Copulation, genital damage and early death in *Callosobruchus maculatus*

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Antagonistic sexual coevolution stems from the notion that male and female interests over reproduction are in conflict. Such conflicts appear to be particularly obvious when male genital armature inflicts damage to the female reproductive tract resulting in reduced female longevity. However, studies of mating frequency, genital damage and female longevity are difficult to interpret because females not only sustain more genital damage, but also receive more seminal fluid when they engage in multiple copulations. Here, we attempt to disentangle the effects of genital damage and seminal fluid transfer on female longevity in the beetle *Callosobruchus maculatus* (Coleoptera: Bruchidae). Males copulating for the sixth time in succession inflicted greater levels of genital damage, but transferred smaller ejaculates in comparison with virgin males. The number of copulations performed by males was negatively related to female fecundity and positively related to female longevity, suggesting a trade-off between fecundity and longevity. However, inclusion of fecundity as a covariate revealed sperm and/or seminal fluid transfer to have a negative impact on female longevity above that caused by the fecundity–longevity trade-off. The consequences of multiple copulations on female longevity were examined. Females that mated twice laid more eggs and died sooner than those that mated once. However, incorporation of fecundity as a covariate into our statistical model removed the effect of female mating frequency on female longevity, indicating that double-mated females suffer greater mortality owing to the trade-off between fecundity and longevity. Males of this species are known to transfer very large ejaculates (up to 8% of their body weight), which may represent a significant nutritional benefit to females. However, the receipt of large ejaculates appears to carry costs. Thus, the interpretation of multiple mating experiments on female longevity and associated functional explanations of polyandry in this species are likely to be complex.

Keywords: copulation; genital damage; longevity; fecundity; *Callosobruchus*

1. INTRODUCTION

Anisogamous sex can result in an uneasy alliance between males and females over sexual reproduction (Bateman 1948; Parker 1979; Rice 1996; Rice & Holland 1997; Arnqvist & Rowe 2002; Chapman *et al.* 2003). In polyandry, males are likely to be favoured by selection if they prevent or delay their sexual partners from remating and/or increase the rate at which their partners produce and lay eggs, as both strategies tend to increase the number of ova fertilized by the male (Simmons 2001). However, such strategies can alter behaviour away from the female optima, resulting in counter selection on females to resist male manipulation and antagonistic coevolution between the sexes ensues (Rice & Holland 1997).

A macabre manifestation of this conflict has recently come to light in the bruchid beetle *Callosobruchus maculatus* (Crudginton & Siva-Jothy 2000) and the dung fly *Sepsis cynipsea* (Blanckenhorn *et al.* 2002). Males of both species have intromittent organs that are tipped with sclerotized spines, which apparently puncture the female reproductive tract during copulation (Crudginton & Siva-Jothy 2000; Blanckenhorn *et al.* 2002; see also Eberhard (1996) for further examples). Crudginton & Siva-Jothy (2000) and Blanckenhorn *et al.* (2002) examined the consequences of

genital damage on female longevity by comparing the lifespan of females that received different numbers of copulations. Crudginton & Siva-Jothy (2000) found that doubly mated females died sooner than singly mated females and Blanckenhorn *et al.* (2002) found that mated females died sooner than virgins, indicating that genital wounding does indeed reduce female longevity. However, experiments such as these are difficult to interpret because females that copulate repeatedly not only sustain more genital damage, but also receive more seminal products derived from the male ejaculates. Given that components of seminal fluid are known to reduce female longevity and increase the rate at which eggs are matured and laid (Chapman *et al.* 1995; Gilliot 2003), the premature death of mated females in the studies of Crudginton & Siva-Jothy (2000) and Blanckenhorn *et al.* (2002) could be attributable to the receipt of elevated levels of seminal fluid.

To examine the effects of seminal fluid transfer on female longevity in *C. maculatus*, we devised a series of experiments based on the observation that males of this species transfer reduced quantities of seminal fluid when mated to females in close temporal succession (Eady 1995; Fox *et al.* 1995). Therefore, we could design treatments in which females received similar amounts of genital damage (as they all mated once), yet received quite different amounts of seminal fluid.

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We also re-examined the effect of copulation frequency on female longevity in this species, given the conflicting results in the literature. For example, Fox (1993) found that double-mated females laid more eggs and lived longer than single-mated females when oviposition resource was abundant. Fox (1993) attributed this finding to the large nutritional donations made by males to females via their ejaculates, which may represent up to 8% of the male's body weight (Fox *et al.* 1995). In contrast, Savalli & Fox (1999) and Crudgington & Siva-Jothy (2000) found that double-mated females had reduced longevity in comparison with single-mated females. Savalli & Fox (1999) attributed this difference to a trade-off between fecundity and longevity (as double-mated females laid more eggs), whereas Crudgington & Siva-Jothy (2000) attributed the reduced longevity of double-mated females to elevated levels of genital wounding.

2. MATERIAL AND METHODS

(a) General methods

Callosobruchus maculatus is a cosmopolitan pest of stored legumes. Females glue eggs to the coat of host seeds. Upon hatching, the larvae burrow into the seed where they complete their development, pupation and finally eclosion approximately four weeks later. Under typical storage conditions, adults do not have access to food or water and thus rely on metabolic water and nutrients acquired during larval development (Bellows 1982). The beetles used in this study were derived from Niamey, Niger and have been cultured by P. Eady for approximately 10 years.

In all the experiments, males and females were initially virgin and between 24 and 48 h from eclosion. All copulations were observed (which can be unambiguously assigned from mounting; Eady 1994) within small perspex pots at 28°C and 35% RH.

(b) Effect of male mating sequence on the extent of female genital damage

To determine the effect of male mating history on the amount of induced genital damage to females, males were mated to six individual females in quick succession (i.e. all six copulations took place within 4 h of the initial copulation). Upon copulation, those females that were either first or last ($n=19$) in the mating sequence were removed from the male and placed in a small ventilated plastic container for 16 h to allow melanized haemocyte plugs to form around the genital wound, before being euthanized. The reproductive tracts were then dissected free under an Olympus SZH stereo-microscope linked to an image analysis workstation running NIH-image software. The extent of genital damage was measured at a magnification of $240\times$ by drawing around the perimeter of each melanized plug and calculating the total area of repair per female as described by Crudgington & Siva-Jothy (2000).

(c) Effect of male mating history on lifetime female fecundity and longevity

(i) Experiment 1: with abundant oviposition sites

Male and female pairs were introduced to individual perspex pots and observed. Upon successful copulation, the female of the pair was removed and replaced by another virgin female. This procedure was repeated until individual males ($n=20$) had copulated with 4–6 females ($n=116$) within 4 h of the

first copulation. Those males and their attendant female partners that failed to achieve 4–6 copulations were excluded from the analysis. Immediately following copulation, females were transferred to individual pots containing 100 black-eyed beans (*Vigna unguiculata*) on which to oviposit. They were then maintained at 28°C within an insectary. The positions of the individual pots were rotated daily to avoid the possibility of microclimate differences affecting female oviposition and longevity. Females were checked daily to ascertain the time of death. Following death, the number of eggs laid was counted and female elytra length (which correlates closely with emergence weight; Wilson & Hill 1989) was measured.

(ii) Experiment 2: no access to oviposition sites

The above experiment was repeated (males, $n=39$; females, $n=223$) except that following copulation females were maintained in individual pots containing no oviposition sites (black-eyed beans). This procedure was followed in order to minimize oviposition differences between the treatments as this is known to covary with female longevity (see §3). The containers were checked for eggs following female death, as females of this species are known to 'dump' considerable number of eggs on the sides of containers (Wilson & Hill 1989; Wang & Horn 2004). Following female death, the number of dumped eggs and elytra length were recorded.

(d) The effect of copulation frequency on female fecundity and longevity

Male and female pairs were observed to copulate. Immediately following copulation, females were transferred to individual pots containing 15 black-eyed beans for 48 h. Half of the females were then provided with a second opportunity to copulate with a virgin male. Those that copulated (the twice-mated treatment, $n=84$) were transferred to individual perspex pots containing no oviposition resource. The females not offered a second opportunity to remate (the single-mated treatment, $n=92$) were transferred to perspex pots containing no oviposition sites at the same time. Female longevity was monitored daily and upon death, elytra length was measured along with the number of eggs dumped on the sides of the container following the second copulation.

(e) Statistical methods

Where the experimental design contained repeated measures, data on fecundity and longevity were analysed using generalized linear models with Poisson and gamma error distributions, respectively, with male identity entered as a random factor (Crawley 2002). Given the large number of explanatory variables, the model simplification approach of Crawley (1993) was followed in which the maximal model (containing all factors, covariates and interactions) was initially fitted and then simplified by a series of step-wise deletion tests, starting with the highest order interaction and progressing to lower order interaction terms and finally the main effects. The critical probabilities for retention were 5% for main effects, 1% for two-way interactions and 0.5% for three-way interactions (Wilson & Hardy 2002). In mixed-effect models, the significance of model simplification was assessed using the maximum-likelihood method (Crawley 2002). Where the experimental design did not contain repeated measures, fecundity and longevity data were analysed using log-linear models and Cox regression, respectively.

3. RESULTS

(a) Effect of male mating sequence on the extent of female genital damage

The extent of genital damage was lower in females mated to virgin males (mean area of damage = $22.4 \mu\text{m}^2 \pm 3.7$ (s.e.), $n=19$) compared with females that copulated with males which had mated five times previously (mean area of damage = $31.7 \mu\text{m}^2 \pm 6.5$ (s.e.), $n=19$; paired $t=1.28$, d.f. = 18, $p=0.02$). The level of male-induced damage during the first and last (sixth) copulation was significantly correlated ($r=0.67$, $n=19$, $p=0.02$), suggesting consistent between-male variation in induced damage.

(b) Effect of male mating history on lifetime female fecundity and longevity

(i) Experiment 1: with abundant oviposition sites

In the analysis of female fecundity, the maximal model contained the factor male mating sequence, the covariate female elytra length and the interaction term. Model simplification revealed fecundity to be positively related to female elytra length (likelihood ratio 8.15, $p=0.004$) and negatively related to male mating sequence (likelihood ratio 124.7, $p<0.001$; figure 1). For the analysis of female longevity, the maximal model contained the factor male mating sequence, the covariates female elytra length and lifetime female fecundity, and all interactions. The minimal model (table 1) revealed female longevity to be positively related to male mating sequence (figure 2).

(ii) Experiment 2: no access to oviposition sites

In the analysis of female fecundity, the maximal model contained the factor male mating sequence, the covariate female elytra length and their interaction. The minimal model contained male mating history only (likelihood ratio = 23.28, $p<0.001$), with a general decline in the number of eggs dumped as male mating history increased (figure 3). For the analysis of female longevity, the maximal model contained the factor male mating sequence, the covariates female elytra length and lifetime female fecundity, and all interaction terms. The minimal model (table 2) revealed female longevity to be positively related to both female elytra length (bigger females live longer) and male mating sequence (figure 4) and negatively related to female fecundity.

(c) Effect of copulation frequency on female fecundity and longevity

(i) Fecundity following the second copulation

The maximal model contained female elytra length, and copulation frequency (i.e. whether females had copulated once or twice) and their interaction. The minimal model revealed a significant effect of female size (Δ deviance = 112, d.f. = 1, $p=0.0005$) and copulatory frequency (Δ deviance = 106, d.f. = 1, $p=0.0007$) on fecundity, with twice-mated females dumping more eggs on the sides of the container than singly mated females (figure 5).

Cox regression (Crawley 2002) revealed female mortality to be negatively related to female size and positively related to copulation frequency (table 3). Thus, double-mated females appear to have reduced longevity. However, this analysis ignores the potentially confounding effect of differences in the number of eggs dumped on the sides of the container. Thus, the maximal model was fitted which contained the factor copulatory frequency, the

Figure 1. Lifetime fecundity (means $\pm$ s.e.) of females mated to males with differing mating history. Male mating sequence 1 represents a male's first copulation (i.e. a virgin male). Male mating sequences 2–6 represent a male's second, third, fourth, fifth and sixth copulations with virgin females, respectively.

Table 1. The minimal mixed-effect model of female longevity following step-wise deletion tests, when females have access to oviposition sites.

	coefficient	<i>t</i>	<i>p</i>
male mating history	2.31	4.12	0.0001
fecundity	0.04	1.14	0.25
male mating history: fecundity	−0.03	3.4	0.001

Figure 2. Percentage survival of females mated to males with differing mating history and allowed access to oviposition resource. Mates 1, 2, 5 and 6 represent males copulating for the first, second, fifth and sixth time, respectively. Survival curves for females mated to third and fourth sequence males were intermediate between the extremes, but are not shown for clarity of presentation.

covariates female size and eggs laid from day 3 till death (i.e. dumped eggs), and all interaction terms. The minimal model (table 4) revealed female mortality to be independent of copulatory frequency (likelihood ratio test = 0.05, d.f. = 1, $p=0.83$), but was found to be negatively related to female size (large females live longer) and positively

Figure 3. Mean number of eggs laid ($\pm$ s.e.) by females on container walls, in the absence of oviposition resource, after being mated to males of differing mating history. Male mating sequences 1–6 represent males mating for the first to sixth time, respectively.

Table 2. The minimal mixed-effect model of female longevity following step-wise deletion tests, when females have no oviposition sites.

	coefficient	<i>t</i>	<i>p</i>
male mating history	0.36	1.92	0.056
female size	8.44	3.06	0.002
fecundity	−0.09	6.99	0.0001

Figure 4. Percentage survival of females mated to males with differing mating history, but denied access to oviposition resource. Mates 1, 2, 5 and 6 represent males copulating for the first, second, fifth and sixth time, respectively. Survival curves for females mated to third and fourth sequence males were intermediate between the extremes, but are not shown for clarity of presentation.

related to the number of eggs laid (females laying more eggs die sooner), even though oviposition sites were not available. Thus, it would appear that double-mated females have reduced longevity owing to their higher rate of egg dumping.

4. DISCUSSION

Our results suggest that the costs and benefits of multiple mating in *C. maculatus* are complex and that a series of factors operate to determine female longevity. Here, we found that females that mated twice had reduced longevity compared with females that mated once, although this difference vanished when female fecundity (associated

Figure 5. The number of eggs laid by females denied access to oviposition sites following the second copulation in relation to female elytra length. Open circles, double-mated females; closed circles, single-mated females.

Table 3. Summary of Cox regression survival analysis following step-wise deletion tests to reveal the minimal model. (Summary refers to the mortality of females denied access to oviposition sites following copulation with either one or two males. Note this model does not contain female fecundity as a covariate.)

	coefficient	<i>Z</i>	<i>p</i>
copulation frequency	3.44	1.19	0.004
female size	−2.36	0.624	0.0001
copulatory frequency: female size	−1.71	0.616	0.006

Table 4. Summary of Cox regression survival analysis following step-wise deletion tests to reveal the minimal model. (Summary refers to the mortality of females denied access to oviposition sites following copulation with either one or two males. Note this model does contain female fecundity as a covariate.)

	coefficient	<i>Z</i>	<i>p</i>
female size	−1.19	−1.75	0.08
fecundity from day 3	0.873	4.15	0.0001
female size: fecundity from day 3	−0.418	0.105	0.0001

with copulation frequency) was incorporated as a covariate. This indicates that the reduced longevity of double-mated females represents a trade-off between fecundity and longevity as suggested (but not formally tested) by Savalli & Fox (1999). Previous studies on the longevity of *C. maculatus* females that mated once or twice have shown contrasting results. Fox (1993) found doubly mated females to have increased longevity, while Savalli & Fox (1999) and Crudgington & Siva-Jothy (2000) found the opposite. Such contrasting results may stem from analytical differences as both Savalli & Fox (1999) and Crudgington & Siva-Jothy (2000) assessed differences in longevity independent of differences in individual female fecundity, although Crudgington & Siva-Jothy (2000) report no overall treatment differences in fecundity.

Understanding these contrasting results requires careful consideration of the costs and benefits to females from engaging in multiple copulations. Female longevity may

be enhanced via multiple mating owing to the nutritional benefits associated with the receipt of large ejaculates (Fox 1993). However, by mating with multiple males, females may suffer reduced longevity via direct harm from the male genitalia and/or seminal fluid compounds, or indirectly via alteration of physiological processes which are known to trade-off against longevity (Williams 1966; Partridge *et al.* 2005). Our finding that female longevity was positively related to male mating history, after differences in fecundity had been incorporated into our statistical model, suggests that compounds in the ejaculate further reduce female longevity beyond that attributable to the fecundity–longevity trade-off.

Given the decreased longevity of females receiving single, large ejaculates, it is unclear as to why females receiving two ejaculates do not die sooner than those mated once. One possibility is that by the time females in our experiment mated for the second time, 48 h after the initial copulation, they were suffering nutritional stress, given that they do not feed or drink as adults. Thus, at this point of time, the nutritional benefits outweigh or at least balance the costs of receiving a second ejaculate. Double-mated females laid more eggs than singly mated females and lived for an equivalent period of time, which would indicate that under the conditions of our experiment, double mating does provide a net benefit to females.

Quite why males harm their mates during copulation remains unclear. The adaptive harm hypothesis suggests that females respond to harm in a manner which is favourable to the male by either having a reduced propensity to remate (Johnstone & Keller 2000) and/or via an elevation in reproductive rate (Lessells 1999). Alternatively, it has been proposed that males harm their mates as a side effect of other adaptations which give males a reproductive advantage, especially in response to sperm competition (Parker 1979). For example, the genital armature of the male may have evolved to prevent dislodgement during copulation (by either the female or rival males) or to increase the efficiency of seminal fluid compounds via punctures within the wall of the female reproductive tract (Eberhard 1996). Recent work by Morrow *et al.* (2003) revealed that female *C. maculatus* subjected to different forms of harm (although not genital harm) did not respond by elevating egg laying rates or by reducing remating propensity, suggesting that harm is a side effect of some other male adaptation. This conclusion was also reached by Edvardsson & Treganza (2005) who found no association between the extent of genital damage (inferred from experimentally induced differences in female kicking ability) and rates of oviposition or remating. The finding that genital damage has little effect on female fecundity is further supported by our observation of a fivefold increase in the fecundity of females (with access to oviposition sites) mated to virgin males in comparison with those mated to males performing their sixth copulation. Because females mated to virgin males sustained lower levels of genital damage, differences in fecundity are most probably attributable to differences in ejaculate size and/or the number of sperm they contain, relegating genital damage to a relatively minor role in affecting female oviposition.

Our results also have a bearing on the relationship between copulation duration and the extent of genital damage. Crudgington & Siva-Jothy (2000) found that

females prevented from kicking at their mates during copulation endured longer copulations (see also Edvardsson & Treganza 2005) and received more genital damage. Our results support a relationship between copulation duration and genital damage as copulation duration is known to increase with male mating history (Brown 2001).

We would like to thank Jonathan Cooper and Charles Deeming for their comments on earlier drafts of the manuscript and Ken Wilson and Ian Hardy for their statistical advice. L.H. was supported by the Nuffield Foundation (grant no. URB/01209/G).

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