model and a test case. *Evolution* **50**: 1024–1033.

Arnqvist G, Edvarrdsson M, Friberg U, Nilsson T. 2000. Sexual conflict promotes speciation in insects. *Proceedings of the National Academy of Sciences, USA* **97**: 10460–64.

Arnqvist G, Nilsson T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour* **60**: 145-164.

Arnqvist G, Rowe L. 2002a. Antagonistic coevolution between the sexes in a group of insects. *Nature* **415**:787-789.

Arnqvist G, Rowe L. 2002b. Correlated evolution of male and female morphologies in water striders. *Evolution* **56**: 936-947.

Arnqvist G, Rowe L. 2005. *Sexual conflict*. Princeton, New Jersey: Princeton University Press.

Bacigalupe LD, Crudgington HS, Hunter F, Moore AJ, Snook RR. 2007. Sexual conflict does not drive reproductive isolation in experimental populations of *Drosophila pseudoobscura*. *Journal of Evolutionary Biology* **20**: 1763-1771.

Bailey NW, Zuk M. 2009. Same-sex sexual behaviour and evolution. *Trends in Ecology and Evolution* **24**: 439-446.

Bateman AJ. 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2**:349-368.

Bergsten J, Miller KB. 2007. Phylogeny of Diving Beetles Reveals a Coevolutionary Arms Race between the Sexes. *PLoS ONE* **2**: e522.

Burdfield-Steel ER, Shuker DM. 2011. Reproductive interference. *Current Biology* **21**: R450-451.

Burdfield-Steel ER, Shuker DM. 2014a. The evolutionary ecology of the Lygaeidae. *Ecology and Evolution* **4**: 2278-2301.

Burdfield-Steel ER, Shuker DM. 2014b. Mate-guarding in a promiscuous insect: species discrimination influences reproductive interference in seed bugs. *Evolutionary Ecology* **28**: 1031-1042.

Butlin, RK, Debelle A, Kerth C, Snook RR, Beukeboom LW, Castillo Cajas RF, Diao W, Maan ME, Paolucci S, Weissing FJ, van de Zande L, Hoikkala A, Geuverink E, Jennings J, Kankare M, Knott KE, Tyukmaeva VI, Zoumadakis C, Ritchie MG, Barker D, Immonen E, Kirkpatrick M, Noor M, Macias Garcia C, Schmitt T, Schilthuizen M. 2012. What do we need to know about speciation? *Trends in Ecology and Evolution* **27**: 27-39.

Carvajal-Rodriguez A, Rolan-Alvarez E. 2006. JMATING: a software for the analysis of sexual selection and sexual isolation effects from mating frequency data. *BMC Evolutionary Biology* **6**: 40.

Chapman T. 2006. Evolutionary conflicts of interest between males and females. *Current Biology* **16**: R744-R754.

Chapman T, Arnqvist G, Bangham J, Rowe L. 2003. Sexual conflict. *Trends in Ecology and Evolution* **18**: 41-47.

Coyne JA, Orr HA. 1989. Patterns of speciation in *Drosophila*. *Evolution* **43**: 362-381.

Coyne JA, Orr HA. 1997. "Patterns of speciation in *Drosophila*" revisited. *Evolution* **51**: 295-303.

Coyne JA., Orr HA. 2004. *Speciation*. Sunderland, MA: Sinauer Associates.

Cutter, AD. 2012. The polymorphic prelude to Bateson–Dobzhansky–Muller incompatibilities. *Trends in Ecology and Evolution* **27**: 209-218.

Deckert J. 1985. Über *Lygaeus simulans* spec. nov. und *L. equestris* (Linnaeus 1758), zwei nahe verwendte paläarktische Lygaeinae (Heteroptera, Lygaeidae). *Mitteilungen aus dem Zoologischen Museum in Berlin* **61**: 273-278.

Dougherty LR, Shuker DM. 2014. Pre-copulatory sexual selection in the seed bug *Lygaeus equestris*: a comparison of choice and no-choice paradigms. *Animal Behaviour* **89**: 207-214.

Dougherty LR, Shuker DM. 2015. The effect of experimental design on the measurement of mate choice: a meta-analysis. *Behavioral Ecology* **26**: 311-319.

Eberhard WG. 1996. *Cryptic Female Choice*. Princeton, New Jersey: Princeton University Press.

Gagnon MC, Turgeon J. 2011. Sexual conflict in *Gerris gillettei* (Insecta: Hemiptera): intraspecific intersexual correlated morphology and experimental assessment of behaviour and fitness. *Journal of Evolutionary Biology* **24**: 1505-1516.

García-González F. 2004. Infertile matings and sperm competition: the effect of "nonsperm representation" on intraspecific variation in sperm precedence patterns. *The American Naturalist* **164**: 457-472.

Gavrilets S. 2000. Rapid evolution of reproductive barriers driven by sexual conflict. *Nature* **403**: 886-889.

Gavrilets S, Arnqvist G, Friberg U. 2001. The evolution of female mate choice by sexual conflict. *Proceedings of the Royal Society of London Series B-Biological Sciences* **268**: 531-539.

Gavrilets S, Hayashi TI. 2005. Speciation and sexual conflict. *Evolutionary Ecology* **19**: 167-198.

Gay L, Eady PE, Vasudev R, Hosken DJ, Tregenza T. 2009. Does reproductive isolation evolve faster in larger populations via sexually antagonistic coevolution? *Biology Letters* **5**: 693-696.

Greenway EV. Dougherty LR, Shuker DM. 2015. Mating failure. *Current Biology*, in press.

Hebets EA, Maddison WP. 2005. Xenophilic mating preferences among populations of the jumping spider *Habronattus pugillis* Griswold. *Behavioral Ecology* **16**: 981-988.

Holland B, Rice WR. 1998. Perspective: Chase-away sexual selection: Antagonistic seduction versus resistance. *Evolution* **52**: 1-7.

Holland B, Rice WR. 1999. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proceedings of the National Academy of Sciences, USA* **96**: 5083-5088.

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

(*Scathophaga stercoraria*). *Proceedings of the Royal Society of London Series B-Biological Sciences* **269**: 1701-1707.

Jennions MD, Petrie M. 1997. Variation in mate choice and mating preferences: A review of causes and consequences. *Biological Reviews* **72**: 283-327.

Kaneshiro KY. 1980. Sexual isolation, speciation and the direction of evolution. *Evolution* **34**: 437-444.

Koene JM, Schulenburg H. 2005. Shooting darts: co-evolution and counter-adaptation in hermaphroditic snails. *BMC Evolutionary Biology* **5**: 13.

Kugelberg O. 1973. Larval development of *Lygaeus equestris* (Heteroptera, Lygaeidae) on different natural foods. *Entomologia Experimentalis Et Applicata* **16**: 165-177.

Kugelberg O. 1974. Laboratory studies on the feeding preference and feeding behaviour in *Lygaeus equestris* (L.) (Het. Lygaeidae). *Entomologica Scandinavica* **5**: 49-55.

Kugelberg O. 1977. Distribution, feeding habits and dispersal of *Lygaeus equestris* (Heteroptera) larvae in relation to food supply. *Oikos* **29**: 398-406.

Lessells CM. 2006. The evolutionary outcome of sexual conflict. *Philosophical Transactions of the Royal Society Series B - Biological Sciences* **361**: 301-317.

Long TAF, Montgomerie R, Chippindale AK. 2006. Quantifying the gender load: can population crosses reveal interlocus sexual conflict? *Philosophical Transactions of the Royal Society Series B - Biological Sciences* **361**: 363-374.

Maklakov AA, Cayetano L, Brooks RC, Bonduriansky R. 2010. The roles of life-history selection and sexual selection in the adaptive evolution of mating behavior in a beetle. *Evolution* **64**: 1273-1282.

Markow TA, Hocutt GD. 1998. Reproductive isolation in sonoran desert *Drosophila*; testing the limits of the rules. In: Howard DJ, Berlocher SH (eds) *Endless Forms; Species and Speciation*. Oxford: Oxford University Press, pp 234-244.

Martin OY, Hosken DJ. 2003. The evolution of reproductive isolation through sexual conflict. *Nature* **423**:979-982.

Maschler B. 2002. Is *Lygaeus simulans* (Heteroptera, Lygaeidae) a "good" species. *Entomologica Austriaca* **5**: 14.

McLain DK, Pratt AE. 1999. The cost of sexual coercion and heterospecific sexual harassment on the fecundity of a host-specific, seed-eating insect (*Neacoryphus bicrucis*). *Behavioral Ecology and Sociobiology* **46**: 164-170.

McLain DK, Shure DJ. 1987. Pseudocompetition: interspecific displacement of insect species through misdirected courtship. *Oikos* **49**: 291-296.

Micholitsch T, Krugel P, Pass G. 2000. Insemination and fertilization in the seed bug *Lygaeus simulans* (Heteroptera: Lygaeidae). *European Journal of Entomology* **97**: 13-18.

Panhuis TM, Clark NL, Swanson WJ. 2006. Rapid evolution of reproductive proteins in abalone and Drosophila. *Philosophical Transactions of the Royal Society Series B - Biological Sciences* **361**: 261-268.

Parker GA. 1979. Sexual selection and sexual conflict. In: Blum MS, Blum NB (eds) *Sexual Selection and Reproductive Competition in Insects.* New York: Academic Press, pp 123-166.

Parker GA, Partridge L. 1998. Sexual conflict and speciation. *Philosophical Transactions of the Royal Society Series B - Biological Sciences* **353**: 261-274.

Perez-Figueroa A, Caballero A, Rolan-Alvarez E. 2005. Comparing the estimation properties of different statistics for measuring sexual isolation from mating frequencies. *Biological Journal of the Linnean Society* **85**: 307-318.

Péricart J. 1998. *Hémiptères Lygaeidae euro-méditerranéens* 1-3 Faune de France 84A-C, vol 84A. Paris: Fédération Français des Sociétés de Sciences Naturelles.

Rhainds M. 2010. Female mating failures in insects. *Entomologia Experimentalis et Applicata* **136**: 211-226.

Rice WR. 2000. Dangerous liaisons. *Proceedings of the National Academy of Sciences, USA* **97**: 12953-12955.

Rice WR, Stewart AD, Morrow EH, Linder JE, Orteiza N, Byrne PG. 2006. Assessing sexual conflict in the *Drosophila melanogaster* laboratory model system. *Philosophical Transactions of the Royal Society Series B - Biological Sciences* **361**: 287-299.

Rolan-Alvarez E, Caballero M. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. *Evolution* **54**: 30-36.

Shuker DM, Underwood K, King TM, Butlin RK. 2005. Patterns of male sterility in a grasshopper hybrid zone imply accumulation of hybrid incompatibilities without selection. *Proceedings of the Royal Society, Series B,* **272**: 2491-2497.

Shuker DM, Ballantyne GA, Wedell N. 2006. Variation in the cost to females of the sexual conflict over mating in the seed bug, *Lygaeus equestris*. *Animal Behaviour* **72**: 313-321.

Shuker DM, Currie N, Hoole T, Burdfield-Steel ER. 2015. The extent and costs of reproductive interference among four species of true bug. *Population Ecology*, in press.

Sillén-Tullberg B. 1981. Prolonged copulation: a male 'postcopulatory' strategy in a promiscuous species, *Lygaeus equestris* (Heteroptera, Lygaeidae). *Behavioural Ecology and Sociobiology* **9**: 283-289.

Solbreck C. 1972. Sexual cycle and changes in feeding activity and fat body size in relation to migration in *Lygaeus equestris* (L.) (Heteroptera, Lygaeidae). *Entomologica Scandinavica* **3**: 267-274.

Solbreck C. 1991. Unusual weather and insect population dynamics: *Lygaeus equestris* during an extinction and recovery period. *Oikos* **60**: 343-350.

Solbreck C, Kugelberg O.1972. Field observations on the seasonal occurrence of *Lygaeus equestris* (L.) (Het., Lygaeidae) with special reference to food plant phenology. *Entomologica Scandinavica* **3**: 189-210.

Solbreck C, Olsson R, Anderson DB, Förare J. 1989. Size, life-history and responses to food shortage in two geographical strains of a seed bug *Lygaeus equestris*. *Oikos* 55: 387-396.

S**olbreck C, Sillén-Tullberg B. 1981.** Control of diapause in a monovoltine insect, *Lygaeus equestris* (Heteroptera). *Oikos* **36**: 68-74.

Stewart AD, Morrow EH, Rice WR. 2005. Assessing putative interlocus sexual conflict in *Drosophila melanogaster* using experimental evolution. *Proceedings of the Royal Society B-Biological Sciences* **272**: 2029-2035.

Sugano YC, Akimoto S. 2007. Asymmetric mating in the brachypterous grasshopper *Podisma sapporensis*. *Ethology* **113**: 301-311.

Tadler A. 1999. Selection of a conspicuous male genitalic trait in the seedbug *Lygaeus simulans*. *Proceedings of the Royal Society of London Series B-Biological Sciences* **266**: 1773-1777.

Tadler A, Nemeschkal HL, Pass G. 1999. Selection of male traits during and after copulation in the seedbug *Lygaeus simulans* (Heteroptera, Lygaeidae). *Biological Journal of the Linnean Society* **68**: 471-483.

Trivers RL. 1972. Parental investment and sexual selection. In: Campbell B (ed) *Sexual selection and the descent of man 1871-1971*. Chicago: Aldine publishing, pp 136-179

Turelli M, Moyle LC. 2007. Asymmetric postmating isolation: Darwin's corollary to Haldane's rule." *Genetics* **176**: 1059-1088.

Wigby S, Chapman T. 2004. Female resistance to male harm evolves in response to manipulation of sexual conflict. *Evolution* **58**: 1028-1037.

Wigby S, Chapman T. 2006. No evidence that experimental manipulation of sexual conflict drives premating reproductive isolation in *Drosophila melanogaster*. *Journal of Evolutionary Biology* **19**: 1033-1039.

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 (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 (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 (*Ipsi*), and estimates of asymmetry (*IApsi*) 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)..

Population Cross	*Ipsi*	SD	*p*	*IApsi*	SD	*p*
Experiment 1						
Morga * Geta	0.001	0.110	0.9922	1.003	0.044	0.935
Morga * Dolomites	0.024	0.109	0.8368	1.009	0.049	0.972
Morga * Tuscany	0.902	0.060	**< 0.0001**	1.505	1.006	0.535
Geta * Dolomites	-0.044	0.107	0.6642	0.998	0.034	0.869
Geta * Tuscany	0.958	0.042	**< 0.0001**	0.438	0.620	0.543
Dolomites * Tuscany	0.753	0.070	**< 0.0001**	4.065	1.235	**0.005**
Total	0.573	0.049	**< 0.0001**			
Experiment 2						
Dolomites * Geta	-0.137	0.197	0.456	1.019	0.121	0.977
Dolomites * Ledro	-0.030	0.195	0.857	1.013	0.164	0.834
Dolomites * Morga	-0.009	0.185	0.948	1.011	0.131	0.888
Dolomites * Predazzo	-0.120	0.183	0.492	0.996	0.116	0.795
Dolomites * Tuscany(1)	0.440	0.217	0.079	1.621	0.525	0.146
Dolomites * Tuscany(2)	0.844	0.131	**0.001**	0.739	0.658	0.947
Geta * Ledro	-0.208	0.177	0.235	0.990	0.120	0.775
Geta * Morga	0.140	0.209	0.513	1.091	0.293	0.892
Geta * Predazzo	-0.069	0.199	0.702	1.014	0.132	0.993
Geta * Tuscany(1)	0.640	0.192	**0.021**	1.423	0.533	0.268
Geta * Tuscany(2)	0.835	0.142	**0.005**	0.725	0.642	0.941
Ledro * Morga	0.011	0.178	0.964	0.988	0.138	0.895
Ledro * Predazzo	-0.086	0.180	0.600	1.001	0.107	0.840
Ledro * Tuscany(1)	0.539	0.176	**0.022**	1.851	0.523	**0.045**
Ledro * Tuscany(2)	0.541	0.144	**0.008**	2.202	0.619	**0.016**
Morga * Predazzo	-0.002	0.179	0.974	1.016	0.142	0.906
Morga * Tuscany(1)	0.647	0.162	**0.010**	1.763	0.584	0.123
Morga * Tuscany(2)	0.589	0.134	**0.001**	2.354	0.642	**0.013**
Predazzo * Tuscany(1)	0.539	0.176	**0.027**	1.853	0.533	**0.047**
Predazzo * Tuscany(2)	0.627	0.145	**0.004**	2.016	0.611	0.056
Tuscany(1) * Tuscany(2)	-0.101	0.187	0.560	0.994	0.134	0.756
Total	0.255	0.081	**0.005**			