



Precopulatory sexual selection in the seed bug *Lygaeus equestris*: a comparison of choice and no-choice paradigms



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Mate choice has long been appreciated as a key component of sexual selection. However, how we measure mate choice, both in the field and in the laboratory, remains problematic. Mating preferences may be tested using either no-choice or choice tests, but explicit comparisons between these two experimental paradigms remain limited. It has been suggested that preferences may be stronger in choice tests as they allow simultaneous comparison, and some studies have indeed found stronger mating preferences in choice tests compared to no-choice tests. Here we explicitly tested the effect of experimental choice paradigm on the measurement of sexual selection on male and female morphology in the promiscuous seed bug *Lygaeus equestris* (Heteroptera, Lygaeidae). We performed mating trials in which we varied the amount of choice presented to each sex, giving four choice treatments: no-choice, male choice, female choice and mutual choice. Overall we found evidence for significant positive directional selection on female body length and stabilizing selection on an overall measure of male body size. However, we found no significant effect of choice paradigm on the patterns of sexual selection for males or females. We suggest this may be because of the method of mate assessment in *L. equestris*, which appears to be primarily via contact cues, which may limit simultaneous comparison between options.

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Mate choice is a central component of sexual selection theory (Andersson, 1994; Darwin, 1871), and understanding mate choice and the underlying preferences that lead to choice are therefore central to understanding sexual selection. However, it is still not clear to what extent mating preferences identified in the laboratory may depend on the experimental paradigms used to test for these preferences, which can lead to difficulties in predicting the strength and direction of preferences, and their consequences, in natural populations (Wagner, 1998). Mating preferences may be tested using two alternative experimental paradigms: no-choice tests or choice tests. In choice tests, subjects are given a choice between multiple (usually two) stimuli presented simultaneously, whereas in no-choice tests each subject is presented with a single stimulus. Choice tests allow for comparisons between options, and so can only test for relative, directional preferences between two stimuli (MacLaren & Rowland, 2006; Wagner, 1998). However, they may allow greater resolving power in terms of determining the strength of behavioural responses (Doherty, 1985; Wagner, 1998). In contrast, no-choice experiments test for absolute

preferences in traits, and have the added complication that the perceived mate encounter rate is lower, which might suggest that rejection of a mate in a no-choice test carries the added risk of lost mating opportunities in the future (Barry & Kokko, 2010; Booksmythe, Jennions, & Backwell, 2011; Werner & Lotem, 2006). This means that choices displayed in these tests may be especially robust in that they persist despite this potential extra cost.

Several studies have tested for mating preferences using both no-choice and choice tests within the same species. In comparing the two paradigms, studies frequently find stronger mating preferences in choice tests (e.g. Barry, Holwell, & Herberstein, 2010; Booksmythe et al., 2011; MacLaren & Rowland, 2006; Owen, Rohrer, & Howard, 2012), although in other cases the level of choice available had little effect on the strength of preference detected (e.g. Gershman & Sakaluk, 2009; Jang & Gerhardt, 2006; Jordan & Brooks, 2011). However, in none of these studies was the effect of choice paradigm explicitly tested statistically, for instance by testing for an interaction between choice paradigm and a trait presumed to be the target of sexual selection.

In this study, we investigated precopulatory sexual selection in the seed bug *Lygaeus equestris* (Heteroptera: Lygaeidae). Both males

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and females mate multiply, and copulations may last in excess of 16 h (Shuker, Ballantyne, & Wedell, 2006; Sillén-Tullberg, 1981), a common duration in the Heteroptera (Alcock, 1994). Sperm transfer occurs mainly in the first few hours of copulation (Micholitsch, Krugel, & Pass, 2000; Sillén-Tullberg, 1981), and long copulations probably serve as a form of postcopulatory mate guarding (Alcock, 1994), with males acting as a 'living mating plug' (Sillén-Tullberg, 1981). Copulation appears to be initiated without much in the way of courtship: the male makes a sudden dash for the female and attempts to couple (Sillén-Tullberg, 1981).

Previous studies of sexual selection in other species of Lygaeidae have shown a general pattern of selection favouring larger males and females. For instance, there is directional selection on male body length in *Neocoryphus bicrucis* (McLain, 1992), male preference for wider abdomens and longer ovipositors in females in *Nysius huttoni*, and female preference for longer antennae and larger genitalia in males in the same species (Yang & Wang, 2004). However, studies in the sister species to *L. equestris*, *Lygaeus simulans*, found no evidence for selection on male body length, but did find evidence for stabilizing selection on a component of the male genitalia, processus gonopori length (Tadler, 1999; Tadler, Nemeschkal, & Pass, 1999). *Lygaeus equestris* does not show obvious sexual ornamentation but there is clear sexual dimorphism in body size, and a previous study has shown a male preference for large females in terms of mating investment via increased copulation duration (Sillén-Tullberg, 1981). A recent study has also shown that males fed sunflower seeds, *Helianthus annuus*, have higher mating success than those fed milkweed seeds, *Asclepias syriaca*, despite sunflower-fed males having shorter bodies on average (Burdfield-Steel, Dougherty, Smith, Collins, & Shuker, 2013).

The aims of our study were (1) to quantify the strength of precopulatory sexual selection on male and female morphology in *L. equestris*, and (2) explicitly to test how this selection varies depending on experimental choice paradigm. We performed mating trials in which we varied the amount of choice available to both males and females, by presenting individuals with either one or two individuals of the other sex. This gave us four experimental treatments allowing: (1) no-choice for either males or females (one male and one female per trial); (2) female choice only (two males and one female per trial); (3) male choice only (one male and two females per trial); and (4) mutual mate choice (two males and two females per trial). We used successful mating, and also subsequent female fertility, as proxies for fitness. Experimental paradigm may influence the patterns of both intersexual choice and intrasexual competition, which will both influence the strength of sexual selection detected. To our knowledge there have been no explicit tests of the effect of experimental paradigm on the measurement of sexual selection. We predicted that large females would be preferred by males, as large females have greater potential fecundity (Honěk, 1993). Males and females exhibit copulatory struggles, and so we also predicted that larger males would gain more matings, leading to sexual selection on male body length and leg length. If the experimental paradigm influences sexual selection, our key prediction was that we should see a significant interaction between experimental paradigm and selection on morphological traits. Specifically, we predicted that sexual selection on both males and females would be stronger in the choice treatments than the no-choice treatment (see above).

By allowing individuals of the same sex to interact, the different paradigms also vary the level of potential intrasexual competition among males and females, so that any sexual selection measured may be the result of both intersexual choice and intrasexual competition. We therefore also recorded behavioural interactions between individuals to attempt to control for this. Comparison of

sexual selection resulting from all four experimental paradigms coupled with behavioural observations should allow us to start to disentangle the effects of choice, competition and conflict on mating success.

METHODS

Insect Husbandry

We maintained populations in the laboratory in continuous culture at 29 °C, with a 22:2 h light:dark cycle to prevent individuals from entering diapause. We separated second-instar nymphs into small plastic deli tubs (108 × 82 mm and 55 mm high), with five nymphs to a tub (mixed sex). Each tub contained at least 10 g of husked sunflower seeds (this represents ad libitum food), a small glass tube filled with distilled water stopped with cotton wool, and dry cotton wool for shelter. Nymphs were moved to a fresh tub with fresh seeds and water once a week, except for the first week when nymphs were too small to move. *Lygaeus equestris* takes around 2 weeks to grow from the second instar to the adult stage, and adults are sexually mature after a further 7 days. All the tubs were checked every day for newly eclosed adults, which were then sexed and moved into new tubs (same-sex) with seeds and water, again containing no more than five individuals per tub.

Mating Trials

All adults were marked with paint at least 1 day before mating trials. The adults were placed in a –13 °C freezer for 3 min, and then marked on the pronotum with a small dot of enamel paint (Plasti-Kote 'Projekt paint' Fast Dry Enamel) using a fine paintbrush, under a dissecting microscope. Individuals were marked on either the right or left side of the pronotum, so that when there was more than one individual of a sex in a dish individuals marked on alternate sides were used. The side on which each individual was marked can be clearly seen during mating trials.

All adults used in mating trials were 7 days old. All trials were started between 0900 and 1000 hours, and performed at room temperature (23–25 °C) under natural light, in small plastic petri dishes (55 mm diameter). On the morning of a trial individual males and females were assigned randomly to one of four experimental treatments: (1) one male and one female per dish: no choice for males or females; (2) two males and one female per dish: no choice for males, choice for females; (3) one male and two females per dish: choice for males, no choice for females; (4) two males and two females per dish: choice for both males and females.

For the mating trials all dishes were watched continuously for 2 h. For each dish we scored the number of mating attempts performed by each male, and towards which female the attempts were directed. Successful copulations were recorded, as well as copulation duration (if any pairs separated within 2 h). Individuals were classed as being in copula when they were first seen in the end-to-end position, and the male aedeagus was properly inserted (copulating pairs were checked by eye to ensure successful intromission; this can be done without disturbing the dish). After 2 h any noncopulating individuals were removed from the dishes and frozen. Copulating pairs were left in their dish and checked every 30 min until they separated naturally, or until 6 h after the trial when they were separated manually (these were classed as 'long copulations', see Results). This was done by gently brushing the pair with a fine paintbrush to initiate uncoupling. All mated males were immediately frozen. Mating trials were performed over 28

(nonconsecutive) days. The trials comprised 688 individuals in total: 344 males and 344 females. We observed 234 dishes in total: 69 dishes of the no-choice treatment and 55 dishes of the remaining three treatments.

Mated females were placed into individual tubs (with water and seeds) to allow oviposition. These female tubs were then checked every day for the presence of fertile eggs (eggs change from white to orange if fertile), to ensure copulation was successful. If no fertile eggs were seen after 2 weeks, copulation was assumed to be unsuccessful. All mated females were frozen after 2 weeks.

Morphometric Measurements

After all trials were finished, the following morphometric traits were measured for all individuals used in mating trials: total body length, antennae length (when possible), and tibia and femur lengths for all three legs on the left-hand side (when viewed dorsally). All lengths were measured using a dissecting microscope with a measuring graticule. Total body length was measured as the tip of the snout to the tip of the underside of the abdomen. Legs and antennae were removed from the body and laid flat before measuring. Sample size for morphological measurements was 613, comprising 303 males and 310 females (some individuals were mistakenly discarded prior to measurement), except for antennae length measures (and measures of 'overall size', see below), where sample size was slightly smaller (605 in total, 300 males and 303 females).

To check the repeatability of our eyepiece measurements, L.R.D. took 30 individuals and remeasured their body length the day after the first measurement, blind to the original measure. This measure was highly repeatable, with a strong significant positive correlation between measures taken on the 2 days (Pearson correlation coefficient: $r_{28} = 0.99$, $P < 0.001$). Repeatability was also assessed using analysis of variance following [Lessells and Boag \(1987\)](#) and shown to be high ($r = 0.99$).

Statistical Analysis

Data on the observed number of mating attempts per male did not follow a normal distribution (including after transformation), and so were analysed using nonparametric statistics. To control for the different numbers of individuals present in each treatment, we randomly allocated one individual of each sex as the 'focal' individual, so that each dish contained a single focal male and female. We used these focal individuals when analysing mating frequency and mating attempts data.

For treatments 2, 3 and 4 each dish contained two individuals of at least one sex. For these dishes the formation of a mating pair could influence the likelihood of mating for the unpaired individual; thus individuals of the same sex cannot be considered independent of each other. To control for this in our sexual selection analysis we also analysed the data using a mixed-model approach, fitting dish as a random effect (i.e. generalized linear mixed-effects models, GLMM) using the software packages SPSS Statistics 21 ([IBM Corp., 2012](#)) and the lme4 package ([Bates, Maechler, Bolker, & Walker, 2013](#)) in R 2.15.1 ([R Development Core Team, 2012](#)). All models gave very small or negligible variance components associated with the random effect.

We tested for the effect of morphology on mating success using two main approaches. Given the strong correlations between morphological traits (including body length: $r > 0.53$, $P < 0.001$ in all cases), we first analysed the data in terms of sexual selection on male and female body length only. We used a generalized linear mixed model with a binary logistic response,

with mating success as the response variable. To examine the effect of choice paradigm, we fitted choice treatment as a main effect with four levels. Significant treatment*morphology interactions would suggest that choice paradigm influences the patterns of sexual selection.

Second, although the morphological measurements were highly correlated, males and females differed in their relationships between body length and other morphological characters: males had relatively longer antennae and leg lengths for their body size (ANCOVAs, main effect of sex after fitting body length as a covariate: antennae: $F_{1,602} = 180.62$, $P < 0.001$; prothoracic legs: $F_{1,610} = 515.42$, $P < 0.001$; mesothoracic legs: $F_{1,610} = 257.48$, $P < 0.001$; metathoracic legs: $F_{1,610} = 41.69$, $P < 0.001$). To understand selection on morphology better, we therefore performed a principal component (PC) analysis to gain an estimate of overall body size that takes these differences into account and also reduces the number of variables in our model. Principal components were extracted from all five morphological traits measured (body length, antenna length, prothoracic leg length, mesothoracic leg length, metathoracic leg length) for males and females separately. Principal component (covariance matrix) scores were extracted using the Anderson–Rubin method. For both males and females only one principal component had an Eigenvalue greater than one. For males, the first principal component explained 70.3% of the variance observed (eigenvalue = 3.51). For females, the first principal component explained 71.5% of the variance observed (eigenvalue = 3.57). For both males and females the principal component loaded heavily on all five morphological traits (all factor loadings above 0.7), with body length loading highest (loading = 0.95 for females and 0.91 for males). These components can therefore be seen as a measure of 'overall size'. We then repeated the mating success analysis as before, but using the principal component of overall size in place of body length for each sex. To facilitate testing quadratic terms for overall size, we made PC values positive by adding 4 to all values.

Finally, we also visualized the shape of sexual selection on male and female body length and overall size using cubic splines ([Schluter, 1988](#); [Schluter & Nychka, 1994](#)). Splines were fitted using a general additive model with mated status as a binary logistic response variable.

We could not follow all copulations fully, and so we classed copulations as either long or short. Long copulations were those that had to be broken up manually at the end of the day. Short copulations were those that finished naturally. Matings shorter than 15 min were excluded from the analysis (sperm transfer takes at least 30 min; [Micholitsch et al., 2000](#)). There were no matings between 15 and 45 min in duration. We tested for determinants of copulation duration using a generalized linear model (GLM) with copulation class (short or long) as a binary logistic response variable, for all mated pairs, using male and female overall size as the only morphological trait in the model. We tested for determinants of female fertility in the same way, using a general linear model with female fertility (presence or absence of fertile eggs) as a binary logistic response variable.

We used a modified model simplification rationale in an attempt to balance the problem of multiple testing associated with model simplification ([Mundry & Nunn, 2009](#); [Whittingham, Stephens, Bradbury, & Freckleton, 2006](#)) with the problem of overparameterizing models, especially when testing several interaction terms. As such, models were first fitted with main effects and any relevant interaction terms (including quadratic terms for our morphological characters of interest, to test for evidence of nonlinear selection). Nonsignificant interactions and quadratic terms were then removed in a stepwise fashion, with all main

effects left in the final model regardless of significance, and the significance of remaining terms tested using type III sums of squares. All statistical analyses were performed in SPSS Statistics 21 (IBM Corp., 2012), except for cubic spline plots and GLMM analyses which were performed in R version 2.15.1 (R Development Core Team, 2012).

RESULTS

Male Mating Attempts

There was no significant difference between treatments in the mean number of total attempts per male (Kruskal–Wallis test: $H_3 = 6.44$, $P = 0.09$). However, the number of mating attempts females received varied with how many males and females were in each treatment ($H_3 = 50.82$, $P < 0.001$). Females received a median of three attempts in treatment 1 and treatment 4 (IQR = 1–6 in both cases), six in treatment 2 (IQR = 2.5–12.5) and one in treatment 3 (IQR = 0–3).

Mating latency varied significantly among the four treatments (ANOVA: $F_{3,150} = 3.34$, $P = 0.021$), with copulation taking longer to initiate on average in treatment 2 (approximately 39 min compared to 25–27 min in the other treatments); observations suggested that this was due to longer male struggles for access to the lone female in treatment 2.

Mating Frequency

Mating trials resulted in 169 mated females (49% of total), with one female mating with two different males, giving 170 matings overall. However 54 females received no mating attempts. Therefore 58% of females that received mating attempts copulated. In treatments with two males present, males were frequently seen to attempt to mate with already mating females, but seemed unable to break up mating pairs.

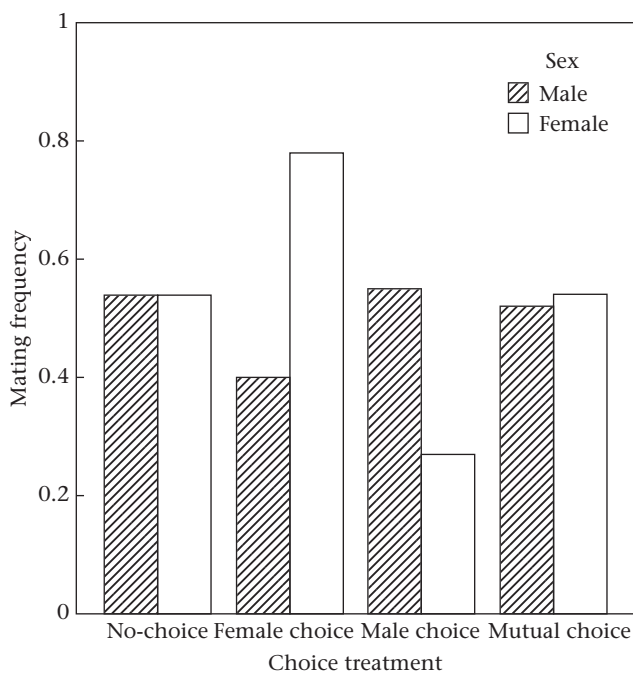


Figure 1. Mating frequency for focal males and focal females for the four experimental treatments. For both sexes, $N = 69$ for treatment 1 (no-choice) and 55 for the other three treatments.

Focal mating frequency was similar for both males and females in the two equal sex ratio treatments (treatments 1 and 4, Fig. 1). Focal male mating frequency was lower in treatment 2 (34%) than in treatments 1, 3 and 4 (around 53%; Fig. 1), but the difference was not significant (chi-square test: $\chi^2_3 = 6.16$, $P = 0.10$). Focal female mating frequency reflects the differences in sex ratio between the treatments (Fig. 1): mating frequency was significantly higher in treatment 2 (78%), where the sex ratio was male biased; and lower in treatment 3 (31%), where the sex ratio was female biased ($\chi^2_3 = 25.13$, $P < 0.001$).

Morphology and Mating Success

Choice treatment did not affect the strength of selection on female body length (binary logistic GLMM, interaction between treatment and female body length: $F_{3,302} = 1.01$, $P = 0.39$). There was significant positive sexual selection on female body length (Fig. 2a, Table 1). Female mating success was significantly affected by choice treatment (Table 1): as in our previous analysis, females were more likely to mate in treatment 2 and less likely to mate in treatment 3 (see above). Male mating success was not associated with treatment (Table 1), male body length (Table 1, Fig. 2b) or their interaction (interaction between treatment and male body length: $F_{3,295} = 0.95$, $P = 0.42$).

When 'overall size' was used in place of body length, there was no sexual selection on female overall size (Fig. 2c, Table 2). Choice treatment again did not affect the strength of selection on female size (interaction between treatment and female overall size: $F_{3,297} = 1.33$, $P = 0.27$) but was significantly associated with female mating success (Table 2). For males, there was significant nonlinear selection on overall size (quadratic term: Fig. 2d, Table 2), and there was a marginally significant effect of choice treatment on the likelihood of mating for males (Table 2). However there was no significant effect of choice treatment on the strength of this selection (interaction between treatment and male overall size: $F_{3,288} = 0.85$, $P = 0.47$; interaction between treatment and male overall size squared: $F_{3,288} = 0.83$, $P = 0.48$).

Copulation Duration

There were 170 matings overall, comprising 69 short copulations (range 50–450 min, median = 290 min, IQR = 130–395 min) and 101 long copulations (range 360–475 min, median = 450 min, IQR = 425–460 min). Separating pairs at the end of the day led to a large spike in frequency at around 7 h, and gives an underestimate of the actual copulation duration (Appendix Fig. A1). There was no significant difference between treatments in the duration of short (Kruskal–Wallis test: $H_3 = 0.34$, $N = 69$, $P = 0.95$) or long copulations ($H_3 = 5.46$, $N = 101$, $P = 0.14$).

We obtained full morphological measurements for 148 mated pairs, comprising 85 long and 63 short copulations. Females with a larger overall size were more likely to have long copulations (Table 3). Male overall size, on the other hand, was not associated with the likelihood of having long copulations (Table 3). Choice treatment also had no effect on the likelihood of having long copulations (Table 3).

Female Fertility

One hundred and six mated females (62% of mated females) laid fertile eggs within 2 weeks of mating (i.e. a 'mating failure' rate of 38%). Females that copulated for longer were more likely subsequently to lay fertile eggs (Table 4). Females that copulated for less than 400 min rarely laid fertile eggs (Appendix Fig. A2). Larger

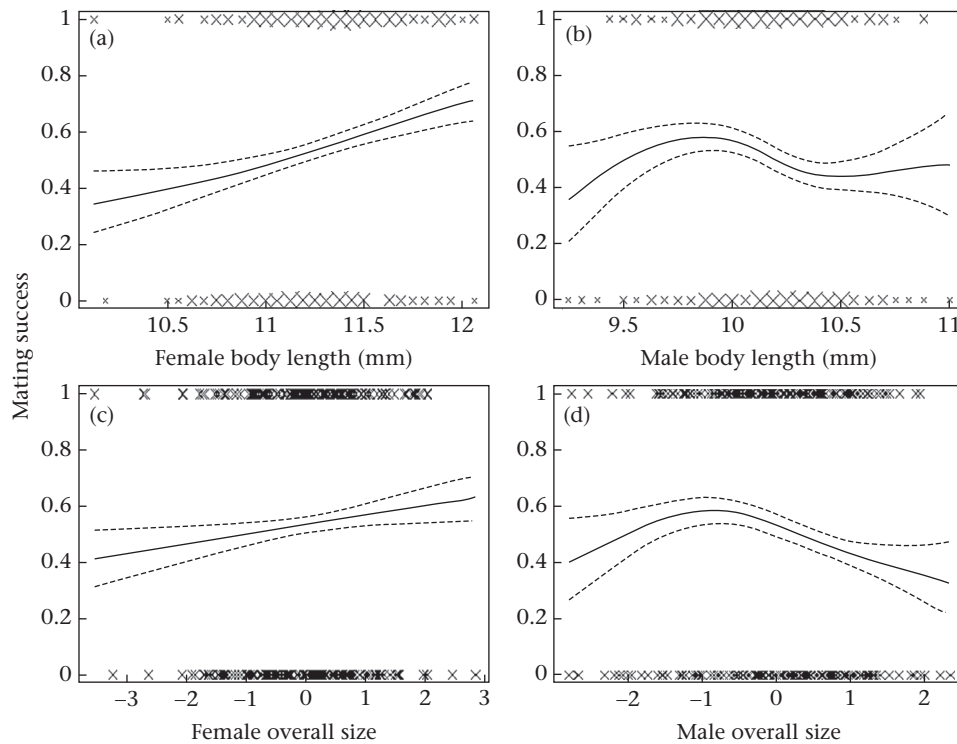


Figure 2. Visualization of sexual selection in terms of mating success on male and female morphology across all four choice treatments (see text for details). Curves were estimated from binomial data (mated or nonmated) using general additive models, and curves drawn using cubic splines. Dashed lines indicate 1 SE above and below the predicted line. Curves show selection on (a) female body length, (b) male body length, (c) female ‘overall size’ (first principal component of five morphological traits, see text for details), and (d) male ‘overall size’. For (a) and (b) cross size indicates the number of data points for each body length increment (between 1 and 20).

females were also more likely to lay fertile eggs, independent of copulation duration (Table 4). Male overall size was not associated with female fertility (Table 4). Males did not have to copulate for as long to fertilize eggs of small females compared to those of large females (interaction between female overall size and copulation duration, Table 4). Finally, there was no significant effect of choice treatment on the fertility of mated females, but there was a significant interaction between treatment and female overall size (Table 4).

DISCUSSION

The aim of our study was to characterize the strength and direction of sexual selection on male and female morphology in *L. equestris*, and explicitly test whether the degree of choice influences the measurement of this selection. We found no

significant effect of experimental paradigm on the strength of precopulatory sexual selection on male or female morphology. This was despite finding significant selection on male overall size and female body length. Visualizing the shape of selection in terms of mating success indicated positive linear selection for larger female body length (Fig. 2a) and weak nonlinear selection on male overall size (Fig. 2d). Male preference for larger females is probably due to the increased female fecundity associated with body size (Honěk, 1993), and is commonly seen in insects (Bonduriansky, 2001). The measure of overall size takes into account body length plus antenna and leg lengths (and so perhaps capture more of the insect’s ‘shape’). Body length in isolation was not a significant indicator of the likelihood of mating for males. However, we predicted that larger males should be more

Table 1

Results of GLMMs testing the effect of body length and choice treatment on mating success for both males and females

	Females				Males			
	F	df1	df2	P	F	df1	df2	P
Corrected model	10.03	4	305	<0.001	1.71	4	298	0.15
Treatment	12.09	3	305	<0.001	1.88	3	298	0.13
Body length	7.48	1	305	0.007	1.55	1	298	0.21

Mated status was included as a binary logistic response variable (mated or nonmated). Dish was fitted as a random effect, and for both models the variance component associated with the random effect was negligible. Models were run separately for males ($N = 303$ males, 209 dishes) and females ($N = 310$ females, 219 dishes). Quadratic terms and interaction terms were all nonsignificant and so were dropped from the models.

Table 2

Results of GLMMs testing the effects of ‘overall size’ and choice treatment on mating success for both males and females

	Females				Males			
	F	df1	df2	P	F	df1	df2	P
Corrected model	9.04	4	300	<0.001	2.59	5	294	0.026
Treatment	11.75	3	300	<0.001	2.54	3	294	0.057
Overall size	1.75	1	300	0.19	3.67	1	294	0.056
Overall size ²	—	—	—	—	4.88	1	294	0.028

Mated status is a binary logistic response variable (mated or nonmated). The variable ‘overall size’ is the first principal component of the five morphological traits measured for each individual. Dish was fitted as a random effect, and for both models the variance component associated with the random effect was negligible. Models were run separately for males ($N = 300$ males, 209 dishes) and females ($N = 305$ females, 219 dishes). Interaction terms were all nonsignificant and so were dropped from the models.

Table 3
Results of a GLM testing for determinants of copulation duration for all mated pairs ($N = 148$)

	Likelihood ratio χ^2	df	P
(Intercept)	3.07	1	0.08
Treatment	6.53	3	0.089
Male overall size	1.75	1	0.19
Female overall size	6.97	1	0.008

Copulation duration is a binary logistic response variable (long or short). The variable 'overall size' is the first principal component of the five morphological traits measured for each individual. Quadratic terms and interaction terms between body lengths and treatment were all nonsignificant, and so were dropped from the model.

Table 4
Results of a GLM testing for determinants of female fertility for all mated pairs ($N = 148$)

	Likelihood ratio χ^2	df	P
(Intercept)	5.9	1	0.015
Treatment	1.29	3	0.73
Copulation duration	40.99	1	0.001
Male overall size	1.78	1	0.18
Female overall size	8.86	1	0.003
Treatment*Female overall size	9.22	3	0.027
Copulation duration*Female overall size	12.06	1	0.001

Female fertility is a binary logistic response variable (presence or absence of fertile eggs). Copulation duration was added to the model as a covariate. The variable 'overall size' is the first principal component of the five morphological traits measured for each individual. All nonsignificant quadratic and interaction terms were dropped from the model, including quadratic effects.

successful in achieving matings because of the observed copulatory struggles in this species. This is clearly not the case, perhaps suggesting that male contributions to mating struggles may not be that important.

Although some previous studies have considered different mate choice paradigms, this is the first experiment to our knowledge that has attempted explicitly to test the effect of experimental paradigm on the measurement of sexual selection statistically, manipulating both male and female choice. A similar design was used by Coyne, Elwyn and Rolán-Alvarez (2005) to study sexual isolation (rather than sexual selection per se) between two species of *Drosophila*. They measured the frequency of conspecific and heterospecific matings observed under the choice paradigms used in this experiment (no-choice, male choice, female choice and mutual choice), but using a mutual choice design with 30 males and 30 females of both species that was not strictly comparable with the other treatments. This mutual choice treatment was designed to mimic mating aggregations observed in other species of *Drosophila* in the wild (Coyne et al., 2005). They found that the frequency of heterospecific matings was higher in the no-choice treatment compared to all three choice treatments, leading to reduced sexual isolation between the two species.

Although differing availability of choice has been shown to influence the measurement of mate choice in other species (see above), in the current experiment we found no significant effect of choice paradigm on any aspects of sexual selection. It is unclear why this is the case. One possible explanation would be that in *L. equestris* simultaneous assessment by either males or females is simply not possible, so that the number of choices available is irrelevant to the choosing process. If this is the case all mates will be assessed sequentially, irrespective of the number of options presented. Possible mechanisms of signalling over short distances have not been investigated in any lygaeid bugs

(although long-range attractant pheromones may be common; Aldrich, 1995; Aldrich, Oliver, Taghizadeh, Ferreira, & Liewehr, 1999). However our observations in this species suggest it does not play a large role. For example, individual males frequently appeared unaware of the presence of females in petri dishes until the pair came into contact with each other. This suggests that conspecific communication is primarily through direct contact only, with recognition of individuals probably determined using cuticular hydrocarbons (CHCs; Howard & Blomquist, 2005). There has been little study of contact communication in the Lygaeidae or Heteroptera in general (but see Drijfhout & Groot, 2001; Jackson, 1983), although CHC composition has recently been shown to mediate mate recognition in a reduviid bug (Cocchiararo-Bastias, Mijailovsky, Calderon-Fernández, Figueiras, & Juárez, 2011).

Thus if contact is needed for mate discrimination, it is important to know whether females in treatments 2 and 4 frequently received mating attempts from both males (allowing comparison through direct contact) and whether males in treatments 3 and 4 made attempts with both available females. If we consider only focal individuals, then 42% (46 of 109) of focal males were observed to direct mating attempts at both females in the dish (treatments 3 and 4), and 54% (59 of 109) of focal females were observed to receive attempts from both males present in the dish (treatments 2 and 4). Therefore in around half of all individuals there was a direct opportunity to compare the two mate options in the dish.

There are two features of our study system that our experimental design did not address. First, individuals of the same sex were allowed to interact freely during choice trials. Although we observed no obvious interactions between members of the same sex (such as overt aggression or contest behaviour), males may interfere with each other while trying to mount females and so we cannot rule out subtle mechanisms of intrasexual competition that might have affected our results (Wong & Candolin, 2005). Although we never saw any obvious aggressive interactions between males (or between females), we did observe male struggles for access to females, which overall led to an increased latency to mate in the male-biased treatment (treatment 2). However, the low frequency of such encounters, plus the fact that there was no significant difference between paradigms, suggests to us that intrasexual competition is a small contributor to the results observed. Second, our measure of preference does not preclude forced copulations by males. In this case male mating success may not arise because of female mating preferences, but may lead to sexual selection on males none the less. Assigning complete agency to males or females is a common problem in insect mating systems in which pairing occurs before any obvious courtship takes place (Shuker & Day, 2002). Indeed, obtaining a measure of mating preferences may be very difficult in this species if mate choice requires that individuals interact (see Martel & Boivin, 2011).

In addition to mating success, we also scored fertilization success. Larger females (in terms of overall size) were more likely to lay fertile eggs irrespective of copulation duration. This may be because larger females are more fecund, and is probably the primary reason males show a preference for larger females. In contrast, we found no significant association between male morphology and fertilization success. Including a measure of fitness beyond mating may be especially important in species such as *L. equestris* in which infertile matings are common, so that

mating success may not correlate with reproductive success. The extent to which females influence this pattern of male fertilization success (through processes of cryptic female choice: Eberhard, 1996) remains unclear and is currently under investigation. We did find a significant interaction between female overall size and copulation duration, such that males did not have to mate as long to achieve similar levels of fertilization with small females compared to large ones. This may be for mechanical reasons, for example if it takes longer for sperm to reach the spermatheca or for the male intromittent organ to travel along the female reproductive tract in larger females.

In conclusion, our results suggest that choice paradigm may not always influence the measurement of mate choice or sexual selection, especially in species in which mate assessment or sampling methods do not require (or may even preclude) simultaneous comparison of mates. Indeed, the extent to which some species compare potential mates simultaneously, even in those for which simultaneous assessment is possible, is still unclear (Bateson & Healy, 2005; Coyne et al., 2005; Gibson, 1996; Kacelnik, Vasconcelos, Monteiro, & Aw, 2011). Experimental design may not influence the level of choice detected in all species, but understanding when it does will be informative, for instance if differences are due to the mechanisms by which different species compare and choose potential mates. Only by identifying and testing the most relevant experimental paradigm or paradigms for a given species will studies of mate choice make real progress.

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Appendix

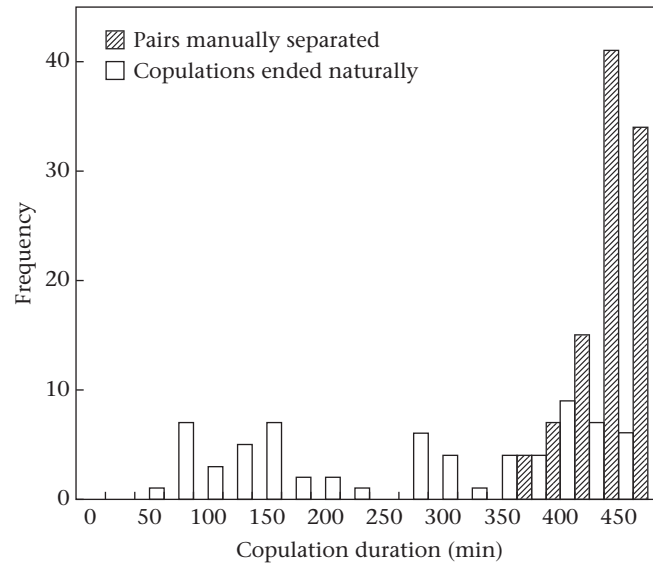


Figure A1. Copulation duration frequency for all mated pairs ($N = 170$), across all treatments. Copulations either ended naturally during the trial (short copulations, $N = 69$) or had to be ended by separating pairs manually (long copulations, $N = 101$).

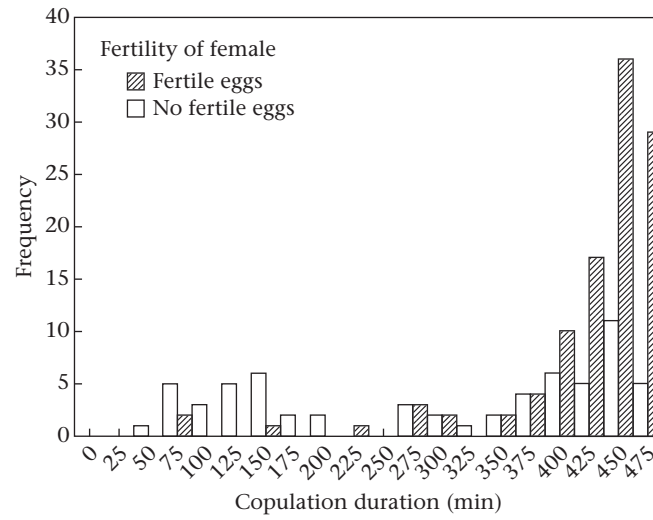


Figure A2. Copulation duration frequency for pairs in which females laid fertile eggs after mating ($N = 106$ pairs) and pairs in which females did not lay fertile eggs ($N = 63$ pairs).