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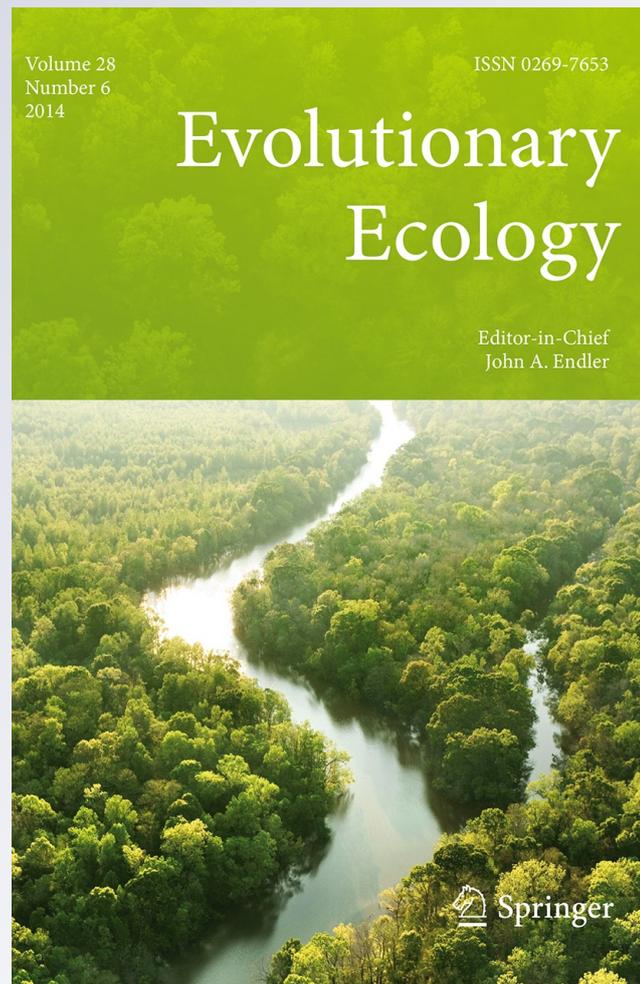
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Mate-guarding in a promiscuous insect: species discrimination influences context-dependent behaviour

Emily R. Burdfield-Steel · David M. Shuker

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Abstract Mating strategy is often informed by social context. However, information on social environment may be sensitive to interference by nearby heterospecifics, a process known as reproductive interference (RI). When heterospecific individuals are present in the environment, failures in species discrimination can lead to sub-optimal mating behaviours, such as misplaced courtship, misplaced rivalry behaviours, or heterospecific copulation attempts. All aspects of mating behaviour that are influenced by social context may be prone to RI, including copulatory behaviours associated with mate-guarding in the presence of possible competitors. Here we investigate the effect of three heterospecifics on the mate-guarding behaviour of male *Lygaeus equestris* seed bugs. We find that, despite previously reported heterospecific mating harassment amongst these species of lygaeid bug, male *L. equestris* are able to effectively distinguish rival conspecific males from heterospecifics. Thus, heterospecific mating attempts in this group may reflect selection on males to mate opportunistically, rather than a failure of species discrimination.

Keywords Species discrimination · Species recognition · Reproductive interference · Mate guarding

Introduction

The ability to distinguish between conspecifics and heterospecifics is an important component of successful mate choice. The failure or breakdown of this species discrimination can have many effects, both on an individual and population level. One particular outcome that has received increasing attention in recent years is reproductive interference (RI). RI occurs when organisms of one species attempt reproductive behaviours with another species, leading to a reduction in fitness for one or both species (Burdfield-Steel and

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Shuker 2011). Examples of RI can range from straightforward heterospecific copulation attempts, (i.e. de Bruyn et al. 2008) to more subtle behavioural changes such as forgoing normal mate-quality assessment in favour of reliable species discrimination (Pfennig and Pfennig 2009). The presence of heterospecific signals in the environment can interfere with sexual signalling (e.g. in the frog, *Allobates femoralis*; Amezcuita et al. 2006, and Neotropical singing mice, *Scotinomys sps.*; Pasch et al. 2013), influencing both mate-attraction and mate-searching. While much of the literature on the effects of RI has focused on responses to heterospecific copulation attempts, including avoidance behaviours leading to changes in habitat use (McLain and Shure 1987), or erroneous mate choice (Butler and Stein 1985; Dame and Petren 2006), heterospecific matings do not actually have to occur for RI to be present. The presence of heterospecifics may influence an organism's reproductive behaviour in more than one way (Miller et al. 2013), especially if they are unable to differentiate between hetero- and conspecifics. For example, studies in *Drosophila melanogaster* have shown that heterospecific song can influence female gene expression and that these changes are broadly comparable to those caused by conspecific song (Immonen and Ritchie 2012).

Context-dependent male mating strategies may be particularly vulnerable to influence from RI. Sperm production and mating both pose non-trivial costs to males (Simmons 2001) and numerous studies have illustrated the interaction between social environment and male sexual behaviour both in insects (Bretman et al. 2009; Wigby et al. 2009; Bailey 2011; Billeter et al. 2012; Bailey et al. 2013; Han and Brooks 2013) and vertebrates (Firman et al. 2013; recently reviewed by Kelly and Jennions 2011). While males may employ a variety of strategies in response to sperm competition (including, but not restricted to: changes in sperm number (Wedell et al. 2002), ejaculation size (Gage 1991; Garcia-Gonzalez and Gomendio 2004); seminal protein composition (Wigby et al. 2009; Perry et al. 2013) and sperm morphology (Gage 1994) behavioural changes have the benefit of being “cheaper” and may allow for more rapid responses to fluctuations in local environment (Bretman et al. 2011). However, failure to discriminate between con- and hetero-specifics may result in males adopting sub-optimal mating strategies, ultimately reducing their fitness.

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

male seed bugs (Hemiptera: Heteroptera: Lygaeidae) to explore both copulation strategies and how males of this group discriminate among competitors.

Lygaeus equestris and *Lygaeus simulans* are closely related species found across mainland Europe. They can be distinguished by the morphology of their antennae (Gusev and Tatarnikov 1992). While the two species can hybridize (Evans 2011) there is evidence for asymmetric pre-zygotic isolation: male *L. simulans* can copulate with *L. equestris* females, but although *L. equestris* males attempt to mate with *L. simulans* females they are usually unable to achieve copulation due to high levels of female resistance (Evans 2011). Additionally, fertilization success is considerably lower in heterospecific crosses; although the exact mechanism behind this remains unknown (Burdfield-Steel et al. 2013, unpublished data). *Spilostethus pandurus* also shares part of its range with *L. equestris* but is far more diverged from *L. equestris* than *L. simulans* and thus completely reproductively isolated. *Oncopeltus fasciatus* is a new world species and would never naturally co-exist with *L. equestris*. While the phylogeny of this group remains to some extent unresolved (Weirauch and Schuh 2011) it is probable that *O. fasciatus* is more distantly related to *L. equestris* than *S. pandurus*. Previous laboratory studies have found evidence of sexual conflict and RI between *L. equestris* and *S. pandurus*. Shuker et al. (in review) showed that *L. equestris* females housed with male *S. pandurus* suffered comparable fitness costs, in the form of reduced egg production and longevity, to those housed with male conspecifics. However, those housed with *O. fasciatus* did not show any such fitness costs. All four species belong to the family Lygaeidae and share a similar, promiscuous, mating system (Sillen-Tullberg 1981). No pre-copulatory courtship has been recorded in these species; instead males rush or jump towards females and grasp them with their legs before initiating genital coupling. If coupling is successful, the pair then moves so they are facing away from each other, end to end (see Fig. 1). Bugs can continue to move and feed in this position, with females typically directing movement and males walking backwards or being dragged along (Walker 1979; Sillen-Tullberg 1981; Shuker et al. 2006); pairs have been observed to remain coupled in this manner for over 15 h (Sillen-Tullberg 1981). As successful insemination has been known to occur from copulations lasting between 1 and 2 h, this prolonged copulation is thought to be a form of mate guarding (McLain 1989; Alcock 1994; Wang et al. 2008). This is further supported by the finding that sperm displacement during second matings may be as high as 90 % and average copulation duration is longer when the local sex-ratio is male-biased (Sillen-Tullberg 1981).

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

If prolonged copulation serves as a form of mate-guarding, we would expect to see longer copulations occurring when the sex ratio is male-biased, due to increased levels of perceived sperm competition. The original paper describing the influence of sex ratio on



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

copulation duration in *L. equestris* (Sillen-Tullberg 1981) used only two treatments, one male with four females, and one female with four males. Thus, it is hard to distinguish the effects of sperm competition versus mate availability on male behaviour in this study. We therefore added a “control” treatment of one male and one female in order to distinguish between these two mechanisms. If male *L. equestris* increase copulation duration only in response to perceived sperm competition then we expect to see no difference in copulation duration between the control and the female-biased treatments. Thus, this “control” treatment is merely to allow us to separate these mechanisms. When looking at male response to heterospecifics the appropriate control is the equivalent sex ratio of conspecifics.

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

Methods

The focal species for this experiment was *L. equestris*. A closely-related species, *L. similans*, and two other Lygaeid species, *S. pandurus* and *Oncopeltus fasciatus*, were used as treatment bugs. We maintained all four species in continuous culture in the laboratory subject to a fixed environmental regime of 22:2 h light:dark cycle and a temperature of 29 °C. These conditions ensure the bugs do not enter reproductive diapause (Shuker et al. 2006). We used 30 cm × 15 cm × 15 cm plastic boxes with ventilated lids as stock cages, and provided organic, dehusked sunflower seeds (*Goodness Direct*, UK) at a depth of 3–4 cm across the bottom of the cage. We also provided two tubes of demineralised water with cotton wool bungs as a water source and changed these tubes weekly. Additionally, a piece of cotton wool was added as three-dimensional substrate for the bugs to hide in.

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

Experiment 1

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

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

Mating time was calculated by adding up the time between the observations. For example, a pair observed copulating at 30 and 60 min were considered to have been copulating for 30 min as this is the minimum time they could have been coupled. Matings that were observed only once (i.e. for only one observation period) were not included. The effect of treatment on the average length of both the first observed copulation, and the longest copulation observed during the 9 h period was tested with a Univariate Analysis of Variance. It should be noted that the first copulations and the longest copulation were sometimes the same so these two measures are not independent. The difference between the treatments was assessed with a LSD post hoc test. All analyses were carried out in PASW Statistics 18 by IBM.

Experiment 2

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

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

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

Results

Experiment 1

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

Experiment 2

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

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

Discussion

Our results suggest that male *L. equestris* are able to correctly distinguish between conspecific rivals and heterospecifics and adjust their behaviour accordingly. However, given that we know that male *L. equestris* do perform interspecific mating attempts, our data show that any form of “species recognition” is context-dependent in this species: males seem to discriminate among species in some contexts, but less so in others. In keeping with

Table 2 Numbers of male and female *S. pandurus* and *O. fasciatus* bugs in each treatment

Treatment	Females (<i>O. fasciatus</i>)	Males (<i>O. fasciatus</i>)	Females (<i>S. pandurus</i>)	Males (<i>S. pandurus</i>)	Total bugs
Female biased (<i>O. fasciatus</i>)	3	0	0	0	5
Male biased (<i>O. fasciatus</i>)	0	3	0	0	5
Female biased (<i>S. pandurus</i>)	0	0	3	0	5
Male biased (<i>S. pandurus</i>)	0	0	0	3	5

All treatments contained one male and one female *L. equestris*

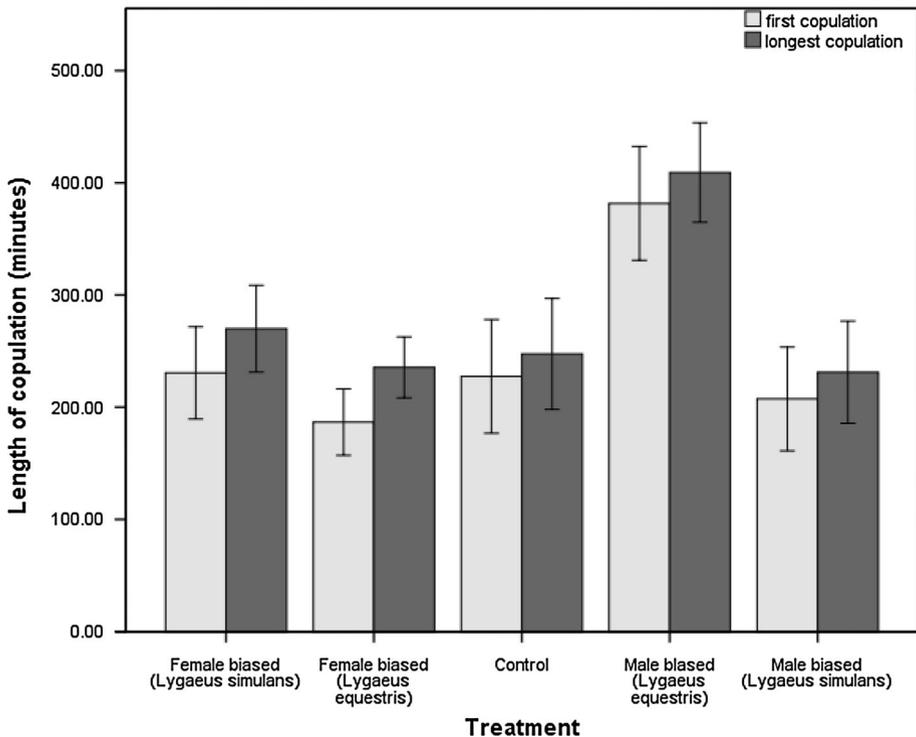


Fig. 2 The mean length of both the first recorded copulations and the longest recorded copulations from each treatment from experiment 1. Error bars indicate ± 1 SE

previous studies (Sillen-Tullberg 1981), male *L. equestris* responded to the presence of multiple male conspecifics by extending the duration of copulation. This supports the hypotheses that extended copulations in *L. equestris* can act as a form of mate guarding (McLain 1989; Alcock 1994). However, no such changes in copulation duration were observed in the presence of multiple male *L. simulans*, *O. fasciatus* or *S. pandurus* despite morphological similarities between *L. equestris* and *L. simulans*, and previous observations that male *S. pandurus* frequently attempt to mate with *L. equestris* females (Shuker et al. in review). Additionally, male *L. equestris* did not adjust copulation duration in response to increased numbers of hetero- or conspecific females. Nor did *L. equestris* males ever

Table 3 Results of a LSD post hoc test on the effect of treatment on the length of first copulation (above the diagonal) and length of longest copulation (below the diagonal)

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

I–J refers to the mean difference between the groups

* Indicates the mean difference is significant at the 0.05 level

successfully pair with *L. simulans* females, although it is possible that heterospecific mating attempts may have occurred during the experiment as not all unsuccessful attempts were recorded.

These findings suggest that heterospecific harassment and mating attempts in this group are likely to be the result of male mating strategy, rather than a breakdown of species discrimination *per se*. As *L. equestris* is a multiply-mating species without parental care, the main limit to male fitness is the number of successful matings they can acquire (Bateman 1948). Thus males are expected to mate opportunistically which may lead them to attempt to mate with females perceived to be of low “quality”, such as heterospecifics, in the absence of other mating opportunities. One way to test this hypothesis would be to include further treatments in which male *L. equestris* were presented with heterospecific, rather than conspecific females. If males are truly opportunistic in their mating behaviour then we would expect to see similar patterns of mate guarding in response to the presence of rival males, regardless of female species. However, clearly males do appear to discriminate between species in some contexts, as suggested here, given that they respond differently to con- and heterospecific males. Thus, it seems that the mating system of encounter promiscuity, rather than species discrimination, may be the driving force behind the RI previously described in this group.

One alternative hypothesis is that the cues used by males to determine female quality or species, and the presence of male rivals are different. If this is the case then males may be more able to distinguish between male con- and heterospecifics than female ones. This is possible given the nature of chemical communication in these insects. Male *O. fasciatus* (as well as those of the related species, *Lygaeus kalmia* and *Neacoryphus bicrucis*) produce a pheromone that attracts both males and females over long distances (Aldrich et al. 1999). It is likely that males of other seed bug species, including those used in this experiment also produce a similar pheromone. Given that males as well as females are attracted to this pheromone it may be that males can also use it to determine the density of rival males in the surrounding area. Further study would be required to determine if males are indeed using these pheromones to inform their mating behaviour, however it presents an intriguing scenario whereby cues used in species discrimination may be sex-specific.

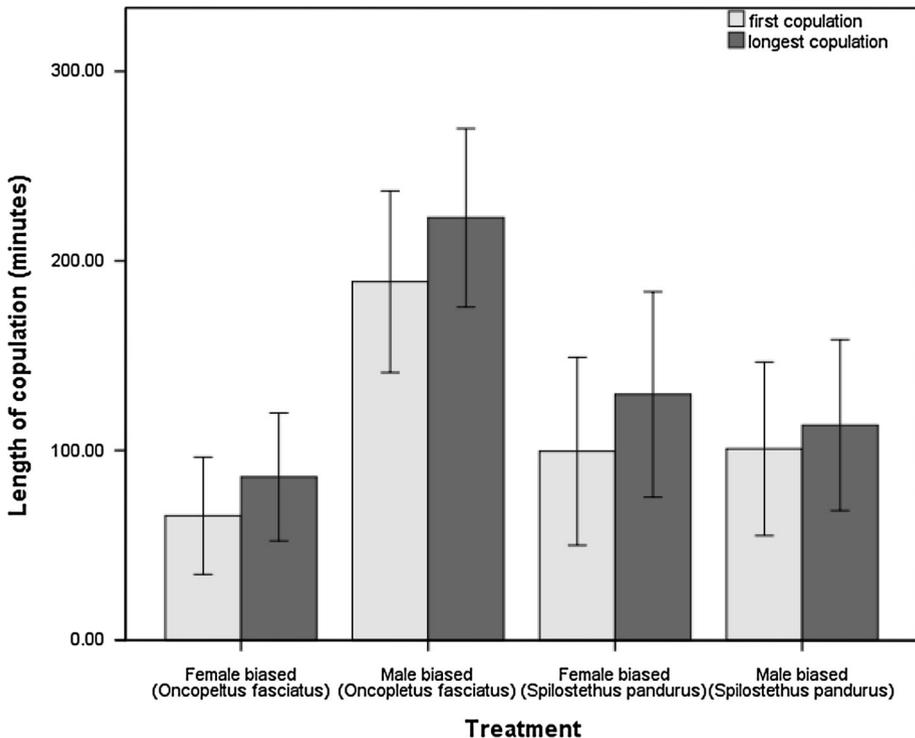


Fig. 3 The mean length of both the first recorded copulations and the longest recorded copulations from each treatment in experiment 2. Error bars indicate ± 1 SE

While male *L. equestris* increased mating duration in the presence of conspecific rivals, they did not show any reduction in copulation duration in the presence of multiple females. Although we might expect to see copulation duration reduced when multiple potential mates are available, there are several possible explanations for the lack of such a pattern in this species. One is that male seed bugs may trade off mating with more females against increased fertilisation rates with each female. Previous studies have found that insemination rates are highest during the first hour of copulation, and matings lasting between 1 and 2 h are sufficient to achieve fertilization in the majority of cases (Sillen-Tullberg 1981, 1985). However, the genitalia of many Lygaeidae, including *L. equestris*, are elongate and complex (Bonhag and Wick 1953; Gschwentner and Tadler 2000; Micholitsch et al. 2000; Higgins et al. 2009; Aspiras and Angelini 2011) and the delay in insemination may be a result of the time taken for the male processus to traverse the female reproductive tract to reach the spermatheca. This, combined with the high levels of mating failure observed in this group (Tadler et al. 1999; Gschwentner and Tadler 2000; Micholitsch et al. 2000), suggests that males may make significant investment in copulation duration due to processes of post-copulatory sexual selection (or “cryptic female choice”: Eberhard 1996). As such, even if other females are in the environment, minimum copulation duration may still represent a significant time investment for males. Patterns of post-copulatory sexual selection are currently being explored in this group. However, the average length of the

first and longest copulations (approximately 200 min) was greater than the 2 h suggested to be necessary for insemination.

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

While this paper has discussed the implications of species discrimination in the context of RI, it should also be noted that many organisms use social information in contexts other than mating. Species discrimination may also play an important role in processes such as choosing feeding or oviposition sites. One example is in the cactus bug, *Narnia femorata*, where the presence of juvenile heterospecifics influences egg laying, overriding the effect of resource quality (Miller et al. 2013). Thus, if organisms do show context-dependant species discrimination, be it as a result of different available cues, or adaptational trade-offs acting on behaviour, then there is the potential for this to impact many aspects of their life-history.

Conclusion

Male *L. equestris* appear to correctly distinguish between rival and heterospecific males in the context of phenotypically plastic copulation behaviour. This, combined with the observations that males will often attempt copulation with female heterospecifics in the absence of conspecific females (Shuker et al. in review), suggests that while males are able to distinguish even closely-related heterospecifics from their own species, they may use this information differently in different contexts. Such context-dependent species discrimination could greatly influence inter-species interactions, both in the context of RI, or more broadly as social information is used by animals to inform many different behaviours. We hope that our work will extend our view of species recognition and the importance of context-dependence.

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