Female Density-Dependent Chemical Warfare Underlies Fitness Effects of Group Sex Ratio in Flour Beetles

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ABSTRACT: In animals, skewed sex ratios can affect individual fitness via sexual (e.g., intersexual conflict or intrasexual mate competition) or nonsexual (e.g., sex-specific resource competition) interactions. Because most analyses of sex ratio focus on sexual interactions, the relative importance of sexual versus nonsexual mechanisms remains unclear. We tested both mechanisms in the flour beetle *Tribolium castaneum*, where male-biased sex ratios increase female fitness relative to unbiased or female-biased groups. Although flour beetles show both sexual and nonsexual (resource) competition, we found that sexual interactions did not explain female fitness. Instead, female fecundity was dramatically reduced even after a brief exposure to flour conditioned by other females. Earlier studies suggested that secreted toxins might mediate density-dependent population growth in flour beetles. We identified ethyl benzoquinone and methyl benzoquinone (quinones) as components of adult stink glands that regulate female fecundity. In female-biased groups (i.e., at high female density), females upregulated quinones and suppressed each other's reproduction. In male-biased groups, low female density and associated low quinone levels maximized fecundity. Thus, females appear to use quinones as weapons for female-specific, density-dependent interference competition. Our results underscore the importance of nonsexual interference competition that may often underlie the fitness consequences of skewed sex ratios.

Keywords: intrasexual competition, interference competition, quinones, ethyl benzoquinone, methyl benzoquinone, *Tribolium castaneum*.

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Introduction

Theoretical models predict that a balanced sex ratio is optimal for most diploid species (Fisher 1930; Emlen and Oring 1977; e.g., Vlad 1989; Kvarnemo and Ahnesjo 1996). However, the adult sex ratio varies substantially across species and across populations, and a large body of research has analyzed the evolutionary consequences of skewed sex ratios. In particular, the impact of sex ratio on reproductive behavior and associated intra- or intersexual conflict has received a lot of attention, since reproductive traits are expected to face strong selection. For instance, male-biased sex ratios can be detrimental because of direct or indirect male harassment, decreasing female longevity (e.g., Nandy et al. 2013), fecundity (e.g., Holland and Rice 1999), or offspring survival (e.g., Sakurai and Kasuya 2008). More generally, increased male-male competition can result in elevated sexual conflict, reducing female fitness (Stockley 1997). On the other hand, under a male-biased sex ratio, females may benefit from the opportunity to selectively mate with higher-quality males (Berglund 1994) or from increased opportunity for polyandry and associated offspring heterogeneity (Arnqvist and Nilsson 2000).

These hypotheses explaining the relationship between adult sex ratio and female fitness largely depend on sexual interactions involving mating and associated behaviors. However, individual fitness is also determined by nonsexual interactions that alter survival and life history (West-Eberhard 1983). Such nonsexual interactions may be especially important for females, who often compete more strongly for resources (such as food or space) rather than for mates (Tobias et al. 2012). A growing body of work on mammals suggests that female competitive interactions may be important for many species (Stockley and Bro-Jørgensen 2011). For instance, in cooperatively breeding meerkats, dominant females increase their reproductive success by suppressing subordinate females' reproduction, reducing competition for resources (Bell et al. 2014). Female parasitoid wasps face intense competition for

suitable hosts and use physiological and chemical mechanisms to determine the outcome in their favor (Harvey et al. 2013). In such cases, resource rather than sexual competition may govern female fitness. More generally, changes in sex ratio may be confounded with changes in the density of one or both sexes, and the intensity of sexual selection may also depend on population density (Kokko et al. 2006). Hence, it is important to quantify the contribution of sexual interactions versus nonsexual competition on individual fitness.

We tested the impact of adult sex ratio and female density on female fitness in the red flour beetle *Tribolium castaneum*, a global pest of stored grains. Typically, populations of *T. castaneum* exhibit a balanced sex ratio (Sokoloff 1977). Both sexes mate multiply and exhibit mate choice (Sokoloff 1977; Arnaud and Haubruge 1999; Lewis et al. 2005; Fedina 2007; Fedina and Lewis 2007), but the degree and fitness consequences of polyandry vary across populations (Pai et al. 2007). Last-male sperm precedence is well known in Tribolium, although there is large variation in male offense capability (Lewis and Austad 1990), which may potentially trade off with sperm defense ability (Bernasconi and Keller 2001). Hence, sexual selection, sexual conflict, and intrasexual competition are important aspects of fitness in flour beetles. In addition, individuals compete strongly for resources, which determines density-dependent population growth rate (Parent et al. 2014). Together, these features make *T. castaneum* an attractive model to analyze the impact of different forms of individual interactions on fitness in the context of imbalanced sex ratios.

We tested the consequences of skewed adult sex ratio on multiple proxies of female fitness: female reproductive fitness (egg production and total surviving offspring), life span, and immune function. We found that, in each case, females from male-biased groups had higher fitness compared to females from either unbiased or female-biased groups. Neither male intrasexual competition nor opportunity for female or male mate choice explained this fitness difference. Instead, we found that negative density-dependent interactions between females were responsible for the fitness effects of group sex ratio. We identified benzoquinones as key chemicals mediating these interactions, suggesting female interference competition. Thus, we present a case where fitness consequences of group sex ratio arise from female-driven nonsexual resource competition rather than sexual interactions.

Methods

Generating Experimental Individuals and Sex Ratio Groups

We used an outbred laboratory population of *Tribolium castaneum* (Khan et al. 2016) maintained on whole wheat flour (henceforth, flour) at 34°C (±1°C) on a 45-day discrete generation cycle. For all experiments, we allowed ~2,000 individuals to oviposit in 750 g of wheat flour for 48 h and collected their offspring at the pupal stage. We housed pupae of each sex separately in 1.5-mL microcentrifuge tubes (three pupae of the same sex in 1 g flour) for 10 days postpupation. The pupal stage lasts for 3–4 days; we thus obtained ~7-day-old (posteclosion), sexually mature virgins. We grouped these adults into three sex ratio treatments ($n = 8$–9 groups per sex ratio): (1) male-biased groups (henceforth, MB: 3 males + 1 female); (2) unbiased groups (henceforth, UB: 3 males + 3 females; or UB1: 1 male + 1 female); and (3) female-biased groups (henceforth, FB: 1 male + 3 females). Each group was maintained in a plastic petri plate (60 mm diameter; Tarsons, India) at a density of 1 beetle per gram of flour (chosen to minimize resource limitation) and placed in a 34°C (±1°C) incubator. Thus, MB and FB groups were given 4 g flour; UB groups received 6 g flour; and UB1 groups received 2 g flour. We chose the UB treatment such that we could determine the effect of skewed sex ratio given the same number of males (UB vs. MB, 3 males each) or females (UB vs. FB, 3 females each). To test whether group size alters the effect of sex ratio, we set up additional groups with 12 beetles/group ($n = 10$ groups per sex ratio), maintaining the same sex ratio and density as earlier (FB: 3 males + 9 females; MB: 9 males + 3 females; each group in 12 g flour). After 1 week, we measured egg production by females from each group as described below.

Measuring Fitness Effects of Group Sex Ratio

Females in each experimental group could oviposit continuously during the experiment, but adult cannibalism would confound estimates of female fecundity. Hence, after constituting the groups described above, we estimated the total offspring produced per female by periodically isolating females: every 5 days for the first 30 days, every 7 days for the next 28 days, and then every 14 days for another 42 days. Thus, we obtained estimates of female reproductive fitness as a function of sex ratio as well as age until females were 102 days old. During each fitness assay, females oviposited individually for 24 h in plastic petri plates (60 mm diameter; Tarsons) containing 5 g flour, after which they were placed again into their original group and provided fresh flour. Thus, offspring from each female developed independently in the oviposition plates. After 3 weeks, we counted the total number of offspring in each plate (including larvae, pupae, and adults) as a proxy for the total reproductive fitness of each female. Note that this measure includes the number of eggs produced (egg quantity) as well as their survival during development (egg quality). To specifically measure egg quantity, we counted the number of eggs laid by 21-day-old females after the 24-h oviposition period. We also monitored mortality of individuals from each group and tested proxies of immune function (see appendix for details).

Quantifying Mating Behaviors

To quantify mating-related behaviors as a function of group sex ratio, we set up additional experimental groups using virgin adults ($n = 15–16$ groups per sex ratio). To identify females in FB and UB groups, we used a paintbrush to mark the elytra with a small dot of nontoxic acrylic (model master) paint, using one of three colors (yellow, green, or red). To control for any effects of the paint on mating behavior, we marked one-third of the MB females with each color. Thus, all females in the experiment were marked regardless of the sex ratio of their group. After marking the elytra, we placed females individually in wells of a 96-well microplate and allowed the paint to dry for 6 h. Since we wanted to quantify mating behaviors directed toward females, we did not mark males. We distributed marked females into sex ratio groups as described in the previous section. A week later, we separated individuals from the flour, placing each group in a 60-mm petri dish whose bottom was covered with filter paper to allow beetles traction for walking. We observed each group for 30 min, noting the number and timing of matings per female. A mating event was recorded when the male mounted the dorsum of the female and attempted or achieved copulation. We quantified (1) total number of copulatory mounts (matings), (2) total time spent in copula, (3) average copulation time, and (4) mating latency (time until the first copulation). Immediately after the behavioral assay, we separated females and measured their reproductive output (as described above) to test whether female fitness was correlated with mating behavior.

Testing the Effect of Conditioned Flour on Female Fitness

Flour used by *Tribolium* beetles gets "conditioned" by the accumulation of secreted quinones, pheromones, and excreta. We tested whether flour conditioned by FB and MB groups differentially affects the fitness of females exposed to the flour. We allowed FB and MB groups (constituted as described earlier) to condition 4 g of flour for 10 days. After this, we removed the adults, froze the flour at −80°C for 15 min, and sifted it through a fine mesh (300-μm pore size; Daigger) to kill and remove juvenile stages. We placed a virgin male and a virgin female (both 7 days old) in 2 g of this conditioned flour for 6 h, 1 day, or 4 days ($n = 12$ pairs per conditioning treatment per duration of exposure). Following this, we isolated the female from each pair and allowed her to oviposit for 24 h in fresh flour. The next day, we counted the number of eggs laid per female as a measure of fecundity. Thus, we obtained female fecundity after 6 h, 1 day, and 4 days of exposure to flour conditioned by either MB or FB groups. In a second experiment, we conditioned the flour using either female-only or male-only groups of beetles (three 7-day-old virgin beetles of the same sex). We then introduced a male and a female (7-day-old virgins) into 2 g of this conditioned flour for either 6 h or 24 h ($n = 11–12$ pairs per conditioning treatment per duration of exposure). Following this, we estimated the number of eggs laid by each female as described above.

Testing the Impact of Stink Gland Contents on Female Fecundity

Flour beetles have paired abdominal and thoracic stink glands that secrete a mix of chemicals including toxic benzoquinones (Markarian et al. 1978). To test whether secreted products from beetles' stink glands directly alter female fitness, we measured female fecundity after a brief exposure to abdominal stink glands. We paired 7-day-old virgins (1 male + 1 female) in 2 g flour for 2 days. We separated the females and transferred them individually into a 35-mm petri dish whose bottom was covered with a filter paper for traction and a thin layer of flour to avoid starving the beetles during the assay. On the same day, we separately dissected abdominal stink glands from 60 MB and 60 FB females (sex ratio groups constituted as described earlier). For each set, we used a micropestle to pool and homogenize the glands in 300 μL hexane and centrifuged the suspension (5,000 rpm, 5 min). Thus, 10 μL of the supernatant was equivalent to the abdominal gland content of 2 females. We then performed serial dilution of the supernatant to obtain abdominal gland content equivalents of 0.75, 0.5, 0.25, 0.1, 0.05, 0.025, and 0.001 females in a total volume of 10 μL. We soaked filter paper discs (10 mm diameter) with 10 μL of the supernatant (or hexane as control), kept them under laminar air flow for 10 min to evaporate the solvent, and then placed the discs at the center of a petri plate containing the experimental female ($n = 5–6$ females/dilution/sex ratio). Twelve hours later, we tested the fecundity of each female as described above.

Next, we wanted to identify the chemical component of female stink glands responsible for reduced female fecundity. Previous analyses show that ethyl benzoquinone (EBQ) and methyl benzoquinone (MBQ) are two major components of *T. castaneum* stink glands (Unruh et al. 1998). Hence, we quantified the amount of EBQ and MBQ (henceforth, for brevity, quinones) produced by females in MB and FB groups. We constituted MB and FB groups as earlier ($n = 10$ groups per sex ratio), and after 7 days we dissected abdominal and thoracic stink glands of a randomly chosen female from each group. For each female, we combined abdominal and thoracic gland samples and homogenized in 60 μL cold methanol. Subsequently, we quantified the amount of EBQ and MBQ in each sample using high-performance liquid chromatography (see appendix for details). For calibration, we used pure MBQ (Sigma) and lab-synthesized EBQ (see appendix).

Finally, to directly test whether quinones regulate fecundity, we exposed 9-day-old test females (reared as a single

mating pair for 2 days before exposure, as described above) to abdominal stink gland extracts of MB females containing varying concentrations of added EBQ and MBQ. We added 10 μL of the gland extract equivalent to 0.5 females, supplemented with 1, 5, or 10 μg of either quinone to a filter paper disc (10 mm diameter), and placed the disc in a 35-mm petri dish containing a female ($n = 7$–10 females per concentration per chemical). As controls, we exposed test females to discs spotted only with solvent or with the MB gland extract without quinones. After exposing females to each chemical mixture for 12 h, we measured fecundity as described earlier.

Data Analysis

Because the number of females varied across sex ratio treatments, we calculated the average trait value for females from each replicate per measurement as the unit of analysis and then compared group means across sex ratio treatments. For MB groups, the group mean was simply the trait value of the single female in each group. We used group means for all analyses, unless noted otherwise. We used a one-way ANOVA to test for all main effects. When residuals were not normally distributed (tested with Shapiro-Wilks normality test), we log transformed the data (e.g., mating behaviors) or used a nonparametric Wilcoxon rank sum test (e.g., phenoloxidase enzyme activity). For all multiple comparisons, we used an ANOVA with Tukey's honest significant difference. For experiments with multiple explanatory variables, we used a two-way ANOVA or an ANCOVA. Further details of all statistical tests are given in the respective tables and figures. We conducted all analyses using the software JMP 10. Data underlying all reported figures are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.p9v3q (Khan et al. 2018).

Results

Male-Biased Sex Ratio Increases Female Fitness

We found that females from male-biased (MB) groups outperformed females from unbiased (UB) and female-biased (FB) groups. Throughout their major reproductive life span, MB females consistently produced more offspring compared to UB or FB females (fig. 1*A*; table 1), resulting in more than twice as many total offspring per female (fig. 1*B*; table 1). Interestingly, we observed that in unbiased groups with 1 female and 1 male, female fitness was similar to that of MB groups rather than to the UB groups with 3 females and 3 males (fig. A1*A*; figs. A1–A6 available online). The differential reproductive success was driven by offspring quantity: MB females laid ~3 times more eggs per female per day, compared to UB or FB females (fig. 1*C*; table 1). We observed a similar fecundity difference in a separate experiment with a larger group size (FB: 9 females + 3 males; MB: 3 females + 9 females; fig. A1*B*). Thus, a male-biased sex ratio generally increased female reproductive fitness by increasing female fecundity. We also found significantly fewer deaths in MB females, compared to both UB and FB females (fig. A2*A*; Fisher's exact test, two-tailed P value: MB vs. FB = 0.009; MB vs. UB = 0.001; UB vs. FB = 0.999). In FB replicates where a female died earlier in the experiment, the sex ratio would become less skewed, leading us to underestimate the impact of the skewed sex ratio. In contrast, female mortality in UB groups would increase the skew in sex ratio. Despite these confounding factors, female reproductive success in FB and UB groups was consistently lower than MB groups, suggesting that early effects of sex ratio were very strong. Finally, MB females also had better immune function (fig. A2*B*–A2*D*). Together, these data indicate that the low fecundity of FB and UB females cannot be explained by trade-offs with other fitness components. In subsequent experiments, we focused on the mechanism underlying the observed impact of sex ratio on female reproductive fitness.

Sexual Interactions Do Not Explain Female Fitness

We evaluated the hypothesis that female fitness increases when females can choose to mate with superior males. This hypothesis predicts that FB females should have low fitness (access to a single male; hence, no mate choice possible) and that UB and MB females should have equal and higher fitness (choice between 3 males in each case). Instead, we found that females in both FB and UB groups had lower fitness than females in MB groups (fig. 1). Thus, increased opportunity for female mate choice was unlikely to explain the higher fitness of MB females. An alternative hypothesis is that male mate choice drives female fitness. This hypothesis predicts that within replicates of UB and FB groups, one female would have higher fitness than MB females. However, when we only considered the female with the highest fitness in each group, we still found that MB females outperformed FB and UB females (fig. A2*E*). Thus, it is unlikely that opportunity for male or female mate choice was responsible for the increased fitness of MB females. Nonetheless, we specifically tested the impact of sex ratio on mating behavior and female fecundity. We found that although MB females received more matings and tended to spend more time in copula (fig. A3; table 2), female fitness was not correlated with either of these behaviors (fig. 2). In other words, adding mating behaviors to a model with group sex ratio as an explanatory variable did not improve the model fit (table 2). Group sex ratio did not affect other aspects of mating behav-

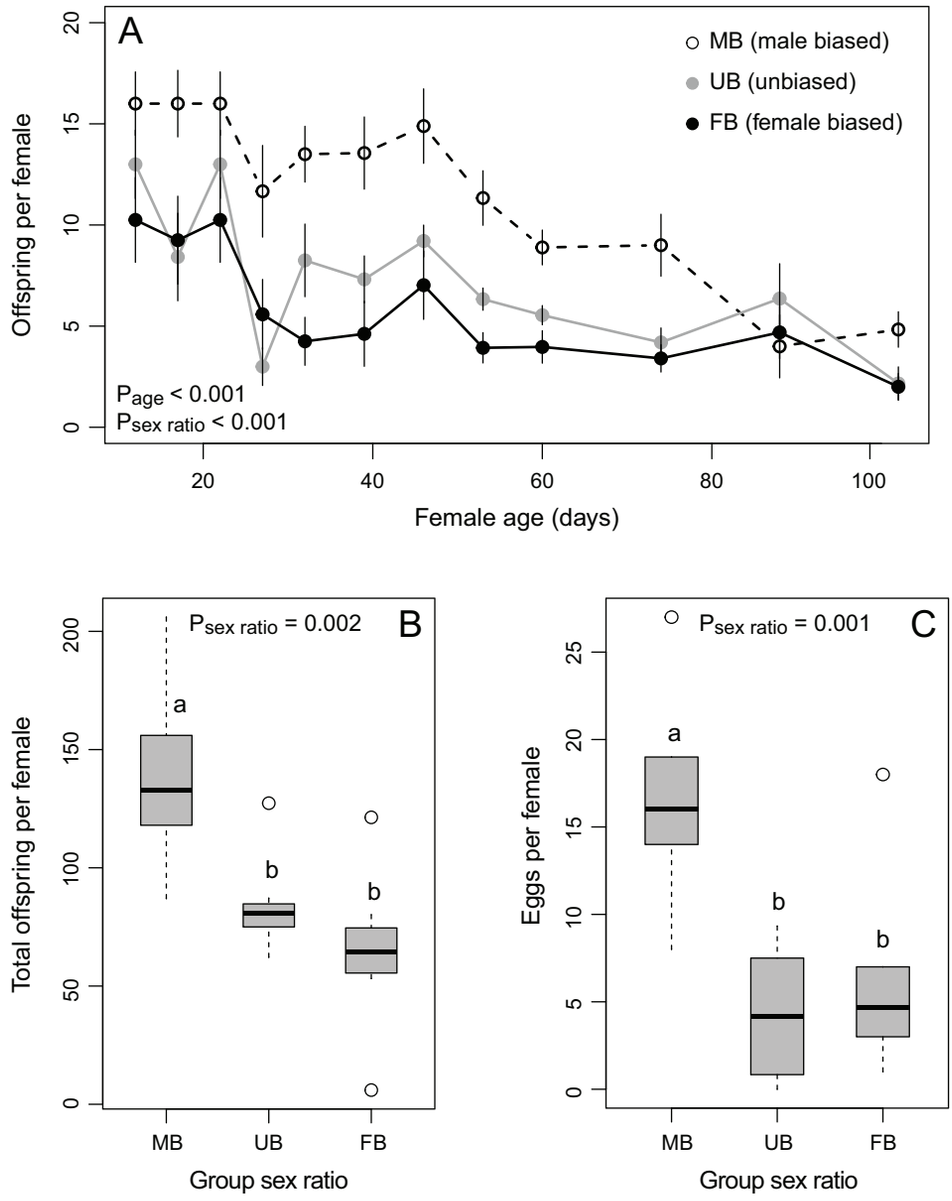

Figure 1: Female fitness as a function of group sex ratio. *A*, Average offspring (±SE) produced per female as a function of age (days posteclosion). *B*, Total offspring produced per female during the experiment. *C*, Fecundity of 28-day-old females as a function of sex ratio. MB = male-biased groups (3 males + 1 female); UB = unbiased groups (3 males + 3 females); FB = female-biased groups (1 male + 3 females). Boxplots show median and quartiles, and lowercase letters indicate significantly different groups inferred from pairwise comparisons; $n = 8$–9 groups per sex ratio.

ior (fig. A3). Thus, sexual interactions between individuals could not explain the higher fitness of MB females.

Female-Secreted Benzoquinones Explain the Fitness Impact of Sex Ratio

Flour beetles secrete toxic compounds in the flour, which are suggested to decrease female fecundity at high concentrations (Park 1934, 1936). Thus, it is possible that greater accumulation of toxins in flour used by FB groups was responsible for the decreased fecundity of FB females. To test this, we measured the reproductive output of mated females exposed to flour previously conditioned by either FB or MB groups. We found that females responded rapidly to cues in conditioned flour, with a large reduction in fecundity after only 6 h of exposure to flour conditioned

Table 1: Summary of analysis of fitness components

Trait, effect	df	SS	*F* ratio	*P*
Offspring per female (fig 1*A*):[a]				
Sex ratio	2	1,784.079	48.771	<.001
Age	11	2,538.146	12.615	<.001
Sex ratio × age	22	540.784	1.344	.144
Error	280	9,482.755		
Total offspring per female (fig 1*B*):[a]				
Sex ratio	2	23,897.369	12.83	.0002
Error	24	44,385.999	…	…
	df	χ^2	*P*	
Fecundity (fig 1*C*):[b]				
Sex ratio	2	14.5294	.001	

Note: Figures with the corresponding data are as indicated.

[a] ANOVA.

[b] Wilcoxon rank sum test.

by FB groups (figs. 3*A*, A4*A*; table 3). Critically, flour conditioned by females also produced a similar decline in fecundity (figs. 3*B*, A4*B*; table 3). These results indicated that the reduced fitness of FB females was mediated by female-secreted chemicals in the flour, rather than via direct inter- or intrasexual interactions.

Previous work shows that female flour beetles secrete more quinones (produced by stink glands) than males (Unruh et al. 1998; Khan et al. 2016). Hence, we tested whether stink gland secretions (rather than other secretions or excreta in the flour) were responsible for the observed effects of female-conditioned flour on female fitness. We found that fecundity of previously mated test females decreased after exposure to abdominal stink gland contents of FB females, whereas gland contents of MB females did not alter fecundity (fig. 3*C*; table 3). These results confirmed that the contents of female stink glands directly affected female fitness. Next, we measured the concentration of benzoquinones (EBQ and MBQ) in abdominal and thoracic stink glands dissected from females from MB versus FB groups. We found that stink glands of FB females had higher amounts of both EBQ and MBQ (fig. 3*D*; table 3), suggesting that these quinones may be responsible for lower fecundity of FB females. Finally, we tested whether adding quinones to gland extracts of MB

Table 2: Analysis of mating behavior

Trait, effect	df	SS	*F* ratio	*P*
N_{mating} (fig 2*A*):[a]				
Sex ratio	2	2.6	5.769	.006
Error	37	8.337		
T_{copula} (fig 2*B*):[a]				
Sex ratio	2	1.324	1.026	.368
Error	37	23.874		
Fecundity (fig 2*A*):[b]				
Sex ratio	2	202.62	4.353	.015
log (T_{copula})	1	27.78	1.193	.538
Sex ratio × log (T_{copula})	2	79.70	1.712	.117
Error	33	784.90		
Fecundity (fig 2*B*):[b]				
Sex ratio	2	251.67	5.42	.009
log (N_{mating})	1	.97	.042	.838
Sex ratio × log (N_{mating})	2	117.17	2.523	.095
Error	34	789.37		

Note: Data were log transformed for normality. T_{copula} = total time spent in copula; N_{mating} = total number of matings per female. Figures showing corresponding data are noted in the first column.

[a] Summary of analysis of mating behaviors as a function of group sex ratio with a one-way ANOVA.

[b] Summary of ANCOVAs of the impact of sex ratio and mating behavior on female fecundity.

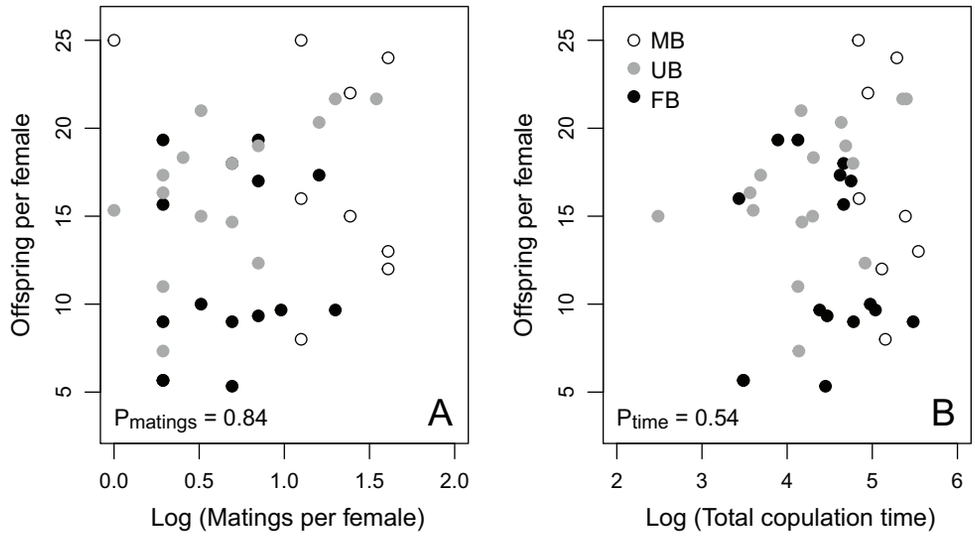

Figure 2: Female reproductive fitness as a function of mating behavior. Average offspring per female, as a function of total number of matings per female in a 30-min observation period (*A*) and total time spent in copula per female (*B*). The *X*-axis was log transformed in both panels. MB = male-biased groups (3 males + 1 female); UB = unbiased groups (3 males + 3 females); FB = female-biased groups (1 male + 3 females); n = 15–16 groups per sex ratio.

females could mimic the effects of glands from FB females. Indeed, we found that adding EBQ or MBQ to stink gland extracts of MB females reduced the fecundity of test females (fig. 3*E*; table 3).

Together, our results show that at high female density (i.e., in UB and FB groups), females upregulate EBQ and MBQ production, suppressing oviposition by all females in the group. In contrast, MB females may secrete less MBQ and EBQ, allowing MB females to maximize oviposition and fitness. We infer that the observed female response to high female density reflects female interference competition for shared dietary and oviposition resources.

Discussion

A rich body of theoretical and empirical work documents the varied consequences of adult sex ratio for individual fitness (Fisher 1930; Vlad 1989; Berglund et al. 1993; Kvarnemo and Ahnesjo 1996). Perhaps due to the obvious implications of skewed sex ratios for sexual conflict and sexual selection, much of this work has been interpreted in light of female mate choice and male-male intrasexual competition. However, we found that neither male mate competition nor increased opportunity for female mate choice explained higher female fitness in male-biased groups of flour beetles. Instead, female competitive interactions were responsible for the observed fitness difference between male-biased (MB) and female-biased (FB) groups, regardless of group size. Similarly, in houseflies, a male-biased sex ratio increased female fecundity and offspring survival (Carrillo et al. 2011). Although male houseflies courted more frequently in male-biased groups, female fitness was not associated with male courtship and was not affected by the opportunity for female mate choice. As discussed by the authors, these results suggest cryptic effects of social, nonsexual interactions between individuals as drivers of increased female fitness under a male-biased sex ratio. Such nonsexual competitive interactions between females may be widespread, with multiple ex amples in mammals (reviewed in Stockley and Bro-Jørgensen 2011) as well as in social insects (discussed in Berglund et al. 1993) and mosquitofish (Smith and Sargent 2006; Smith 2007). Thus, nonsexual interactions between females may frequently mediate the outcome of altered sex ratio in multiple taxa.

Importantly, we identified female-secreted benzoquinones as mediators of female competitive interactions in flour beetles. Our experiments with synthetic EBQ and MBQ constitute the first direct demonstration of the fitness impact of benzoquinones, since previous studies were typically carried out with conditioned flour and, hence, confounded the impact of benzoquinones with other secretions (including excreta). At high density, juvenile stages show developmental abnormalities attributed to increased quinone concentrations (Sokoloff 1977); quinones may thus represent a form of interference competition at high density. Indeed, beetles typically avoid flour containing high amounts of quinones (Loconti and Roth 1953). Thus, we hypothesized that quinones may be responsible for the observed differences in female fitness as a function of sex ratio. We expected that MB groups should

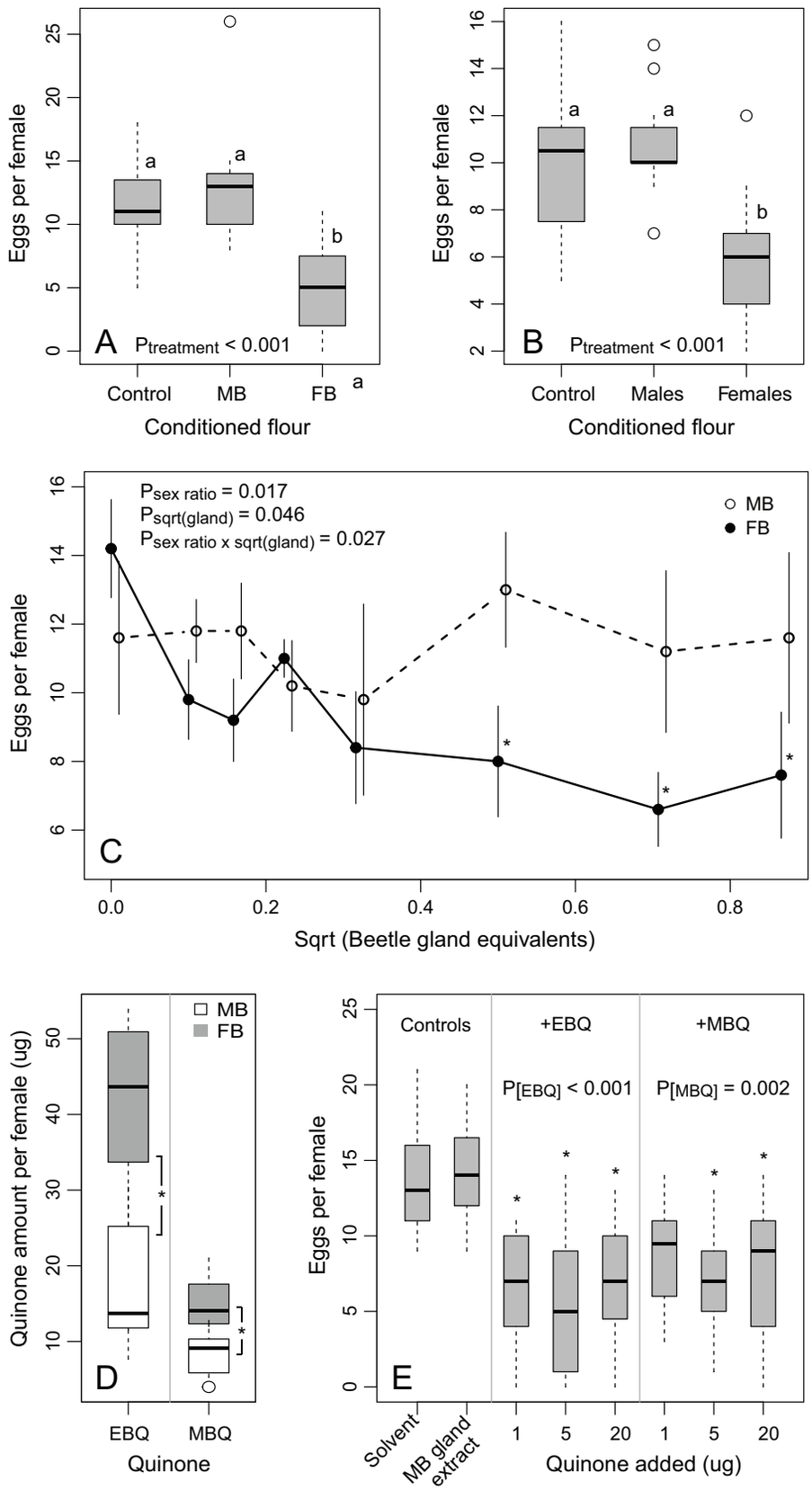

Table 3: Summary of analysis of fitness as a function of conditioned flour and quinones

Experiment, effect	df	SS	*F* ratio	*P*
Fecundity after exposure to flour conditioned by sex ratio groups (MB vs. FB groups; fig 3*A*):[a]				
Treatment	1	539.215	50.269	<.0001
Duration	2	41.809	1.949	.151
Treatment × duration	2	81.561	3.802	.028
Error	69	1,358.642	…	…
Fecundity after exposure to flour conditioned by virgins (males vs. females; fig 3*B*):[a]				
Treatment	1	189.851	19.469	<.0001
Duration	1	5.328	.546	.464
Treatment × duration	1	6.884	.706	.406
Error	39	550.775	…	…
Fecundity after exposure to stink gland contents of MB vs. FB females (fig 3*C*):[b]				
Sex ratio	1	82.01	5.99	.017
Square root (gland concentration)	1	55.52	4.05	.048
Sex ratio × square root (gland concentration)	1	69.87	5.1	.027
Error	64	932.4		
Fecundity after exposure to stink gland of MB females with added EBQ or MBQ (fig 3*E*):				
EBQ concentration	4	538.45	7.59	.0002
Error	32	567.44	…	…
MBQ concentration	4	368.81	5.497	.0018
Error	31	512.93	…	…
	df	χ^2	*P*	
Quinones in stink glands of MB vs. FB females (fig 3*D*):				
Sex ratio, EBQ	1	5.906	.0151	
Sex ratio, MBQ	1	4.835	.0279	

Note: Figures showing corresponding data are as indicated. MB = male-biased groups; FB = female-biased groups; EBQ = ethyl benzoquinone; MBQ = methyl benzoquinone.

[a] Summary of ANOVA for fecundity as a function of exposure to flour conditioned by virgins or sex ratio groups (treatment) and duration of exposure.

[b] Summary of ANCOVA for fecundity of focal females exposed to increasing concentrations of stink gland extracts from females from MB versus FB groups.

produce less quinones than FB or UB (unbiased) groups and that exposure to more quinones should decrease female fecundity. We found support for both these predictions in independent experiments: MB females had less quinones in their stink glands, and adding quinones to gland extracts of MB females mimicked the fitness impact of gland extracts of FB females. Our results also suggest that females are particularly sensitive to conditioned flour and respond by modulating their fecundity. Previous experiments support this finding: under a balanced sex ratio, net female fecundity decreased rapidly with increasing population density, but the decline was slower in male-biased groups containing a single fe-

Figure 3: Quinones in female stink glands mediate fitness effects of group sex ratio. Fecundity of test females exposed for 6 h to flour conditioned by male-biased (MB) versus female-biased (FB) groups ($n = 12$ pairs per treatment; *A*) or three virgin females versus three males (controls: unconditioned flour; $n = 11$–12 pairs per treatment; *B*). Lowercase letters indicate significantly different groups inferred from pairwise comparisons. *C*, Mean fecundity (±SE) of test females exposed to increasing concentrations of stink gland extracts of females from MB versus FB groups ($n = 5$–6 mated females per treatment). MB data are slightly displaced along the *X*-axis for clarity. Asterisks indicate treatments that are significantly different from the respective control for each treatment (concentration 0). *D*, Amount of ethyl benzoquinones (EBQ) and methyl benzoquinones (MBQ) in stink glands of females from MB versus FB groups ($n = 10$ groups per sex ratio). *E*, Fecundity of test females exposed to MB stink gland extracts containing varying amounts of either EBQ or MBQ (controls: solvent used for gland extraction and MB gland extract without added quinones; $n = 7$–10 females per concentration per chemical). Boxplots show median and quartiles; asterisks indicate groups that are significantly different from the control (MB gland extract).

male (Birch et al. 1951). Note that the fecundity of all females within a group decreases, either due to females' own quinones (potentially spiteful interactions) or due to quinones secreted by other females (reciprocal suppression). However, a single female does not produce enough quinones to reduce fecundity; rather, females respond to high total quinone concentration, which scales with female density. Hence, it is unlikely that our results represent a case of spite.

More generally, our results support the idea that quinones mediate negative density-dependent population growth in *Tribolium* (Park 1937; Park and Woollcott 1937; Sonleitner and Gutherie 1991), a suggestion that was never explicitly tested. Quinones are secreted from abdominal and thoracic glands (Markarian et al. 1978) and are thought to accumulate in flour in a density-dependent manner (Sokoloff 1977). The individual response to major quinone components also increases as a function of population density (Duehl et al. 2011). For instance, exposing a pair of focal beetles to olfactory cues from crowded beetles (presumably, largely quinones) for 3 days decreased female oviposition rate by up to 25% (Sonleitner and Gutherie 1991). Previous work also shows that sensitivity to conditioned flour is a heritable trait that can rapidly evolve under selection in laboratory populations and is controlled by a single (or few) genes (Lavie et al. 1978). Thus, density dependence itself may arise due to sensitivity to quinones. While previous studies investigated the impact of total population density, our work shows for the first time that female fecundity is largely regulated by female density rather than total beetle density. Higher quinone sensitivity in females may also explain our observation that male mortality was not affected by group sex ratio (data not shown). Although our results strongly suggest that quinones mediate negative density dependence in flour beetles, we note that further work is necessary to test whether females control the secretion rate of stink gland contents in a density-dependent manner and whether other stink gland components also affect female fitness. In addition, the precise mechanism through which benzoquinones suppress fecundity remains to be elucidated; possibilities include direct effects on egg maturation or oviposition behavior or indirect effects via physiological costs of producing quinones.

Although we infer competitive interactions between females, an alternative explanation for our results is that reducing fecundity at high density is adaptive, and females use quinones as a density signal. Previous work suggests that quinones are toxic for *Tribolium castaneum* juveniles (Sokoloff 1977) and cause high adult mortality in related species such as *Tribolium destructor* (Palm 1946). Beetles also grow faster and larger in male-biased groups (Ellen et al. 2016), possibly due to relatively lower amounts of toxic quinones. Hence, it may indeed be beneficial to avoid laying eggs under high quinone concentrations and wait to find a less crowded resource patch. However, we observe that females upregulate quinone production up to four-fold at high female density, with enormous consequences for multiple aspects of female fitness including survival and immune function. Thus, quinones are toxic not only for juveniles but also for females themselves, arguing against its use as a density signal. Therefore, we suggest that quinones are actively used for competition rather than as passive density-dependent accumulation of a signal. However, we acknowledge that explicit experiments are necessary to test the possibility that quinone-induced fecundity reduction is adaptive.

Our finding that female fitness increases as a function of male-biased sex ratio contradicts previous work with *T. castaneum* showing intersexual conflict: in experimentally evolved male-biased populations, male competitive ability and mating success increased significantly (Michalczyk et al. 2010). In that study, increasing the number of males in mating trials decreased the fitness of evolved FB females but not of evolved MB females. As the authors discuss, these data suggest that females became more sensitive to (as yet unknown) deleterious effects of multiple mating, indicating underlying sexual conflict. However, unlike in *Drosophila*, where sexual conflict is mediated via mating-related harm to females (discussed in Michalczyk et al. 2010), there is no direct evidence for such harm in *T. castaneum*. Furthermore, the impact of female-female interactions in the evolved FB lines in the previous study is unclear. Testing quinone production and sensitivity in these evolved male-biased and female-biased lines may help to understand the conflicting results. In contrast, two other studies with *T. castaneum* corroborate our results, showing increasing female fitness with increasing male-biased sex ratios. First, populations selected for female-biased sex ratios showed a reduction in fitness (Lavie and Beiles 1981). Second, Wade measured the total number of offspring produced by groups with sex ratios ranging from 0.2 to 0.8 after 50 days (Wade 1984). Reanalyzing these data, we found that per capita female fitness decreased as a function of the proportion of females in the group (fig. A5*A*). Similarly, in another set of experiments (M. J. Wade, unpublished data), per capita female productivity declined as a function of the number of females in groups with different sex ratios maintained at a constant density (fig. A5*B*). Together, these results suggest that female resource competition may be the most important determinant of female fitness in *T. castaneum*.

Although we focus on the role of quinones as mediators of interference competition, quinones play multiple roles in the biology of flour beetles. For instance, o-quinone molecules are involved in the biosynthesis of melanin and are important for cuticle sclerotization and melanization (reviewed in Sokoloff 1977). *Tribolium castaneum* stink gland secretions largely contain p-benzoquinones (ethyl-1,4 benzoquinone and methyl-1,4 benzoquinone) and 1-pentadecene, as well as a number of other compounds in relatively low quan-

tities (Loconti and Roth 1953; Unruh et al. 1998; Villaverde et al. 2007). The physiological and fitness effects of these components remain unclear. Benzoquinones also show antimicrobial activity (Prendeville and Stevens 2002), and a recent study suggested a coevolutionary arms race between tenebrionid benzoquinone secretions and a common pathogenic fungus, *Beauveria bassiana*, that degrades benzoquinones (Pedrini et al. 2015). Thus, experiments that explicitly test the various physiological and ecological impacts of quinones are necessary to pinpoint the evolutionary significance of the observed responses to quinones. Regardless of the evolutionary history of quinones, our experiments clearly show that quinones suppress reproduction, directly determining the fitness impacts of group sex ratio.

In closing, we suggest that the role of females in the evolution and consequences of group sex ratio has generally been underappreciated (Stockley and Bro-Jørgensen 2011). This is unfortunate because imbalanced sex ratios provide a context where both sexual selection and resource competition may be altered. Our work demonstrates that fitness consequences of male-biased sex ratios in flour beetles are driven by chemically mediated female nonsexual competition. Specifically, we show that females use toxic benzoquinones as a key weapon in this warfare, although the mechanism responsible for the resulting suppression of fecundity remains unclear. We suggest that such effects of intrasexual female interference competition may be widespread and may frequently confound the effects of sex ratio on female fitness.

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