

Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ

Klaus Reinhardt*, Richard Naylor and Michael T. Siva-Jothy

Department of Animal and Plant Science, The University of Sheffield, Sheffield S10 2TN, UK

The frequent wounding of female bedbugs (*Cimex lectularius*: Cimicidae) during copulation has been shown to decrease their fitness, but how females have responded to this cost in evolutionary terms is unclear. The evolution of a unique anatomical structure found in female bedbugs, the spermalege, into which the male's intromittent organ passes during traumatic insemination, is a possible counteradaptation to harmful male traits. Several functions have been proposed for this organ, and we test two hypotheses related to its role in sexual conflict. We examine the hypotheses that the spermalege functions to (i) defend against pathogens introduced during traumatic insemination; and (ii) reduce the costs of wound healing during traumatic insemination. Our results support the 'defence against pathogens' hypothesis, suggesting that the evolution of this unique cimicid organ resulted, at least partly, from selection to reduce the costs of mating-associated infection. We found no evidence that the spermalege reduces the costs of wound healing.

Keywords: bacterial infection; sexual conflict; traumatic insemination

1. INTRODUCTION

Sexual conflict is a powerful evolutionary force that can lead to particularly rapid coevolution of male and female reproductive traits (Parker 1979; Rice & Holland 1997; Holland & Rice 1998; Johnstone & Keller 1999; Kokko *et al.* 2003). In general, sexual conflicts result in male traits that enhance male fitness at the females' expense (only direct costs have been identified so far). These costs are often imposed by male anatomical adaptations, e.g. in water striders (Arnqvist 1997), haglid orthopterans (Sakaluk *et al.* 1995), bedbugs (Stutt & Siva-Jothy 2001), and bruchid beetles (Crudgington & Siva-Jothy 2000). Because the costs imposed upon females are often considerable (e.g. Chapman *et al.* 2003; Crudgington & Siva-Jothy 2000; Stutt & Siva-Jothy 2001; Arnqvist & Rowe 2002), the selection pressure for female counteradaptations to those male traits is also very strong (Holland & Rice 1998). Although it has been argued that the imposition of such costs need not necessarily decrease overall fitness (Cordero & Eberhard 2003; Kokko *et al.* 2003), these direct costs give an indication of how much females must benefit indirectly in order to outweigh the direct costs. Despite the relative wealth of examples of male adaptations resulting from sexual conflict, there are relatively few examples of female counteradaptations (but see Crudgington & Siva-Jothy 2000; Arnqvist & Rowe 2002).

Bedbugs (*Cimex lectularius*: Cimicidae) provide an ideal study system to examine potential female adaptation resulting from sexual conflict. During mating, the male pierces the abdomen of the female with a sclerotized, needle-like paramere (the male's intromittent structure that transfers sperm to the female) and inseminates into her body cavity despite the presence of a fully functional female reproductive tract (Carayon 1966). However, during several thousand observations it was never recorded

that a male bedbug intromits his paramere into the vagina (reviewed Carayon 1966; R. Naylor, K. Reinhardt, M. Siva-Jothy and A. Stutt, unpublished observation). Mating frequencies appear to be determined by males and occur at a higher rate than is required to maintain maximum fertility in females. The result of natural rates of traumatic insemination is a 25% reduction in female lifespan (Stutt & Siva-Jothy 2001).

Male bedbugs do not/can not pierce females at any site on their abdomen. The females of *C. lectularius* (and most other cimicids) possess an organ called the spermalege (Carayon 1966). It has two embryologically discrete parts: the ectodermal 'ectospermalege' and the mesodermal 'mesospermalege'. The ectospermalege consists of a groove in the right-hand posterior margin of the fifth sclerite (e.g. Stutt & Siva-Jothy 2001) overlying a structurally modified pleural membrane. During traumatic insemination, male bedbugs insert their intromittent organ into this groove, pierce the pleural membrane and so gain access to the female's haemocoel (body cavity). The piercing produces wounds that are quickly repaired but leave melanized scars (Carayon 1966). Attached to the wall of the haemocoel, directly underneath the external groove, lies its second component: the mesospermalege. During traumatic insemination, sperm and seminal fluid are ejaculated into this haemocyte-containing membrane-bound sac (Carayon 1966). Sperm travel out of the posterior part of the mesospermalege into the female's haemolymph (blood) from where they migrate to specialized sperm storage structures (the seminal conceptacles) and then on to the ovaries where fertilization takes place (Carayon 1966). There appear to be two main morphological types of haemocytes in the mesospermalege (Klein & Kallenborn 2000; Steigner 2001). One type appears to be phagocytic and may absorb seminal fluid, while the other has been suggested to digest spermatozoa (Klein & Kallenborn 2000; see also Carayon 1966).

The presence of haemocytes in the mesospermalege, the possibility that sperm and seminal fluids are digested

*Author for correspondence (k.reinhardt@sheffield.ac.uk).

there, and the damage associated with traumatic insemination as well as its fast repair have stimulated several hypotheses for the function of this unique structure. Eberhard (1985, 1996) suggested that because sperm and seminal substances remain in the mesospermalege for 2–4 h (Carayon 1966) the mesospermalege may enable females to exercise cryptic choice of paternity either directly (through some unidentified compatibility mechanism) or indirectly by digesting, and hence selecting, a subset of sperm. If females select sperm from certain males to increase their fitness one would expect to see differences in offspring fitness from multiple versus single mating. However, no such differences have been found in terms of egg size, development rate, or imaginal size of the offspring (Stutt & Siva-Jothy 2001).

A second hypothesis proposes that the spermalege functions to defend against opportunistic pathogens introduced during traumatic insemination (Stutt & Siva-Jothy 2001). Bedbugs spend most of their life in 'refugia' (cracks in the walls and floors of the host's sleeping area), often at high densities. These cramped spaces are not only full of bedbugs, but are replete with exuvia, dead bugs, and the digested remnants of their blood meals. These unsanitary conditions will favour the growth of microbes that may be introduced into the female during traumatic insemination. A third hypothesis also relates to the naturally selected consequences of traumatic insemination. If the costs of wound healing are high, females may reduce those costs with the spermalege. This may be achieved because the ectospermalege directs, and so localizes, the wounding associated with traumatic insemination to one place (Klein & Kallenborn 2000), and/or because the mesospermalege reduces the costs of wound healing (Stutt & Siva-Jothy 2001).

In this paper, we test the 'resistance to pathogens' and 'reduction of wound healing costs' hypotheses for the function of the spermalege in the context of the conflict that arises from traumatic insemination. If either is supported it would suggest that the spermalege evolved, at least in part, as a female counteradaptation to traumatic insemination.

2. MATERIAL AND METHODS

(a) *Insect cultures*

Stock cultures of bedbugs were sourced from the Medical Entomology Centre, Cambridge, UK in 1998. The bugs were maintained in an incubator at 26 ± 1 °C, at 70% relative humidity with a cycle of 12 L : 12 D. Bugs were fed weekly on rabbit blood using the protocol of Davis (1956). All procedures involving rabbits adhered to the UK's Animals (scientific procedures) Act 1986, and were covered by UK Home Office licences.

Penultimate instar, virgin bugs were sexed and isolated prior to allocation to the experimental protocols outlined below.

(b) *Experimental protocols*

Female bedbugs were randomly assigned to one of four treatments ($n = 21$ per treatment). All experimental bedbugs were fed once per week to avoid mortality differences due to the large metabolic differences between fed and non-fed individuals (Mellanby 1939). We mated the bug females once every fourth week to avoid sperm depletion in females (Abraham 1934;

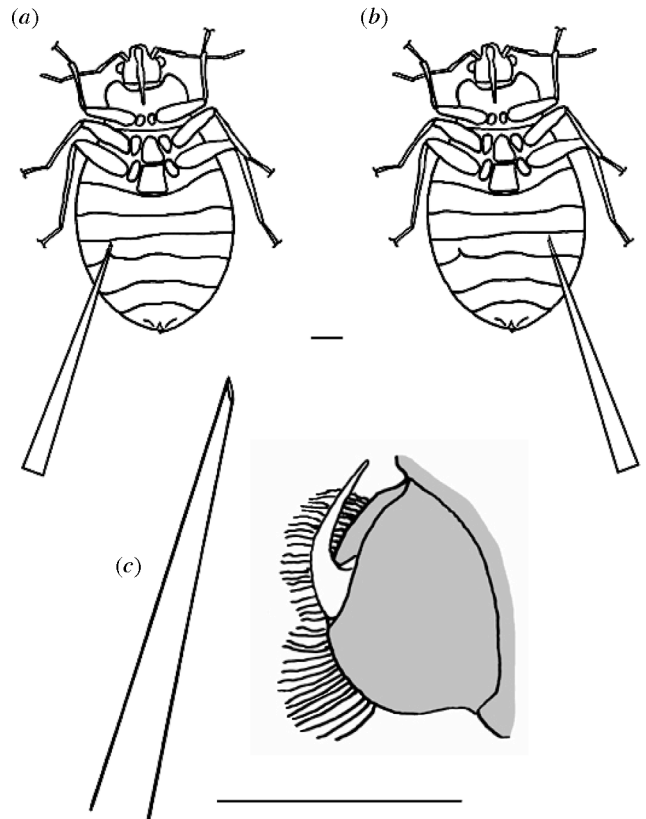


Figure 1. (a) The site of piercing in the sterile/spermalege and contaminated/spermalege treatments (i.e. wounding in the spermalege). The female bedbug was immobilized on her back and the needle introduced with a mechanical micromanipulator to the depth indicated in the figure. (b) The site of piercing in the sterile/abdomen and contaminated/ abdomen treatments (i.e. abdomen). The needle was introduced to the same depth as in the 'spermalege' treatment. (c) A comparison of the needle tip (left) and the male's intromittent organ (right, unshaded). Although the tip diameters differ, the diameter of the intromittent organ at the most anterior part to enter the female is the same in the male's organ and the needle. Scale bars represent 0.5 mm.

Mellanby 1939; Khalifa 1952; Davis 1964; Stutt & Siva-Jothy 2001). Matings were interrupted after 60 s. This procedure standardizes ejaculate size because there is a linear relationship between copulation duration and sperm number (Siva-Jothy & Stutt 2003). A standardized sperm number was desirable since the spermatozoa trigger the release of an oviposition stimulating hormone from the corpora allata (Davis 1965) and hence potentially influence lifespan through differential egg production.

On the day after feeding, each female was immobilized on her dorsum on a microscope slide and the experimental treatment administered (see below). Females received either a sterile-needle trauma or a contaminated-needle trauma (see below) into either their spermalege, or into an abdominal site opposite the spermalege (see figure 1). This resulted in four treatment groups: sterile/spermalege (SS), sterile/abdomen (SA), contaminated/spermalege (CS) and contaminated/abdomen (CA).

The ventral surface of the females, as well as the needle, in both sterile-needle treatments (SS and SA) were swabbed with absolute ethanol and left to be airdried before each artificial piercing. Females from the contaminated treatments (CS and CA) were not swabbed. Each female was pierced with a hollow

glass needle that mimicked the dimensions of the male's intromittent organ in the region that entered the female (see figure 1). Females in treatments CS and CA were pierced with a needle that had been sterilized, then dipped into a bacterial solution (prepared by incubating filter paper, on which bedbugs were cultured for several generations, in Hepes buffer 24 h prior to the experiment). Examination of the bacteria present in this solution revealed the near-exclusive presence of *Bacillus cf. licheniformis* (M. Wainwright, personal communication). SS and CS females were pierced through the spermalege (i.e. mimicking natural mating), whereas SA and CA females were pierced through the cuticle on the opposite side of the abdomen (figure 1*b*) thereby avoiding the spermalege.

Female survival was monitored every 2 days: after 10 weeks this was reduced to two surveys per week. The number of eggs laid by each female was monitored every seventh day. We terminated the experiment 24 weeks (170 days) after the start of treatments.

(c) Experimental design and statistics

To determine the relationship between longevity and egg number, we forced a linear regression between these traits through the origin. We did this because, biologically speaking, these traits cannot have a positive or negative intercept. One SS female was excluded from this analysis because she laid no eggs for 13 weeks and then died. Two SA females were accidentally pierced through the spermalege and hence excluded from the analysis of egg numbers and censored from the day of occurrence in the survival analysis.

If the spermalege functions to reduce wound-healing costs we predict a significant reduction in fitness of SA females compared with SS females. If the spermalege has evolved as an effective means to remove pathogens, but has no anti-wounding function, we predict that CS and SS will not differ from one another. The fitness of CS females should be lower than SS females if the spermalege is not perfectly efficient in removing pathogens. If the spermalege is associated with anti-pathogen activity during wounding, the fitness differences in CS females should be larger than the difference seen in SA and SS females (wounding effect). Statistically, this would be apparent as a significant interaction between 'bacterial contamination' and 'site of piercing' in a factorial design.

We investigated the effects of the 'bacterial contamination' and 'site of piercing', as well as their interaction, on female survival using Cox regression. For this analysis, we first ran the full model (including thorax width: see below) and then applied a backwards mode of variable exclusion, based on *F*-values.

Unlike most other body-size parameters, thorax width in bedbugs is not affected by feeding (Hase 1917; K. Reinhardt and M. Siva-Jothy, personal observation). Thorax width was measured by placing an immobilized female on a microscope slide, capturing a digital image of her thorax and then measuring the maximum width from the digital image using image analysis software (OPTIMAS 6.1).

3. RESULTS

The mean egg number in SA females was reduced by 12.5% (mean effect of wounding) compared with SS females. In the contaminated treatment, egg numbers were reduced by 89.7% if females were pierced through the abdomen, but only by 69.9% when pierced through the spermalege (figure 2). Lifespan almost entirely

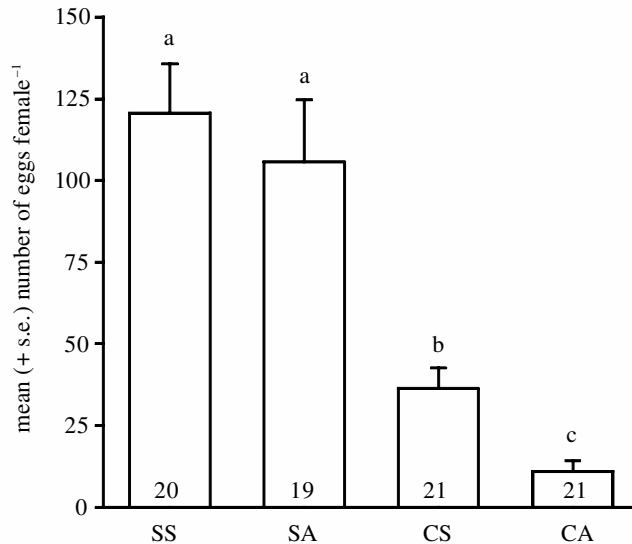


Figure 2. The mean cumulative egg production of females in each treatment group showing the significant effect of treatment on egg production (ANOVA, $F_{3,80} = 20.17$, $p < 0.0001$). Treatment bars with the same letter above them do not differ at $p < 0.05$ (Games-Howell *post hoc* comparisons). Sample sizes are shown at the base of the histogram.

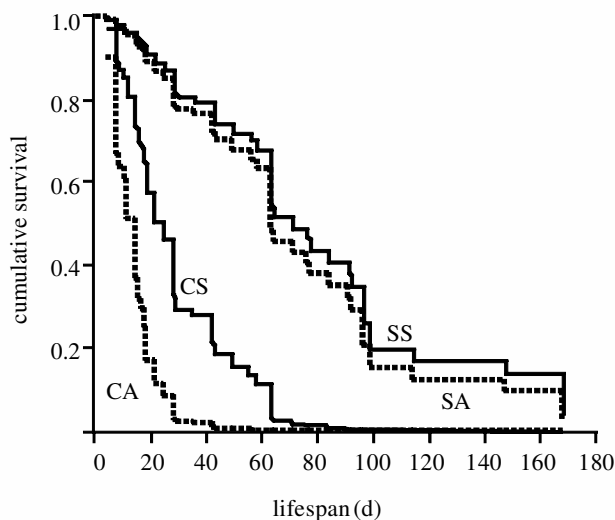


Figure 3. The survival curves for females in each experimental treatment. Females received a wound from either a sterile or contaminated needle into either their spermalege (solid lines) or their abdomen (broken lines) (see figure 1). See § 2 for detailed description of treatments.

predicted egg production ($r^2 = 0.93$, $t = 38.028$, $p < 0.001$) with 1.4 eggs laid per female per day. We conclude that lifespan is the major determinant of the differences in egg production between treatment females.

Thorax width had no effect on survival in the full model (slope $b = 0.514$, Wald $\chi^2 = 0.094$, $p = 0.759$) and so was excluded in the backwards procedure.

There was no effect of whether or not the stabbing was carried out through the spermalege (slope $b = 0.230$, Wald $\chi^2 = 0.441$, $p = 0.507$; figure 3). We conclude that the costs of wound healing are not statistically significant.

In the backwards exclusion procedure, contaminated-needle treatments led to a fivefold reduction in survival

(slope $b = 1.611$, Wald $\chi^2 = 18.970$, $p < 0.001$). There was an additional 3.2-fold reduction in survival due to the effect of the interaction of site of piercing and contamination (slope $b = 1.163$, Wald, $\chi^2 = 11.767$, $p = 0.001$). We conclude that the spermalege plays a role in defending the female against bacteria introduced into the spermalege, a process that is likely to occur during natural traumatic inseminations.

4. DISCUSSION

Our results show that lifespan explains 93% of the variation in egg production: consequently, lifespan is a good surrogate for fitness in bedbugs. We show that survivorship in female bedbugs was higher when wounding occurred with a contaminated needle inserted into the spermalege compared with wounding elsewhere in the abdomen. This result supports the 'defence against pathogens' hypothesis for the function of the spermalege, but does not exclude the possibility that the spermalege has additional functions. However, our results suggest that reducing the costs of wound healing is less likely to be one of these additional functions.

Female counteradaptations to harmful male traits comprise several behavioural, morphological and physiological responses. If, for example, females are coerced to mate they have potentially a very wide range of means to reduce the paternity by that male (e.g. Eberhard 1996). Likewise, in bruchid beetles, females kick away males after a certain period of copulation time, a behaviour that effectively increases their lifespan (Crudginton & Siva-Jothy 2000). A third example is the genital clasper armament and leg movement behaviour by female water striders enabling them to withstand pre- or postcopulatory male coercive behaviours (e.g. Arnqvist 1997; Arnqvist & Rowe 2002). Unique in the bedbug system is the evolution of a novel organ type with immune function. Although immune organs are very rare in insects in general (e.g. Chapman 1998; but see Poulsen *et al.* 2002), the discrete spatial concentration of haemocytes is potentially very effective given the fact that haemocytes are one of the major pathogen-defence devices in the insects (e.g. Chapman 1998).

The amount of bacteria introduced during our contaminated treatment was difficult to quantify. When the intromittent organs of bedbug males were cultured on agar plates it was apparent that the size of the colony was, on average, nearly four times larger when the parameres were dipped into the bacterial solution than when cultured without previous treatment (intromittent organs airdried after dipping them into ethanol did not produce any bacterial colonies; R. Naylor and K. Reinhardt, unpublished data). It is, therefore, very likely that the needle in our treatment was more heavily contaminated than intromittent organs found in nature. We are currently unable to test whether this higher than normal bacterial dose enabled us to uncover the antipathogenic function of the spermalege.

There are several, non-exclusive, reasons why confining wounding to the spermalege may increase survival if pathogens are introduced during traumatic insemination. First, females are frequently wounded at this site (i.e. they are polyandrous), which could select for an increased efficiency of the systemic immune response at this site, for

example by faster transport of haemocytes, or other effector systems, to the spermalege. Second, the haemocytes that constitute the spermalege (Carayon 1966; Klein & Kallenborn 2000; Steigner 2001) may be responsible for the rapid response to pathogens introduced during mating. Third, frequent mating-associated wounding may have selected for an increased density of receptors that provide faster detection of pathogens and subsequently trigger either or both of the above more efficiently. A fourth possibility is closely related to our methodology. Although the needles used by us closely mimicked the male's paramere (figure 1), there may be other cues associated with mating that we could not mimic. For example, the slightly curved intromittent organ of the male (figure 1) may wound the female in a slightly different way, different angle or perhaps much more precisely at a localized site within the spermalege than we did in our treatment. Likewise, if the female has ultrastructural cuticle adaptations to these precise wounding locations our treatment may have been more severe than in nature and may have enabled an easier introduction of bacteria (although this would rather strengthen our conclusions). If the lifespan of our treatment females is compared with those reported previously in the literature, we find that females in our sterile-needle treatment survived less well than females under culture conditions in previously published studies (Girault 1917, Cragg 1923; Titschak 1930; Janisch 1933, 1935; Mellanby 1939; Khalifa 1952; Stutt & Siva-Jothy 2001). Our attempts failed to produce a formal control group for females that were mated normally but pierced by males on the other side of the abdomen (see Abraham (1934) and Ludwig & Zwanzig (1937) for a description of such females). However, a group of 21 females run in parallel to our experiment that were mated every fourth week and pierced by the male four times without sperm transfer had survived better than even our sterile spermalege treatment: by day 170 only 38% had died. However, because of the aforementioned difficulties, we were unable to use those females as a proper control to our treatment.

In conclusion, our experimental evidence lends very strong support to the hypothesis that the spermalege of *C. lectularius* (and, by extension, the spermalege of other cimicid bugs) has evolved as a female counteradaptation to reduce costs imposed by pathogens introduced during traumatic insemination.

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