Current Biology

Sex Ratio Bias Leads to the Evolution of Sex Role Reversal in Honey Locust Beetles

Highlights

- Females evolved to be more attractive and competitive under strong mate competition

- Under biased sex ratios, female traits evolved more than male traits

- Females with more intense courtship displays did not produce more offspring

- We found no evolution of male investment in ejaculates across treatments

Authors

Karoline Fritzsche, Isobel Booksmythe, Göran Arnqvist

Correspondence

fritzsche.karoline@gmail.com

In Brief

Fritzsche et al. present the first experimental evidence that female-biased sex ratios generate the evolution of female-specific mating behavior and that this can even modify sex roles, as hypothesized by Charles Darwin. They also demonstrate that female sexual behavior evolved more than male behavior.

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Report

Sex Ratio Bias Leads to the Evolution of Sex Role Reversal in Honey Locust Beetles

Karoline Fritzsche,[1,4,*] Isobel Booksmythe,[2] and Göran Arnqvist[3]
[1]Department of Zoology, University of Graz, Universitätsplatz 2, 8010 Graz, Austria
[2]Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[3]Animal Ecology, Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18D, 752 36 Uppsala, Sweden
[4]Lead Contact
*Correspondence: fritzsche.karoline@gmail.com
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SUMMARY

The reversal of conventional sex roles was enigmatic to Darwin, who suggested that it may evolve when sex ratios are female biased [1]. Here we present direct evidence confirming Darwin's hypothesis. We investigated mating system evolution in a sex-role-reversed beetle (*Megabruchidius dorsalis*) using experimental evolution under manipulated sex ratios and food regimes. In female-biased populations, where reproductive competition among females was intensified, females evolved to be more attractive and the sex roles became more reversed. Interestingly, female-specific mating behavior evolved more rapidly than male-specific mating behavior. We show that sexual selection due to reproductive competition can be strong in females and can target much the same traits as in males of species with conventional mating systems. Our study highlights two central points: the role of ecology in directing sexual selection and the role that females play in mating system evolution.

RESULTS AND DISCUSSION

> If we might assume that the females have become much more numerous than the males [...], then it is not improbable that the females would have been led to court the males, instead of being courted by them. — Charles Darwin, *The Descent of Man and Selection in Relation to Sex*, 1871

The conventional animal mating system, with showy males and choosy females, is reversed in a minority of taxa. In such sex-role-reversed mating systems, females instead court males and are sometimes equipped with elaborated ornaments [2] while males are the more discriminating sex and may reject female mating attempts [3]. Darwin [1] postulated that such reversals are the evolutionary result of female-biased operational sex ratios (OSR), where females compete for access to resources provided by males [4]. There is comparative evidence

for this tenet [5–8], and studies of plasticity in sex roles also implicate OSR [9–11]. Changes in sex roles in insects have also been associated with changes in OSR due to different environmental factors, such as food availability or bacterial infections [12, 13]. Here, we employ experimental evolution in an insect to provide a direct test of Darwin's fundamental prediction: that sex role reversal evolves under female-biased OSR.

In the honey locust beetle (*Megabruchidius dorsalis*), studies of wild populations have shown that sex roles in courtship are reversed compared to other seed beetles: females search for males, actively initiate mating, and court males vigorously, whereas males often reject female mating attempts [14–16]. Male ejaculates provide females with essential nutrients that increase females' fecundity and longevity [17], and females that secure more matings live longer and produce more offspring [8, 14, 18]. In contrast, matings carry substantial direct costs to males [16, 17], and males prefer to mate with females that perform more vigorous courtship displays [14, 16].

We allowed replicated honey locust beetle populations to evolve for 19 generations under two adult sex ratios (female biased, 1:5, or male biased, 5:1) and food regimes (abundant food or no food during adulthood) using a full factorial design. Due to male investment in ejaculates, the inter-mating interval is slightly longer in male than in female honey locust beetles [17, 18], so the potential reproductive rate [8] is somewhat lower in males. We predicted competition between females over access to males to be intensified under female-biased and greatly relaxed under male-biased sex ratios [19–21]. Our experimental design enabled us to directly measure the evolution of female courtship behavior as a response to increased and relaxed sexual selection in females. Inclusion of a food treatment allowed us to assess whether male-provisioned resources can compensate for normal feeding, in which case we expected the evolutionary effects of sexual selection in females to vary with resource availability. Following post-selection rearing under common garden conditions, we quantified male-female courtship interactions and the fitness effects of mating using replicated sex-specific mating system assays that paired beetles from the selection lines with standard reference individuals of the opposite sex.

Females that evolved under female-biased OSR (elevated reproductive competition) significantly outperformed females from male-biased lines in courtship and mating success. Females from female-biased lines made earlier contact with prospective mates, started courting males sooner, showed higher
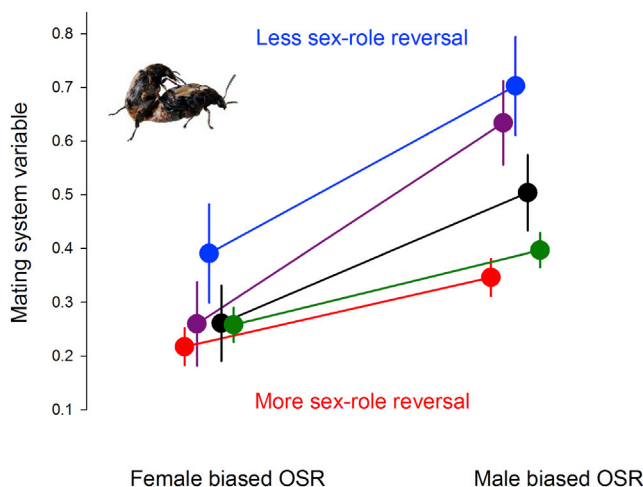
Figure 1. Females Evolving under Female-Biased OSR Become More Sex-Role-Reversed

Mean evolutionary responses (±SEM) under male- or female-biased OSR for the five behavioral variables that showed a significant effect of sex ratio, in assays where selection line females were paired with standard reference males (Table 1): time to first encounter (red), time to first courtship (blue), time to mating (black), number of mounts needed before mating occurred (purple), and probability that the male rejected the female in the first courtship attempt (green). High values are associated with less pronounced sex role reversal for all variables. For the purpose of visualization, the first four variables are scaled here to fit within a range of 0 to 1 (scaling factors used are 0.005, 0.002, 0.001, and 0.2, respectively). See also Figure S2 and Movie S1.

courtship efficacy (i.e., the probability that the first courtship attempt results in mating), and achieved successful copulation sooner and after fewer male pre-copulatory mountings (Figure 1; Table 1). Thus, as predicted, increased sexual selection in females led to the evolution of a more sex-role-reversed mating system: females courted males more intensely and at a higher rate. As a result, these females were effectively more attractive to reference males, who more readily accepted them as mates.

In contrast to the marked evolutionary response seen in female behavior, male-specific traits showed little evolution as a result of altered reproductive competition (Table 2). Males from male-biased lines were as slow to mate as those from female-biased lines. Multivariate analyses of variance of our eight behavioral mating system variables collectively provided evidence for overall evolution in females (Pillai trace = 2.23, $F_{24,18}$ = 2.18, p = 0.047), but not in males (Pillai trace = 1.99, $F_{24,18}$ = 1.49, p = 0.193). Furthermore, Bartlett's sequential residual root test revealed two significant orthogonal multivariate evolutionary response dimensions in females (root 1-3: χ^2_{24} = 43.42, p = 0.009; root 2-3: χ^2_{14} = 23.72, p = 0.049), but none in males (root 1-3: χ^2_{24} = 35.23, p = 0.065; root 2-3: χ^2_{14} = 14.67, p = 0.401). We conclude that alteration of the sexual selection regime affected the evolution of sex-specific behaviors more in females than in males.

Several studies have proposed that sexual selection should differ fundamentally between the sexes due to sex-specific investment trade-offs [2, 22]. This is often held to be a consequence of anisogamy and the larger cost of egg production relative to sperm production [23, 24]. However, this inference is problematic when males provide females with direct benefits

Table 1. Response to Experimental Evolution in Females

Response Variable	Source	ndf	ddf	F	p
Time to first encounter	feeding regime	1	13	0.84	0.377
	sex ratio	1	13	5.81	0.032
Time to first courtship[a]	feeding regime	1	12	0.02	0.903
	sex ratio	1	12	5.75	0.034
	male weight	1	12	5.33	0.040
Number of turns in first courtship bout[a]	feeding regime	1	13	5.96	0.030
	sex ratio	1	13	0.88	0.365
First courtship results in mating[a]	feeding regime	1	12	3.78	0.076
	sex ratio	1	12	9.55	0.009
	feeding regime × sex ratio	1	12	13.40	0.003
Number of mounting attempts[a]	feeding regime	1	11	0.13	0.721
	sex ratio	1	11	10.69	0.007
	female weight	1	11	10.28	0.008
	male weight	1	11	5.51	0.039
Time to mating[a]	feeding regime	1	12	1.82	0.203
	sex ratio	1	12	6.71	0.024
	male weight	1	12	13.07	0.004
Total number of turns[a]	feeding regime	1	13	11.51	0.005
	sex ratio	1	13	0.09	0.775
Courtship occurrence	feeding regime	1	13	0.02	0.882
	sex ratio	1	13	0.74	0.406
Ejaculate weight[a]	feeding regime	1	13	0.22	0.644
	sex ratio	1	13	1.01	0.332
Female body weight	feeding regime	1	13	1.89	0.192
	sex ratio	1	13	0.16	0.691
Mating duration[a]	feeding regime	1	13	0.38	0.549
	sex ratio	1	13	1.70	0.215

Analyses of variance/covariance of the effects of selection regime (food treatment and sex ratio) on mating system parameters in assays where females from selection lines were paired with standard reference males. ndf, numerator degrees of freedom for the chi-square; ddf, denominator degrees of freedom for the chi-square of the F-distribution. See also Table S1 and Movie S1.

[a]Analysis excluded pairs that did not court and/or mate.

that impose similar or even greater costs relative to female parental investment (e.g., [17, 19]). Males of many species provide substantial parental investment or nuptial gifts (e.g., [25]), and intrasexual competition for these direct benefits can result in strong sexual selection in females [26–28]. In order to better

Table 2. Response to Experimental Evolution in Males

Response Variable	Source	ndf	ddf	F	p
Time to first encounter	feeding regime	1	13	1.68	0.217
	sex ratio	1	13	2.82	0.117
Time to first courtship[a]	feeding regime	1	13	4.87	0.046
	sex ratio	1	13	3.60	0.080
Number of turns in first courtship bout[a]	feeding regime	1	13	0.07	0.790
	sex ratio	1	13	2.06	0.175
First courtship results in mating[a]	feeding regime	1	13	6.74	0.022
	sex ratio	1	13	0.43	0.524
Number of mounting attempts[a]	feeding regime	1	13	3.48	0.085
	sex ratio	1	13	0.31	0.586
Time to mating[a]	feeding regime	1	13	2.89	0.113
	sex ratio	1	13	0.89	0.364
Total number of turns[a]	feeding regime	1	13	0.13	0.727
	sex ratio	1	13	0.60	0.451
Courtship occurrence	feeding regime	1	12	0.07	0.799
	sex ratio	1	12	0.07	0.799
	feeding regime × sex ratio	1	12	11.64	0.005
Ejaculate weight[a]	feeding regime	1	13	1.05	0.324
	sex ratio	1	13	0.05	0.825
Male body weight	feeding regime	1	13	1.49	0.243
	sex ratio	1	13	0.27	0.611
Mating duration[a]	feeding regime	1	13	0.03	0.869
	sex ratio	1	13	0.11	0.748

Analyses of variance/covariance of the effects of selection regime (food treatment and sex ratio) on mating system parameters in assays where males from selection lines were paired with standard reference females. ndf, numerator degrees of freedom for the chi-square; ddf, denominator degrees of freedom for the chi-square of the *F*-distribution. See also Table S2 and Figures S1 and S2.
[a]Analysis excluded pairs that did not court and/or mate.

Figure 2. Females Evolving under Male-Biased OSR with Abundant Food Become Less Successful in Converting Courtship to Mating
Mean (±SEM) probability that the first courtship resulted in mating in assays where selection line females were paired with standard reference males (Table 1). Unfed, black bars; fed, gray bars.

in females may often have been underestimated [2, 26]. In a previous study [8], we found that the opportunity for sexual selection was as high, or even higher, in female than male honey locust beetles.

The fact that we found few main effects of food treatment (Table 1) suggests that food resources are not entirely exchangeable with male-provided resources in this system. In fact, females rarely feed as adults in the wild, while males forage frequently [12] and can mate more frequently when they do so [18]. We did find a significant interaction between sex ratio and feeding regime for the evolution of female courtship success (Figure 2; Table 1). Females from female-biased lines were very successful in achieving mating during their first courtship attempt under both feeding regimes. In contrast, females from male-biased lines achieved comparable success only if they evolved under low food availability. This suggests that copulations can to some extent compensate for food shortage: females evolved elevated courtship displays either when males were a limiting resource or when feeding regime likely slowed male remating rates even further.

We found no significant evolution of male investment in ejaculate size across treatments. Reference females did, however, produce less offspring after mating with males from lines that evolved under low food availability with a male-biased sex-ratio (Table S1; Figure S1). Interestingly, these males were also least attractive to females (Table 2; Figure S2). This suggests that ejaculate composition evolved in response to food limitation, but that this effect is contingent upon the prevailing pattern of reproductive competition. In a similar experiment in the closely related *M. tonkineus*, males responded to elevated mate competition by evolving increased ejaculate size, and females benefitted from receiving large ejaculates [33]. Our results thus suggest that responses to reproductive competition in males can be multifaceted, presumably reflecting the complexity of ejaculate composition and the diversity of functions of various ejaculate components in seed beetles [34].

understand the drivers and consequences of sexual selection in females, we should direct attention to such species. We found that the evolutionary responses to altered OSR in females were well aligned with those typically observed in males in species with conventional mating systems [29, 30]. Females not only evolved to become more attractive to males, they also showed more rapid evolution of reproductive behavior than did males. Three experimental evolution studies of taxa with conventional sex roles have also found significant female trait evolution in response to sex ratio manipulation, without a measurable response in males [30–32]. This accords with recent reevaluations of theory suggesting that the strength of sexual selection
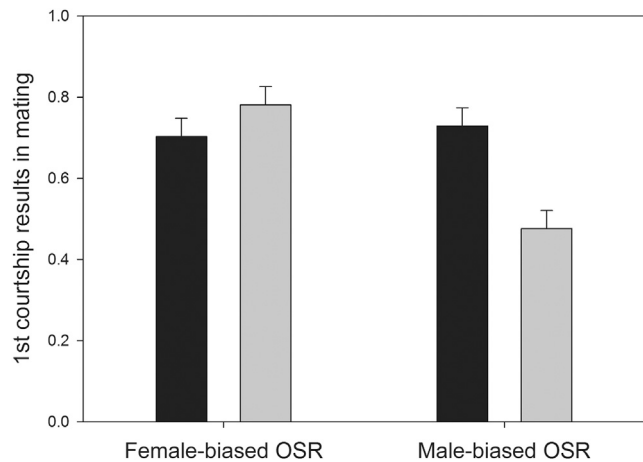
Our results indicate that the evolution of sexually selected traits in females need not signal fecundity, as is sometimes assumed [26]. Females with increased courtship efficacy (i.e., from female-biased lines) neither produced more offspring nor lived longer (Table S2). Although this may reflect inferential limitations of our study, it is also consistent with the possibility of female "sensory exploitation" of males [35] or possibly indirect genetic benefits to males from mate choice (a "sexy daughters" effect) [36, 37].

Our study (see also [33]) is the first experimental evolution study of a sex-role-reversed species. We found that female adaptations that increase female mating success evolved rapidly under strong reproductive competition among females. This led to the predicted evolution of intensified sex role reversal, thus validating our general understanding of mating system evolution [3]. Behavioral traits evolving in females were not associated with significant fecundity benefits to males, suggesting that male mate choice may not be adaptive. Our study also highlights the essential but sometimes overlooked role that females play in mating system evolution [2, 26, 38].

EXPERIMENTAL PROCEDURES

Stock Population
We established a large (>500 individuals) laboratory stock population from a sample of a natural population of *M. dorsalis* (Inogashira Park, Tokyo, Japan; ≈3000 adults, June 2009). Beetles were reared on seeds of the honey locust *Gleditsia triacanthos* in climate-controlled chambers at 26°C and 70% ± 10% relative humidity on a 16:8 light:dark cycle. All beetles were maintained in replicate 1 L glass containers and were fed 20% sucrose solution, pollen, and water. Under these conditions, *M. dorsalis* has a generation time of approximately 6–7 weeks. We generated virgin individuals to start our experimental evolution lines, and to initiate each new generation of our experimental evolution, by isolating single beans with larvae in 24-well culture plates. We collected individual beetles as they hatched from the beans and kept them isolated until the onset of experiments.

Experimental Evolution
We initiated 16 experimental populations, which were exposed to one of four treatment combinations (factorial 2 sex ratios × 2 food regimes design), using four replicate lines per treatment combination (n = 16 selection lines in total). The experimental lines were allowed to evolve in the laboratory for 19 generations. Lines were kept either at male-biased (125M:25F) or female-biased (25M:125F) sex ratio conditions, and beetles were either fed or left unfed. Under female-biased conditions, female mating rate will be relatively low, male mating rate will be high, and reproductive competition between females will be intense. Food availability has profound effects in honey locust beetles, affecting for example lifespan, the cost of reproduction, and male ejaculate size [15, 16].

Every generation, 150 virgin adults per line were placed at the selected sex ratio in clean 1 L glass jars containing a breeding substrate of *G. triacanthos* beans. Adults were allowed to mate and lay eggs in these jars for 2 weeks and were then removed and discarded. Females cement their eggs to the inside of the jar; the larvae hatch after 5–7 days and seek out a bean, which they bore into to complete their development (3–4 weeks). "Fed" lines were provided with 20% sucrose solution, an ample supply of pollen granules (Bee Pollen Capsules, Manuka Health New Zealand), and distilled water. "Unfed" lines were provided with distilled water only.

During the larval development period, prior to the emergence of new beetles from the beans, we distributed beans from each line individually in single wells of 24-well culture plates to allow the collection of virgin adults for the next generation. We note that, unlike in seed beetles with conventional sex roles, sex-specific variances in reproductive fitness are statistically indistinguishable in *M. dorsalis* [8]. Thus, the two sex ratio treatments used here show the same estimated effective population size ($N_e \sim 83$).

We terminated the experimental conditions at generation 19. To ensure that parental environmental effects, which can be transmitted for up to two generations in seed beetles [39], did not confound our results, the lines were maintained under common garden conditions of equal sex ratios without access to adult food for four subsequent generations prior to the assays described below.

Mating System Assays: Behavioral Responses to Experimental Evolution
M. dorsalis shows sex role reversal in courtship, in that females show active and extended courtship of males [14]. Typical courtships last from less than a minute to several minutes and consist of multiple repetitions of female turns [14, 16]. Males determine the outcome of courtship and reject their prospective mate in approximately 50% of female courtship attempts [14, 16].

In order to independently assay evolution of mating system variables in males and females, we paired focal individuals from selection lines with randomly selected opposite-sex individuals from a standard reference population (i.e., our outbred stock population). Each particular cross was replicated 7–8 times per line and sex. Virgin adults were collected from each of the 16 experimental evolution lines, as well as from the reference population, and were paired on the seventh day following emergence. All individuals were weighed to the nearest 0.1 mg prior to the assays (Sartorius AG ME/SE analytical micro balance). Pairs were introduced together in 6 cm plastic Petri dishes and filmed using a digital camera (Sony HDR CX250E). After 30 min, pairs were separated and each individual was re-weighed. In pairs that mated, the difference in male body weight before and after mating provides an accurate estimate of ejaculate weight [40]. We note that *M. dorsalis* males have a long refractory period (12–24 hr) such that each pair mated either once only or not at all. Males were placed individually in 6 cm plastic Petri dishes and females in 12 cm glass Petri dishes containing 100 g *G. triacanthos* beans for oviposition. We checked females and males daily until death to record lifespan and recorded the number of emerged offspring in each female dish after 8 weeks, when all offspring had emerged, as a measure of reproductive success.

We used video playbacks of all mating interactions to record courtship and mating behavior. Videos were analyzed by one observer blind to treatments. We recorded eight distinct aspects of male-female interactions, courtship behavior, and mating: time to first encounter, time to first courtship event, number of turns performed in the first courtship bout, whether the first courtship resulted in mating or not, the total number of male mountings, time to mating, the total number of turns, and whether courtship occurred or not. Here, "turns" (the number of times a female turns in front of a male to initiate mating) are a measure of courtship effort. In addition, we measured the mating duration in all trials where mating occurred.

Data Resources
All data reported in this paper have been published in Mendeley Data and are available at http://dx.doi.org/10.17632/htm5c76w5f.2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, two tables, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.07.018.

AUTHOR CONTRIBUTIONS

K.F. and G.A. conceived and designed the study, analyzed and interpreted data, and wrote the manuscript. K.F. and I.B. designed and performed experiments. All authors reviewed and approved the manuscript.

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