A matter of taste: direct detection of female mating status in the bedbug

Michael T. Siva-Jothy* and Alistair D. Stutt

Evolution and Behaviour Group, Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK

Males of the bedbug, *Cimex lectularius*, traumatically inseminate females by inserting a needle-like intromittent organ (penis) through the female's abdominal wall after she has fed. We demonstrate that: (i) mating duration determines ejaculate size; (ii) a female's first copulation in a bout of copulations always lasts longer than subsequent copulations; (iii) the intromittent organ bears sensillae; (iv) males use their intromittent organ to 'taste' whether their current mate has recently copulated; and (v) the consequence of detecting female mating status is the reduction of copulation duration and ejaculate size. We discuss why male bedbugs might show this pattern of ejaculate-size adjustment.

Keywords: *Cimex lectularius*; ejaculate-size adjustment; contact chemoreceptors; copulation duration; penis

1. INTRODUCTION

The males of many animals adjust their ejaculate size according to the probable mating status of females (Simmons & Siva-Jothy 1998; Simmons 2001) because relative sperm numbers can determine paternity outcomes (Parker 1990; Ball & Parker 2000) when females mate more than once. Adjustment of ejaculate size occurs in insects in response to socio-sexual cues such as the operational sex ratio and female size (e.g. Gage & Baker 1991; Simmons *et al.* 1993; Gage & Barnard 1996) and, potentially, because of the need for males to conserve sperm (Wedell *et al.* 2002). Males also adjust copulation duration in relation to female mating status (e.g. Wedell 1992; Cook & Gage 1995; Andrés & Cordero-Rivera 2000) but it is far from clear how males detect whether or not their mate has mated recently.

Bedbugs (family Cimicidae) have a unique mode of copulation: despite the fact that female bedbugs have a genital tract, males push the hardened needle-like intromittent organ (a modified paramere; figure 1a) through the female's abdominal wall and inseminate directly into her body cavity (Carayon 1966). For this reason, copulation in bedbugs is termed 'traumatic insemination', and female cimicids have evolved a unique secondary genital system, called the paragenital system, through which traumatically inseminated sperm move (Carayon 1966). During traumatic insemination the male deposits sperm into a membrane-covered organ (the mesospermalege) in which it remains for *ca.* 4 hours (Carayon 1966). Sperm then migrate through the blood into a discrete paired sperm-storage organ from where they migrate up the oviducts and fertilize eggs in the ovaries (Carayon 1966). The trauma associated with the mode of insemination in *Cimex lectularius* is anatomically localized on the female's abdomen because of the presence of a groove in the female's fifth abdominal sternite (the ectospermalege; see Carayon 1966; Stutt & Siva-Jothy 2001). Bedbugs show a remating

cycle that is closely allied to their natural feeding regime (Mellanby 1939): immediately after feeding there is a period of high mating activity during which each female mates approximately five times, with a remating interval of *ca.* 17 min (Stutt & Siva-Jothy 2001). Because ejaculates remain localized in the female's mesospermalege for several hours after insemination (Carayon 1966), and because they are deposited in the same structure over a relatively short space of time (Mellanby 1939), it is highly probable that the intromittent organs of subsequent males will come into contact with the ejaculate(s) of their predecessor(s).

Given that mating duration is intimately linked with ejaculate size in many insects (Simmons & Siva-Jothy 1998; Simmons 2001), that ejaculate size can affect paternity outcome (Parker 1990; Ball & Parker 2000), and that theory predicts males should adjust ejaculate size in accordance with sperm-competition risk (Simmons & Siva-Jothy 1998), there is good reason to suppose selection should favour male physiological adaptations that enhance the ability to allocate optimal ejaculate volumes. We examined several aspects of the mating behaviour and anatomy of the bedbug and conducted an experiment that revealed that male bedbugs use their intromittent organ to detect directly the ejaculates of rivals inside the female.

2. MATERIAL AND METHODS

(a) *Insect culture*

Bedbug cultures were maintained at 26 ± 1 °C and 70% relative humidity, and fed weekly on rabbit blood using standard protocols (Davis 1956). Penultimate instar bugs of similar size were separated from the stock cultures weekly and kept in individual containers until they became imagines. This protocol ensured that adult bugs had not copulated.

(b) *Observation of copulatory behaviour*

Copulations were carried out immediately after the adult female had received a blood meal and took place under dim red light (60 W) at 26 ± 1 °C. All copulations were observed continuously until natural termination, or until they were experimentally interrupted.

* Author for correspondence (m.siva-jothy@sheffield.ac.uk).

Figure 1. Scanning electron micrographs of the male bedbug's intromittent organ. (a) A view of the posterior end of the intromittent organ showing its hollow needle-like structure. The open rectangles encompass the fields shown in (b) and (c). The dorsal region is at the bottom of the picture. (b) Enlargement of the ventral surface anterior to the ejaculatory duct; arrows indicate putative contact chemoreceptors (peg height *ca.* 2 µm). (c) Enlargement of the tip of the intromittent organ; arrows indicate putative contact chemoreceptors (peg height *ca.* 1 µm). Scale bar, 40 µm. SEMs by Andrew Syred, Microscopix.

(c) *Measurement of sperm volumes*

Freeze-killed mated females were dissected in 0.15 M Hepes buffer (pH 7.2). The mesospermalege was removed and placed on a microscope slide in a droplet of buffer. A coverslip bridge preparation was made by placing a 0.1 mm thick coverslip either side of the mesospermalege and then covering the preparation with a 0.2 mm coverslip. The slide was viewed with a compound microscope (Leitz Diaplan) and a digital image of the flattened sperm mass was captured with a digital camera (PULNiX) and its area assessed using image-analysis software (Optimas 6.0). The volume (in mm³) of the sperm mass was calculated as the area (in mm²) × 0.1.

When assessing the volumes of ejaculates in the experiment outlined in § 2c, it is important to bear in mind that the males were mating with virgins. This, and the very small amounts of ejaculate painted onto their intromittent organs, enabled us to measure the ejaculate volume of 'second matings' without the confounding effects of the presence of a preceding ejaculate.

(d) *Scanning electron microscope examination*

Samples for scanning electron microscope examination were fixed in 2.5% paraformaldehyde, critical-point dried and sputter-coated with gold before being examined and photographed (Cambridge stereoscan 250).

(e) *Experiment to test modality of mating-status detection*

We tested whether contact chemoreceptors on the intromittent organ mediated adjustment of copulation duration and ejaculate size as follows. We allocated virgin males to one of three experimental groups. One group of males (treatment 1) had their intromittent organs painted with an ejaculate dissected from a just-mated female and diluted in 50 µl of Grace's medium. Males allocated to the second group (treatment 2) had their intromittent organs painted with an ejaculate dissected from a male's seminal vesicles and diluted in 50 µl of Grace's medium. Males allocated to the third group (control) had their intromittent organs painted with the supernatant from the dissection of a virgin female's mesospermalege conducted in Grace's medium (i.e. obtained under similar conditions to treatment 1). Each male was immediately introduced to a recently fed vir-

gin female: copulation began within *ca.* 1 min of introduction. We recorded copulation duration and, after termination of copulation, collected the ejaculate from the female's mesospermalege and measured its volume as outlined in § 2c.

(f) *Statistical analysis*

All analyses were performed using STATVIEW 5.0 for the Macintosh platform. All data were checked for normality and homogeneity of variances. They are presented as means ± s.e.

3. RESULTS

(a) *Copulation duration and ejaculate volume*

The measurement of the volume of an ejaculate in the mesospermalege after matings between virgin males and virgin females revealed a significant positive relationship ($r^2 = 0.75$, $F = 53.8$, $p < 0.0001$, $y = -0.417 + 0.015x$) between ejaculate volume and copulation duration in experimentally interrupted copulations (figure 2).

(b) *Copulation duration and female mating status*

Comparison of the copulation durations of first and second matings after the first adult feeding bout, and first and second matings in the next feeding bout (i.e. the third and fourth matings with a 7 day gap between the second and third matings) revealed that the first male to mate within the mating bout spent significantly longer in copulation than the subsequent mate (ANOVA, $F_{3,116} = 16.82$, $p < 0.0001$) regardless of whether he was mating with a virgin or was the first to mate with a female in her second mating bout (first male and virgin female: 129.3 ± 13.6 s; second male 66.8 ± 5.6 s; first male in the female's second mating bout 135.1 ± 10.8 s, second male/in the second mating bout 64.7 ± 4.1 s). Since no sperm remain in the mesospermalege 2 days after insemination (Carayon 1966), the mesospermalege is effectively empty at the start of the subsequent mating bout approximately 7 days later. Measurement of the durations of five successive copulations in a bout (figure 3) revealed a significantly (ANOVA, $F_{4,55} = 76.3$, $p < 0.0001$) longer first

Figure 2. The relationship between copulation duration and ejaculate volume in experimentally interrupted matings between virgin males and females.

Figure 3. Copulation durations (mean $\pm$ s.e.) for virgin males sequentially mated to females. Bars with different alphanumeric characters are significantly different (Fisher's PLSD, $p < 0.0001$).

copulation duration compared with the subsequent four copulations.

(c) Does the intromittent organ bear putative chemosensory sensillae?

Scanning electron microscope examination of the surface of the intromittent organ revealed short pegs (figure 1b,c) that ranged in height from 1 to 2 μm . These pegs have a morphology that is consistent with a contact chemosensory function (Chapman 1998).

(d) Does the presence of seminal fluid on the surface of the intromittent organ affect mating?

A virgin male whose intromittent organ was painted with seminal fluid collected from a mated female (treatment 1) or from a male (treatment 2) showed a significant effect of the treatment on copulation duration and ejaculate volume (MANOVA, Wilk's lambda = 0.259, $p < 0.0001$) (ANOVA for copulation duration,

Figure 4. Copulatory responses of virgin males to the treatment of their intromittent organs with topical application of ejaculates (see text for details). Bars with the same alphanumeric characters do not differ. (a) Copulation duration (mean $\pm$ s.e.) in the control group was significantly (Fisher's PLSD, $p < 0.0001$) longer than in treatment groups 1 (virgin males that had their intromittent organ painted with ejaculate removed from a female) and 2 (virgin males that had their intromittent organ painted with ejaculate removed from a male). (b) Ejaculate volume (mean $\pm$ s.e.) in the control group was significantly (Fisher's PLSD, $p < 0.0001$) larger than in treatment groups 1 and 2 (groups as in (a)).

$F_{2,27} = 38.4$, $p < 0.0001$; figure 4a) (ANOVA for ejaculate volume, $F_{2,27} = 25$, $p < 0.0001$; figure 4b). Furthermore, a comparison of the insemination rates in the three experimental groups and the data on insemination presented in figure 2 revealed a significant difference between insemination rates (ANOVA, $F_{3,46} = 13.7$, $p < 0.0001$) (figure 5). The shorter copulation durations resulting from sperm detection were associated with slower insemination rates.

4. DISCUSSION

Our results show that male bedbugs detect the presence of an ejaculate(s) in their current mate with chemoreceptors on the intromittent organ and consequently reduce copulation duration and ejaculate size. We have also identified structures on the intromittent organ that may be responsible for the chemodetection of female mating status. Since there was no difference between the copulatory outcomes when virgin males were treated with an ejaculate removed from a male and an ejaculate removed

Figure 5. Insemination rates (mean $\pm$ s.e.) for virgin males that were experimentally interrupted during copulation (data from figure 2) or had had their intromittent organs treated with topical application of supernatant saline or ejaculates (see § 2e for details). Bars with the same alphanumeric do not differ. Interrupted-insemination rates were significantly greater than insemination rates in treatments 1 and 2 combined (Fisher's PLSD, $p < 0.0001$) and control insemination rates were significantly greater than insemination rates in treatments 1 and 2 combined (Fisher's PLSD, $p < 0.005$).

from a once-mated female it is unlikely that females produce mating-induced compounds that signal their mating status to males. We conclude that males use the presence of male-derived compounds in the ejaculate(s) within females to gauge female mating status.

The consequence of this mechanism of mating-status detection is ejaculate-size adjustment, which is usually interpreted in the context of sperm-competition risk (Simmons & Siva-Jothy 1998). Recent work on bed bugs (Stutt & Siva-Jothy 2001) revealed a 68% second-male sperm precedence when he copulated immediately after the first male, despite the longer copulation of the first male. Theory predicts a range of potential ejaculate-size adjustment outcomes depending on the nature of the mating system: in some conditions the male in the disfavoured role under sperm competition should produce an equal or larger ejaculate (Parker 1990), while under other conditions (e.g. sperm limitation) the disfavoured male should produce a smaller ejaculate (Mesterton-Gibbons 1999; Ball & Parker 2000). One explanation for the large ejaculate produced by male *C. lectularius* that mate with virgin females stems from another unique aspect of this insect's reproductive biology. The female's mesospermalege is full of phagocytic cells that are reported to ingest sperm after mating (Carayon 1966). When assessed two weeks after a controlled double mating, sperm-precedence patterns (A. D. Stutt and M. T. Siva-Jothy, unpublished data) showed equal fertilization success for the first and second males. This parity occurred despite the fact that the first male copulates for ca. 50% longer than the second and inseminates a larger ejaculate. If the first male's ejaculate is subjected to disproportionate phagocytic attack, selection via sperm competition, as well as any antagonistic

female physiological responses to sperm, may favour first males who have a compensatory increase in ejaculate size.

The authors thank Allison Davids and Heather Jenkins for technical support, Andrew Brooks (Environmental Health Agency, Liverpool) for his help with 'wild-caught' bugs, and Andrew Syred (Microscopix) for his SEM work. Sophie Armitage, Jon Ryder, Dave Coltman, Yannick Morret and Jens Rolff provided comments that improved the manuscript. M.T.S.-J. was supported by the Leverhulme Trust (grant no. F/00118/Q); A.D.S. was supported by a BBSRC studentship.

REFERENCES

- Andrés, J. A. & Cordero-Rivera, A. 2000 Copulation duration and fertilization success in a damselfly: an example of cryptic female choice? *Anim. Behav.* **59**, 695–703.
- Ball, M. A. & Parker, G. A. 2000 Sperm competition games: a comparison of loaded raffle models and their biological implications. *J. Theor. Biol.* **206**, 487–506.
- Carayon, J. 1966 Paragenital system. In *Monograph of the Cimicidae* (ed. R. Usinger), pp. 81–167. Philadelphia, PA: Entomological Society of America.
- Chapman, R. F. 1998 *The insects: structure and function*. Cambridge University Press.
- Cook, P. A. & Gage, M. J. G. 1995 Effects of risks of sperm competition on the numbers of apyrene and eupyrene sperm ejaculated by the moth *Plodia interpunctella* (Lepidoptera, Pyralidae). *Behav. Ecol. Sociobiol.* **36**, 261–268.
- Davis, N. T. 1956 The morphology and functional anatomy of the male and female reproductive systems of *Cimex lectularius* L. (Heteroptera, Cimicidae). *Ann. Entomol. Soc. Am.* **49**, 466–493.
- Gage, A. R. & Barnard, C. J. 1996 Male crickets increase sperm number in relation to competition and female size. *Behav. Ecol. Sociobiol.* **38**, 349–353.
- Gage, M. J. G. & Baker, R. R. 1991 Ejaculate size varies with socio-sexual situation in an insect. *Ecol. Entomol.* **16**, 331–337.
- Mellanby, K. 1939 The physiology and activity of the bed bug (*Cimex lectularius* L.) in a natural infestation. *Parasitology* **31**, 200–211.
- Mesterton-Gibbons, M. 1999 On sperm competition games: incomplete fertilization risk and the equity paradox. *Proc. R. Soc. Lond. B* **266**, 269–274. (DOI 10.1098/rspb.1999.0632.)
- Parker, G. A. 1990 Sperm competition games—sneaks and extra-pair copulations. *Proc. R. Soc. Lond. B* **242**, 127–133.
- Simmons, L. W. 2001 *Sperm competition and its evolutionary consequences in the insects*. Princeton University Press.
- Simmons, L. W. & Siva-Jothy, M. T. 1998 Sperm competition in insects: mechanisms and the potential for selection. In *Sperm competition and sexual selection* (ed. T. R. Birkhead & A. P. Møller), pp. 341–432. London: Academic Press.
- Simmons, L. W., Craig, M., Llorens, T., Schinzig, N. & Hosken, D. 1993 Bush-cricket spermatophores vary in accord with sperm competition and parental investment theory. *Proc. R. Soc. Lond. B* **251**, 183–186.
- Stutt, A. D. & Siva-Jothy, M. T. 2001 Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. *Proc. Natl Acad. Sci. USA* **98**, 5683–5687.
- Wedell, N. 1992 Protandry and mate assessment in the wart-biter *Decticus verrucivorus* (Orthoptera, Tettigonidae). *Behav. Ecol. Sociobiol.* **31**, 301–308.
- Wedell, N., Gage, M. J. G. & Parker, G. A. 2002 Sperm competition, male prudence and sperm-limited females. *Trends Ecol. Evol.* **17**, 313–320.