Sperm competition in the scorpionfly *Panorpa communis* (Mecoptera, Insecta)

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Sperm competition

Darwin himself extended his theory of natural selection (1859) by the concept of sexual selection (1871) as elaborated male traits seemed to be contradictory to natural selection. Therefore, selection is not only non-random differential survival but also non-random differential reproduction. Sexual selection can arise in two ways. First, intersexual selection favours preferences for particular traits in the other sex as, for example female preference for elaborated tails in male peacocks. And second, intrasexual selection comprises competition between members of one sex for access to mates or the resources those mates require. This latter sort of selection can lead to obvious traits like the antlers of male deer for fighting or the enlarged mandibles of male stag beetles. But, traits favoured by intrasexual selection can also be very cryptic if competition between males arises on the level of sperm. In fact, intrasexual selection is the fundamental process on which theory of sperm competition is based on.

Sperm competition is defined as competition within a single female between the sperm from two or more males for the fertilisation of the ova (Parker 1970). The occurrence of sperm competition has been noticed for a wide range of animals (for review see: Birkhead & Møller 1998, Simmons 2001) for which the main question was which of the several males a female mated with sired the offspring. There are several hypotheses which mechanisms control sperm competition (e.g. Parker 1990, Parker et al. 1990, Ball & Parker 1997, Harvey & Parker 2000). Two of the main types of sperm competition currently known are sperm mixing in which sperm of different males is mixed inside a sperm storage organ and sperm displacement with one male actively removing sperm of a former male.

Hypotheses about various sperm competition mechanisms can be made from the knowledge of anatomy of the male and female reproductive tract and the mechanism of sperm transfer (Simmons 1987, Dickinson 1986). Assuming the knowledge of sperm transfer mechanisms, Parker et al. (1990) proposed simple mathematical models for testing these hypotheses of sperm competition. If there is complete mixing of sperm derived from different males in the female's storage organ, this can result in each sperm from

each male having equal chances to fertilise an egg. This scenario has been termed a ‘fair raffle’ (Parker et al. 1990). Thereby, winning paternity resembles a true raffle or lottery with single sperm being the ‘tickets’ for fertilisation (Parker 1981). But, males transferring sperm of greater competitive ability may be favoured by sperm competition. Consequently, chances for gaining fertilisation are shifted towards a special male even though the same amount of sperm is transferred. This is what Parker et al. (1990) called a ‘loaded raffle’. In contrast, the ‘sperm displacement model’ describes the possibility that sperm of a former male is displaced by the next male. Thereby, sperm may be mixed instantaneously during displacement or no mixing takes place until displacement is complete. Result is, however, the last male siring all or the majority of the offspring. Essentially is that there are several different mechanisms like, for example, incomplete mixing of ejaculates (Harvey & Parker 2000), age effects of females (Mack et al. 2003) or also sperm age (Reinhardt & Siva-Jothy 2005) which diverge from the simple models Parker et al. (1990) suggested.

Since there is a female biased investment into production of gametes (Bateman 1948) or more general into parental care (Trivers 1972, Andersson 1994) females are expected to be choosy. Males, however, are suggested to increase their reproductive success by mating with as many females as possible (Clutton-Brock & Vincent 1991). But, for males mating is not as cheap as assumed on the first glance. Males never utilise only one spermatozoon to fertilise one egg, they produce a seminal fluid containing up to thousands of spermatozoons. Therefore, the production of an ejaculate itself can be costly (Pitnick & Markow 1994) as well as the production of several ejaculates within a given time (Dewsbury 1982, Parker 1990). Consequently, males should invest their sperm strategically, in particular, if they face sperm competition. If the possibility increases that sperm has to compete with rival sperm (risk of sperm competition theory), also, ejaculate expenditure is expected to increase (Parker et al. 1997). Otherwise, if the intensity of sperm competition increases, namely the absolute number of males engaged in competition, males are expected to decrease ejaculate expenditure (Parker et al. 1996). Actually, there are strategies of males to avoid sperm competition like postcopulatory mate guarding (see for review: Simmons 2001), insertion of mating plugs (e.g. Parker 1970) or sperm removal (e.g. Gack & Peschke 1994, Simmons & Siva-Jothy 1998, Cordoba-Aguilar 1999).

For females, advantages of multiple mating and sperm storage are less obvious.

It is often stated that a single copulation is sufficient for obtaining enough sperm to ensure fertilisation of all eggs. While this may sometimes be true, there is evidence for many insects species that females run out of sperm if not allowed to remate (Ridley 1988). But, storing sperm of different males may also protect females from infertile sperm as it has been shown that infertility is a current factor in sperm competition (García-González 2004). Due to sperm degenerating inside the storage organ (Bernasconi et al. 2002, Snook & Hosken 2004) females may replenish their sperm reserve via multiple mating. In addition, stored sperm from several males enable cryptic female choice (e.g. Simmons 1987, Bussière et al. 2006) if females are not able to choose before copulation. In particular, this is relevant if females are able to influence the amount of sperm they receive and to detect, however, a male's quality during copulation.

In insects, high levels of sperm competition can be found because of the strong remating tendencies of females and, perhaps even more important, because females store and maintain sperm internally within specially adapted sperm storage organs. The considerable evolutionary diversity in mechanisms of sperm transfer, storage and utilisation in insects makes them unique models for exploring the evolutionary consequences of sperm competition. In particular, the comparison of mechanisms developed in related species might shed more light on the evolution of sperm competition mechanisms within a taxonomic group. In the thesis in hand, I investigated the mode of sperm competition in an insect model organism, the scorpionfly *Panorpa communis*.

Sperm competition in scorpionflies

The holarctic distributed *Panorpa*-group is well investigated with respect to the taxonomic structure in general (Misof et al. 2000, Pollmann & Sauer unpublished data) and the characteristics of life history and reproductive behaviour in the European species in particular (e.g. Sauer 1977, Sauer et al. 1997, Gerhards 1999, Aumann 2000, Engqvist & Sauer 2003, Kullmann & Sauer 2005). To my knowledge, males of all investigated species are polygynous, whereas females of most species are polyandric to different degrees and partly monandric. It is known that scorpionfly females have a sperm storage organ, the spermatheca, in which they can store sperm of several males until fertilisation (Kaltenbach 1978, Gack & Peschke 1994). Although, sperm competition occurs in all species of this group if females mate multiple, the exact mode of sperm

competition was detected by Sauer et al. in 1999 for *Panorpa vulgaris*. If females of *P. vulgaris* are paired to two different males the outcome of sperm competition is raffle based. Males gain fertilisations proportional to their contingent of sperm in the spermatheca. However, for the slightly polyandric species *Panorpa germanica* the mode of sperm competition differs from a fair raffle (Kock et al. 2006) as paternity is biased to the last male. Hence, it is of interest how sperm competition mechanisms generally work in scorpionflies, how these mechanisms evolve and which might be the primal one. Therefore, more detailed knowledge of sperm competition mechanism for different scorpionfly species is needed.

In my thesis, I investigated the mechanism of sperm competition in *P. communis*. This species is very frequent in central Europe and depending on the geographical dispersal of populations one or two generation per year are developed. All individuals I used for my experiments were F_1-offspring from wild caught adults of a population near Freiburg (i.Br., Germany) where two annual generations are developed.

Simulating natural conditions in the laboratory, *P. communis* females mate up to ten times (Aumann 2000). They allow a male to initiate copulation if it offers a nuptial gift like a salivary mass or, more uncommonly a piece of prey. Copulations without a gift are very rare and terminated by the female soon (Sauer et al. 1998, Sauer 2002, Aumann 2000). Mostly, females terminate copulations by heavy kicks with their hind pair of legs, if the male offers no more salivary masses. Consequently, as it is known that the number of transferred sperm increases constantly with increasing copulation duration (Aumann 2000) females thereby control sperm transfer.

In order to detect the mode of sperm competition, specific pairings between individuals with known genotype are reasonable for testing the paternity of the resulting offspring. A frequently used method for paternity tests is the application of microsatellite markers. Microsatellites are tandemly repeated DNA sequences of one to six bases that ideally consist of a single repeat motif which is not interrupted anywhere by a base that does not match the repeat pattern (Hancock 1999). So far analysed they have been detected within the genomes of every organism at very high frequencies (Hancock 1999). The most useful microsatellites are highly polymorphic in particular, if a relatively low number of loci are available and a large number of parents are unknown. Models predict that for population assignment studies an allelic diversity of six to ten alleles per loci is sufficient (Bernatchez &

Duchesene 2000). For parentage assignment with low numbers of potential parents and known genotype, less polymorphic markers may also be sufficient.

Since mostly microsatellites are species-specific I established new microsatellite markers for *P. communis* which are introduced in *Chapter I*.

In *Chapter II*, a first experiment for detecting the mode of sperm competition in *P. communis* is described. Females were paired with two different males and paternity of the offspring was determined. By the proportion of sired offspring and the estimation of transferred sperm of the two potential fathers, I was able to draw conclusions pointing out the existing mode of sperm competition.

Since *P. communis* females are polyandric the former experiment (*Chapter II*) using doubly mated females, does not reflect the natural competition situation. Therefore, the detected mechanism may not be the one actually occurring in nature. To exclude this effect, I discuss a continuative experiment in *Chapter III* with females mated to three different males, supporting the results from *Chapter II*.

Finally, in *Chapter IV*, with sperm transfer rate being equal in subsequent copulations of single males I tested whether varying numbers of transferred sperm might influence the varying paternity values. Again, females were mated doubly but for a standardised duration and paternity of the offspring was assigned. Additionally, after the first copulation males were paired to a new female which was dissected afterwards for sperm counting. On the background of standardised copulation duration, I draw conclusions regarding the number of sperm transferred in the first copulation.

The separate chapters of this thesis should be comprehensive as they are. Consequently, recurrent descriptions and explanations are occasionally inevitable. References to citations are given separately for each section

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Chapter III

The fair sperm raffle remains fair if females of the scorpionfly *Panorpa communis* mate with more than two males

The fair sperm raffle remains fair if females of the scorpionfly *Panorpa communis* mate with more than two males

Sperm competition is a widely discussed subject in insects. In nature females of numerous species mate multiply. Contrary, most laboratory studies regarding sperm competition use only doubly mated females. However, since sperm competition mechanism revealed in a two-male mating design might change if females are paired to more than two males, results of a two-male mating design might not reflect the natural situation.

For doubly mated females of the scorpionfly *Panorpa communis* I showed before that the outcome of sperm competition depends on the amount of sperm the males transferred and the fertilisation mode corresponds to a fair raffle. Since females of this species also mate multiply in nature, I extended the former experiment by pairing females with three males. Measuring the outcome of sperm competition verified the results of the two-male mating design showing that even if females mate with three males, the fertilisation mode corresponds to a fair raffle principle.

Introduction

In many insect species with internal fertilisation, females have a special organ to store sperm (for review see: Parker 1970, Simmons 2001). Not only the morphology of this spermatheca differs across various taxa, sperm can also be stored for different periods of time (for review see: Parker 1970, Walker 1980, Simmons 2001). In some species the spermatheca contains only sperm of a single male (e.g. Parker 1990, Eady 1994) whereas in other species sperm of several males can be stored until fertilisation (e.g. Eberhard 1996, Simmons 2001). In the latter case sperm competition occurs.

Sperm competition is defined as the competition between the sperm of different males for the fertilisation of the ova of one female (Parker 1970). The outcome of sperm competition varies widely across species (for review see: Simmons & Siva-Jothy 1998, Simmons 2001). Some of the influencing factors are mating shortly before copulation (Müller & Eggert 1989), the amount of sperm different males transfer (e.g. Parker 1990, Wedell & Cook 1998, Sauer et al. 1999, Kock et al. 2006), the ability of males to remove sperm of other males (e.g. Helversen & Helversen 1991, Gack & Peschke 1994, Cordoba-Aguilar 1999, Arnaud et al. 2001) or any kind of sperm stratification in the spermatheca (e.g. Lewis & Jutkiewicz 1998, Lewis et al. 2005). In addition, mating order implies a potential effect on the outcome of sperm competition with, occasionally, the last copulating male siring all or the majority of the offspring (for review see: Simmons & Siva-Jothy 1998, Simmons 2001). This last male sperm precedence has been regarded as the prior mechanism in sperm competition in insects for a long time (Parker 1970). But, a rising interest in this subject entailing an increasing number of studies dealing with sperm competition have shown, that sperm mixing is frequently occurring in insects (e.g. Parker 1990, Sakaluk & Eggert 1996, Sauer et al. 1999, Harvey & Parker 2000, Simmons 2001, Eggert et al. 2003). If sperm is mixed completely in the spermatheca and the quantity of gained fertilisations for one male is proportional to its contingent of sperm inside the spermatheca, this is often called a fair raffle or honest raffle (Parker et al.1990). If special males have advantages in competition irrespective the amount of sperm transferred or sperm is mixed incompletely this is called a loaded raffle (Parker et al. 1990).

In the laboratory, sperm competition studies are mostly arranged with doubly mated females, although, in many cases females mate several times in nature. This is a fundamental problem as it has been shown that sperm competition mechanisms revealed in a two male mating design can differ if females are paired with more males. For a pseudoscorpion Zeh & Zeh (1994) showed that strong last-male sperm precedence can change to sperm mixing if females mate with three instead of two males.

Also complete sperm mixing detected in doubly mated females may change to other strategies in a multiple mating design. Particularly, if the spermatheca is filled after one or two copulations as it has been shown for the yellow dung fly *Scatophaga stercoraria* after a single copulation (Parker et al. 1990) and in the red floor beetle

Tribolium castaneum after two copulations (Lewis & Jutkiewcz 1998). Generally, there are two possible strategies if assuming that a spermatheca is filled after two copulations and sperm received in these copulations is mixed completely. First, sperm of a third male may be unable to enter the storage organ and flow out. This would result in mixed paternity of the first and second male. Second, sperm of a third male displaces the mixed sperm of the former males which would result in high paternity for the third male and low fertilisation rate for the first two males. In both cases the results of a two-male experiment are not conferrable to a three-male design. Accordingly, it is crucial to test for the outcome of sperm competition in multiply mated females if it is known that they mate more than twice in nature.

In the study species, the scorpionfly *P. communis*, which is common in central Europe (Sauer 1970), females are polyandric; in semi-natural conditions they mate up to 10 times (Aumann 2000). Sperm of different males is stored in a kidney-shaped spermatheca and fertilisation takes place during egg desposition (Grell 1942; Kaltenbach 1978). Obviously, there is sperm competition in *P. communis* (Aumann 2000, *Chapter II*). Since it is known that copulation duration is strongly related to the number of transferred sperm (Aumann 2000) I estimated the number of transferred sperm by the duration of copulation. In a former experiment (*Chapter II*) I found that in females of *P. communis*, mated to two different males, sperm is mixed completely in the spermatheca and the outcome of competition represents a fair raffle. Since this may not reflect the natural situation where females mate multiple, in the present study I paired females with three different males and assigned offspring paternity via microsatellites to analyse the sperm mixing mode.

Material and Methods

Breeding of P. communis

Adults of *P. communis* used in this experiment were F_1-offspring of field caught individuals collected near Freiburg (i. Br.Germany) in spring of 2004 and reared following a protocol given in Sauer (1970, 1977) and Thornhill & Sauer (1992).

After hatching in summer of 2004, adults were reared separately in plastic tubes (∅: 3.5 cm, height: 8 cm) in an environmental chamber with light-dark cycle of 18h : 6h at 20°C at light and 18°C at dark, respectively. All individuals were well nourished by feeding them one segment of last-instar mealworm (*Tenebrio molitor*) every third day. Adequate supplemental with water was assured by moist tissue paper at the ground of the plastic tubes. Sexual

maturity was reached approximately after 10 days. Only pubescent individuals were used for the experiment.

Experimental methods

Females were mated to three different males on consecutive days in a plastic box (10 cm × 10 cm × 7 cm) which was covered with moist tissue paper on the ground. All individuals were controlled for being no siblings. Pairs were observed for copulation duration as a measurement for the amount of transferred sperm, as copulation duration correlates well with number of transferred sperm (Aumann 2000). Every male was paired only once and was transferred to 100% alcohol after copulation for subsequent DNA isolation. Between the trails females were put back into their plastic tubes.

Females paired three times were transferred to a new plastic box (10 cm × 10 cm × 7 cm) containing moist tissue paper and a Petri dish (∅ 5.5 cm) filled with peat for egg deposition. The boxes were controlled for eggs every day. For each female, egg clutches were removed carefully and transferred to a Petri dish (∅ 5.5 cm) containing moist tissue paper. Clutches were controlled every day for hatched larvae. Larvae of one clutch were transferred to a small Eppendorf tube filled with 100% alcohol for subsequent DNA isolation. Females were allowed to lay eggs for 20 days. Afterwards they were also transferred to 100% alcohol individually. If females died before this time limit they were conserved earlier.

Paternity detection & statistics

Genomic DNA was extracted by using a 10% solution of Chelex®- 100 sodium forum in sterile water. 500 µl Chelex was added to a tube containing a piece of leg-musculature of an adult or the whole larva, respectively and incubated 15 min at 95°C. After vortexing 15 sec the specimens were spun down and stored at -18°C. The five microsatellites used for detection of paternity as well as conditions for PCR amplification can be found in *Chapter I*. PCR fragments were applied on a polyacrylamidgel, resolved with an ABI 377 DNA-Sequencer and scored with ABIPrism GeneScan analysis software.

Parentage analysis was arranged with CERVUS 2.0 (Marshall et al. 1998). Statistical analysis were arranged with SPSS 12.0. If possible, parametic tests were used but since in some cases data were not normally distributed and transformation failed, nonparametric tests were applied.

In the following sections, the abbreviation P_2 representing the proportion of offspring sired by the second male

(Boorman & Parker 1976) and hence P_1 and P_3 for the proportion of offspring sired by the first male and the third male, respectively, will be used.

Results

Each of the 20 females used in the experiment produced between two and five clutches. All females produced a first and a second clutch, whereas only nine of them produced a third one, three a fourth one and two a fifth one. For each clutch 17 larvae were included in the parenthood analysis or all if less than 17 larvae hatched.

Including all clutches and larvae, I first tested with a one sample t-test whether P_1, P_2 or P_3 differed significantly from 0.33, representing equal paternity for each of the males. Mean P_1 did not differ significantly from 0.33 (t-test: t_{19}=0.71, p=0.48; Figure 1) nor did P_2 (t-test: t_{19}=-0.18, p=0.90; Figure 1) or P_3 (t-test: t_{19}=-0.58, p=0.57; Figure 1).

Then, I analysed the consecutive clutches of females separately. P_1 did not differ significantly from 0.33 in first, second and third clutches (first clutches: t-test, t_{19}=0.30, p=0.77, Figure 2a; second clutches: Mann-Whitney-Test, N=20, Z=-1.16, p=0.29, Figure 2b; third clutches: t-test, t_8= 0.80, p=0.45, Figure 2c). The same was true for P_2 (first clutches: Mann-Whitney-Test, N=20,

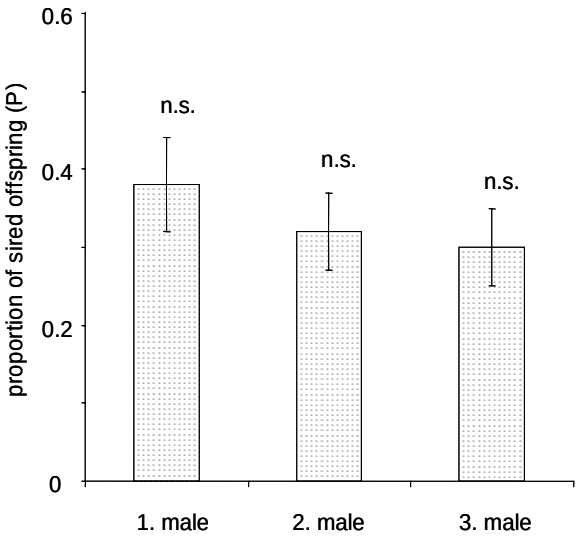

Figure 1 Comparison of the proportion of sired offspring of first (P_1), second (P_2) and third (P_3) males with 0.33, pooled data from all clutches (mean±S.E.: P_1=0.38 ±0.06, P_2=0.32±0.05, P_3=0.3 ±0.05)

Z=-1.16, p=0.29, Figure 2a; second clutches: t-test, t_{19}=0.01, p=0.99, Figure 2b; third clutches: t-test, t_8= -0.97, p=0.36, Figure 2c). Furthermore, P_3 did not differ significantly from 0.33 in second and third clutches (second clutches: t-test, t_{19}=-0.35, p=0.73, Figure 2b; third clutches: t-test, t_8= 0.04, p=0.97, Figure 2c) but it differed significantly from 0.33 in first clutches (Whitney-Test, N=20, Z=-2.63, p=0.01; Figure 2a). Separate analysis for fourth and fifth clutches was not possible because of the very small sample size.

I performed an ANCOVA with the particular proportion of sired offspring as dependent variable, in order to analyse the effect of the proportional copulation duration (continuous variable) and mating

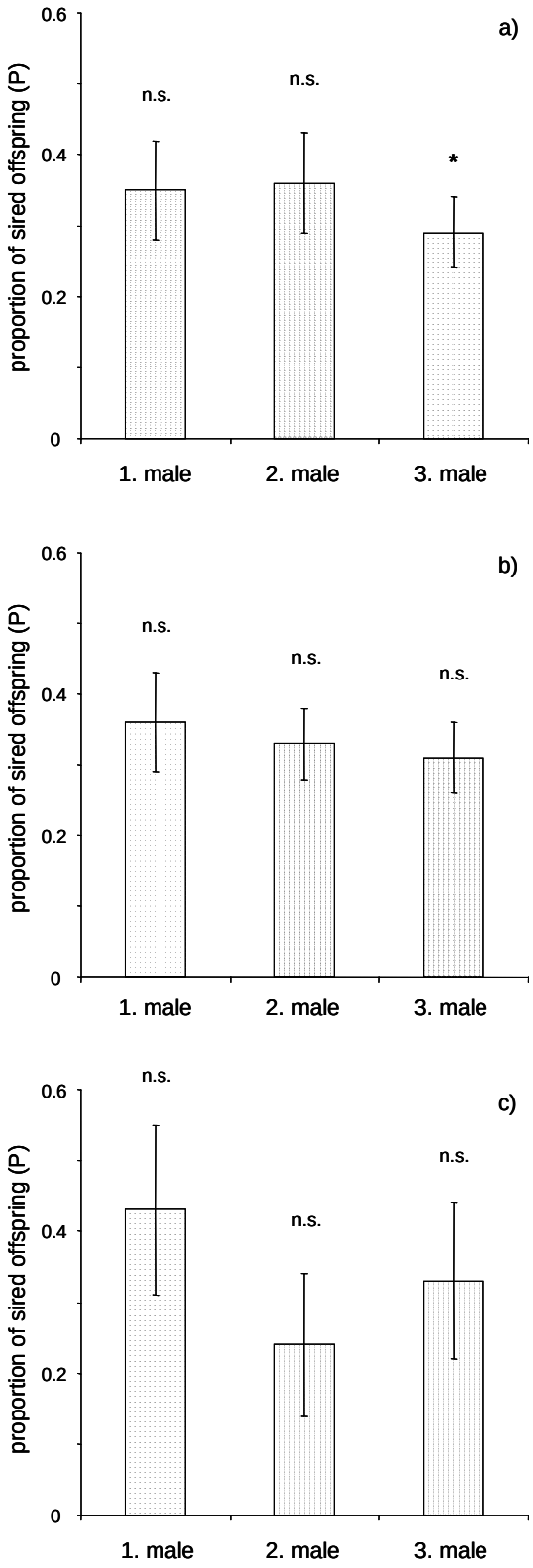

Figure 2 Comparison of the proportion of sired offspring of first (P_1), second (P_2) and third (P_3) males with 0.33 in a) first clutches (mean±S.E.: P_1=0.36 ±0.07, P_2=0.29±0.05, P_3=0.35 ±0.07), b) second clutches (mean±S.E.: P_1=0.36 ±0.07, P_2=0.33±0.05, P_3=0.31 ±0.05)and c) third clutches (mean±S.E.: P_1=0.43 ±0.12, P_2=0.24±0.09, P_3=0.33 ±0.11)

order of males (factor). As three males copulated with one female, neither the proportional copulation duration nor the proportion of sired offspring of a male is independent from those of the other males. Thus, each female is represented by data of only one male within the analysis. Females were randomly divided in three groups: the first group (N=6) contained data of the first males, the second group (N=7) data of second males, and the third group (N=7) contained only data of third males. Proportion of sired offspring was not effected by mating order (ANCOVA: $F_{2,16}$=0.36, p=0.706) whereas the proportional copulation duration showed a highly significant influence (ANCOVA: $F_{1,16}$=20.81, p<0.001). The regression lines of the three correlations did not differ significantly underlining that there is no effect of mating order on proportion of sired offspring (slope group1: b=1.12±0.26; slope group2: b=1.28±0.63; slope group3: b=0.79±0.36; ANCOVA $F_{2,14}$=0.365, p=0.7; Figure 3).

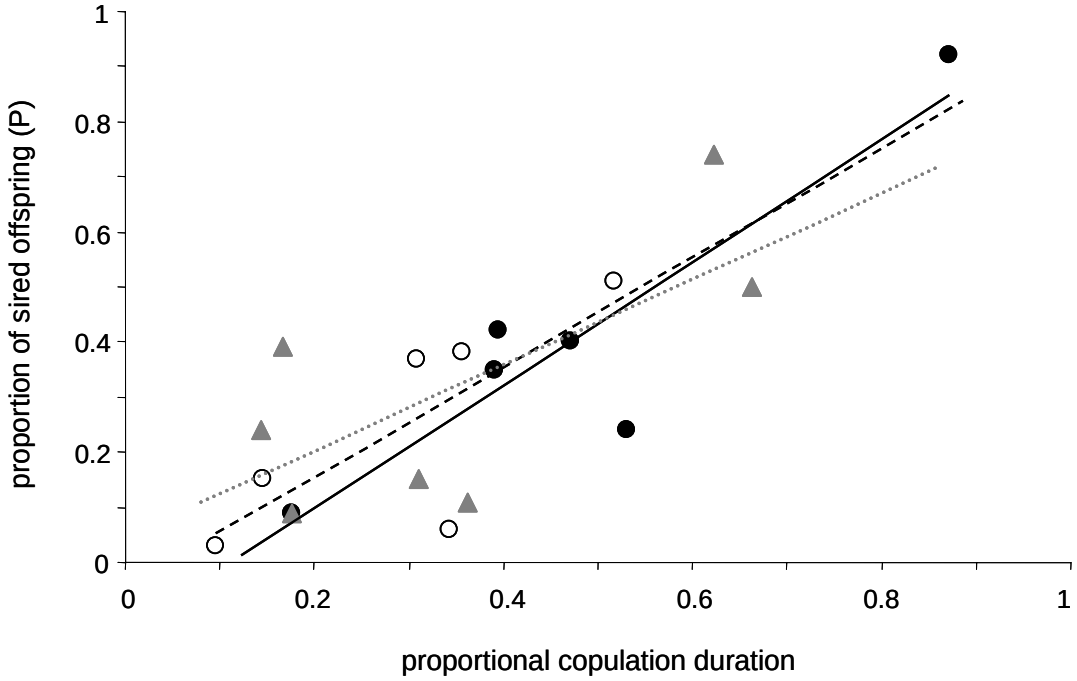

Figure 3 Correlations between the proportional copulation duration and the proportion of sired offspring for three different groups of females. Filled circles and solid lane: females where only first male is considered; empty circles with dashed line: females where only second male is considered; triangles with dotted line: females where only third males are considered

Discussion

In this study the outcome of sperm competition for triply mated females of *P. communis* was determined for the first time. I investigated, whether the proportion of sired offspring of first males (P_1), second males (P_2), and third males (P_3) differed from the hypothesised equal paternity. Assuming sperm stratification in the kidney-shaped spermatheca of female *P. communis*, sperm of the last male would lie nearest to the point of fertilisation (Grell 1942, Kaltenbach 1978). If so, I would expect the last male, in this study the third one, to sire all or the majority of the offspring. However, I could not find a significant difference from equal paternity for any of the males when I pooled the data of consecutive clutches. The usage of stored sperm could change with increasing interval between the last copulation and egg deposition as found in the red flour beetle *Tribolium castaneum*, for example (Lewis & Jutkiewicz 1998, Arnaud et al. 2001). In this species there is strong last male sperm precedence because of sperm stratification for offspring sired two days after the last copulation. Contrary, after one to two weeks sperm is mixed and paternity is randomly distributed in *T. castaneum*. Similar mechanisms could occur in *P. communis* but

this effect could be hidden in my study by using a combination of all clutches of respective females. Particularly, if considering that there are up to 20 days between the last copulation of the females and production of their last clutch. Consequently, I tested consecutive clutches separately for differences between P_1, P_2 and P_3 ascertaining equal paternity and could exclude any form of sperm stratification, accordingly.

Furthermore, I could exclude sperm precedence with a last male removing sperm of a former male out of the female's spermatheca for three possible reasons. First, there are no structures in the genital tract of the male known that allow the removal of sperm (Grell 1942, Kaltenbach 1978). Second, if a male displaces sperm of former males by its own sperm I would expect the factor 'mating order' to influence the proportion of sired offspring. Third, males should gain a higher fertilisation rate in comparison to former males irrespective of copulation duration as estimator for the number of transferred sperm. In fact this is the case. However, my analysis revealed the proportional paternity differing from equality for third males in first. But finally, there is a strong effect of 'copulation duration' on the proportion of sired offspring but no effect of 'mating order'. Therefore, I state that these results indicate the absence of last male sperm precedence. Anyway, a replacement of rival sperm is expected if a spermatheca is completely filled. I assume the spermatheca of *P. communis* not being filled after three copulations because females do mate more often in nature (Aumann 2000) and can contain very high numbers of sperm (unpublished data).

In my experiment, the amount of sperm a male contributes to the totality of sperm in the spermatheca of the female was crucial for the outcome of sperm competition. In particular, this is important because copulation duration of males varied largely in this study, actually between 22 and 443 minutes and accordingly the number of sperm the different males transferred varied largely. I pooled the two possible parameters influencing the outcome of sperm competition, 'mating order' and 'copulation duration' in one analysis, verifying that 'mating order' had no influence whereas 'copulation duration' had. The longer a male copulates in comparison with the two rival males, the higher is its percentage of gained fertilisations, irrespective of the male's mating position. This result underlines that the number of sperm a male is able to transfer to the female is the deciding factor in sperm competition in *P. communis*. This mechanism has been called a fair raffle in sperm competition by Parker (1982) and

Parker et al. (1990) who stated that a male's fertilisation success is proportionally to its contingent of sperm in the spermatheca of the female. Consequently, numerical superiority should be advantageous for males and therefore males should try copulating long enough to optimise fertilisation success. Actually, this is the fact in *P. communis*, where courting males offer a nuptial gift, the salivary mass, to the female (Aumann 2000). The more salivary masses a male offers the longer the female will copulate and the more sperm the male is able to transfer (Aumann 2000). Accordingly, the male increase its contribution of sperm in the spermatheca and therefore its portion of fertilisations. Although females seem not to be able to detect a males' quality before copulation, it has been shown for *P. vulgaris* that the salivary masses indicate the quality of a certain male (Thornhill & Sauer 1992; Sauer et al. 1998, Engels & Sauer 2006) and I presume the same for *P. communis*. Therefore, a fair raffle system constitutes a form of cryptic female choice (*Chapter II*).

Possibly different males transfer varying amount of sperm in the same time or the same male transfers varying amount if mating with different females (e.g. Engqvist & Sauer 2003). I do not suspect this to influence the results of this study because males and females were chosen randomly and differences in the amount of transferred sperm depending on the individuals should be balanced on average.

These findings are consistent with the results of a former study (*Chapter II*) that showed the outcome of sperm competition in *P. communis* follows the principle of a fair raffle in a two-male mating design. Yet, a sperm competition mechanism detected for a polyandric species, can be misleading if revealed in a two-male mating design and possibly change if females are paired with more than two males. This has been show for a pseudoscorpion by Zeh & Zeh (1994). Conversely, a complete sperm mixing may change if females mate with several males instead of two. In the actual study I excluded this possibility for *P. communis* because in the three-male mating design sperm competition follows the same principles as in the two-male mating design.

To conclude, in the present study, I proofed that in *P. communis* the mechanism of sperm competition revealed by a two-male mating design in a former study was retained if females are paired with three males. Also, in a three-male mating design the fertilisation mode is raffle-based.

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Chapter I

First set of microsatellite markers for the scorpionfly *Panorpa communis* L. (Mecoptera, Insecta)

First set of microsatellite markers for the scorpionfly *Panorpa communis* L. (Mecoptera, Insecta)

Microsatellites are nowadays routinely used as a basic and effective instrument in parentage studies. Here, five new microsatellite markers for *Panorpa communis* L. (Mecoptera, Insecta) are presented which were found to be polymorphic. Expected and observed heterozygosity and derivations from Hardy-Weinberg equilibrium have been calculated for a population near Freiburg (Germany). I additionally checked for compatibility with Mendelian inheritance and preformed a test for linkage disequilibrium between the loci. With these five microsatellites it was possible to reliably detect relatedness between individuals and particularly parenthood of individuals.

Panorpa communis (Mecoptera, Insecta) is one of five scorpionfly species in central Europe. Its well-investigated life history (Sauer & Hensle 1977, Sauer 1986, Sauer et al. 2003) makes it a suitable model organism in evolutionary biology. Females are polyandric and sperm of different males occur simultaneously in the spermatheca resulting in sperm competition. This reproductive behaviour is known for several other scorpionfly species (e.g. Sauer et al. 1998, Sauer et al. 1999, Kock et al. 2006). In contrast to the closely related species *Panorpa vulgaris* and *Panorpa germanica* (Misof et al. 2000, Pollmann & Sauer unpublished data), however, there is nothing known about the mode of sperm competition in *P. communis*. In the highly polyandric species *P. vulgaris* the sperm mixes completely (Sauer et al. 1998, Sauer et al. 1999) whereas in the lightly polyandric *P. germanica* mixing is incomplete (Kock et al. 2006). Particularly, for the understanding of the evolution of mating behaviour in scorpionflies and the evolution of mating tactics in general it is of

Table 1 Characteristics of five microsatellites for *Panorpa communis*. Shown for each locus: sequences of both primers, repeat motif, allele size range (bp), number of different alleles, observed heterozyhosity *(H_O)*, expected heterozygosity (H_E), optimal annealing temperature in °C (T_A) and number of individuals.

locus	primer sequences	repeat motif	allele size range (bp)	number of alleles	H_O	H_E	T_A	number of individuals
Pcm 2	F: (FAM) 5'-tagaacaattctgcgcagc R: 5'-tcattctgacggagctacg	$(AC)_{12}$	100 - 110	4	0.450	0.468	51	87
Pcm 3	F: (HEX) 5'-acaaagtacactgttcacgctg R: 5'-gtacgagtatgtaccaatgcacc	$(AC)_{11.5}$	194 – 204	5	0.410	0.425	57	69
Pcm 8	F: (HEX) 5'-gaacagatccagcacgag R: 5'-atgcatctgcagaagcag	$(AC)_{10}$	116 – 134	8	0.500	0.534	57	76
Pcm 10	F: (FAM) 5'-ccccaatcatttcacccgtat R: 5'-tttggtcatgttccctcag	$(AC)_{14}$	126 – 134	3	0.560	0.522	57	88
Pcm 15	F (FAM) 5'-agaacgcatggaagaggtg R: 5'-tcatcttcaagaaaagacatagg	$(CT)_9$	148 – 166	6	0.390	0.414	60	84

high interest to compare closely related taxa in respect to their mode of sperm competition. To clarify the outcome of sperm competition it is necessary to estimate paternity of the offspring of a given female. A frequently used technique for such kinship studies is the application of microsatellites. So far, however, no polymorphic microsatellites were available for *P. communis*. Here I present a first set of five microsatellite markers with which the detection of parenthood in the laboratory was able in *P. communis*. This is a necessary precondition for the further investigation of sperm competition modes in this species which will help to shed more light on the evolution of different mating systems in the *Panorpa* group.

Genomic DNA was extracted from adults by using a 10% solution of Chelex®-100 sodium-forum in sterile water. 500 µl Chelex were added to a tube containing a piece of leg-musculature and incubated 15 min at 95°C. After vortexing for 15 sec the samples were spun down and stored at -18°C until further analysis. For library construction a protocol presented by Nolte et al. (2005) was applied. This is the first time this method was applied to an insect and it appeared to be successful. The positive clones obtained in the cloning were sequenced without further screening and the fragments were examined for whether they carried microsatellites. 14 microsatellite loci were received for which primers were designed using FASTPCR (http://www.biocentre.helsinki.fi/bi/bare-1_html/oligos.htm). The optimal annealing temperature was determined on a temperature gradient thermocycler. In a prescreening five loci were selected which were found to be polymorphic. The PCR amplification for these five loci was carried out in a total volume of 10 µl using 0.5 µl DNA for the

loci Pcm2, Pcm8, Pcm10 and Pcm15 and 2 µl DNA for the locus Pcm3, respectively. The PCR mix contained 1× reaction buffer (peqlab), 200 µM dNTP mix, 10 pmol/ µl of each forward (labeled; Table 1) and reverse primer, 1× EnhancerSolP (peqlab) and 0.25U Taq polymerase. Thermal cycling started with 94°C for 5 min followed by 32 cycles for 30 sec at 94°C, 1 min at the optimal annealing temperature (Table1) and 1 min at 72°C. The process was finished by final extension period for 5 min at 72°C. The PCR products were applied on a polyacrylamidgel, resolved with an ABI 377 DNA-Sequencer and scored with ABIPrism GeneScan analysis software.

Number of alleles, observed and expected heterozcygosity (Table1) as well as derivation from Hardy-Weinberg equilibrium were determined and a control for linkage disequilibrium was calculated with GENEPOP (http://wbiomed.curtin.edu.au/genepop/). Individuals used for microsatellite characterisation (Table 1) belonged to the same population located near Freiburg (Germany) and were caught in spring 2003 and spring and summer 2004. There was no linkage disequilibrium, and for none of the five loci a deviation from Hardy-Weinberg equilibrium was detected. Locus Pcm8 showed a heterozygote deficit which implies the occurrence of null alleles. Nevertheless this locus was retained in the analysis as Mendelian inheritance was demonstrated. To test for segregation according to the Mendelian hypothesis full-sib progeny was achieved from laboratory pairings of males and females hatched in the laboratory in summer 2005. All observed progeny ratios of each primer pair were tested against the expected Mendelian segregation ratios using g-squared test (Table 2). All loci in all families conformed to the Mendelian expectations except Pcm8 in family 4. Since in the other five families Pcm8 conformed to the Mendelian expectations I retained this marker for my analysis.

The presented microsatellite loci are not as highly polymorphic as desirable in general nor are there especially many of them. Nevertheless this is the very first set of microsatellite markers available for *P. communis*. Incidentally even using the simplified method to find microsatellites proposed by Nolte et al. (2005) this procedure is still time-consuming. Hence, it is expedient to focus on a limited number of loci which will conform to special questions. However, in combination the five loci presented here enabled me to estimate parenthood very exactly in the laboratory. Given this requirement it is now possible to arrange experiments to investigate the mechanism of sperm competition in this

Table 2 Segregation of loci in *Panorpa communis* larvae from six full-sib families

familiy	locus	maternal genotype	paternal genotype	observed number of offspring[1] in each genotype class				N_I	G	*p*
4	Pcm2	102/104	102/102	102/102	102/104			87	0.47	0.49
				48 (43.5)	39 (43.5)					
	Pcm3	198/198	194/198	194/198	198/198			89	0.05	0.82
				43 (44.5)	46 (44.5)					
	Pcm8	116/126	126/126	116/126	126/126			89	4.23	0.04*
				31 (44.5)	58 (44.5)					
	Pcm10	126/126	126/126	126/126				89	-	-
				89 (89)						
	Pcm15	148/152	162/164	148/162	148/164	152/162	152/164	89	2.49	0.48
				30 (22.25)	19 (22.25)	16 (22.25)	24 (22.25)			
16	Pcm2	102/102	102/104	102/102	102/104			113	0.36	0.55
				61 (56.5)	52 (56.5)					
	Pcm3	196/198	196/198	196/196	196/198	198/198		115	0.35	0.84
				31 (28.75)	53 (57.5)	31 (28.75)				
	Pcm8	126/126	116/134	116/126	126/134			114	0.63	0.43
				63 (57)	51 (57)					
	Pcm10	126/128	126/128	126/126	126/128	128/128		117	1.2	0.55
				29 (29.25)	52 (58.5)	36 (29.25)				
	Pcm15	152/162	148/162	148/152	152/162	148/162	162/162	117	0.15	0.99
				30 (29.25)	27 (29.25)	31 (29.25)	29 (29.25)			
17	Pcm2	104/104	102/104	102/104	104/104			91	3	0.08
				34 (45.5)	57 (45.5)					
	Pcm3	198/198	194/198	194/198	198/198			92	0.17	0.66
				43 (46)	49 (46)					
	Pcm8	134/134	116/134	134/134	116/134			88	1.47	0.23
				52 (44)	36 (44)					
	Pcm10	128/134	126/128	126/128	128/128	126/134	128/134	94	3.18	0.37
				17 (23.5)	29 (23.5)	30 (23.5)	18 (23.5)			
	Pcm15	162/162	148/162	148/162	162/162			93	0.005	0.94
				46 (46.5)	47 (46.5)					
26	Pcm2	102/104	102/104	102/102	102/104	104/104		82	1.94	0.38
				15 (20.5)	49 (41)	18 (20.5)				
	Pcm3	-	194/198							
	Pcm8	-	126/134							
	Pcm10	126/128	126/126	126/126	128/126			83	0.3	0.59
				45 (41.5)	38 (41.5)					
	Pcm15	148/162	162/164	148/162	148/164	162/162	162/164	82	3	0.39
				25 (20.5)	24 (20.5)	12 (20.5)	21 (20.5)			
30	Pcm2	104/104	102/102	102/104				103	-	-
				103 (103)						
	Pcm3	194/196	198/198	194/198	196/198			99	0.13	0.72
				52 (49.5)	47 (49.5)					
	Pcm8	116/134	126/126	116/126	134/126			100	0.18	0.67
				53 (50)	47 (50)					
	Pcm10	126/128	126/128	126/126	126/128	128/128		97	1.76	0.42
				30 (24.25)	40 (48.5)	27 (24.25)				
	Pcm15	148/162	162/162	148/162	162/162			101	1.13	0.29
				43 (50.5)	58 (50.5)					
40	Pcm2	102/110	102/102	102/102	102/110			128	0	1
				64 (64)	64 (64)					
	Pcm3	196/198	196/198	196/196	196/198	198/198		136	0.19	0.91
				37 (34)	65 (68)	34 (34)				
	Pcm8	126/134	126/134	126/126	126/134	134/134		122	0.34	0.89
				34 (30.5)	59 (61)	29 (30.5)				
	Pcm10	126/128	126/126	126/126	126/128			125	0.49	0.49
				57 (62.5)	68 (62.5)					
	Pcm15	162/162	148/152	148/162	152/162			134	0.73	0.39
				60 (67)	74 (67)					

[1] value in parentheses is the expected number according to Mendelian expectation, * Significant ($p<0.05$), N_I: number of larvae

species to further the knowledge on the evolution of different mating systems in a closely related species.

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Chapter II

The invisible fight: sperm competition and cryptic female choice in a scorpionfly

The invisible fight: sperm competition and cryptic female choice in a scorpionfly

An intensively discussed subject in evolutionary biology is the question why females mate multiply. Whereas the advantage for males to mate more than once seems obvious, there are various reasons for females. Females may, for example, gain genetic benefits and replace sperm of a previous male with sperm of a genetically superior male. Females of the polyandric species *Panorpa communis* store sperm inside the reproductive tract until fertilisation takes place. In this study, the progeny of double mated females were tested for paternity using five microsatellite markers. Males which copulated longer and, consequently, transferred more sperm sired proportionately more of the progeny. My data were in agreement with a fair raffle model of sperm competition. In this way, females of *P. communis*, which are unable to detect a males' quality before copulation, can influence the paternity of their offspring by controlling copulation duration. Thereby, they may adopt a form of cryptic female choice.

Introduction

In most species, females choose among potential partners, whereas males compete among each other for the access to females (Andersson 1994). While in these cases males usually invest more in precopulatory competition, females invest more in parental care (Trivers 1972, Andersson 1994). Production of eggs is costly (Bateman 1948) but production of sperm can be costly as well (Dewsbury 1982, Olsson et al. 1997). Additionally, in many species, females mate more than once during a reproductive cycle which can signify enormous costs for the male because it has to face sperm competition.

It was Parker (1970) who amplified Darwin's (1871) concept of sexual selection on the gametic level by defining sperm competition as 'the competition within a

single female between the sperm of two or more males' which obviously is a form of postcopulatory sexual selection. In different species sperm competition can have very different occurrences. For some species, it has been suggested, that the observed predominance of the last male in sperm competition can be explained by the structure of the spermatheca, because the sperm of the last male should lie closest to the point of fertilisation (Parker 1970). A predominance of a last or second male, respectively, can also be explained by sperm displacement where a last male removes sperm of previous males out of the spermatheca before transferring the own sperm. This has been shown for several Odonata (e.g. Waage 1979, Cordoba-Aguilar 1999, Siva-Jothy & Hooper 1995, Siva-Jothy & Tsubaki 1989), some Coleoptera (e.g. Gack & Peschke 1994) and some Orthoptera (e.g. Simmons & Siva-Jothy 1998), for example.

Furthermore, sperm of different males can be mixed inside the spermatheca with the fertilisation mode in sperm competition assumed to be raffle-based, mostly (Parker 1982). Parker (1970) proposed that this raffle can be a fair raffle at which the male fertilisation success is proportional to the contingent of its sperm within spermatheca. Alternatively, the raffle may be loaded. In this case, the sperm of special males have an advantage in competition. There is evidence for both, the basic model (e.g. Parker 1990, Parker et al. 1990, Sakaluk & Eggert 1996, Eggert et al. 2003) and also for supplementary proposals like 'sloppy' sperm mixing where sperm is transferred in packages (Harvey & Parker 2000).

To better understand the evolution of mating behaviour and sexual selection, knowledge of different sperm competition mechanisms in various species is necessary. Especially in insects, sperm competition is quite common and a popular subject of research (Parker 1970, Simmons & Siva-Jothy 1998, Simmons 2001).

Scorpionflies are an excellent organism for studying sperm competition. In many species females are polyandric (Sauer et al. 1998, Sauer et al. 1999, Aumann 2000, Engqvist & Sauer 2003) and store sperm in a kidney-shaped spermatheca until fertilisation which occurs during egg disposition (Grell 1942, Kaltenbach 1978). Hitherto, the mode of sperm competition is known for two European species. In *Panorpa vulgaris* females are highly polyandric (Sauer et al. 1998) and sperm competition follows the fair raffle principle (Sauer et al. 1999). Contrary, in *Panorpa germanica* sperm mixing is incomplete and paternity is biased to the last

male (Kock et al. 2006). To access deeper insight into the evolution of sperm competition in the Panorpidae I chose a third species to detect the mode of sperm competition. The selected species is *Panorpa communis* which is closely related to *P. vulgaris* (Misof et al. 2000, Pollmann & Sauer unpublished data). Also, females of *P. communis* are polyandric and mate with seven to ten males (Aumann 2000).

In mating experiments females were paired with two different males. Paternity of the offspring was assigned with five newly developed microsatellites (*Chapter I*). On the basis of these data, I was able to draw conclusions about the mechanism of sperm competition for *P. communis*.

Materials and methods

Breeding of P. communis

Individuals of *P. communis* used in this experiment were F_1-offspring of field caught adults, caught in summer of 2003 in Freiburg (i. Br.). Breeding was accomplished following a protocol given in Sauer (1970, 1977) and Thornhill & Sauer (1992). After diapause, adults hatched in spring 2004.

Individuals were reared separately in plastic tubes (∅: 3.5 cm, height: 8 cm) and fed every third day with one segment of last-instar mealworm (*Tenebrio molitor*), simulating high food availability. Only adults that were at least 10 days old were used for the experiment to secure sexual maturity. The breeding as well as the experiment were performed in an environmental chamber with a 18 h light : 6 h dark photoperiod at 20°C at light respectively 18°C at dark.

Experimental methods

For the experiment, one male and one female were put into a plastic box (10 cm × 10 cm × 7 cm) containing moist tissue paper. Pairs were chosen randomly but mating with siblings was avoided. The duration of the copulations was determined. After copulation, males were frozen at -80°C for later DNA isolation. Singly mated females were allowed to remate the following day. The male for a females' second copulation was also chosen randomly. However, siblings to either the female or the female's first male were avoided. Again, the copulation duration was determined. Similarly, the second male was frozen at -80°C after copulation. If a female did not copulate, mating trials were repeated. Females with more than two days between pairings where excluded from the analysis.

Doubly mated females were transferred to a new plastic box (10 cm × 10 cm × 7 cm)

containing moist tissue paper and a Petri dish (∅ 5.5 cm) filled with peat for egg deposition. Every day the boxes were controlled for clutches. Egg clutches were transferred carefully into Petri dishes (∅ 5.5 cm) containing moist tissue paper (one clutch per dish). After hatching the larvae were transferred to small plastic tubes filled with 100% ethanol (larvae of one clutch together in a single tube). Females were allowed to produce eggs up to three weeks after their second copulation before they were also frozen at -80°C. If a female died before this time limit, it was frozen earlier. Females produced one to three clutches.

Laboratory methods

Genomic DNA was extracted using a 10% solution of Chelex®- 100 sodium-forum in sterile water. 500 µl Chelex were added to a tube containing a piece of leg-musculature of an adult or the whole larva, respectively, and incubated 15 min at 95°C. After vortexing 15 sec the specimens were spun down and stored at -18°C.

All mothers, potential fathers, and 17 larvae per clutch were genotyped using five species specific microsatellites markers. PCR conditions as well as the five microsatellites used for detection of paternity are given in *Chapter I*. PCR fragments were applied on a polyacrylamidgel, resolved with an ABI 377 DNA-Sequencer and scored with ABIPrism GeneScan analysis software.

Statistics

Parentage analysis was performed with CERVUS 2.0 (Marshall et al. 1998). Statistical analysis was performed with SPSS 12.0. Parametic tests were used if data were normally distributed or discrepancies from normal distribution were small.

Results

As common in literature I use the abbreviation P_2 for the proportion of offspring sired by the second male and P_1 for the first male, respectively. I wanted to test for the proportional paternity differing between first and second males in order to control for mating order effects in sperm competition. As P_1 and P_2 are not independent from each other, I preformed a

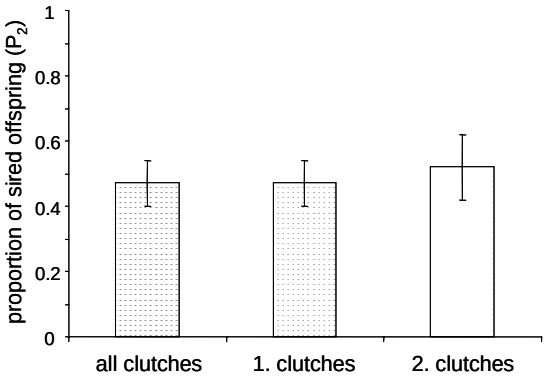

Figure 1 P_2 of all clutches, first and second clutches compared with 0.5; all clutches: mean±S.E.=0.48±0.07, first clutches: mean ±S.E.=0.47±0.07, second clutches: mean±S.E.=0.52±0.1

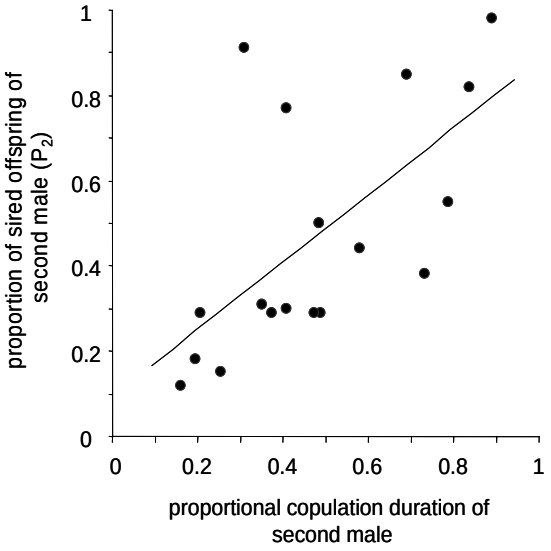

Figure 2 Correlation between proportional copulation duration and proportion of sired offspring of second male (P_2)

one-sample test to the P_2-value of merged data from all clutches and, separately, to those of first and second clutches. Because the proportional paternity for both males is expected to be 50% if there is no effect of mating order, I tested against the value 0.5. P_2 did not differ significantly from 0.5 for merged data of all clutches (t-test: N=18, t=-0.49, p=0.63; Figure 1). The same was true if considering only data from first clutches (t-test: N=18, t=-0.50, p=0.63; Figure 1) and second clutches (t-test: N=10, t=-0.15, p=0.88; Figure 1). The number of females within the separate analyses varied because only 10 females of the original sample size of 18 produced a second and only 5 a third clutch. Accordingly, a separate analysis for the third clutch was inapplicable.

There was a positive correlation between P_2 and the proportional copulation duration of the second male (pooled data from all clutches: N=18, r_s=0.688, p=0.002; Figure 2).

The data were fitted to the fair raffle model proposed by Parker (1990). The linear form of the model is given by the equation $1/P_2 = (S_1/S_2) + 1$ in which, in the original model, S_1 and S_2 is the number of sperm transferred by the first or the second male, respectively. In my study, I used copulation duration as a measurement for the number of transferred sperm (Aumann 2000, *Chapter IV*). For a fair raffle, two requirements have to be fulfilled within the model: the correlation $1/P_2$ against S_1/S_2 has to be significant and the slope as well as the intercept of the regression line has to be +1.

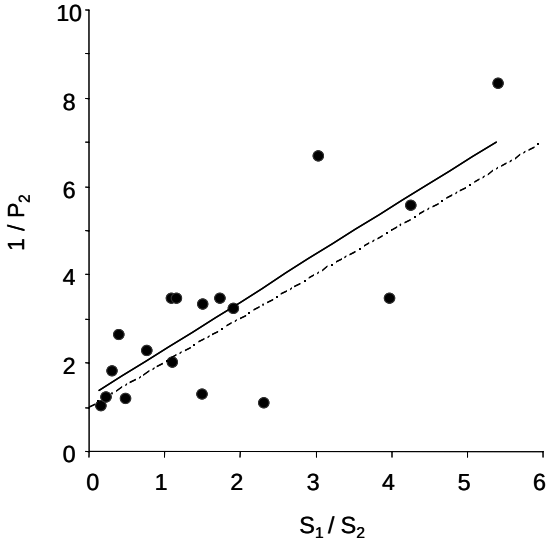

Figure 3 Pooled data from all clutches fitting to Parkers fair raffle model. Regression line is presented as solid lane, relationship predicted from the model is presented as broken line.

My data confirmed both of the predictions from this model: first, the correlation $1/P_2$ against S_1/S_2 was highly significant (N=18; r_s=0.688, p=0.002; Figure 3). And second, the intercept was +1 (intercept = 1.241±0.443; t_{16}=2.801; p=0.604; Figure 3) as well as the slope (slope: b=1.073 ± 0.196; t_{16}=5.477; p=0.715; Figure 3).

Discussion

Up to now the mechanism of sperm competition is known only for two scorpionfly species. In *P. vulgaris* sperm competition is based on a fair raffle (Sauer et al. 1999) whereas in *P. germanica* mixing of sperm is incomplete and shifted to the last male (Kock et al. 2006). In this study on the European scorpionfly *P. communis*, I was able to show that fertilisation success does not depend on mating order. In a two male mating design with varying copulation duration, the proportion of offspring of male two (P_2) did not differ from equal paternity for all males, irrespective of analysing consecutive clutches separately or altogether. Accordingly, I suggest random sperm mixing with proportional fertilisation due to the number of transferred sperm. Actually, my data fitted very well to the fair raffle model proposed by Parker et al. (1990).

Females of *P. communis* store sperm in a kidney-shaped spermatheca (Grell 1942, Kaltenbach 1978), which contains sperm of all males a female mated with. Females can mate several times before laying eggs and fertilisation takes place during oviposition. Thus, the question arises if sperm of different males is mixed in some way or stratified and how this is reflected in the distribution of paternity. In my analysis, P_2 did not differ from 0.5 in any case. This indicates that sperm of all males is mixed and paternity is distributed evenly between the two males. However, sperm of the first male may die inside the spermatheca due to storage duration, whereas sperm of the second male is still viable and might fertilise the majority of eggs in later clutches (Tsubaki & Yamagishi 1991). This may result in misleading conclusions concerning the mode of sperm competition. To rule out this problem and to secure freshness of both males' sperm, only females in which the second copulation followed one or at most two days after the first one were included in the analysis.

The outcome of sperm competition may be different if looking at P_2 within subsequent clutches, separately. In first clutches, P_2 may be higher than in subsequent clutches as a result of sperm stratification, which can lead to a

preferential use of last male sperm via a ‘last-in-first-out’-mechanism (Parker 1970, Birkhead & Hunter 1990). This can imply the usage of first male’s sperm in later clutches because of sperm depletion of second male’s sperm.

Accordingly, pooling the data of all offspring can also mislead to the conclusion of sperm mixing. Therefore, I performed the same analyses for consecutive clutches separately. Treating first and second clutches separately, P_2 also did not differ from 0.5. As a result, we can refuse the possibility of sperm stratification and exclude any form of last or first male sperm precedence for a two male mating scenario.

I found that the longer a male copulated, the higher was the proportion of offspring sired. In a former study (Aumann 2000), it has been shown that there is a strong correlation between the duration of copulation and the number of sperm transferred during the copulation. In addition, sperm transfer rate does not vary within copulation. Accordingly, I used copulation duration as an estimate for the amount of transferred sperm.

In my analysis, there was no effect of mating order on paternity, whereas P_2 depended on the proportional amount of sperm transferred by second male. Hence, I concluded that sperm is mixed inside the spermatheca of the female and, furthermore, that sperm was used in proportion to their numerical representation. Parker (1982, 1984) termed this the “raffle principle” and Parker et al. (1990) proposed a simple mathematical model to test biological data for this model. The application of Parker’s model to my data confirms that a fair raffle might also be the mechanism in *P. communis*. However, I did not test the “loaded raffle” model and the sperm displacement model (Parker et al.1990) because there are variables needed which were not detectable from the available data. Nevertheless, there are no hints for the displacement of sperm in *P. communis*.

As far as known females of *P. communis* are not able to detect a male’s quality before copulation (Aumann 2000) meaning that there might be no female choice before copulation. Yet, there is strong indication that females can detect a male’s quality during copulation. As in several scorpionfly species (Sauer et al. 1998, Gerhards 1999, Engqvist & Sauer 2001) *P. communis* males offer a nuptial gift before copulation, usually they secrete a salivary mass (Aumann 2000). As soon as the female starts feeding on this salivary mass, it allows the male to initiate copulation. During copulation the male has to provide further salivary masses otherwise the female terminates copulation by heavy

kicking with its hind leg pair (Aumann 2000, Sauer 2002). It has been shown for another scorpionfly species, *P. vulgaris*, that the salivary masses are a reliable indicator of a male's quality (Thornhill & Sauer 1992, Sauer et al. 1998, Engels & Sauer 2006) because producing salivary masses is costly for males (Sauer et al. 1998, Sauer 2002, Engqvist & Sauer 2001, Engels & Sauer 2006). The same can be assumed for *P. communis* since males of this species, raised under limited food supply, are able to produce only few or even no salivary masses and gain shorter copulation durations than well nourished males (Aumann 2000, personal observation). Thereby, via salivary masses, females may have the chance to judge a males quality and control the amount of sperm it transfers. Combining this fact and my finding that there is complete sperm mixing in the spermatheca, I hypothesise that this female strategy could be a form of cryptic female choice in *P. communis*. Since there is a controversial discussion about an appropriate definition of cryptic female choice (Birkhead 2000, Eberhard 1996) I adopt a definition including copulatory female processes which allow a female to control sperm transfer (Thornhill 1983, Eberhard 1996). Thus, *P. communis* females seems to be able to influence the proportional paternity of a male by controlling copulation duration since the proportion of sired offspring depends on the contingent of sperm a male contributes to the totality of sperm in the spermatheca.

In this study, I did not investigate male traits that influence sperm competition which may also play a role for differences in paternity. Even though there was a strong correlation between duration of copulation and P_2 there was visible variation in P_2. Additionally, if sperm transfer rate during copulation is constant not every male may transfer the same amount of sperm per time (e.g. Sauer et al. 1997, Engqvist & Sauer 2003). But, not only male traits, also female traits like size in relation to male size could affect this variability, as Vermeulen (2004) found for *P. vulgaris*. Furthermore, it has been shown that sperm competition mechanisms investigated in two male designs can change down if females are paired with three or more males (Zeh & Zeh 1994). Since females of *P. communis* normally copulate with several males, it is necessary to control for changes in sperm competition in multiple mated females, which was not possible with the experimental design I used.

To sum up, my results reveal that there is sperm mixing in double mated *P. communis* females and paternity is distributed in

proportion to the number of sperm a male transferred during the copulation. Thereby, females of this species seem to adopt a form of cryptic female choice.

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Chapter IV

Variability in paternity patterns is not induced by different sperm transfer rates

Variability in paternity patterns is not induced by different sperm transfer rates

Even though the mechanism of sperm competition is known for a species there may be unexplained variance in paternity patterns. Out of various explanations, the most obvious may be that the inter-male rate of sperm transfer differs.

To test this hypothesis, I used the scorpionfly *Panorpa communis* as in this species there is a fair raffle in sperm competition but still unexplained variance in paternity. In a controlled mating experiment females were paired to two successive males for fixed copulation duration. Afterwards, the same males were paired to a further female for independent sperm counting. I found variance in the number of sperm transferred by different males, but there was no correlation between sperm transfer and proportional paternity. Furthermore, there was no indication for sperm transfer rates manipulated by males, nor by females. The number of sperm transferred within 90 minutes is rather small, compared to the number transferred in natural copulations. Therefore, I expect the observed variance being smaller than appearing in this experiment. I assumed this to be the reason that no effects on paternity patterns could be detected.

Introduction

In insects, males as well as females show a tendency for multiple mating (for review see: Parker 1970). In many cases, females mate with different partners and store sperm in a storage organ, namely the spermatheca, until fertilisation (for review see: Parker 1970, Simmons 2001). Thus, the sperm of different males might overlap temporally and locally resulting in sperm competition (Parker 1970). The outcome of sperm competition can depend on different mechanisms out of which one is the fair raffle. Here, the proportion of sired offspring of a male depends on its contingent of sperm stored in the spermatheca (Parker et al. 1990). Via sperm selection, this provides the opportunity for cryptic female choice

(Simmons 2001). Thereby, I apply a definition of cryptic female choice including copulatory female processes which allow a female to control sperm transfer (Thornhill 1983, Eberhard 1996). Representing an arena for male sperm competition, females could ensure that only the most competitive sperm fertilises the eggs or at least a predominant part of them. The result is a sexual conflict with females selecting sperm of particular males and males trying to ensure fertilisation with their own sperm.

Suggesting a fair raffle mechanism in sperm competition, females are able to select sperm by controlling the amount of sperm a male transfer (Simmons 2001), for example, by controlling copulation duration. Thus, for males it would be advantageous to transfer more sperm in the same amount of time than a rival male. Furthermore, larger males may have higher rates of sperm transfer as has been shown for the dung fly *Scatophaga stercoraria*, for example (Simmons & Parker 1992). Not taking differences in sperm transfer rate into account, this could result in unexpected variance, which may differ from the expected values in the outcome of sperm competition.

As has been shown in *Chapter II* & *III* the polyandric scorpionfly *Panorpa communis* shows a fair raffle in sperm competition. Even though paternity patterns can be explained by the estimated number of sperm transferred in general, there is still notable variance. One reason for this variance could be the between male difference in sperm transfer rates. Furthermore, females may differ in their ability to influence sperm transfer, beyond the control of copulation duration. Practically, the muscle controlling the spermatheca of scorpionflies might be a prerequisite for this ability, since it is suggested to build a counterpressure against intruding sperm (Vermeulen 2004). Females in better condition may be more successful in achieving the resulting counterpressure.

To detect the outcome of sperm competition, I performed an experiment in which females where paired to two males for a fixed copulation duration. Since there is sperm mixing and a fair raffle in sperm competition (*Chapter II* & *III*) the proportion of sired offspring for first and second males should be equal, consequently. To control for differences in sperm transfer rates of different males, all males were paired with a further female afterwards, and a male’s sperm transfer ability was estimated.

Material & Methods

Breeding of P. communis

Individuals reared for this experiment were F_1-offspring of adults captured from a field population near Freiburg (i.Br. Germany) in summer 2004. The breeding was arranged following a protocol given in Sauer (1970, 1977) and Thornhill & Sauer (1992). Adult animals hatched after diapause in spring 2005 and were reared individually in plastic tubes (∅: 3.5 cm, height: 8 cm) in an environmental chamber with light-dark cycle of 18h : 6h at 20°C at light respectively 18°C at dark. They were fed with one segment of last-instar mealworm (*Tenebrio molitor*) every third day, to achieve well nourished individuals. Adequate water supply was assured by moist tissue paper on the ground of all tubes. Sexual maturity was reached approximately after 10 days and only pubescent individuals were used in the experiment. Pubescent males can be identified when they start courting even without a present female.

Competition experiment

Adult females were paired successively with two different males. All individuals were controlled for being no siblings. Before mating trials were arranged, individuals were weighted with an analytical balance (Satorius BP 110S). Mating trials were performed in small plastic boxes (10 cm × 10 cm × 7 cm) which were covered with moist tissue paper. Copulations were interrupted after 90 minutes. Individuals that did not copulate were put back in their plastic tubes and offered to a different partner on the following day. Individuals that copulated less than 90 minutes were excluded from the experiment.

Doubly mated females were put individually into a plastic box (10 cm × 10 cm × 7 cm) containing moist tissue paper and a Petri dish (∅ 5.5 cm) filled with peat for egg disposition. For each female, existing egg clutches were removed daily and transferred carefully in a Petri dish (∅ 5.5 cm) containing moist tissue paper. Every day, hatched larvae were transferred in a small Eppendorf tube (one tube for each clutch) filled with 100% alcohol for subsequent DNA isolation. Females were allowed to lay eggs for 20 days. Afterwards, they were also transferred to 100% alcohol. Females that died before this expiration were preserved earlier.

Control copulations & sperm dissection

To estimate male sperm transfer rate, males that were paired once within the regular experiment were offered to a second, virgin, and not related female. Copulations were interrupted after 90 minutes and, afterwards

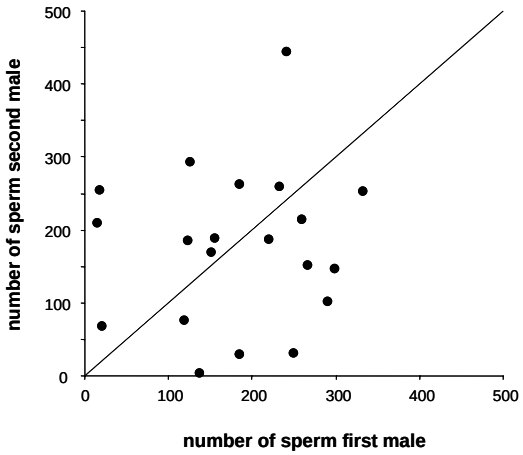

Figure 1 Numbers of transferred sperm for first and second males in the control trial. Each point represents one female. The line indicates the case of equal sperm numbers for both males mated with one female.

males were transferred to 100% alcohol. The spermatheca of a female was dissected directly after copulation and transferred in a drop of the DNA-specific flurochrome DAPI (4', 6-diamidino-2-phenylindole; Cal Biochem GmbH, Frankfurt, Germany; concentration 5g/ml Trisbuffer [0.1 molar, pH7]) on a glass slide and then ruptured to release the sperm content. Thereby, DNA carrying regions were stained by the fluorochrome. After three minutes a drop of glycerine was added to avoid draining. Spermatozoa were counted using an Orthoplan-fluorescence microscope (magnification 200 x).

Paternity detection

Genomic DNA was extracted using a 10% solution of Chelex®-100 sodium forum in sterile water. 500 µl Chelex were added to each tube containing either a piece of leg-musculature of an adult or the whole larva, respectively, and then incubated for 15 min at 95°C. After vortexing 15 sec the specimens were spun down and stored at -18°C.

Paternity was detected using five microsatellite markers and PCR amplification as introduced in *Chapter I*. PCR fragments were applied on a polyacrylamidgel, resolved with an ABI 377 DNA-Sequencer and scored with ABIPrism GeneScan analysis software.

Parentage analysis was performed using CERVUS 2.0 (Marshall et al. 1998). Statistical analysis was done with SPSS 12.0.

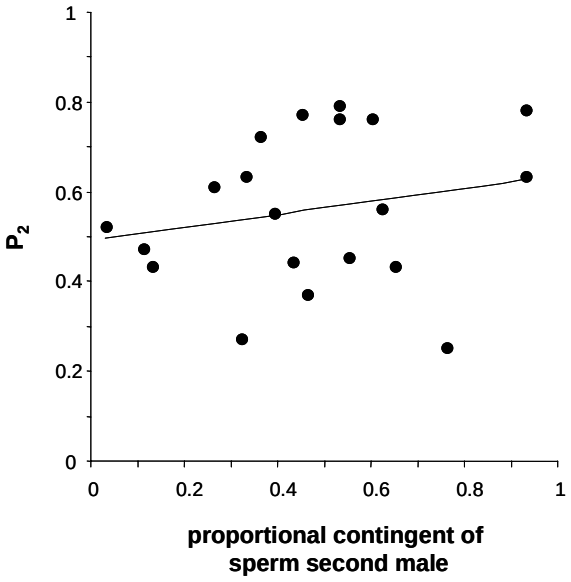

Figure 2 Correlation between the potential proportional contingent of sperm a second male contributes to the spermatheca of a female in the competition trial and the sperm numbers of control copulations for the same male.

Results

Within the competition trial, 20 females mated with two males. The subsequent control trial showed that the number of sperm transferred within the 90 minutes of the test varied strongly between males (mean±S.D: 183 ± 97, minimum: 4, maximum: 443). There was no difference between first males and second males for the number of sperm transferred in control copulations (t-test: N=20, t=0.186; p=0.85; Figure 1).

I intended to detect whether the variance of the proportional paternity of first (P_1) and second (P_2) males depends on differences in sperm transfer rates. Thus, I calculated a potential proportional contingent of sperm a second male contributes to the spermatheca of a female in the competition trial and related this to the paternity success of males in the control copulations. Thereby, I suggested that a male's rate of sperm transfer does not differ between subsequent copulations (unpublished data). However, no correlation between the two factors could be found (Product Moment correlation: N=20, r_P=0.21, p=0.37; Figure 2).

To test for any effects of male size on the number of sperm it is able to transfer, I tested for a correlation between male weights on the day of copulation in the control trial and the number of sperm transferred in this copulation. No correlation could be detected (Product Moment correlation: N=39, r_P=-0.03, p=0.88; Figure 3).

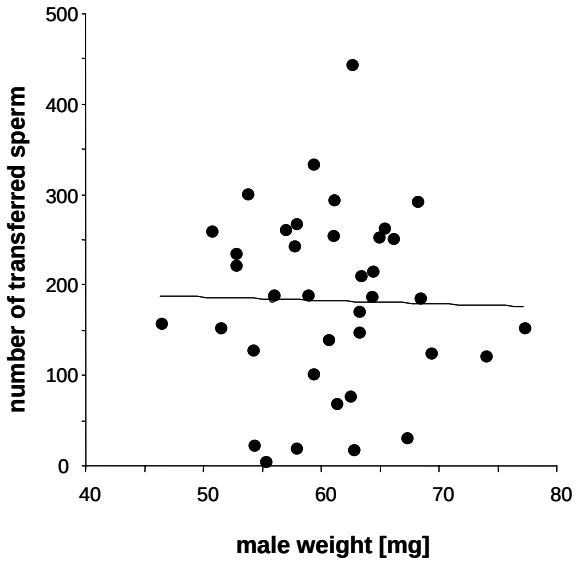

Figure 3 Effect of male copulation weight on number of transferred sperm (control copulation)

To test for any effects of female size on the number of sperm a male is able to transfer, I tested for a correlation between female weight on the day of copulation and the number of sperm transferred in this copulation. No correlation could be detected (Product Moment correlation: N=40, r_P=0.18, p=0.27; Figure 4a). Also no correlation between the difference of female and male weight at the day of the control copulation and the number of transferred sperm could be detected (Product Moment correlation: N=40, r_P=0.01, p=0.97; Figure 4b).

Discussion

Considering the absolute numbers of sperm males transfer to females in control copulations, I found that there is considerable variance between males, even though copulation duration was equal. This seems to be contradictory to the assumptions of my former experiments (*Chapter II* & *III*). There, I used copulation duration as an estimator for the number of transferred sperm. But, this is problematic if the rate of sperm transfer is different between males as the current results implied.

To exclude this possible effect, I calculated a correlation between the potential proportion of transferred sperm and the proportion of sired offspring for first as well as for second males. As there is no significant correlation, the differences in the rate of sperm transfer between males seems to be too small to induce the variance I found in paternity patterns. Differences in the amount of sperm associated with differences in paternity patterns seem to be achieved mainly by varying copulation duration. Hence, it is possible to use copulation duration as a parameter to estimate the amount of transferred sperm.

In general, sperm transfer in scorpionflies starts shortly after initiation of copulation, and is constant during copulation (Sauer et al. 1997, Sauer et al. 1998, Aumann 2000). Therefore, all males in this experiment were expected to transfer sperm and I assume an artificial effect being responsible for very low rates of sperm transfer in my study. Sometimes the genital tract of males is damaged during hatching for the male is not able to transfer any sperm at all.

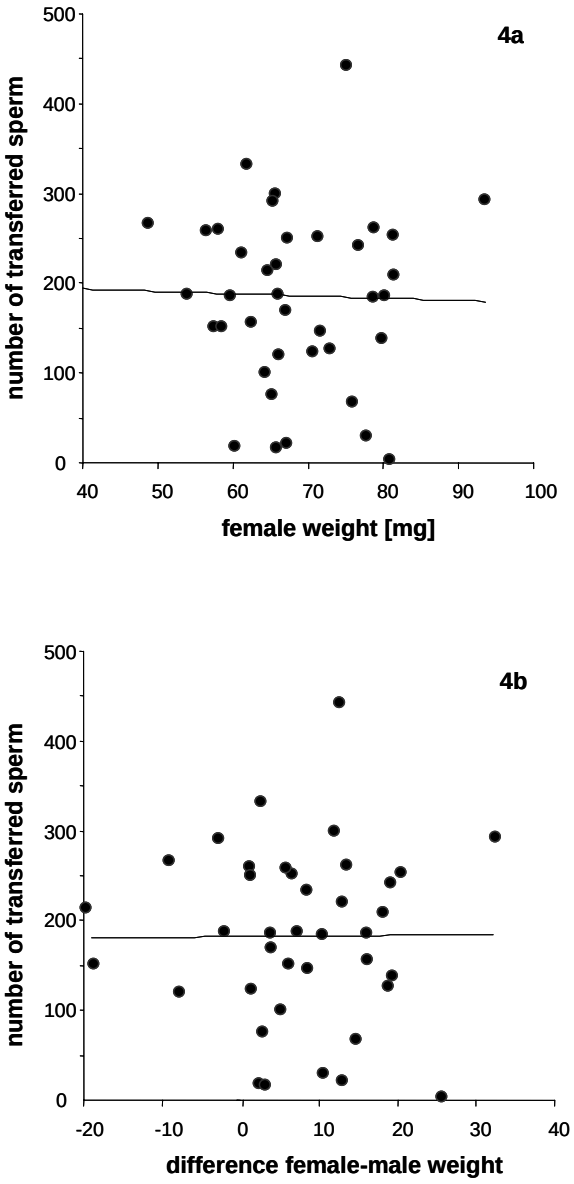

Figure 4 Effect of female weight (4a) and difference of female-male weight (4b) on number of transferred sperm (control copulations)

Nevertheless, the obvious range in sperm numbers can be caused by different factors. Generally, larger males or males in better condition are expected to have a higher rate of sperm transfer (see for review: Simmons 2001). In an experiment with fixed copulation duration Ward (1993) showed for the dungfly *Scathophaga stercoraria* that larger males have higher proportional paternity than smaller males irrespectively mating sequence. Furthermore, larger males have higher constant rates of sperm transfer in *Scathophaga* in general (Simmons & Parker 1992, Ward 1993, Parker & Simmons 1994, Parker & Simmons 2000). But, in my experiment, using male weight at the day of control copulation as a measurement for male size, there is no effect of weight on the number of sperm transferred. This might be a hint that male size alone does not influence the rate of sperm transfer. In addition, there are no data about the amount of sperm a male is able to produce overall. Yet, scorpionflies, generally, are not suspected to be sperm limited as males can remate directly or the day after a copulation without observed reduction in fertilisation success (unpublished data). However, larger scorpionfly males are able to produce more respectively larger salivary masses to gain longer copulations (Sauer et al. 1998, Aumann 2000, Engqvist & Sauer 2001) and they influence the amount of transferred sperm via copulation duration (Sauer et al. 1997, Engqvist & Sauer 2003, Kock et al. 2006). Hence, male size might effect sperm transfer rates more indirectly. Furthermore, sperm quality might cause differences in sperm transfer rates as has been shown for various insect species. Sperm viability can be different between males (Bernasconi et al. 2002) and can be responsible for inter-male variances in paternity success (García-González & Simmons 2005). Even the presence of sex chromosome meiotic drive has been shown to influence the outcome of sperm competition. In the stalk-eyed fly *Crytodiopsis whitei* drive males showed reduction in production of viable sperm and reduction in competitive ability (Fry & Wilkinson 2004). There are no available data for differences in sperm quality in scorpionflies at all except a hint in a study of Vermeulen (2004) for *P. vulgaris*. There, sperm transfer rates were influenced by larval nutrition in first annual generation males. As my current study was arranged with individuals of the first annual generation also, such effects are possible for *P. communis*. Also, infertility of males is assumed to be an important factor in explaining variances in paternity patterns (García-González 2004). There are no available data in order to test this hypothesis for *P. communis* but, I suggest male infertility or sterility being a possible

explanation for unexplained variances in paternity patterns. Finally, possible inter-male differences in sperm quality or male infertility have to be considered if discussing variances in sperm transfer rates.

Also, females may be responsible for the range in sperm numbers since the female controls sperm storage and usage and therefore offers the potential for sperm selection. Vermeulen (2004) showed clearly that females of the scorpionfly *P. vulgaris* are able to influence the rate of sperm transfer. These results indicated that well nourished females exerted muscle contractions to counteract male sperm transfer. Particularly, the compression muscle associated to the spermatheca seems to be responsible for this restriction in sperm transfer. The genital tract of *P. communis* is very similar to the one of *P. vulgaris* and therefore similar mechanisms can be expected in this species. In the current study, I used female weight at copulation as an estimator for the female ability to resist sperm intrusion. But, since there is no correlation between female weight and the number of sperm transferred it seems unlikely that *P. communis* females can restrict sperm transfer.

Since all individuals used for the experiment were around the same age, I do not expect age effects being responsible for the observed variance in sperm transfer as has been described for some other insects species (e.g. Mack et al. 2003, Reinhardt & Siva-Jothy 2005).

However, the design of the current experiment was not meant to detect how males or females manipulate sperm transfer. I was able to show that there is variance in the number of transferred sperm between males in copulations of same duration. But, this variance does not seem to influence paternity patterns, assuming males of the control copulations transferred comparable amount of sperm in the competition trail. Considering that wild caught females can contain up to 10000 spermathozoa (unpublished data) the observed variance in the current study seems to be negligible. One general conclusion of this experiment is the confirmation that copulation duration is a good estimator for the number of transferred sperm during copulation. To analyse what exactly might be the mechanisms inducing the inter-male variance could be substance of further experiments.

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General discussion

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Summary

General discussion

The aim of the present study was to investigate sperm competition in the scorpionfly *Panorpa communis*. It has been assumed for several years that there is sperm competition in this species because of the high remating frequencies of females and sperm storage in the spermatheca. But, nothing was known about the mechanisms that control which male fertilises the eggs. Hence, I concentrated on the detection of the basal mechanism of sperm competition in *P. communis*.

In *Chapter I*, I presented five newly designed microsatellite markers for *P. communis* which enabled me to assign paternity. Two experiments, presented in *Chapter II* and *Chapter III*, showed that in *P. communis* complete sperm mixing occurs, based on the principle of a fair raffle. In *Chapter IV* I tested for sperm transfer rates of different males, with the result that relatively small variances seem not to influence paternity in this species.

Microsatellites for *P. communis*

Five microsatellite markers for *P. communis* were introduced in *Chapter I*. Microsatellites are a simple and practicable method for paternity detection (Balding 1999). Since the existing markers available for *Panorpa vulgaris* (Epplen et al. 1998) did not work for *P. communis*, which first seemed to be an option because of the close phylogenetic relation of both species (Misof et al. 2000), the detection of new markers was essential.

I successfully applied the method described by Nolte et al. (2005) for detecting microsatellites for the first time in insects. The five markers I finally used for my analysis are not highly polymorphic. Yet, they are absolutely sufficient for paternity assignment in the laboratory, in particular, if the genotype of all candidate parents is known.

Finally, a first set of microsatellite markers for *P. communis* is now available which enabled me to detect paternity in my experiments and which will be useful also in further studies.

Sperm competition in *P. communis*

With my investigations, I was able to clarify the mechanism of sperm competition for the scorpionfly *P. communis*. In two laboratory experiments (*Chapter II* & *Chapter III*) females were paired with two or three different males, respectively, and

copulations were not interrupted artificially. As a result, copulation durations strongly varied. Due to a continuous sperm transfer during copulation the number of sperm transferred to the female's spermatheca increases continuously during copulation (Aumann 2000). Therefore, the number of sperm males transferred in the experiments also showed strong variation. Hence, a male's paternity is influenced by two components: copulation duration and sperm transfer rate.

It has been shown for *Panorpa vulgaris* that sperm transfer rate is influenced by male weight, meaning it is higher in heavier males. But, this effect decreases if males are well nourished (Vermeulen 2004). Since *P. vulgaris* and *P. communis* are closely related species (Misof et al. 2000) similar can be expected for *P. communis*. As, in all experiments of the current study males were well nourished, I assumed sperm transfer rate of males to be equal. In contrast to this assumption, I found variance in sperm transfer rates (*Chapter IV*). But, since the amount of sperm a male transfers in a copulation of natural duration (Aumann 2000) is much higher than in my experiment, I suggest this variance to be negligible. Mainly, because the variance in sperm transfers rates did not influence paternity patterns. Consequently, using copulation duration as an estimate for the number of transferred sperm is possible in particular, if all individuals are well nourished.

Considering the results of *Chapter I* to *IV*, I conclude sperm of different males being mixed inside the spermatheca of *P. communis* females. The outcome of sperm competition is based on the principle of a fair raffle, i.e. sperm compete numerically.

Due to the fact that this fair raffle occurs in sperm competition, females have the ability for postcopulatory female choice. Yet, applying a definition of cryptic female choice including copulatory female processes which allow a female to control sperm transfer (Thornhill 1983, Eberhard 1996), I suppose this to be a form of cryptic female choice. Females of *P. communis*, seemingly, are not able to detect a male's quality before copulation (Aumann 2000). In general, scorpionfly females are able to terminate copulation anytime by heavy kicks with their hind leg pair if the nuptial gift is consumed or no further is offered (Thornhill & Sauer 1991, Thornhill & Sauer 1992, Bockwinkel & Sauer 1994, Aumann 2000, Engqvist & Sauer 2003). As a consequence, it is the female which influences the amount of transferred sperm indirectly in response to male quality which is indicated by the number or size of the nuptial gift (Engqvist & Sauer 2001, Sauer 2002, Engels & Sauer

2006). This is also true for *P. communis* (Aumann 2000). Therefore, since high quality males transfer more sperm than males of lower quality and sperm compete numerically inside the spermatheca of *P. communis*, high quality males gain more fertilisations proportional to the higher representation of their sperm. Finally, the female controls the proportion of offspring a male sire by controlling copulation duration. Hence, these two mechanisms underline that female *P. communis* indeed adopt cryptic female choice.

Sperm competition in scorpionflies

In general, one main intention to investigate sperm competition in scorpionflies is to explain the evolution and maintenance of such mechanisms in the group Panorpidae. So far, the mode of sperm competition is known for three different scorpionfly species, namely *Panorpa germanica* (Kock et al. 2006), *P. vulgaris* (Sauer et al. 1999) and *P. communis* (*Chapter II* & *Chapter III*). In all those species the mode of sperm competition is closely connected to life history traits and, in particular, reproductive behaviour. As mentioned above, females of *P. communis* are able to adopt cryptic female choice by accepting more sperm from high quality males and sperm competing numerically. Similar is known for the highly polyandric species *P. vulgaris* (Sauer et al. 1999, Sauer 2002), which also is not able to detect a male's quality before copulation. Both species, *P. communis* and *P. vulgaris*, are phylogenetical closely related (Misof et al. 2000, Pollmann & Sauer unpublished data).

Having a closer look at the third species, *P. germanica*, which is phylogenetically more distant to *P. communis* and *P. vulgaris* (Misof et al. 2000, Pollmann & Sauer unpublished data), differences in reproductive behaviour and the mode of sperm competition can be detected. In contrast to the other two species, which court and mate during daytime, males of *P. germanica* start courting at dusk and night (Gerhards 1999). Consequently, acquiring mates via visual signals, as it is known from diurnal scorpionflies, is impossible. To compensate for this lack of visual choice, *P. germanica* males release a pheromone attracting females (Gerhards 1999, Rathmann-Schmitz 2000) which is suspected to act as a quality indicator for the female (Kock & Sauer unpublished data). This hypothesis is supported by the fact that females of *P. germanica* are extremely choosy in selecting a male and, additionally, they mate only with one or two males (Gerhards 1999). It is not surprising, to find the mode of sperm competition for *P. germanica* being concordant with its

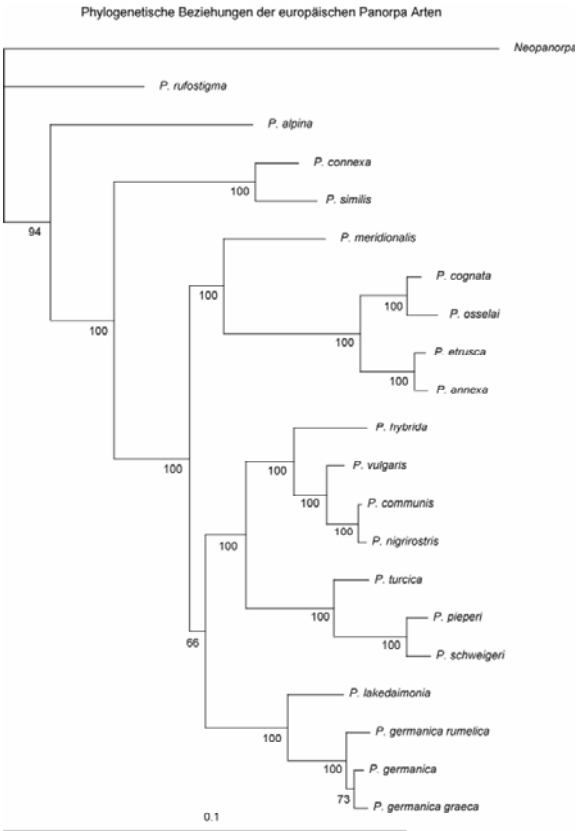

Figure Phylogenetic relations of the European Panorpa species; with friendly permission of Pollmann & Sauer

mating behaviour. In this species, sperm mixing seems to be incomplete and paternity of the offspring is shifted to the last male (Kock et al. 2006). Also, females of *P. germanica* do not allow a male to initiate copulation if no nuptial gift is offered and they terminate copulation if no more nuptial gifts in form of salivary masses are offered by the male and, necessarily, mate with a further male. Therefore, cryptic female choice seems to be existent if females of *P. germanica* remate but possibly, do not play a crucial role as in *P. communis* and *P. vulgaris*. To conclude briefly, this comparison of three Panorpa species shows that if females are not able to detect a male's quality before copulation a fair raffle in sperm competition connected with strong polyandry seems to be maintained.

For the Panorpidae in general, it is more difficult to discuss the evolution of sperm competition as the mechanism of sperm competition is known only for the three species I described above. Considering the phylogenetic trees of European scorpionflies or also considering North American species (Misof et al. 2000, Pollmann & Sauer unpublished data, Figure), the already discussed species are indeed relatively close related. Therefore, no assumptions of the original mechanism of sperm competition can be made. Both strategies, mating multiple with complete sperm mixing and mating less frequent with incomplete sperm mixing, could possibly be the original one. Having a closer look at *P. germanica* again, I discussed that copulation duration is influenced by the number of male salivary masses, although this is not necessary for the female to detect a male's quality. This may be a hint that the mating behaviour of *P. germanica* developed secondary. Therefore, at least for this group, sperm mixing combined with multiple mating of females would be the original mechanism, at least for this small group.

Nevertheless, with the available data I only can hypothesise about the evolution of sperm competition in the European Panorpidae. However, postulating that the mechanism of sperm competition is associated to the female tendency of multiple mating and the mating behaviour in general, conclusions can be drawn about the mechanisms that occur in groups where these facts are also known. As an example, for two Caucasian species P. *connexa* and *P. similis*, unfortunately, nothing is known about female remating frequencies and only little about the mating behaviour in general (Kullmann & Sauer 2005). Out of this two, only for *P. similis* it is knowm that females seem to control sperm transfer directly by rejecting sperm of males offering no salivary mass during copulation (Kullmann & Sauer 2005). Yet, complete sperm mixing and a raffle based outcome of sperm competition are possible if females do mate multiple. A different example comes from *P. alpina* in which life history is well investigated (unpublished data). Here, females mate multiply and males release a pheromone while courting. But, it is not known if the pheromone is used for acquiring males or also acts as a quality indicator. If the latter is true, I would expect females to mate less frequent and more specific like in *P. germanica*. If the pheromone is used only to attract females multiple mating with a fair raffle in sperm competition would be a possible system in *P. alpina*. This could result in postcoulatory or cryptic female choice in this species.

Although most is only speculation, these examples underline that multiple mating and cryptic female choice via sperm mixing combined with a fair raffle could be the original mechanism in the European Panorpidae. However, further studies are required for definitive conclusions about the evolution of sperm competition in scorpionflies. In particular, the European species *P. alpina*, as a closely related species to the North American species (Misof et al. 2000, Pollmann & Sauer unpublished data), should be analysed for the mechanism of sperm competition. This, as well as more data from North American or Asian species could be helpful to generalise the knowledge we got from the well investigated European scorpionflies.

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Summary

The aim of the present study was to investigate the basal mechanism of sperm competition in the European scorpionfly *Panorpa communis*. Within three laboratory experiments, I used five newly developed microsatellite markers to detect the mechanism of sperm competition in the scorpionfly *P. communis*.

In general, sperm competition occurs if sperm of different males compete for the fertilisation of the ova of one female. As *P. communis* females mate multiple with different males and store sperm in a storage organ until fertilisation, there is sperm competition in this species. To clarify the outcome of sperm competition, paternity detection of offspring of a multiple mated female is necessary. The most practicable way for paternity detection is the application of microsatellite markers. As a first step of my work, I established five new markers to achieve species specific microsatellites for *P. communis*.

Then, I arranged two experiments in order to detect the mechanism of sperm competition, Here, females were paired to two or three different males, respectively. Since copulation duration, generally, is known to be a good estimator for the number of transferred sperm, I was able to draw conclusions from the proportion of sperm a male contributed to the spermatheca of the female in relation to rival males. I was able to show that the outcome of sperm competition is not influenced by the mating order of males. Consequently, any form of last male sperm precedence for *P. communis* could be excluded. But, paternity patterns were influenced by copulation duration and therefore by the proportion of sperm represented in the spermatheca. Both experiments conclude that, in *P. communis*, sperm of different males is mixed and compete numerically for fertilisations, i.e. there is a fair raffle in sperm competition.

In a further experiment, I analysed if sperm transfer rates of different males are equal. Although, males were slightly different respective their sperm transfer rates, these differences did not influence the outcome of sperm competition. Accordingly, using copulation duration as a general estimator for the number of transferred sperm is possible.

Finally, I discussed the role of sperm competition for scorpionflies in general and, how it may be maintained in this group. Furthermore, I hypothesised how the

remarkable mating system in combination with different sperm competition mechanisms in scorpionflies may have evolved.

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