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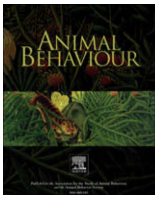

Condition-dependent female remating resistance generates sexual selection on male size in a ladybird beetle

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Behavioural resistance to remating by females is common, but the causes and consequences of resistance are rarely explained. Prominent hypotheses include resistance as a means of avoiding costly and superfluous mating, or as a means of biasing mating towards high-quality males. In species in which males produce nutritious nuptial gifts, females may further modulate resistance according to their need for nutrition. We investigated these hypotheses in the ladybeetle *Adalia bipunctata*, in which females frequently display vigorous resistance before copulation and ingest a spermatophore after copulation. In two experiments, we manipulated female nutritional state, depriving or satiating females for a short (16 h) or long (96 h) interval before a remating trial. We found that food-deprived females resisted mating more frequently and for longer periods than satiated females and consequently remated less frequently. This condition dependence of resistance supports the hypothesis that resistance functions to reduce superfluous and costly mating. Our finding that food-deprived females were more resistant suggests that mating imposes energetic costs, and that nuptial feeding does not offset these costs. In a third experiment, we investigated whether the extent of resistance depended on male size or whether resistance itself biased mating towards large males. The extent of female resistance was independent of male size, but resistance itself resulted in a mating bias towards large males. In summary, our results support the hypotheses that females resist mating simply because it is costly and superfluous, and that a side effect of resistance is sexual selection for large male size.

Behavioural resistance to mating by females is a common feature of mating systems. It may function to reduce female mating frequency, and in some cases, it biases mating success of males towards phenotypes that can overcome resistance (Arnqvist & Rowe 2005). Forms of resistance range from vigorous struggles with males (e.g. Rowe et al. 1994; Day & Gilburn 1997; Jormalainen 1998; Blanckenhorn et al. 2002) to avoidance of males through habitat switching (e.g. Krupa et al. 1990; Stone 1995; Rowe et al. 1996). In several of these examples, experiments have shown that resistance is costly to females. These costs include physical harm or elevated mortality (e.g. Mesnick & Le Boeuf 1991; Rowe 1994; Mühlhäuser & Blanckenhorn 2002) and missed opportunities such as foraging (Rowe 1992; Stone 1995). Evidence that females may pay a cost for resistance implies that some direct or indirect benefit offsets these costs.

There are several nonexclusive hypotheses that may account for female resistance to mating, yet there have been few attempts to experimentally distinguish among them. First, females may resist simply because additional mating is superfluous for fertilization and is costly. Although there is substantial support for the existence of costs to superfluous matings (reviews in Thornhill & Alcock 1983; Gwynne 1989; Choe & Crespi 1997; Arnqvist & Nilsson 2000), experimental support for the hypothesis that these costs account for female resistance is minimal. Direct support comes from economic studies where the costs (or benefits) of mating to females are manipulated, and the extent of resistance is then monitored (e.g. Lauer 1996; Blanckenhorn et al. 2002; Hosken et al. 2003; Teuschl & Blanckenhorn 2007). For example, in water striders, hungry females tend to increase resistance to mating, as expected because mating conflicts with female foraging (Rowe 1992), and females with stored sperm are more resistant than those depleted of sperm (Ortigosa & Rowe 2003).

The economics of female resistance to mating in species with nuptial gifts may be a particularly interesting case. In these species,

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it has been argued that some mating occurs as a means of acquiring resources from males (Gwynne 1984). If so, then one would expect resistance to decline when females are hungry, the opposite of the pattern observed in water striders. The evidence here is mixed. In some species with nuptial gifts, nutritionally deprived females do tend to be less resistant (e.g. Thornhill 1984; Gwynne 1990; Simmons & Bailey 1990; Bilde et al. 2007). However, in other species, nutritional state appears to have no effect on willingness to mate (Engqvist 2007b), suggesting that either nuptial gifts are not valuable to females as food items (see Vahed 1998), or some other factor is determining resistance.

A second hypothesis for female resistance is the male screening hypothesis, which relies on indirect rather than direct selection on resistance (West-Eberhard 1983; Wiley & Poston 1996; Eberhard 2002; Kokko et al. 2003). In short, females may resist males selectively so that mating is biased towards males of high genetic quality; females pay a direct cost of resisting males to obtain the indirect benefit of improved offspring quality. In some species, resistance does favour certain male phenotypes; however, there is little evidence that females modulate their level of resistance based on male phenotype (Arnqvist & Rowe 2005). If female resistance does not depend upon male phenotype, but male phenotype does affect the success of males in overcoming resistance, then biases may simply be a by-product of a general resistance by females to costly mating as per the first hypothesis above. In two well-studied systems (seaweed flies and water striders) the by-product hypothesis is supported: resistance depends on ecological circumstance rather than the phenotype of the male (Crean & Gilburn 1998; Shuker & Day 2001; Ortigosa & Rowe 2002). The by-product hypothesis can account for resistance and biases in male mating success (i.e. direct selection on male phenotypes that help males overcome resisting females), but does not speak to any positive (or negative) indirect selection that may result from these biases (i.e. a good genes process is not necessarily predicted).

In this study we examine each of these hypotheses for female resistance in a species with so-called nuptial gifts, the two-spot ladybird beetle *Adalia bipunctata*. Following copulation, females eject a spermatophore and consume it (Perry & Rowe 2008a). Nevertheless, females often vigorously resist remating by kicking at or running from males or by bending the abdomen to prevent genital contact. Ladybirds are known to face food-limited conditions in nature (Sloggett & Majerus 2000), and we reasoned that mating would conflict with female foraging. Mating involves females carrying males for several hours, and it is likely that foraging efficiency would decline and energy consumption would be elevated during this period. If females resist because mating interferes with foraging and is energetically costly, then hungry females should resist mating more than satiated females. Alternatively, if energy acquired through consuming spermatophores offsets these costs, then we would expect the opposite effect of hunger on resistance. In two experiments of the current study, we manipulated short-term and long-term female nutritional state to test these predictions.

In a third experiment, we determine whether resistance is dependent on the phenotype of males, and whether resistance tends to bias mating success of males. There are mixed reports of a mating advantage to large males in *A. bipunctata*, but the origin of these size biases have not been investigated (e.g. Tomlinson et al. 1995; Yasuda & Dixon 2002). One possibility is that females resist less with larger males as a means of biasing mating success towards them (i.e. the male screening hypothesis, Eberhard 2002). Another is that larger males are simply better able to overcome female resistance. To distinguish these hypotheses, we determined whether females altered their level of resistance based on male size, and whether resistance per se favoured larger males.

METHODS

Experimental Animals

Adalia bipunctata is an aphid predator widespread in temperate habitats (Omkar & Pervez 2005). Both males and females mate multiply. Females are typically larger than males and there is substantial variation in mass (range: female, 5.22–17.83 mg; male, 5.34–13.95 mg; this study). The beetles used in this study were from the F1 generation reared in our laboratory, from stock obtained from Natural Insect Control (Stevensville, Ontario, Canada). During maintenance periods, animals were provided daily with moistened cotton as a water source and fed pea aphids (*Acyrthosiphon pisum* reared on broad bean, *Vicia faba*) and UV-sterilized flour moth eggs (*Ephestia kuehniella*).

Assessing Female Remating Resistance

We investigated the effect of food level on female remating behaviour in two experiments. Each experiment consisted of an initial mating, followed by a feeding treatment and then a remating trial in which we monitored mating resistance. Females were housed individually in petri dishes (50×12 mm) throughout. For the initial mating, virgin females of similar age were mated once to a male from the laboratory stock. We did not interfere with spermatophore consumption after mating, which meant that most females probably ingested some or all of the spermatophore (>90%; Perry & Rowe 2008a). Following this mating and before the feeding treatment, females were fed excess flour moth eggs for several days. During this period, we monitored oviposition and discarded females that did not oviposit because it may have indicated a failure of sperm transfer.

For the remating trial, females were paired with a test male from the laboratory stock. All males had mated at least once previously. Males were maintained on excess flour moth eggs and kept isolated from females for at least 2 days before the trial. Males that did not attempt to mount the female within 10 min were replaced. We recorded whether the female resisted a male's mating attempt, and when resistance occurred, we measured the duration of resistance behaviour until mating began or until the male was dislodged. Males often remount females immediately after being dislodged. If the male did not remount the female within 1 min, we ended the trial. When males remounted within 1 min and females again resisted, we timed the duration of resistance and added it to the initial resistance time. We repeated this measurement of resistance until mating occurred or the female successfully eluded the male for at least 1 min. This design accounts for the likelihood that, in nature, a male may be able to immediately remount a female if dislodged but, we conjecture, would be less likely to remount if the female puts some distance between them. We separately analysed the remating responses of females considering only the first bout of resistance, but as the results were similar we do not report them here.

Short-term Hunger

To test the hypothesis that short-term hunger influences resistance, we began the food treatment 16 h before the mating trial. Females were transferred to new petri dishes, provided with moistened cotton as a water source, and either deprived of food ($N = 16$) or fed an excess of flour moth eggs ($N = 18$). Sixteen hours should have been sufficient time for gut clearance, which occurs in 2–12 h (McMillan et al. 2007). After 16 h, females were transferred to a new dish and paired with a male for the remating trial.

Long-term Hunger

Here the food treatment began 4 days before the remating trial. Low-food females were fed one adult pea aphid daily, and high-food females were provided excess pea aphids replenished three times a day. Food treatments are often used as surrogates for condition (Cotton et al. 2004; Bonduriansky & Rowe 2005), and there was evidence that our food treatment affected female condition. After 4 days of differential feeding, low-food females oviposited significantly fewer eggs (mean ± SE = 16 ± 9.1 versus 93 ± 8.0; $F_{1,30} = 40.2$, $P < 0.0001$) and gained less weight, compared to high-food females. High-food females gained mass (3.33 ± 0.42 mg), whereas low-food females maintained or lost mass (−0.54 ± 0.48 mg), and this difference was significant ($F_{1,30} = 37.1$, $P < 0.0001$). The experiment began with 18 females in each treatment group; four low-food females died before the remating trial.

Resistance and Male Size

By pairing resistant and less resistant females with small or large males, we tested two hypotheses about the relationship between resistance, male size and mating success. Specifically, we asked first whether either resistant (low condition) or less resistant (high condition) females altered their resistance as a function of male size. Second, we asked whether male size affected the probability of mating at either resistance level.

We generated small and large males by subjecting third-instar larvae to a low(one aphid daily) or high (excess aphids) diet treatment until emergence. Prior to the third instar, all larvae were fed excess flour moth eggs.

Prior to the female feeding treatment, virgin females were fed excess flour moth eggs daily and mated once to a male from the laboratory stock. Following this initial mating, we monitored oviposition and discarded females that did not oviposit before the remating trial. We generated females that were more or less resistant to remating by applying a food treatment after the initial mating: a low (0.1 mg of flour moth eggs for 2 days) or high (excess flour moth eggs) food diet for 8 days before the mating trial.

This food treatment was substantial enough to detect a difference in female condition between treatments. Low-food females experienced a smaller mass increase than high-food females (1.00 ± 0.28 mg versus 3.57 ± 0.29 mg; ANOVA: $F_{1,50} = 40.5$, $P < 0.0001$) and laid fewer eggs before the remating trial (23 ± 8.1 versus 85 ± 8.5; $F_{1,49} = 28.2$, $P < 0.0001$). Three low-food females died before the remating trial. The male food treatment also affected condition. Low-food males emerged as significantly smaller adults compared to high-food males (for a random subset of males: 6.50 ± 0.25 mg versus 7.83 ± 0.26; $F_{1,61} = 13.5$, $P = 0.0005$). All adult males were fed flour moth eggs and pea aphids ad libitum. Despite this, low-food males remained lighter in mass when the experiment began (7.40 ± 0.11 mg versus 10.04 ± 0.12 mg; $F_{1,178} = 267.4$, $P < 0.0001$).

For the remating trial, females were randomly paired with a small or large male (low-food, 52 and 39 pairs, respectively; high-food: 47 and 46 pairs). Female resistance behaviour was recorded as described above. The experiment was conducted in two blocks, the first with 53 pairs (28 low-food and 25 high-food) and the second with 131 pairs (63 low-food and 68 high-food).

Analyses

Each experiment generated two categorical and one continuous measure of female resistance: whether resistance occurred, whether remating occurred and the duration of resistance. For the two food-level experiments, the female food treatment was the sole factor in the chi-square goodness-of-fit tests or *t* tests. For the food and male size experiment, the categorical responses were analysed with multiple logistic regression and the continuous response by ANOVA. Both types of models included food, male size, their interaction, and experimental replicate. Resistance duration data was analysed only for females that displayed resistance. Where appropriate, we applied a transformation to the resistance duration data to meet the assumptions of parametric statistics; backtransformed least squares means are presented with confidence intervals. The analyses were conducted using JMP 6.0.3 (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Condition and Resistance

Following the long-term hunger treatment, low-food (low condition) females were more likely to display remating resistance, and when they did resist, they resisted longer, with both effects contributing to reduced mating frequency (Table 1). Females that had experienced long-term hunger engaged in mating struggles that were seven times as long as those of satiated females, resulting in a 57% decrease in remating frequency. These effects were significant ($P < 0.01$), while the increase in the frequency of remating resistance was not (Table 1). We did not detect a significant difference in mating behaviour from the short-term hunger treatment, although the pattern of responses was consistent with the long-term experiment. Relative to the satiated females, females that had experienced short-term hunger were twice as likely to resist a mating attempt, and when resisting would struggle for over twice as long, resulting in a 28% decrease in mating frequency. This contrast suggests that an effect of short-term hunger was of lower magnitude, but may have been detectable with a larger sample.

Resistance and Male Size

Female condition had effects on resistance that were consistent with the other experiments. Low-food females were significantly more likely to resist remating (Table 2), and when they did resist, they resisted more than two-fold longer than high-food females (backtransformed means: low-food females: 461 s (329, 644), high-food females: 153 s (98, 234); analysis performed on log-transformed data: $F_{1,118} = 17.3$, $P < 0.0001$; Fig. 1).

Male size had no detectable influence on whether females showed remating resistance (Table 2) or the duration of resistance

Table 1

Female remating behaviour in ladybeetles after a short (16 h) or long (4 day) food treatment

	Low-food females	High-food females	Test
Short-term experiment			
Resistance frequency	9/16 (56%)	5/18 (28%)	χ^2_1=2.7, P=0.10
Resistance duration (s)*, †	100 (67, 201)	82 (53, 181)	t_{12}=0.3, P=0.61
Remating frequency	9/16 (56%)	14/18 (78%)	χ^2_1=1.7, P=0.19
Long-term experiment			
Resistance frequency	11/14 (79%)	9/18 (50%)	χ^2_1=2.6, P=0.11
Resistance duration (s)*, ‡	657 (348, 1239)	139 (69, 280)	t_{18}=10.4, P=0.005
Remating frequency	5/14 (36%)	15/18 (83%)	χ^2_1=6.8, P=0.009

* Least squares mean resistance durations were calculated for females that displayed resistance (i.e. excluding zeroes). Backtransformed means are presented with confidence intervals.

† Inverse-transformed in the analysis.

‡ Log-transformed in the analysis.

Table 2
The remating behaviour of female ladybeetles exposed to a low- or high-food diet for 8 days and paired with a small or large male

	Resistance frequency	Remating frequency	
		Resisting females	Nonresisting females
Treatment group			
Low-food female, small male	47/52 (90%)	26/47 (55%)	5/5 (100%)
Low-food female, large male	33/39 (85%)	24/33 (73%)	4/6 (67%)
High-food female, small male	22/47 (47%)	15/22 (68%)	23/25 (92%)
High-food female, large male	21/46 (46%)	18/21 (86%)	21/25 (84%)
Analysis*			
Food level	χ^2_1=31.2, $P < 0.0001$	χ^2_1=2.5, P=0.11	χ^2_1=0.0, P=0.94
Male size	χ^2_1=0.6, P=0.46	χ^2_1=4.5, P=0.03	χ^2_1=0.0, P=0.93
Food level × male size	χ^2_1=0.6, P=0.45	χ^2_1=0.1, P=0.74	χ^2_1=0.0, P=0.93
Experimental replicate	χ^2_1=4.1, P=0.04	χ^2_1=2.8, P=0.09	χ^2_1=2.2, P =0.14

* *P* values for the chi-square statistic are presented.

when it occurred ($F_{1,118} = 0.0$, $P = 0.99$; Fig. 1; food level × male size interaction: $F_{1,118} = 0.2$, $P = 0.68$). There was, however, an effect of male size on the frequency of remating that was dependent on female resistance. When males were paired with females that displayed remating resistance, large males were more likely to achieve copulation than small males: 59% of small males copulated, compared to 78% of large males (Table 2). In contrast, male size did not influence the mating outcome when males were paired with nonresisting females: 93% of small males copulated, compared to 81% of large males (Table 2). These data suggest that female resistance per se favours large males.

The overall level of resistance was higher in the first replicate of this experiment compared to the second, for both resistance frequency (Table 2) and resistance duration ($F_{1,118} = 5.7$, $P = 0.02$).

DISCUSSION

An understanding of the causes and consequences of costly female resistance to mating is crucial for distinguishing among competing models of sexual selection (Arnqvist & Rowe 2005; Rowe & Day 2006). Our data support the hypothesis that female resistance to mating depends on their ecological setting: females that were deprived of nutrition increased their level of resistance, presumably because mating is costlier for low-condition females. This result suggests that any nutritional gain from edible spermatophores that come with mating is not sufficient to offset the cost of mating. Our data further suggest that resistance favours large, high-condition males. However, this bias did not occur because females modulated their resistance according to male phenotype; instead, large males simply seem better able to overcome resistance.

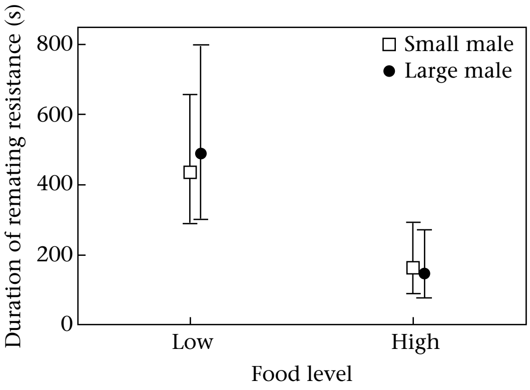

Figure 1. Duration of female resistance to a second mating for female ladybeetles fed low- and high-food diets and paired with a small or large male. Backtransformed least squares means are presented. Bars indicate 95% confidence intervals.

Condition Dependent Remating Resistance

Vigorous and lengthy behavioural mating resistance (>5000 s; unpublished data) presumably carries some energetic costs for female *A. bipunctata*, and therefore suggests some benefit of resistance to females. Two broad hypotheses may account for resistance: (1) it is a means of reducing the rate of costly and superfluous mating (a direct benefit), or (2) it is a means of screening for high-quality males as fathers (an indirect benefit; West-Eberhard 1983; Eberhard 1996; Wiley & Poston 1996; Cameron et al. 2003; Chapman et al. 2003; Eberhard & Cordero 2003; Kokko et al. 2003). Our results are consistent with the former hypothesis and are not predicted by the latter. In particular, our finding that females in poor nutritional condition displayed increased resistance implies that the costs of mating, and benefits of resisting mating, are greater for low-condition females. Clearly, a potential source for such differential costs is that low-condition females have a more pressing need to forage and mating may entail reduced foraging efficiency. Mating may also impose energetic costs that low-condition females have a reduced ability to tolerate. Both types of costs have been found in other species (e.g. Rowe 1992; Fairbairn 1993; Watson et al. 1998; Plaistow et al. 2003). Furthermore, the need to forage no doubt increases as time passes without food, and in our study, the length of food deprivation influenced the extent of female resistance. Condition-dependent resistance is not predicted by the mate screening hypothesis (Ortigosa & Rowe 2002). In fact, it is more likely that if resistance serves to screen males and is energetically costly, then high-nutrition females should resist more because they are better able to pay these costs. We observed the opposite.

Our finding that females in good nutritional condition showed some resistance and did not always accept remating attempts from males may imply other costs of mating in addition to energetic costs, such as a risk of predation or pathogen transmission (e.g. Rowe 1994; Hurst et al. 1995). Direct harm from males is another possible cost, but recent laboratory studies of *A. bipunctata* have detected no such costs from additional matings to female longevity or reproductive success (Haddrill et al. 2007; Perry & Rowe 2008b). Potential costs (e.g. reduced foraging, increased predation) that are not detectable in the laboratory should be a topic of further investigation.

The extent of mating resistance observed is a joint outcome of female resistance and male persistence. Accordingly, two of the responses we measured, resistance duration and remating frequency, may have been influenced by male behaviour. Males may modulate persistence depending on female quality or on female resistance (and, thus, their chance of success). Both hypotheses predict that males will persist more with high-quality, less-resistant females. However, inspection of the mating frequency data in Table 2 indicates that the remating rate among nonresisting females was very similar for low- and high-food females. Furthermore, male persistence is not expected to influence the initial occurrence of female resistance to a mating attempt, and this measure was not substantially different from the other responses.

Resistance and Nuptial Gifts

We found no support for the hypothesis that hungry females seek additional matings to forage for edible ejaculates, suggesting that any nutritional benefit of the ejaculate does not offset the cost of mating. Past results have been mixed on this issue. In some orthopterans, poor food conditions result in females that are more receptive to mating (Gwynne 1990; Simmons & Bailey 1990). In contrast, female scorpionflies on a low-food diet are no more or less likely to accept nuptial gifts or to copulate with males than are females fed a high-food diet (Engqvist 2007b; but see Thornhill 1984). We have previously found that female *A. bipunctata* have little to gain nutritionally from consuming spermatophores (Perry & Rowe 2008a). In fact, the failure to detect any benefit from consuming nuptial gifts is a common outcome in other species, and hypotheses addressing the maintenance of nuptial feeding in the absence of any benefit have been discussed elsewhere (e.g. Sakaluk 2000; Vahed 2007).

We have previously shown that spermatophore ingestion quickly and substantially increases female remating resistance (Perry & Rowe 2008a). It is noteworthy that our present results suggest that providing females with extra food diminishes remating resistance. Taken together, these results suggest that ingested ejaculates are distinct from food in their effect on resistance. An interesting possibility is that spermatophores transfer signals or stimulant proteins from males; the most likely source of such proteins is the male accessory glands, from which spermatophores are derived. Accessory gland proteins are well known to induce remating resistance in several species (Gillott 2003). A similar increase in remating resistance following nuptial gift ingestion has been found in other insects (Sakaluk et al. 2006; Engqvist 2007a). The present study suggests that in *A. bipunctata*, the increase in resistance from spermatophore ingestion cannot be explained as a food effect.

Sexual Selection as a By-product of Resistance

Large, high-condition males achieved a mating advantage over small males when females were resistant to mating. This result suggests that large males are better able to overcome the vigorous resistance of females, as has been indicated in other species (Crean & Gilburn 1998; Shuker & Day 2001; Ortigosa & Rowe 2002). Indeed, higher mating success for larger males was reported for *A. bipunctata* in a study in which heavier and lighter males were placed together with a single female (Tomlinson et al. 1995). Our experiment suggests that the basis of the size advantage is in overcoming female resistance rather than success in intrasexual competition. A second study of resistance in *A. bipunctata* found that small males had an advantage in mounting females when competed against large males, but small males did not achieve more copulations (Yasuda & Dixon 2002).

The mate screening or selective resistance hypothesis suggests that resistance to mating does not arise from a general cost of mating, but from a cost of mating with low-quality males (e.g. West-Eberhard 1983; Wiley & Poston 1996; Eberhard 2002; Kokko et al. 2003). Thus, a large (high-quality) male mating advantage might arise from females selectively screening out the small (low quality) males. Two lines of evidence argue against this hypothesis for our results. First, females did not modulate their resistance with respect to the size of the male attempting to mate. This negates the simplest means of using resistance to selectively screen males: reducing resistance to favoured males. Second, resistance behaviour was condition dependent, and the selective resistance hypothesis cannot account for the finding that high-condition females were less resistant than low-condition females.

In summary, this study suggests an important role for direct selection on female resistance, and points to sexual selection on male size as a consequence of resistance. Future work should investigate the generality of these patterns in other species in which females display conspicuous mating resistance.

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