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Costly traumatic insemination and a female counter-adaptation in bed bugs

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Male bed bugs pierce females through the body wall and inseminate directly into the body cavity. It has previously been shown that such traumatic insemination carries costs for females, and sexual conflict regarding the mode of insemination should thus propel male–female coevolution. Since males accumulate sexually antagonistic adaptations, females should evolve counter-adaptations that efficiently abate the costs to females of sexual interactions. Yet, unambiguous experimental evidence for female counter-adaptations is lacking. In bed bugs, the spermalege (a highly modified region of the abdomen where the male usually pierces the female) may represent a female counter-adaptation. We assess the female costs of traumatic insemination by varying the rate of insemination on the one hand, and the rate and mode of piercing trauma to females on the other. Our results show that female mating costs are not extreme—elevated mating rate shortened female lifespan but had no significant effect on lifetime egg production. More importantly, additional abdominal piercing in the spermalege had no effect on females whereas even a very low rate of such piercing outside the spermalege reduced female lifetime egg production by 50%. Thus, females are well counter-adapted to the intrusive mode of insemination exhibited by male bed bugs and the costs of elevated mating are comparable with those in other insects, as predicted by theory. We therefore demonstrate that the spermalege efficiently reduces the direct costs of piercing trauma to females, and hence provide experimental evidence for a female counter-adaptation to a sexually antagonistic male trait.

Keywords: sexual selection; multiple mating; *Cimex lectularius*; costs of mating; sperm competition; coevolutionary arms race

1. INTRODUCTION

Differences in the evolutionary interests of the sexes are rife in sexually reproducing organisms (Parker 1979; Chapman & Partridge 1996; Rice 1996, 2000). Such sexual conflict is predicted to lead to coevolutionary 'arms races' between the sexes (Parker 1979; Rice & Holland 1997). Such arms races proceed over evolutionary time where adaptations in one sex select for counter-adaptations in the other sex, where the latter are aimed at ameliorating the costs imposed by the former. Thus, as Dawkins & Krebs (1979, p. 57) phrased it, 'as swords get sharper, so shields get thicker, so swords get sharper still'. Sexually antagonistic coevolution plays a significant role in the evolution of sexual dimorphism in general (Rice 1984), as well as in the evolution of reproductive morphology (Arnqvist & Rowe 2002*b*), physiology (e.g. seminal fluid proteins; Rice 1996) and behaviour (e.g. courtship; Arnqvist & Rowe 2002*a*). It has also been implicated as an important engine of speciation (Parker & Partridge 1998; Rice 1998; Arnqvist *et al.* 2000; Gavrilets 2000).

Despite the fact that sexually antagonistic coevolution generates adaptations, in both sexes, which are costly for the other sex, the process is notoriously difficult to detect empirically. This is because we expect adaptation in one sex to be balanced by counter-adaptation in the other, thus effectively hiding the costs and conflict from the observer (Chapman & Partridge 1996). Thus, counter to naive expectations, we do not expect sexual interactions to become more costly as antagonistic adaptations become more escalated in a given sex, because matching counter-adaptations will occur in the other sex (Arnqvist & Rowe 2002*a*). Nevertheless, convincing empirical (Rice 1996) and comparative (Arnqvist & Rowe 2002*a*) evidence now exists to demonstrate that such arms races do occur.

Male bed bugs (Heteroptera; Cimicidae) do not copulate via the genital opening of the female. Instead they pierce the female abdominal wall with their sharp intromittent organ (paramere) and inject sperm and accessory gland fluids directly into the blood, leaving visible melanized scars (Carayon 1966). This unusual mode of copulation, termed traumatic extragenital insemination, occurs in only a small number of taxa but it is found in all members of the bed bug family (Usinger 1966). In many bed bugs, the area at which the male pierces the integument of the female shows several unilateral modifications called the spermalege. For example, in the human bed bug *Cimex lectularius* a specialized notch and a cuticular thickening of the right side of the fifth sternite (the ectospermalege) lies directly over a distinct pocket filled with haemocytoid cells on the inner surface of the abdominal wall (the mesospermalege) (Carayon 1966).

Mating at very high rates is costly to female insects in general (see Arnqvist & Nilsson 2000 for a review) and bed bugs are no exception (Stutt & Siva-Jothy 2001). Despite the apparently damaging nature of traumatic insemination, we do not expect male–female interactions

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in bed bugs to carry extreme costs for two reasons. First, because of continual sexually antagonistic coevolution, females should be well counter-adapted to cope with any damaging effects of mating. The spermalege is an obvious candidate for such a female counter-adaptation. The evolution of the spermalege within the Cimicidae was first considered by Carayon (1966) who explicitly suggested that the spermalege evolved to reduce direct costs to females associated with the trauma of insemination (see also Stutt & Siva-Jothy 2001). Such costs may include: (i) repair of the wounded cuticle; (ii) leakage of haemolymph; (iii) increased risk of infection through the puncture wound; and (iv) immune defence against sperm or accessory gland fluids that are introduced directly into the blood of the female. The protection afforded by the spermalege to the female during and after copulation may operate in a number of ways: (i) by localizing damage to one area on the abdomen; (ii) by restricting the diffusion of the ejaculate inside the female; (iii) by reducing leakage of blood through the wound site; and (iv) by restricting entry of pathogens into the bloodstream. It should be noted, however, that the degree of elaboration of the spermalege varies tremendously across different heteropterans exhibiting traumatic insemination (Carayon 1966).

Second, all else being equal, males will suffer from harming their mates (Parker 1979). There should therefore be selection in both sexes to reduce the direct costs of traumatic insemination to females, favouring males that inseminate females in a less costly manner and leaving only those costs that are more or less 'unavoidable' to persist over evolutionary time (see Hosken *et al.* 2003; Morrow *et al.* 2003). For these reasons, the costs of mating in bed bugs should not be extraordinary.

The purpose of this reported work is twofold. By independently varying the rate of insemination and the type of puncture trauma in a bed bug species, we first quantify female mating costs and second we dissect the proximate causes of such costs. We predict that the costs of mating should not be dramatic. Further, the cost to females of mating trauma in the spermalege should be minimal, in contrast to extra-spermalege trauma, whereas the costs of insemination may be significant (cf. Arnqvist & Nilsson 2000).

2. MATERIAL AND METHODS

(a) *Rearing conditions*

We used the human bed bug (*C. lectularius*) for the experiments described below. All animals were taken from a stock population (CB strain) that has been reared in constant environment chambers (Sanyo) at 27 ± 1 °C and 70% relative humidity in continual darkness for the past two years. Both the stock and experimental animals were fed weekly. Experimental animals were isolated from the stock population as either fourth- or fifth-instar nymphs and kept individually in glass test tubes with a slip of filter paper, plugged with a piece of foam. All nymphs were fed communally but were returned to their individual tubes after feeding, ensuring the virginity of any eclosed adults. The tubes were checked weekly for any eclosed adults, which were then sexed and placed into either a male or a female holding tube prior to use in the experiment. Any females not used within a week of eclosion into adulthood were excluded from the experiment.

(b) *Experimental treatments*

We independently varied the following factors: (i) the rate of insemination; (ii) the rate of piercing trauma; (iii) the site of piercing trauma; and (iv) the probability of infection during piercing trauma. The first variable was based upon mating regime, using two treatments. Females were either housed with four virgin males (high mating rate) or were housed with four virgin males, three of which had had their paramere glued into the paramere groove (low mating rate), using a small amount of quick-drying glue (Super Attak, Loctite). This gluing treatment had no observable effect on male behaviour. The latter treatment controls for any confounding effects of exposure to males or male harassment, thus isolating the effect of copulation, since three out of the four males were unable actually to copulate. Any males that died during the period of the experiment were replaced with a male of the same type (glued or normal). Each replicate of four males and a single female was housed in a well (volume of 6 ml) of a modified cell-culture plate fitted with a gauze panel in the lid. A small slip of filter paper was provided as an oviposition substrate. Females were included in the experiment until their death.

In addition to the trauma experienced during mating, all females were subjected to additional experimentally imposed abdominal piercing trauma. Females were either: (i) simply handled for the same amount of time that the treatments below took to complete (*ca.* 1 min); (ii) pierced through the ectospermalege with a sterilized pin (rinsed briefly in 98% ethanol and allowed to dry prior to piercing); (iii) pierced through the ectospermalege with a non-sterilized pin that, prior to piercing, had been wiped over the surface of a contaminated filter paper taken from a jar containing a bed bug colony; (iv) pierced outside of the spermalege with a sterilized pin, through the integument between the fifth and sixth segments of the ventral surface of the abdomen on the left side of their body. This position mirrors exactly the position of the ectospermalege on the ventral surface of the right side of the female abdomen. These treatments were carried out once a week, and allowed us to evaluate statistically the effects on females of rate of piercing trauma, site of piercing trauma and probability of infection during piercing trauma.

All piercings were carried out under a stereo binocular microscope (×20 magnification) at room temperature and humidity during daylight hours. The female was held ventral side uppermost in a spring-loaded clamp, the jaws of which were cushioned with pieces of closed-cell foam. The pins used for puncturing the females were stainless steel entomological pins (size A1; Watkins and Doncaster, UK) that had been glued into a pipette tip for easy handling. This tool was then mounted on a micromanipulator (Narishige MM-3), which enabled the fine and precise movements of the pin tip necessary for the piercing procedure. The dimensions of the tip of this grade of pin correspond very closely to that of the male paramere (maximum diameter of 0.05 mm at 0.2 mm from the tip of the needle). Using the micromanipulator, the pin tip could be inserted through the abdominal wall of a female at an oblique angle and to a depth that closely simulated the length of the male paramere that is normally inserted (*ca.* 0.2 mm; personal observation).

(c) *Female fecundity and lifespan*

The number of eggs laid by each female on the substrate was counted every week, and the substrate was replaced. The date that each female died was recorded, and female lifespan as an adult was calculated as beginning from the date from which the female was included in the experiment.

Table 1. Analysis of deviance of the effects of mating rate and experimental piercing on adult female lifespan ($n = 76$) (test of model: $\chi^2 = 11.71$, d.f. = 4, $p = 0.019$).
(Addition of the two-way interactions between mating rate and the other variables to this model did not further improve model fit ($\chi^2 = 3.32$, d.f. = 3, $p = 0.345$).)

factor	d.f.	χ^2	p
mating rate	1	5.99	0.014
pierced	1	2.33	0.127
piercing site	1	4.20	0.040
contaminated piercing	1	0.14	0.712

Table 2. Analysis of deviance of the effects of mating rate and experimental piercing on female lifetime fecundity ($n = 76$) (test of model: $\chi^2 = 4.51$, d.f. = 4, $p = 0.341$).
(Addition of the two-way interactions between mating rate and the other variables to this model did not improve model fit ($\chi^2 = 0.40$, d.f. = 3, $p = 0.940$).)

factor	d.f.	χ^2	p
mating rate	1	0.27	0.603
pierced	1	1.00	0.317
piercing site	1	3.70	0.054
contaminated piercing	1	0.13	0.718

3. RESULTS

(a) *Adult female longevity*

The effects of our experimental variables on female longevity and lifetime fecundity were analysed in a generalized linear model, with Poisson errors and a logit-link function, using GLIM (version 3.77; Payne 1986). Since both of these response variables represent count data, this model is preferable to conventional linear models that assume a continuous response variable (McCullagh & Nelder 1989). Statistical inferences were based on an analysis of deviance (ANDEVA), a procedure that is analogous to an analysis of variance that follows traditional linear models (Crawley 1993). To compensate for overdispersion, an empirically derived scale factor was used in the analysis (Aitkin *et al.* 1989). This analysis (see table 1 and figure 1) revealed a significant effect of mating rate upon female longevity. Females included in the high mating rate treatment suffered a *ca.* 30% reduction in adult lifespan ((average in days ± s.e.): low 36.53 ± 3.24, $n = 38$; high 25.84 ± 2.65, $n = 38$). Neither the sterility of the pin used nor whether females were experimentally pierced had any significant effects, whereas females pierced outside of the spermalege suffered a significantly reduced lifespan (24.06 ± 4.09) compared with those pierced through the spermalege (36.05 ± 3.33).

(b) *Lifetime fecundity*

The effects of our treatment variables on female lifetime fecundity were analysed in a generalized linear model, identical to the one described above. The overall model was not significant (table 2), suggesting that our treatments did not collectively affect female lifetime fecundity significantly. However, the prediction that the spermalege functions to reduce the direct fitness cost of the piercing trauma *per se* was supported by our analyses: when comparing females experimentally pierced outside of the spermalege with those pierced in the spermalege with a focused test, the latter suffered a 50% reduction in lifetime fecundity compared with the former (9.0 ± 3.2 versus 18.8 ± 3.2; $\chi^2 = 3.72$, d.f. = 1, $P_{\alpha/2} = 0.027$).

4. DISCUSSION

Despite the apparently dramatic nature of the mode of mating in bed bugs, our results show that traumatic insemination does not carry extreme costs to females. This may seem surprising, but it is actually what we would expect based on sexually antagonistic coevolutionary theory (see § 1). Despite the fact that our mating-rate treatment inflated the potential mating rate by a factor of four, no detectable effects on female lifetime egg production were detected although female lifespan was reduced by 30%. These results are similar to those of other experiments involving insects without traumatic insemination, which show an average effect size of 0.84 ± 0.08 95% confidence limits (see Arnqvist & Nilsson 2000 for a review), and suggest that an increased rate of insemination in bed bugs elevates female reproductive rate without significantly reducing female fitness. In the only earlier experimental study of the costs of mating in a traumatically inseminating species of which we are aware, Stutt & Siva-Jothy (2001) performed an experiment where they varied mating rate for female bed bugs. They documented a lifespan reduction due to increased mating rate that was quantitatively very similar to that reported here, but did not find any effects on reproductive rate, and consequently found that lifetime fecundity was reduced by frequent mating. The differences between our results and those of Stutt & Siva-Jothy (2001) can be attributed to the facts that the absolute mating rates were lower in their experiment and their experimental design did not allow a separation of the effects of copulation *per se* from those of the trauma of insemination.

It is clear from our study that female bed bugs are well adapted to both the physical trauma of piercing of the abdominal wall that occurs during copulation and to any associated complications that may arise from pathogens gaining entry through the wound site. Instead, the costs of a high mating rate documented here (see also Stutt & Siva-Jothy 2001) apparently derive primarily from the receipt of the ejaculate into the haemolymph, which could be problematic to females for two reasons. First, sperm and/or other ejaculate components are likely to initiate a female immune response since they will appear as foreign matter. Second, male bed bugs transfer accessory gland products to females at mating (Davis 1956, 1966). Such seminal substances may be manipulative, and negative effects to females of receiving large amounts have been documented in several insects without traumatic insemination (e.g. Das *et al.* 1980; Arnqvist & Nilsson 2000; Chapman 2001). Interestingly, in bed bugs, aggregations of phagocytic cells present within the female become active directly after insemination, and apparently take up and digest seminal fluid material (Davis 1956; Carayon 1966). However, the fact that the magnitude of the negative effects to female bed bugs of elevated mating rates are comparable with those in insects without traumatic

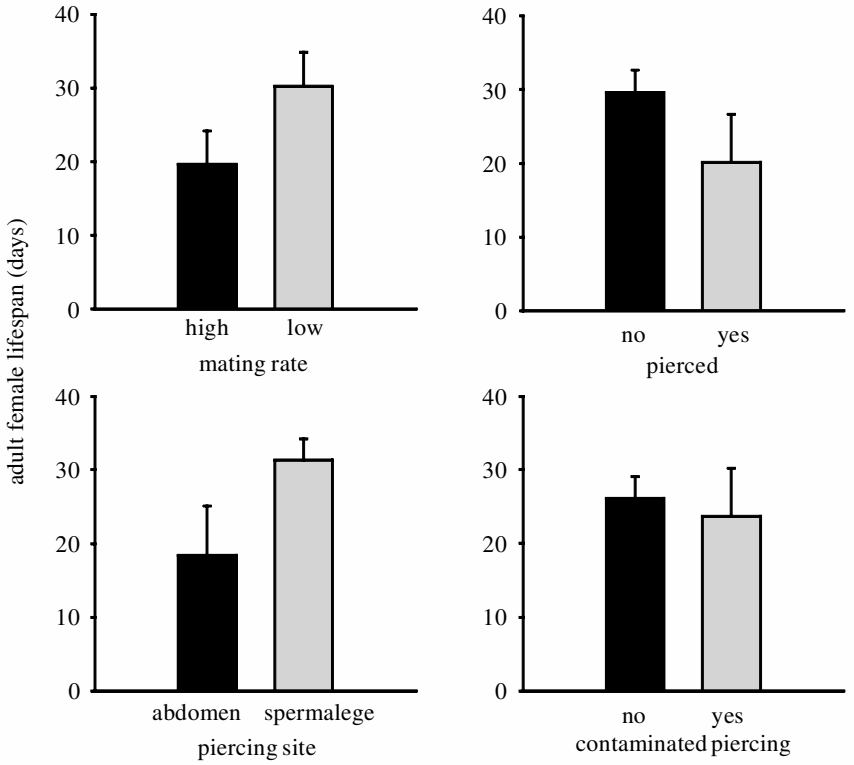

Figure 1. The effects of various experimental treatments on adult female lifespan. Figure shows least-squares means (± s.e.) from a general linear model analogue of the model presented in table 1.

insemination (cf. Arnqvist & Nilsson 2000) suggests that females are well adapted to the special challenges that may be associated with receiving the ejaculate in the body cavity. Moreover, the costs to female bed bugs of elevated mating rates are primarily due to the effects of the ejaculate, a phenomenon not unique to traumatically inseminating species.

Our results reveal that experimental traumatic piercing in the spermalege, closely mimicking that occurring during natural copulation, had no significant effects on female performance. By contrast, when females were subjected to such piercing outside the spermalege, it caused a dramatic decrease in both lifespan and lifetime egg production despite a very low rate of piercing (once a week). This result unambiguously shows that the spermalege effectively reduces the direct costs of trauma to females during insemination in bed bugs, and hence provides support for the suggestion that the spermalege represents a female counter-adaptation to an antagonistic adaptation in males (traumatic insemination) (Davis 1956; Carayon 1966; Thornhill & Alcock 1983; Stutt & Siva-Jothy 2001). Although such counter-adaptations are no doubt very widespread and appear in many different forms (e.g. Holland & Rice 1998; Chapman *et al.* 2003) our study is, to our knowledge, the first to provide experimental evidence showing that a female morphological trait reduces the fitness cost of exposure to an antagonistic male trait. Earlier experimental studies of putative counter-adaptations have either not measured net fitness costs to females or have been unable to identify the trait in females that mediates resistance to males (Arnqvist & Rowe 1995; Holland & Rice 1999).

It has previously been suggested that the bed bug ectospermalege is a female counter-adaptation to cope with the costs of physical piercing trauma and that the mesospermalege functions to minimize the costs of receiving ejaculates by restricting diffusion of sperm and/or seminal fluid within the female body (Davis 1956; Carayon 1966; Stutt & Siva-Jothy 2001). Here, we have at least provided experimental support for the former suggestion. The fact that females have invested in the evolution of counter-adaptations to an antagonistic behaviour in males raises two relevant issues. First, given our results, it is easy to see why males should evolve to pierce and inseminate females in the spermalege rather than in any other part of the body. All else being equal, males will benefit from harming their mates as little as possible (Morrow *et al.* 2003) and the spermalege clearly offers an opportunity for less traumatic piercing. Second, it is interesting to note that the degree of elaboration of the spermalege varies tremendously within the Cimicidae (see Carayon 1966). At one extreme, we find the genus *Primicimex* where females lack any traces of a spermalege altogether. At the other, we find the genus *Stricticimex* in which the ectospermalege is well developed and the mesospermalege essentially forms a complete and complex copulatory tube leading to the oviducts. In the latter case, this derived and unique reproductive tract ensures that the ejaculate does not come into direct contact with the haemolymph. If this variation is indeed the result of sexually antagonistic coevolution, we expect the degree of elaboration of the spermalege to covary with antagonistic traits in males (cf. Arnqvist & Rowe 2002*a*). Unfortunately, we currently lack both a well-resolved phylogeny for this group and a clear idea of which male traits might antagonistically coevolve with the female spermalege. Future comparative studies promise to shed light on the dynamics of intersexual coevolution in this group.

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