Behav Ecol Sociobiol (2002) 52:426–433
DOI 10.1007/s00265-002-0524-9

ORIGINAL ARTICLE

Gerhard Schöfl · Michael Taborsky

Prolonged tandem formation in firebugs (*Pyrrhocoris apterus*) serves mate-guarding

Received: 10 March 2002 / Revised: 25 June 2002 / Accepted: 26 June 2002 / Published online: 21 August 2002

Abstract When copulating, firebugs (*Pyrrhocoris apterus*) form tandems for prolonged periods. Half of the copulations of marked individuals in the field lasted longer than 12 h, and some lasted up to 7 days. We found that: (1) females mate usually with several males prior to an oviposition bout, and (2) they store sperm from each mating for a large proportion of their reproductive lives. This causes a high potential for sperm competition to occur within female firebugs. We studied whether prolonged tandem formation is a male adaptation to this situation by testing five alternative hypotheses: (1) mate-guarding, (2) sperm-loading, (3) mate monopolization for future clutches, (4) prevention of ejaculate removal, and (5) mechanical sperm displacement. The sperm-utilization pattern was determined using a genetic marker. The second male to mate had a slight but significant fertilization advantage (P_2=0.59). In laboratory experiments, copulation duration varied systematically with the operational sex ratio, from a median duration of 7.3 h with a female-biased sex ratio to 15.3 h with a male-biased sex ratio. Sperm transfer commenced from the beginning of copulation, but the number of sperm in the female spermatheca reached an asymptote after 3–4 h. Smaller males had longer copulation durations than large ones, while there was no relationship between female size and copula duration. From our results, we exclude hypotheses 2–5 as possible explanations for prolonged tandem formation. Rather, males prolong copulations as a form of ejaculate-guarding under high competition with other males. Sperm displacement by prolonged sperm transfer may act in addition to this function, although this was not tested in this study.

Keywords Sperm competition · Prolonged copulation · Sperm transfer · Sex ratio · Heteroptera

Communicated by L. Simmons

G. Schöfl (✉) · M. Taborsky
Konrad Lorenz-Institut für Vergleichende Verhaltensforschung (KLIVV),
Austrian Academy of Sciences, Savoyenstraße 1a,
1160 Vienna, Austria
e-mail: gerhard.schoefl@i122server.vu-wien.ac.at
Tel.: +43-1-250775614, Fax: +43-1-250775693

Present address:
G. Schöfl, Institut für Tierzucht und Genetik,
Veterinärmedizinische Universität Wien, Josef Baumann Gasse 1,
1210 Vienna, Austria

Present address:
M. Taborsky, Department of Behavioural Ecology,
Institute of Zoology, University of Bern, Wohlenstrasse 50a,
3032 Hinterkappelen, Switzerland

Introduction

Reproductive competition favours adaptations in males that prevent their mates from receiving sperm from rival males, which would diminish their own fertilization success (Parker 1970, 1974; Smith 1984; Birkhead and Møller 1998). Males may attempt to avoid competition with future ejaculates by extending their mating association with a female beyond the transfer of sperm. There are several possibilities how this may be achieved, ranging from contact mate-guarding before, during or after copulation, to non-contact postcopulatory "guarding" by means of mating plugs or induced female non-receptivity (Danielsson 1998; Simmons and Siva-Jothy 1998).

Prolonged mating associations are among the most widespread male tactics to overcome sperm competition (reviewed by Alcock 1994). From a male point of view, they may serve primarily: (1) to impede subsequent copulations of their mate (mate-guarding) (McLain 1980; Sillén-Tullberg 1981; Radwan and Siva-Jothy 1996); (2) to reduce competition with previous ejaculates if by long copulations males transfer more sperm than their rivals have done (sperm-loading) (Dickinson 1986; Boiteau 1988; Tsubaki and Sokei 1988); (3) to monopolize mates for further clutches (Carroll 1991); (4) to prevent the female from removing their ejaculate or spermatophore prematurely (Bateman and MacFadyen 1999); or to enable the male to displace sperm donated by previous partners (5) either mechanically (Siva-Jothy

1987; Siva-Jothy and Tsubaki 1989; Cordero 1990), or (6) indirectly via flushing of the spermatheca with the male's ejaculate (Parker and Simmons 1991; Simmons et al. 1999). Each hypothesis makes predictions about the timing of sperm transfer. When the function of prolonged copulations is mainly to dilute rival sperm (sperm-loading), sperm transfer should continue for a large proportion of copulation time (Simmons 1987); when the function is to displace rival sperm, mechanical sperm displacement will occur prior to the transfer of own sperm (Waage 1979, 1984), although ejaculation may continue throughout copulation under indirect displacement (sperm-flushing; Parker and Simmons 1991; Simmons et al. 1999); when long copulations function primarily as a means of ejaculate-guarding, sperm transfer will occur before a guarding phase (e.g. Sillén-Tullberg 1981; Barnett and Telford 1994; Radwan and Siva-Jothy 1996).

Female insects often mate multiply and frequently they maintain sperm in their spermathecae for a large proportion of their reproductive lifespan. Hence, insects are predisposed to high levels of sperm competition (Parker 1970; Simmons and Siva-Jothy 1998). In seed-feeding Heteroptera for instance, there is strong evidence that sperm competition plays an important role because a number of species show prolonged copulations (McLain 1980, 1989; Sillén-Tullberg 1981; Carroll and Loye 1990; Carroll 1991; Tsukamoto et al. 1994). Typically, these bugs are gregarious, and some tend to have strongly male-biased adult sex ratios which increase the competition among males for mating partners (Carroll and Loye 1990; Carroll 1993). One of the most widely distributed heteropteran species in the western Palaearctic is the red firebug, *Pyrrhocoris apterus* (L.), which has been used extensively as a model species in physiological, endocrinological and genetic research (Socha 1993). Its reproductive behaviour has not been studied extensively (but see Hellwig and Ludwig 1951; Žd'árek 1970). However, conspicuously persisting copulations that may last for days (personal observations) indicate that sperm competition is likely in this species.

In this study, we examined the mating behaviour and the potential for sperm competition in *P. apterus* in its natural habitat. In particular, we aimed to discriminate between the different hypotheses explaining the adaptive function of prolonged copulations, and we studied male behavioural adaptations in the context of this trait. For technical reasons, we had to confine our tests to the first five of the six mentioned hypotheses. We asked: (1) do females remate under natural conditions and, if so, how often? (2) Do females retain sperm between ovipositions? (3) Is sperm transferred until the end of copulation or is there a postinsemination, pure guarding phase? (4) What is the relative fertilization success of males in a mating sequence? (5) Does copulation duration vary with sex ratio in the manner predicted by sperm competition theory? Our approach combined direct observations in the field with experiments under standardized and natural conditions.

Methods

Study species

In Central Europe, *P. apterus* (L.) (Heteroptera: Pyrrhocoridae) is closely associated with lime trees (*Tilia cordata* Mill. or *T. platyphyllos* Scop.), whose seeds form the basic component of its food (Socha 1993). Even solitary lime trees provide enough food to support high-density populations consisting of several thousand individuals. Firebugs are at least partially bivoltine under the climatic conditions of Austria (personal observation). They overwinter as adults in the leaf litter at the base of lime trees and usually do not disperse far from the immediate vicinity of their food plants during the rest of their life-cycle (Tischler 1959). The ovarian development of females moulting later in the year is halted by a photoperiodically induced diapause, and resumed after hibernation in spring (Hodek 1971; Socha and Šula 1992). Thus, the majority of adult bugs start to mate at the hibernation sites on the first warm days in early spring (March, April; Žd'árek 1970). Bugs from the same generation have been observed to stay reproductively active until July (Tischler 1959). Žd'árek (1970) distinguished three phases in the mating behaviour of this species: (1) a male reacts to female presence at a short distance (~1 cm) mainly by responding to olfactory stimuli; he approaches the female quickly and mounts her. (2) Whilst orientating his body alongside the female, the male antennates her vigorously. After reaching the proper position, the male extends his genital capsule and taps repeatedly the valvae covering the female genital opening with the protruding tip of his penis. Receptive females respond by opening the valvae. (3) When the male has introduced the penis and achieved a firm genital coupling, the pair assumes a "tail-to-tail" position. From time to time, either the pair folds up ventrally and the male antennates the female, or the male initiates a vigorous jerking of the abdomen. Copulations have been reported to last from a few minutes to several hours under laboratory conditions (Žd'árek 1970).

Study site and seasons

We made most field observations on a firebug population sustained mainly by two solitary old lime trees in the grounds of the Konrad Lorenz-Institut für Vergleichende Verhaltensforschung (KLIVV) in Vienna, between May and July 1998 and in May 1999. The laboratory stock used in the experiments was derived from approximately 80 bugs collected at KLIVV in March 1998. For breeding, we kept the animals in 0.5-l jars at a density of approximately 40 bugs per jar in mass culture. They were supplied with linden seeds ad libitum, and glass vials plugged with cotton wool provided drinking water. The stock was maintained at 25±2°C under a light regime of LD 18:6 h in order to prevent the bugs from entering diapause. During laboratory experiments, we kept the experimental animals in plastic petri dishes (9 cm in diameter), which we lined with dry filter paper. They were provided with linden seeds and water ad libitum. To ensure virginity, the experimental animals were sexed and kept separately from the fifth instar onwards. Animals were used in experiments only after at least 7 days from imaginal moult, ensuring that gonad development was completed and individuals of both sexes had become sexually active (Žd'árek 1970). For paternity tests, we used a mutant strain (*yolk body*). Approximately 60 individuals of this strain were provided by R. Socha (Institute of Entomology, České Budějovice, Czech Republic), which formed the basis of a laboratory stock maintained under the same conditions as described above.

Mating frequency and copulation duration

We observed mating behaviour in a field arena covering 10 m^2, which we placed at the base of a lime tree. The arena was enclosed by plastic lawn edging, whose upper rim was covered with Vaseline to prevent exchange of bugs between arena and surround-

ings. *P. apterus* is flightless, even in its macropterous morph. Since all activities of firebugs are confined to the vicinity of lime trees, we assumed that the establishment of an arena would not change the behaviour of the bugs in any fundamental way.

In total 249 bugs were found and captured in the fenced arena (128♀♀,121♂♂; density=24.9 bugs/m^2). We sexed and weighed them to the nearest 0.01 mg. Differently coloured, prefabricated bee tags (numbered 0–99) were glued onto the pronotum and scutellum using cyanoacrylate glue. The tags did not appear to handicap the bugs. After marking, we separated the bugs by sex and kept them in plastic boxes supplied with food and water ad libitum, before returning them to the arena in the evening of the same day. Censuses at which we determined the proportion of bugs in copula commenced 12 h later. Some bugs vanished from the arena because they died, got over the fence or lost their tags, at a relatively constant rate of 3.7% (mean) per day. There was no difference in dropout rate between the sexes. We censused all individuals in the arena twice per day, from 0800 to 1000 hours and from 1800 to 2000 hours, for twelve consecutive days, and estimated mating frequencies and mating durations by monitoring the copulatory behaviour of marked bugs during these surveys. When the same two mates were seen in tandem in two successive observations, we regarded this as resulting from prolonged copulation (the probability of a copulating pair breaking up and resuming copulation with the same partner was less than 1.4×10^{-4}, as estimated from the number of possible pairwise combinations of available partners and with an average of 69% of all bugs being unmated at each census). On 3 days during the observation period, we took additional censuses in the early afternoon, at 1300 hours, in order to test this assumption. We detected no instance of an interruption and resumption of a copulation between identical partners. During each scan, we recorded the mating status (paired or single) of each bug and the identity of its mate. Unmarked individuals (immigrants) were discovered occasionally and removed from the arena upon discovery.

Sperm storage

Virgin females that mated successfully with only one male were isolated after this copulation for about 4 weeks. We recorded egg number and fertilization rate of all clutches laid by these females during this period to confirm whether females retain sperm between ovipositions.

Sperm transfer

To determine the starting point and course of sperm transfer during copulation in the laboratory, we counted the number of sperm transferred to the spermatheca in copulations that were experimentally interrupted at different time intervals from 10 min to 6 h from start of copulation. Pairs were randomly assigned to different copulation durations. For each mating, a virgin male and a virgin female were placed together in a petri dish. Some pairs were observed continuously for up to 1 h after copulation commenced, while others were checked every half hour. When the previously chosen copulation interval was over, the pair was carefully separated, and the female was frozen immediately in an ultra-deep freezer at –75°C in order to immobilize the sperm in her genital tract. The dead bugs were dissected under insect saline using a stereomicroscope with cold-light illumination.

To determine the number of spermatozoa in the spermatheca, the latter was detached at the junction of the spermathecal duct (ductus receptaculi) with the bursa copulatrix, and placed in a depression dish with 200 µl deionized water, to which Labosol detergent had been added. The sperm was first pressed out of the spermatheca by squeezing, and finally the spermatheca was ruptured with a pair of pincers. The deionized water caused the extraordinarily long spermatozoa (950 µm according to Furieri 1965) to curl up, which in combination with the detergent resulted in a homogeneous dispersion of sperm in the solution. After stirring with an entomological pin for at least 1 min, a drop of DNA-binding fluorochrome Hoechst 33342 was added to stain the nuclei of the spermatozoa. Subsequently, the solution was stirred for a further minute, washed into a glass tube with about 10 ml deionized water, and filtered through a Millipore filter (25 mm diameter, 0.22 µm pore width) by means of a vacuum pump. The filter was mounted on a glass slide and the stained sperm nuclei were examined using a fluorescence microscope. The total number of sperm present on the filter was estimated from a count of the number of nuclei in 25 focal areas that were distributed regularly over the entire filter. The magnifications used were 1:100 or 1:200 depending on sperm density on the filter, with the total filter surface covering 9.1% or 2.3% by the combined focal areas, respectively. Sperm was spread evenly on the filters and sperm numbers did not differ significantly between central and peripheral counting areas (Wilcoxon test, Z=0.764, n=10, P=0.44). Sperm on each filter was counted three times. The mean deviation from sample mean was 4.9±3.1% (n=55) in either direction.

Sperm utilization

We examined the pattern of sperm utilization by determining the proportion of offspring sired by the second male to mate in a double-mating trial (P_2), using wild-type *P. apterus* from our laboratory stock in combination with the autosomal recessive body-colour mutant *yolk body* (*yb*) (Socha and Nemec 1996) as a paternity marker. *Yb* individuals can be distinguished easily from the wild-type from the first instar stage due to their conspicuous yellow coloration. They exhibit approximately the same viability and reproductive capacity (i.e. number and hatchability of eggs) as wild-type individuals (Socha 1997). We performed reciprocal crosses in order to control for possible differences in the competitive ability of sperm or in mating preferences between strains. In this experiment, we were not able to check for possible effects of copulation duration on sperm utilization. We took care, however, that copulations lasted long enough (≥4 h) for transferred sperm quantities to reach the maximum level as determined from sperm-transfer experiments with virgin females.

For each double mating, a single virgin *yb* female was placed with a single virgin male (*yb* or wild-type) into a petri dish provided with linden seeds and water ad libitum. Each pair was observed continuously until copulation commenced. Bugs failing to achieve copulation within 1 h were removed. We checked the copulating pairs at half-hour intervals during the next 4 h. Every mating continuing until the end of this period was labelled "successful" and the pair was left in the petri dish overnight. Pairs still in copula the next day were carefully separated. About 24 h after the start of the first mating and at least 6 h after the pair had been separated, a male from the opposite genetic strain was introduced to each female, and the same procedure was followed as with the first mating. Females that had oviposited in the meantime or refused to mate the second time were excluded from analysis. Following the second mating, the male was removed and the females were kept isolated in their petri dish for a period of about 4 weeks, during which time they oviposited. Last-male fertilization success was determined for all ensuing oviposition bouts by scoring the body colour of the progeny at the second instar stage, and calculating the proportion of progeny sired by the last male to mate. The first clutch laid by each female after the double mating was used to assess relative paternities of the two males.

Copulation duration under varying sex ratios

In the laboratory, three set-ups with different sex ratios were employed: 3:1, 1:1 and 1:3 (males:females). Petri dishes served as "mating arenas". For each of 4 trials, we established 39 arenas, 13 of which were assigned to 1 of the 3 sex-ratio treatments. Each trial was recorded by time-lapse video for 24 h. During the experiment, the arenas were permanently illuminated. On the morning of a trial, either we picked bugs from the mass culture and assigned them at random, either to perform as a mating pair, or to be added to a mating pair in pairs of two males or two females. Individuals

assigned to be mating pairs were marked with a dot. Trials began at 0900 hours when we placed one female and one male into an arena. As soon as the pair had acquired the tail-to-tail position, two males, two females or no bugs at all were added to the arena. We recorded the duration of all copulations to the nearest minute, but used only the first copulation that took place between the marked individuals for statistical analyses. All copulations lasting from the beginning of the trial to its very end were pooled in one time category (>24). Following the trial, the experimental animals were weighed to the nearest 0.01 mg, and body length and pronotal width were measured.

In the field, we employed a set-up similar to the observational procedure outlined above, except that now two arenas covering 5 m^2 each were established. Instead of bee tags, we used self-made, numbered white and yellow plastic tags that were both lighter and smaller than the prefabricated tags and proved to be more durable under field conditions. All *P. apterus* were removed from either arena before we placed 150 individually marked bugs with sex ratios of 1:2 and 2:1 (male:female), respectively, into each arena (density=30 bugs/m^2). Subsequently, the bugs were censused for 16 consecutive days as outlined above. We measured weight, body length and the pronotal width of all remaining bugs directly after the experiment.

Statistical procedures

Statistical analyses of field and experimental data were performed using the STATISTICA 5.1 for Windows software package. Normality of data was tested in all instances using the Kolmogorov-Smirnov test, and homogeneity of variances was tested using Levene's test. H_0 was rejected at a level of significance of P=0.05. When data could not be transformed to conform to the requirements of parametric statistics, or sample size seemed too small to justify application of normality tests or a transformation of data, non-parametric procedures were applied. Descriptive statistics are given as arithmetic means±1 SD, unless stated otherwise.

Results

Field observations

In the field, we found frequent multiple matings by both females and males. Fifty-nine marked females were sighted regularly during the entire observation period of 12 consecutive days. They mated with up to 6 different males (mean number of copulation partners per female=2.75±1.8, n=59). This is a minimum estimate as we spotted on average only about half of the experimental animals at each survey, and therefore some copulations probably went unnoticed. Furthermore, unrecorded short copulations might have occurred during the 12-h intervals between two surveys. When we experimentally biased the sex ratio towards more males (2:1), females mated with an average of 4.76±2.6 males (n=34, range=0–9). When we biased the sex ratio towards more females (1:2), they mated with an average of 2.64±1.85 males (n=69, range=0–6; difference between both treatments: Mann-Whitney U=621, P<0.001).

In females that mated more often than once under an unbiased sex ratio, the interval between two matings ranged from immediate re-copulation to 8 days, with a median of 49.5 h (q_1=33, q_3=74, n=56). When the sex ratio was male biased (2:1), the median interval between two matings decreased significantly to 32 h (q_1=24, q_3=28, n=35; Mann-Whitney U=676, P=0.013). When the sex ratio was female biased, the interval between two matings was similar to the unbiased situation (median=49.5 h, q_1=36, q_3=67 h, n=58). For the same reason as explained above, these intervals may have been overestimated. We found no correlation between copula duration and the interval to the next copulation (Pearson's product moment correlation, r=–0.204, n=59, P>0.1 at the unbiased sex ratio, r=–0.15, n=58, P>0.1 at the female-biased sex ratio, and r=–0.23, n=35, P>0.1 at the male-biased sex ratio).

Copula durations were highly variable and ranged from a few minutes to 7.5 days, with 48.7% of all copulations lasting longer than 12 h (i.e. these pairs were observed in copula at two or more consecutive surveys; this percentage is a rough estimate, as on average only about half of the experimental animals were spotted at each survey). Copulations initiated later in the day lasted longer. One hundred and thirteen copulations were first detected at the morning surveys while 273 copulations were first detected at evening surveys. The latter lasted longer than 1 survey interval, more often than copulations first detected at a morning survey [176 copulations out of 273 (65%) vs 45 copulations out of 113 (40%), χ^2=19.8, P<0.001], even though the intervals between morning and evening checks were slightly shorter (around 10 h) than the intervals between evening and morning surveys (around 14 h).

Sperm storage

Following a single insemination, the average number of eggs per clutch remained approximately the same in four successive oviposition bouts (one-way repeated-measures ANOVA, $F_{3,66}$=1.17, P=0.33). Overall, the mean clutch size was 42.2±7.38 (n=130, range=23–60). The fertilization rate declined significantly over the four successive oviposition bouts (Friedman ANOVA $\chi^2_{3,20}$=11.16, P=0.011). There was no difference between the first two clutches, but a clear decline of fertilization rate thereafter (Table 1). Post-hoc comparisons revealed that rates in the first and second clutches were significantly higher than in the fourth clutch (P<0.05, Wilcoxon-Wilcox multiple comparisons). We found no significant difference in fertilization rates between singly ($\bar{x}$=0.74±0.24, n=37) and doubly ($\bar{x}$=0.79±0.17, n=23) mated females (Kruskal-Wallis H=0.41, n=60, P=0.52). These data show that females do retain sperm between ovipositions, and sperm from a single insemination is sufficient to ensure a high fertilization rate for at least two clutches. The mean interval between clutches was 5.0±0.76 days (n=36, range=2–10).

Sperm transfer

Males cannot inseminate directly into the spermatheca as their penis is not shaped to fit into the long and thin sper-

Table 1 Clutch size and fertilization rate of four consecutive oviposition bouts following a single insemination ($\bar{x}$±SD)

	1st clutch ~ 1 day after copulation	2nd clutch ~ 6 days after copulation	3rd clutch ~ 11 days after copulation	4th clutch ~ 16 days after copulation
Clutch size	43.6±7.89, *n*=37	42.8±7.68, *n*=37	41.4±6.3, *n*=33	40.3±6.75, *n*=23
Fertilization rate	0.74±0.24, *n*=37	0.74±0.23, *n*=37	0.67±0.27, *n*=32	0.57±0.24, *n*=20

mathecal duct. Instead, the penis hooks to a sclerotized anchor-shaped structure of the dorsal vaginal wall directly at the orifice of the spermathecal duct, where the sperm is released (Ludwig 1926; Merle 1969). The sperm must then migrate through the spermathecal duct towards the spermatheca where it is stored. In five out of eight cases we found a small amount of sperm in the spermathecal duct and in the spermatheca, following a copulation lasting only 10 min. This indicates that sperm transfer may commence almost instantaneously or at least shortly after the onset of copulation. After a copulation period of 4 h, there appeared to be no further increase in sperm numbers in the spermatheca (Fig. 1), suggesting that the amount of sperm transferred is either not time dependent after an initial insemination phase of approximately 4 h, or that ejaculation is continuous but with simultaneous indirect displacement from the spermatheca. The mean total number of sperm transferred after 4 h was 5,387±1,290 (*n*=12, range=3,387–7,452).

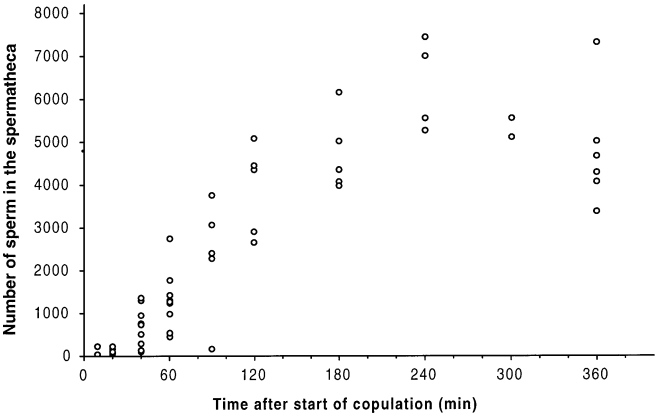

Fig. 1 Time pattern of sperm transfer in *Pyrrhocoris apterus*. Virgin females were interrupted at different times during copulation and the number of sperm in the spermatheca was counted (*n*=60)

Sperm utilization

The proportion of offspring fathered by the second male to mate when a female copulated first with a wild-type male and subsequently with a *yb* mutant was 0.572±0.241 (*n*=9, range=0.375–0.979), and 0.603±0.322 (*n*=14, range=0.021–0.975) in the reversed sequence. These two P_2 values did not differ significantly from each other (Mann-Whitney *U*=58, *P*=0.75). The combined overall P_2 value of 0.591 (±0.287, *n*=23) was significantly different from 0.5 (meta-analysis of probabilities from two-tailed Fisher's exact tests for each trial; Sokal and Rohlf 1995, χ^2_{46}=77.8, *P*<0.01). There was no difference between this P_2 value and P_2 values obtained from the subsequent two clutches (Kruskal-Wallis *H*=0.48, *n*=44, *P*=0.79).

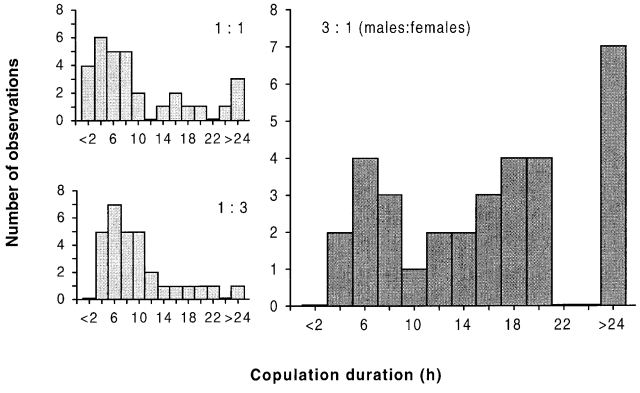

Fig. 2 Frequency distributions of copulation durations at three different sex ratios under standardized laboratory conditions. Copulations lasted significantly longer in the male-biased condition (see text)

Copulation duration under varying sex ratios

Our laboratory experiments showed that operational sex ratio affects copulation duration under controlled conditions [medians for a sex ratio (males:females) of 1:1 (*n*=31) were 6.58; for a ratio of 1:3 (*n*=30) 7.33; and for a ratio of 3:1 (*n*=32) 15.29; Kruskal-Wallis *H*=11.41, *n*=93, *P*=0.003, Fig. 2], with copulations being significantly longer when the operational sex ratio was male biased than when unbiased (Dunn's multiple comparison, *Q*=3.13, n_1=32, n_2=31, *P*<0.01) or female biased (*Q*=2.64, n_1=32, n_2=30, *P*<0.05). Copulation durations did not differ between the unbiased and female-biased groups (Dunn's multiple comparison, *Q*=0.46, n_1=31, n_2=30, *P*>0.5). The frequent courtship of single males directed towards copulating pairs [218 attempts in total during the first 6 h; median=5 (q_1=3, q_3=8.75), *n*=32 experiments] never led to successful take-over, showing that prolonged copulations effectively reduce the probability that females will remate.

In the field arenas, mating durations were estimated by assigning a copulation that was witnessed only during a single census, a duration of 12 h. Mating durations did not differ significantly between arenas with different sex ratios of 1:2 and 2:1 (Z_U=–1.56, n_1=254, n_2=241, *P*=0.119, Mann-Whitney *U*-test, Fig. 3).

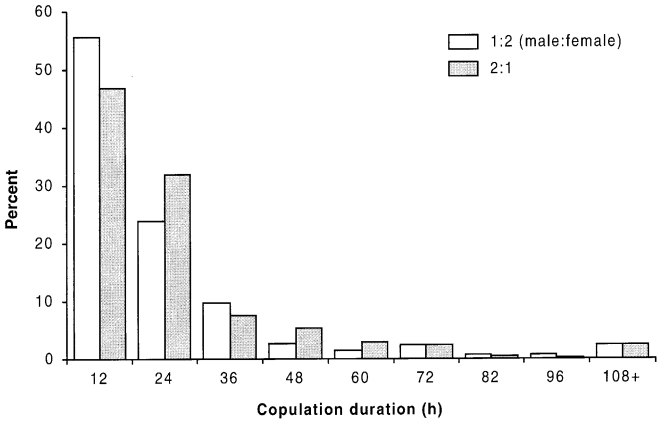

Fig. 3 Relative frequency of copulation durations of marked pairs of *Pyrrhocoris apterus* estimated by monitoring the bugs at 12-h intervals in field arenas at two different sex ratios. The sampling period was 16 days

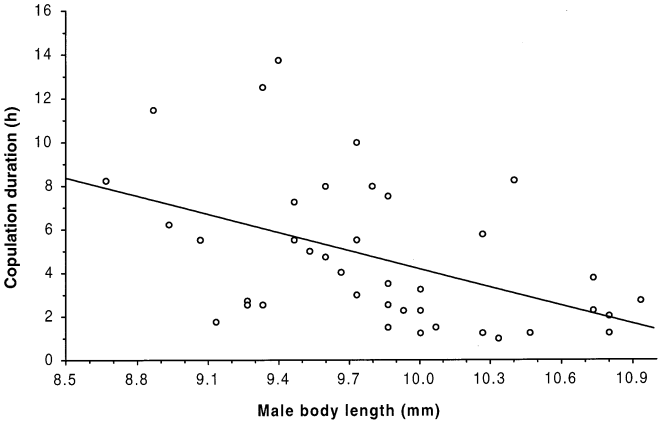

Fig. 4 Relationship between male body length and copulation duration. Pearson product-moment correlation r=–0.47, P<0.003, n=39

In the laboratory, copulation duration correlated negatively with male size in undisturbed pairs (Pearson's product moment correlation, r=–0.47, n=39, P<0.003 for body length, r=–0.41, n=39, P=0.01 for body weight, and r=–0.37, n=39, P=0.02 for pronotal width). The correlations between copulation durations and male body length (Fig. 4) and weight were significant (α=0.05) after also employing sequential Bonferroni adjustment (Rice 1988). No significant correlation was found between any female size measures and copulation duration.

Discussion

Under natural conditions, it is very likely that an unguarded female firebug will be inseminated by more than one male prior to any egg-laying. In the field, *P. apterus* females copulate on average every 2.1 days with a different mate. Intervals were even shorter with a male-biased sex ratio. Egg-laying follows a constant gonadotropic cycle of approximately 5 days, independent of insemination (laboratory data Žďárek 1970; Sláma 1971; this study). Under field conditions, the average oviposition intervals may be longer (8.4 days as estimated from the number of clutches produced throughout a season; Honěk 1986). Our laboratory data show that a single insemination was sufficient to fertilize, on average, three complete, successive clutches.

Female receptivity drops just before egg-laying and a female is non-receptive for about 1 day thereafter. Otherwise, there is no indication that sexual activity ever decreases after a mating in either sex (Žďárek 1970). The great variation in the female refractory period after copulation between a few minutes and 8 days is probably not related to the time spent in copula with the previous male. After some of the longest matings, females quickly engaged in copulations with a new partner, whereas after brief matings some females remained unmated for several days. Copulations starting late in the day lasted longer than those starting in the morning, which suggests that daylight hours rather than total time in copulation may be important.

Patterns of sperm utilization

When females were successively mated with two males in our laboratory experiment, paternity was mixed in subsequent clutches. Sperm from later inseminations had a slight but significant advantage over previously stored sperm (P_2=nearly 60%). This P_2 value is close to the lower end of values found in other mate-guarding Heteroptera (*Nezara viridula*: P_2=0.51, McLain 1985; *Jadera haematoloma*: P_2=0.65, Carroll 1991; *Aquarius remigis*: P_2=0.65, Rubenstein 1989; *Neacoryphus bicrucis*: P_2=0.78, McLain 1989; *Lygaeus equestris*: P_2=0.92, Sillén-Tullberg 1981). Relatively low P_2 values may still favour the evolution of mate-guarding (McLain 1980; Dickinson 1995; Radwan and Siva-Jothy 1996). Yamamura (1986) showed that it may pay a male to prevent his mate from remating at high population densities, high mate-searching efficiencies and a male-biased OSR even if P_2 is below 0.5.

Variance in P_2 was high in *P. apterus* (SD=0.29, range=0.02–0.98), just as in many other insects (Simmons and Siva-Jothy 1998). Due to our experimental setup, the starting points of the two successive copulations were spaced by 24 h. Passive sperm loss from the spermatheca between copulations is probably very low in *P. apterus*, as females retained fertility for extended periods of time after a single copulation. The observed variation in P_2 values may be due mainly to the considerable variability of transferred sperm numbers between males.

Mate-guarding should increase with male bias in OSR, which was confirmed in our laboratory experiments, in which copulations lasted twice as long with a male bias compared to either a female bias or in the unbiased situation. In the field, however, when we varied sex ratios to less extent than in the laboratory, this effect was not significant. This result may have been affected

by the large sampling interval applied in the field (ca. 12 h).

Alternative hypotheses to explain prolonged mating associations

Which of the six hypotheses given at the beginning of this article best explains the prolonged mating association in *P. apterus*? Direct displacement of rival sperm (hypothesis 5) is not an option in *P. apterus* as no part of the male genital apparatus could possibly reach into the sperm storage organ of the female. Also, we found no evidence that females passively lose or actively remove sperm, as a large proportion of eggs in four subsequent clutches were fertilized after only one copulation. So prolonged copulations are unlikely to serve to prevent ejaculate removal (hypothesis 4). Nor do males monopolize females for future clutches, as pairs part before egg-laying and females will typically mate with a different male thereafter (hypothesis 3). Sperm-loading (hypothesis 2) would be beneficial when complete sperm mixing occurs in the female sperm-storage organs (Parker 1990). Our data indicate at least some sperm mixing and a great variation in ejaculate size. Sperm transfer, however, commences right from the start of copulation in *P. apterus*, and the amount of transferred sperm reaches an asymptote after just 3–4 h. This appears similar to the pyrrhocorid, *Dysdercus cingulatus*, where the total ejaculate was transferred to the female after about 2.5–4 hs (Pluot 1970). Therefore, sperm-loading is unlikely to explain prolonged copulations in *P. apterus* either.

Two hypotheses remain as possible explanations for prolonged copulations in this firebug. First, already donated ejaculates are protected from competition with sperm of potential future males of the female (mate-guarding, hypothesis 1). Second, males may displace sperm of rivals indirectly by continuous sperm transfer (sperm-flushing, hypothesis 6; Simmons et al. 1999). This would cause the number of sperm in the spermatheca to remain constant once it was completely filled with sperm. Our data show that prolonged copulations in firebugs are an effective mate-guarding mechanism. We lack data, however, on the relationship between copula duration and sperm utilization, which would allow us to test whether sperm-flushing occurs in this species. We should point out that, in our experiments, males copulated with virgin females, so there were no sperm to be displaced. However, it may be impossible for males to recognize the mating history of their mates, as appears to be the case in yellow dung flies (Parker et al. 1993).

The price males pay for prolonged copulations is a reduced opportunity for additional matings. Our laboratory data suggest a bimodal distribution of copulation durations (cf. Fig. 2). Apparently, males do not respond to potential sperm competition by gradually increasing copulation times, but by switching from an emphasis on frequent remating, with copulations lasting only long enough to transfer an amount of sperm sufficient for fertilizing a clutch, to a prolonged mate-guarding tactic. Copulation duration was negatively correlated with male size, so that either smaller males were more inclined to mate-guard than larger males, or larger males usually displace sperm at faster rates than smaller males (Parker and Simmons 1994). These two possibilities are not mutually exclusive. Smaller males may suffer from diminished chances to find mates in the scramble competition polygyny mating system (as defined by Thornhill and Alcock 1983) of *P. apterus*, if small size confers some disadvantage in mobility or courtship. Also, larger males may be able to produce larger ejaculates. Unfortunately, our data do not allow us to test whether male size correlates with ejaculate size.

Why do female *P. apterus* mate multiply? There may be little intersexual conflict over mating frequency or duration due to a possible reduction of predation risk by tandem formation (instead of an increase; Fairbairn 1993; Rowe 1994), as females may benefit from enhanced conspicuousness while in tandem due to aposematism. Also, mating does not impede feeding, as copulating females were observed to feed often on linden seeds. For female firebugs, multiple matings may be "copulations of convenience" (Thornhill and Alcock 1983) which reduce male harassment and also ensure a sufficient sperm supply for future clutches, so that rejecting copulations may be more expensive than accepting them.

Acknowledgements We thank Radomír Socha for providing bugs and helpful information, and Leigh W. Simmons and two anonymous referees for valuable comments on the manuscript. The experiments in this study comply with the current laws of Austria.

References

Alcock J (1994) Postinsemination associations between males and females in insects: the mate-guarding hypothesis. Annu Rev Entomol 39:1–21

Barnett M, Telford SR (1994) The timing of insemination and its implications for sperm competition in a millipede with prolonged copulation. Anim Behav 48:482–484

Bateman PW, MacFadyen DN (1999) Mate guarding in the cricket *Gryllodes sigillatus*: influence of multiple potential partners. Ethology 105:949–957

Birkhead TR, Møller AP (1998) Sperm competition and sexual selection. Academic Press, London

Boiteau G (1988) Sperm utilization and post-copulatory female-guarding in the Colorado potato beetle, *Leptinotarsa decemlineata*. Entomol Exp Appl 47:183–188

Carroll SP (1991) The adaptive significance of mate guarding in the soapberry bug, *Jadera haematoloma* (Hemiptera: Rhopalidae). J Insect Behav 4:509–530

Carroll SP (1993) Divergence in male mating tactics between two populations of the soapberry bug. I. Guarding versus non-guarding. Behav Ecol 4:156–164

Carroll SP, Loye JE (1990) Male-biased sex ratios, female promiscuity, and copulatory mate guarding in an aggregating tropical bug, *Dysdercus bimaculatus*. J Insect Behav 3:33–48

Cordero A (1990) The adaptive significance of prolonged copulations of the damselfly, *Ischnura graellsii* (Odonata: Coenagrionidae). Anim Behav 40:43–48

Danielsson I (1998) Mechanisms of sperm competition in insects. Ann Zool Fenn 35:241–257

Dickinson JL (1986) Prolonged mating in the milkweed leaf beetle *Labidomera clivicollis* (Coleoptera: Chrysomelidae): a test of the "sperm-loading" hypothesis. Behav Ecol Sociobiol 18:331–338

Dickinson JL (1995) Trade-offs between post-copulatory riding and mate location in the blue milkweed beetle. Behav Ecol 6:280–286

Fairbairn DJ (1993) Costs of loading associated with mate-carrying in the waterstrider, *Aquarius remigis*. Behav Ecol 4:224–231

Furieri P (1965) Osservazioni preliminari sulla ultrastruttura dello spermio di *Pyrrhocoris apterus* Fall. (Hemiptera, Pyrrhocoridae). Redia 48:179–187

Hellwig H, Ludwig W (1951) Versuche zur Frage der Arterkennung bei Insekten. Z Tierpsychol 8:456–462

Hodek I (1971) Termination of adult diapause in *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) in the field. Entomol Exp Appl 14:212–222

Honěk A (1986) Enhancement of fecundity in *Pyrrhocoris apterus* under alternating natural conditions (Heteroptera, Pyrrhocoridae). Acta Entomol Bohemoslov 83:411–417

Ludwig W (1926) Untersuchungen über den Copulationsapparat der Baumwanzen. Z Morphol Ökol Tiere 5:291–380

McLain DK (1980) Female choice and the adaptive significance of prolonged copulation in *Nezara viridula* (Hemiptera: Pentatomidae). Psyche 87:325–336

McLain DK (1985) Male size, sperm competition, and the intensity of sexual selection in the southern green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae). Ann Entomol Soc Am 78:86–89

McLain DK (1989) Prolonged copulation as a post-insemination guarding tactic in a natural population of the ragwort seed bug. Anim Behav 38:659–664

Merle J (1969) Anatomie des appareils génitaux mâle et femelle et fonctionnement ovarien de *Pyrrhocoris apterus* (L.) (Hemiptera, Pyrrhocoridae). Acta Entomol Bohemoslov 66:275–288

Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. Biol Rev 45:525–567

Parker GA (1974) Courtship persistence and female-guarding as male time investment strategies. Behaviour 48:157–184

Parker GA (1990) Sperm competition games: raffles and roles. Proc R Soc Lond B 242:120–126

Parker GA, Simmons LW (1991) A model of constant random sperm displacement during mating: evidence from *Scatophaga*. Proc R Soc Lond B 246:107–115

Parker GA, Simmons LW (1994) Evolution of phenotypic optima and copula duration in dung flies. Nature 370:53–56

Parker GA, Simmons LW, Ward PI (1993) Optimal copula duration in dungflies – effects of frequency-dependence and female mating status. Behav Ecol Sociobiol 32:157–166

Pluot D (1970) La spermathèque et les voies génitales femelles des Pyrrhocoridés [Hemiptera]. Ann Soc Entomol Fr 6:777–807

Radwan J, Siva-Jothy MT (1996) The function of post-insemination mate association in the bulb mite, *Rhizoglyphus robini*. Anim Behav 52:651–657

Rice WR (1988) Analyzing tables of statistical tests. Evolution 43:223–225

Rowe L (1994) The costs of mating and mate choice in water striders. Anim Behav 48:1049–1056

Rubenstein DI (1989) Sperm competition in the water strider, *Gerris remigis*. Anim Behav 38:631–636

Sillén-Tullberg B (1981) Prolonged copulation: a male "postcopulatory" strategy in a promiscuous species, *Lygaeus equestris* (Heteroptera: Lygaeidae). Behav Ecol Sociobiol 9:283–289

Simmons LW (1987) Sperm competition as a mechanism of female choice in the field cricket, *Gryllus bimaculatus*. Behav Ecol Sociobiol 21:197–202

Simmons LW, Siva-Jothy MT (1998) Sperm competition in insects: mechanisms and the potential for selection. In: Birkhead TR, Møller AP (eds) Sperm competition and sexual selection. Academic Press, London, pp 341–434

Simmons LW, Parker GA, Stockley P (1999) Sperm displacement in the yellow dung fly *Scatophaga stercoraria*: an investigation of male and female processes. Am Nat 153:302–314

Siva-Jothy MT (1987) Variation in copulation duration and the resultant degree of sperm removal in *Orthetrum cancellatum* (L.) (Libellulidae: Odonata). Behav Ecol Sociobiol 20:147–151

Siva-Jothy MT, Tsubaki Y (1989) Variation in copulation duration in *Mnais pruinosa pruinosa* Selys (Odonata: Calopterygidae). 1. Alternative mate-securing tactics and sperm precedence. Behav Ecol Sociobiol 24:39–45

Sláma K (1971) Hormonal control of metabolism in *Pyrrhocoris*. Entomol Exp Appl 5:85–90

Smith RL (1984) Sperm competition and the evolution of animal mating systems. Academic Press, London

Socha R (1993) *Pyrrhocoris apterus* (Heteroptera) – an experimental model species: a review. Eur J Entomol 90:241–286

Socha R (1997) Genetics of a yolk body-color mutation in *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Ann Entomol Soc Am 90:358–362

Socha R, Nemec V (1996) Coloration and pteridine pattern in a new, *yolk body* mutant of *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Eur J Entomol 93:525–534

Socha R, Šula J (1992) Voltinism and seasonal changes in haemolymph protein pattern of *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) in relation to diapause. Physiol Entomol 17:370–376

Sokal RR, Rohlf FJ (1995) Biometry, 3rd edn. Freeman, New York

Thornhill R, Alcock J (1983) The evolution of insect mating systems. Harvard University Press, Cambridge, Mass

Tischler W (1959) Zur Biologie der Feuerwanze (*Pyrrhocoris apterus* L.). Zool Anz 163:392–396

Tsubaki Y, Sokei Y (1988) Prolonged mating in the melon fly, *Dacus cucurbitae* (Diptera: Tephritidae): competition for fertilization by sperm-loading. Res Popul Ecol 30:343–352

Tsukamoto L, Kuki K, Tojo S (1994) Mating tactics and constraints in the gregarious insect *Parastrachia japonensis* (Hemiptera: Cydnidae). Ann Entomol Soc Am 87:962–971

Waage JK (1979) Dual function of the damselfly penis: sperm removal and transfer. Science 203:916–918

Waage JK (1984) Sperm competition and the evolution of odonate mating systems. In: Smith RL (ed) Sperm competition and the evolution of animal mating systems. Academic Press, London, pp 251–290

Yamamura N (1986) An evolutionary stable strategy (ESS) model of postcopulatory guarding in insects. Theor Popul Biol 29:438–455

Žďárek J (1970) Mating behaviour in the bug, *Pyrrhocoris apterus* L. (Heteroptera): ontogeny and its environmental control. Behaviour 37:253–268