

Sperm depletion, male mating behaviour and reproductive 'time-out' in *Gammarus pulex* (Crustacea, Amphipoda)

Jean-François Lemaître*, Thierry Rigaud¹, Stéphane Cornet¹, Loïc Bollache¹

Laboratoire BioGéoSciences, CNRS UMR 5561, Université de Bourgogne

ARTICLE INFO

Article history:

Received 26 May 2008

Initial acceptance 27 June 2008

Final acceptance 23 August 2008

Published online 30 October 2008

MS. number: 08-00347

Keywords:

female quality

Gammarus pulex

male mate choice

reproductive 'time-out'

sperm depletion

In *Gammarus pulex*, male–male competition is generally intense because the operational sex ratio (OSR) is strongly biased towards males; however, studies have shown possible fluctuation in this intrasexual competition, which could be caused by sperm depletion, a phenomenon recently found in gammarids. Sperm depletion may also affect male mating behaviour. We therefore tested the influence of sperm depletion on the OSR in *G. pulex*. Two sets of experiments were conducted: first, to find out the number of sperm in the testis before and after mating events (sperm depletion), and second, to test the implications of sperm depletion for the mating behaviour of male *G. pulex*.

We found substantial sperm allocation to each reproductive event but also a relatively fast replenishment. However, contrary to one of our hypotheses, sperm depletion had no impact on the male reproductive 'time-out' and therefore on the OSR, since depleted males could engage in a precopula within a few hours of a previous copulation. The decision to initiate an amplexus de novo was more dependent on indicators of the female's quality such as her time left to moult. Depletion status also did not affect male competitive ability. Indeed, in a competitive context, recently mated *G. pulex* males were more likely to pair again than those males that had not mated recently, independently of sperm reserves, male size and energy storage. Consequently, some males had better access to reproduction than others, which could be explained by various hypotheses.

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Differences between the sexes in the variance of reproductive success (Bateman 1948) are classically explained by the asymmetry in the cost of gamete production (Parker et al. 1972). Owing to the substantial energy and time devoted by females to ova production and parental investment (Trivers 1972) and also the traditional view of 'cheap sperm production', males are almost always considered to allocate a greater proportion of their time to sexual competition than females (Clutton-Brock & Parker 1992). Consequently, the operational sex ratio (OSR) is generally male biased, which means that at any one time there are more males than females ready to mate in the population (Emlen & Oring 1977; Kvarnemo & Ahnesjö 2002). Thus, males should strive to increase their fitness by maximizing the number of sperm produced and the number of matings obtained (Parker 1984). Nevertheless, in many species, recently mated males have a low level of sperm (Preston et al. 2001; Velando et al. 2008). This sperm depletion phenomenon leads these males to be restrained in the number of females they

can inseminate and is one of the main reasons why males may not have access to reproduction at some point in their lifetime (Dewsbury 1982; Nakatsuru & Kramer 1982). Thus, by decreasing male abilities, sperm competition and fertilization success, sperm depletion could affect male reproductive behaviour (Van Son & Thiel 2006).

The amphipod *Gammarus pulex* is a relevant biological model for studying sexual selection (Bollache & Cézilly 2004a; Hume et al. 2005), mainly because, like many amphipods, it shows precopulatory mate guarding (amplexus), allowing researchers to test different mate choice strategies. In *G. pulex*, females are asynchronously receptive for copulation during a brief period, whereas males are considered available for mating during most of their moult cycle (Sutcliffe 1992). Therefore, the OSR is predicted to be strongly biased towards males who are in competition for access to females and in particular for 'high-quality' females (Elwood et al. 1987; Bonduriansky 2001). Precopulatory mate guarding behaviour is thought to have evolved as a male competitive strategy in response to the brief period of female receptivity (Parker 1974; Grafen & Ridley 1983). Empirical studies have demonstrated that in *G. pulex* precopula pairs tend to be matched for size (i.e. assortative mating), with large males pairing with large females, and smaller males with smaller females (Strong 1973; Birkhead & Clarkson 1980; Adams & Greenwood 1983; Elwood et al. 1987; Elwood

* Correspondence and present address: J.-F. Lemaître, Mammalian Behaviour and Evolution Group, Department of Veterinary Preclinical Science, University of Liverpool, Leahurst, Neston CH64 7TE, U.K.

E-mail address: Jean-Francois.Lemaître@liv.ac.uk (J.-F. Lemaître).

¹ T. Rigaud, S. Cornet & L. Bollache are at the Laboratoire BioGéoSciences, CNRS UMR 5561, Université de Bourgogne, 6 Boulevard Gabriel, F-21000 Dijon, France.

& Dick 1990; Bollache et al. 2000). However, this magnitude of size-assortative pairing, which is positively correlated with the intensity of sexual selection on male body size, can vary according to the degree of male–male competition (Bollache & Cézilly 2004a).

In gammarids, sperm depletion is poorly documented except for the recent study of Dunn et al. (2006), on *Gammarus duebeni*. In this species, sperm depletion has an impact on male and female fitness by limiting the number of offspring produced after repeated mating. Consequently, *G. duebeni* males strategically allocate sperm by investing more in high-quality females (in their study, unparasitized) than in low-quality (infected) females (Dunn et al. 2006). In *G. pulex*, the male pairing decision to engage in a precopula (i.e. mate guarding) is known as a state-dependent behaviour since it is negatively influenced by parasitism and the proximity of a moult event (Bollache et al. 2001; Bollache & Cézilly 2004b), but positively influenced by prior pairing status (a male separated from his female before copulation is more likely to re-pair than a previously unpaired male; Bollache & Cézilly 2004b). However, after mating, males could be unavailable for a certain period because they must replenish their sperm. In crustaceans, no work has specifically investigated the influence of such sperm depletion on pairing decisions and male competitive ability.

Our aim in this study was therefore to evaluate the behavioural and evolutionary consequences of sperm depletion in *G. pulex*, a species closely related to *G. duebeni*. We first quantified sperm depletion intensity and the kinetics of sperm replenishment, to determine the level of sperm investment in *G. pulex*. We then investigated whether there was any effect of a putative male refractory, or 'time-out', period (Clutton-Brock & Parker 1992) by studying males' propensity to initiate an amplexus after a previous reproductive event. We then investigated the influence of sperm depletion on male mate choice in a competition context. During all tests, we controlled for different biological features known to influence the male's decision to initiate an amplexus: male and female size, female time left to moult, and glycogen and lipid storage (Elwood et al. 1987; Dick & Elwood 1990; Plaistow et al. 2003; Bollache & Cézilly 2004b).

METHODS

All individuals were collected from the river 'Suzon' (Burgundy, France; 47°24,215' N, 4°52,974' E) by kick sampling (Hynes 1955). One sample was collected between January and March 2006, and a second in August 2006. We used this last sample to evaluate male competitive ability (see below). In all experiments, individuals were maintained in the laboratory in oxygenated tap water previously conditioned using coal active filter and UV treatment to eliminate chlorinate residues and pathogenic organisms. Paired individuals were kept in dishes (diameter 10 cm, height 8 cm, volume 30 ml) and unpaired males were kept in a larger tank (35 × 23 cm and 20.5 cm high). In all tanks, water was changed every 2 days and food (elm leaves) was provided ad libitum. The temperature of the room was 15 ± 1 °C and the photoperiod was 12:12 h light:dark.

At the end of the experiments, individuals were killed in 70° ethanol and measured (body height at the level of the base of the fourth coxal plate, e.g. Bollache et al. 2002) using a Nikon SMZ 1500 stereoscopic microscope and the Lucia G 4.81 software (Laboratory Imaging, Prague, Czech Republic). Female fecundity was evaluated after dissection by counting eggs in the ventral incubating pouch.

Sperm Count

We counted sperm using a variation of the technique described by Rigaud & Moreau (2004), with one major modification: in our

study, we estimated the remaining quantity of sperm in the testis, whereas Rigaud & Moreau (2004) worked on the sperm quantity in ejaculates. Because fertilization is semiexternal in *G. Pulex*, it was impossible to collect male ejaculates. Consequently, we obtained an indirect estimate of each male's investment. One testis per individual was isolated in a watch glass, in 1000 µl of Crustacean Ringer (Van Harrevelde 1936). After isolation, the gonad was dissected, and divided into small fragments with forceps, under a binocular microscope. Then, these pieces of gonad were submitted to 10 s of ultrawave treatment to separate the membranes from the sperm (Ultra-waves tank, Branson 2200 Branson Cleaning Equipment Company, Shelton, Co, U.S.A.) and homogenized. For each male, four samples of 10 µl were placed on one slide and dried for 10 min at 37 °C. Each drop was marked with an indelible pen and then gently rinsed with demineralized water to eliminate Ringer's crystals. We counted all sperm in each drop under optic microscopy (Nikon Eclipse E600, magnification × 100). Statistical analyses were carried out using the sum of the sperm numbers of the four drops, after the repeatability between drops had been established (analysis not shown).

Sperm Depletion and Replenishment

In the first experiment, three groups of males, varying in pairing status, were established for the evaluation of sperm depletion. The first one consisted of males collected in amplexus in the field, for which sperm were counted before mating (hereafter called 'paired males'). The second group consisted of males collected unpaired in the field (hereafter called 'unpaired males'). The third group consisted of males collected in amplexus in the field which were allowed to mate before we counted sperm (hereafter called 'mated males'). In this last group, fertilization of the eggs was identified by the presence of female moult and dividing eggs in the ventral brood pouch. Males of the three groups spent the same time in the laboratory (18 h) to control for possible differences in sperm replenishment. Sperm were counted in one testis for each male (preliminary analyses revealed no differences between the two testes dissected from single males; results not shown).

In the second experiment, we estimated sperm replenishment kinetics for three groups of mated males. These recently mated males were maintained in isolation in the laboratory, and were dissected 2 days (D2), 4 days (D4) or 6 days (D6) after their previous reproductive event. The experiment was stopped after 6 days to avoid the loss of individuals and because sperm replenishment was found to be maximal at this time (see Results).

Sperm Reserves and Male Pairing

A series of laboratory experiments was conducted to study the inclination of 'mated males' to pair with a receptive female to investigate how low sperm reserves influence the male's mating decision (i.e. whether males with low sperm reserves can be considered to be in 'time-out'). The use of mated males allowed us to be sure that the tested male would have only a small quantity of sperm before the experiment. All individuals were collected in the field around 1700 hours on Day 1. At 1000 hours on Day 2, males that had mated with a female ('mated males') were placed in small cups (diameter 10 cm, height 8 cm, volume 30 ml) filled with well-aerated water at 15 ± 1 °C with a new receptive female which was sampled in amplexus on Day 1 ($N = 56$ pairs isolated). Four hours later, we noted whether the mated males had initiated a new amplexus or not. Since we wanted to evaluate male mating propensity after sperm depletion over a short period, we did not add alternative choices such as food. All males were then measured and dissected and sperm in their testis were counted. To evaluate

the female time left to moult (TLM), females under test were placed with the original male with which they had been caught in the field and kept until their moult (therefore until mating). After fertilization, females were measured (body height at the level of the base of the fourth coxal plate).

Sperm and Energy Reserves and Male Competitive Ability

To examine whether sperm depletion alters male competitive capacity, 64 pairs of male (one 'mated male' and one 'unpaired male') matched for size ('mated male': $N = 64$, mean length \pm SE = 2.65 ± 0.02 mm; 'unpaired male': $N = 64$, 2.68 ± 0.03 mm; Student's t test: $t_{126} = 0.451$, $P = 0.65$) were maintained in small cups (diameter 10 cm, height 8 cm, volume 30 ml) filled with well-aerated water at 15 ± 1 °C. They were acclimated to the cups for 1 h before the introduction of a receptive female. All females used in the experiments had previously been caught in amplexus to control for their receptivity. For identification, one male was marked by a small spot of white typographic correction fluid (Tipp-Ex) applied with a tiny brush on the male's cuticle. The marking was alternately made on the mated male and on the unpaired male. Four hours after adding the female, we observed whether an amplexus had occurred, and we noted which male was involved in this pairing. Sixty-four trios were tested. Among them, 24 were used to determine the influence of level of sperm storage on male competitive ability. The remaining 40 trios were used to compare the lipid and sugar contents of mated and unpaired males, allowing us to estimate the influence of energy reserves on their competitive ability.

Both lipid and sugar (glucose) contents were quantified using a colorimetric assay following a modified protocol from [Rivero & Fergusson \(2003\)](#). Gammarids were crushed using a micro-centrifuge tube pestle into 100 μ l of a solution of 2% sodium sulphate and 750 μ l of a chloroform/methanol (1/2) solution. Samples were centrifuged (4 °C, 4000 rpm, 4 min) and two fractions of the supernatant (350 μ l each) were transferred into clean culture tubes for lipid and sugar analyses, respectively. Samples were placed into a water bath at 95 °C to enable solvent to evaporate. For lipid determination, 40 μ l of sulphuric acid was added and samples were reheated for 2 min at 95 °C and then 960 μ l of vanillin-phosphoric acid reagent ([Rivero & Fergusson 2003](#)) was added. Samples were vortexed and 150 μ l transferred into a 96-microplate well. The optical density was read at 525 nm in a microplate reader (Versamax, Molecular Devices Corporation, Sunnyvale, CA, U.S.A.) and analysed using the software SOFT-MaxPro 4.0 (Molecular Devices Corporation). Lipid concentration was obtained from a standard curve ranging from 0.5 to 32 μ g/ml of a commercial vegetable oil. For sugar determination, residues after evaporation were heated for 15 min at 95 °C with 1000 μ l of anthrone reagent ([Rivero & Fergusson 2003](#)). After cooling, 150 μ l were transferred into a 96-microplate well and optical density was read at 625 nm. Sugar concentration was obtained from a standard curve ranging from 3 to 200 μ g/ml of a glucose solution. Both sugar and lipid contents were corrected for our measure of male size prior to analysis, by analysing the residuals of their correlation with size, as described in [Plaistow et al. \(2003\)](#).

Statistical Analysis

For analyses we used JMP 5.0 software (SAS Institute Inc, Cary, NC, U.S.A.). The normality of the distribution was tested by Shapiro–Wilk tests and variance homoscedasticity by Bartlett tests. When possible, the sperm counting distribution was normalized by a Box–Cox transformation ([Sokal & Rohlf 1995](#)). Otherwise, nonparametric tests were used.

RESULTS

Sperm Depletion and Replenishment

Sperm reserves (Box–Cox transformed) were significantly affected by male pairing status (ANOVA: $F_{2,74} = 27.01$, $P < 0.0001$; [Fig. 1](#)), but not by male size ($F_{1,74} = 0.08$, $P = 0.78$) or by the interaction between male status and size ($F_{2,74} = 0.06$, $P = 0.94$; Global model: $F_{5,74} = 11.81$, $P < 0.0001$, $R^2 = 0.46$). Post hoc comparisons showed that mated males had far fewer sperm than unpaired or paired males, but also that paired males had more sperm than unpaired ones (Fisher's highly significant difference post hoc tests; [Fig. 1](#)). During the 6 days following copulation, sperm number increased significantly with time after fertilization ($F_{3,94} = 37.20$, $P < 0.0001$) and also tended to be higher in larger males ($F_{1,94} = 4.05$, $P = 0.047$; [Fig. 2](#)). Nevertheless, it was not affected by the size of the fertilized female ($F_{1,94} = 0.82$, $P = 0.37$). All interactions were nonsignificant and were removed from the model (global model: $F_{5,94} = 25.58$, $P < 0.0001$, $R^2 = 0.55$). Similar results were obtained when female size was replaced by the number of eggs in the model (analysis not shown).

Sperm Reserves and Male Pairing

Among the 56 pairs isolated in this experiment, 24 males entered into amplexus after 4 h (42.8%). Logistic regression showed that sperm number had no effect on male pairing inclination (Wald $\chi^2_1 = 0.07$, $P = 0.79$; [Fig. 3a](#)), and that only male size (Wald $\chi^2_1 = 6.90$, $P = 0.009$; [Fig. 3b](#)) and female time left to moult (TLM; Wald $\chi^2_1 = 5.11$, $P = 0.02$; [Fig. 3c](#)) had a significant effect (global model, likelihood ratio: $\chi^2_3 = 14.33$, $P = 0.002$). Thus, males in amplexus were larger than those remaining unpaired, and TLM was significantly shorter for paired females than for unpaired females. Female size and all interactions were nonsignificant and were removed from the model (backward elimination procedure). Among the pairs formed during this experiment, we found a significant positive correlation between male size and female TLM ($r_{21} = 0.41$, $P = 0.04$), so that larger males were more inclined to pair with females furthest from their moult. No correlation was found between female TLM and female size ($r_{21} = 0.25$, $P = 0.25$).

Sperm and Energy Reserves and Male Competitive Ability

Among the 64 trios set up in this experiment, 40 males initiated an amplexus (62.5%). The pairing success of males was mainly influenced by their previous mating status. Among the 40 trials

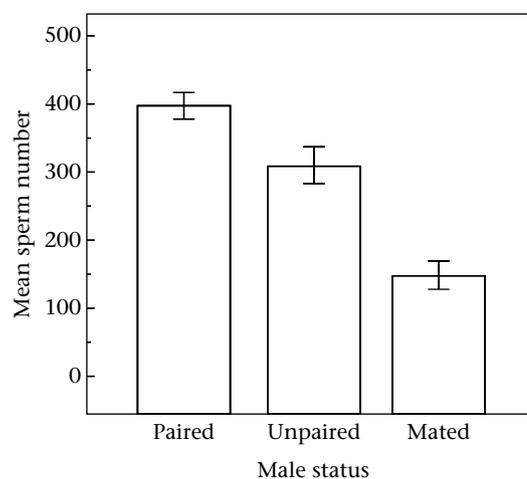


Figure 1. Mean \pm SD sperm numbers for the three groups of male ($N = 25$ in each).

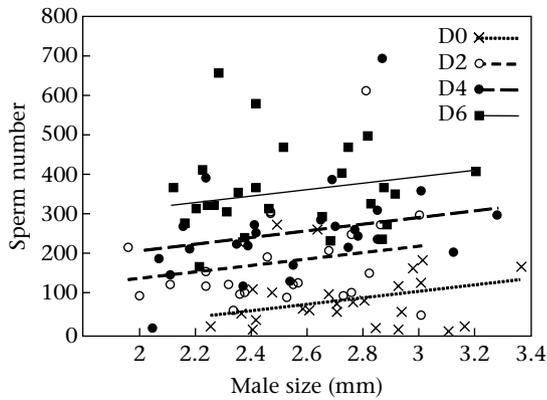


Figure 2. Relationship between number of sperm and male length, according to the time passed after a copulation event (D0: the same day as the copulation; D2–D6: 2–6 days after copulation).

where one male was paired at the end of the experiment, mated males entered precopula more often than unpaired males (binomial test: $P = 0.006$). No effect of Tipp-Ex marking was detected on the probability of pairing (binomial test: $P = 0.15$). The relative size of males did not influence this pairing pattern, since there were no males of a given category ('mated male' and 'unpaired male') that were larger than males of the other category, either in trios where no amplexus was found (Wilcoxon signed-ranks test: $Z = 19.5$, $N = 23$, $P = 0.56$) or in trios where amplexus was found ($Z = 20.2$, $N = 40$, $P = 0.79$). Female size also had no influence; there was no significant difference in size between paired and unpaired females ($t_{61} = 1.25$, $P = 0.21$).

We analysed testis contents in relation to competitive ability for 24 trios. Since the sample of animals was collected later in the season than for all previous experiments (August instead of January–March), we again compared sperm number between mated and unpaired males (Fig. 4). Contrary to previous results, the difference in sperm number between these two groups was not significant (ANOVA on Box–Cox-transformed data: $F_{1,47} = 0.06$, $P = 0.80$). More specifically, unpaired males appeared to have fewer sperm than in winter samples (Figs. 1, 4). Only 14 of these males entered into amplexus. Males paired at the end of the experiment did not have more sperm than unpaired males (Wilcoxon signed-ranks test: $Z = -22.50$, $N = 14$, $P = 0.17$).

For the analysis of the effect of energy reserves on competitive ability we used the 40 remaining trios. Male status (mated or unpaired in the field) had no significant effect on lipid content corrected for size (ANOVA: $F_{1,77} = 0.32$, $P = 0.57$) or sugar content ($F_{1,77} = 0.37$, $P = 0.55$). When considering only the trios where one male initiated an amplexus ($N = 26$), we found no effect of sugar content on competitive ability (Wilcoxon signed-ranks test: $Z = 15.50$, $N = 26$, $P = 0.70$). However, paired males had lower lipid reserves than their unpaired competitors ($Z = -80.50$, $N = 25$, $P = 0.03$).

DISCUSSION

This study provides evidence that *G. pulex* males invest a large quantity of sperm in each reproductive event relative to their sperm reserve (approximately 50% of the reserve) which is common in crustaceans (e.g. Kendall et al. 2001). In *G. pulex* the precise fertilization site remains unclear. It probably lies in the semiclosed incubating pouch (Borowsky 1991), in consequence diluting the number of sperm invested in the copulation. This dilution effect may be compensated for by the large allocation of sperm (Levitan 1998). The risk of sperm competition, reported in *G. pulex* by Birkhead & Pringle (1986), may also explain the large investment in sperm

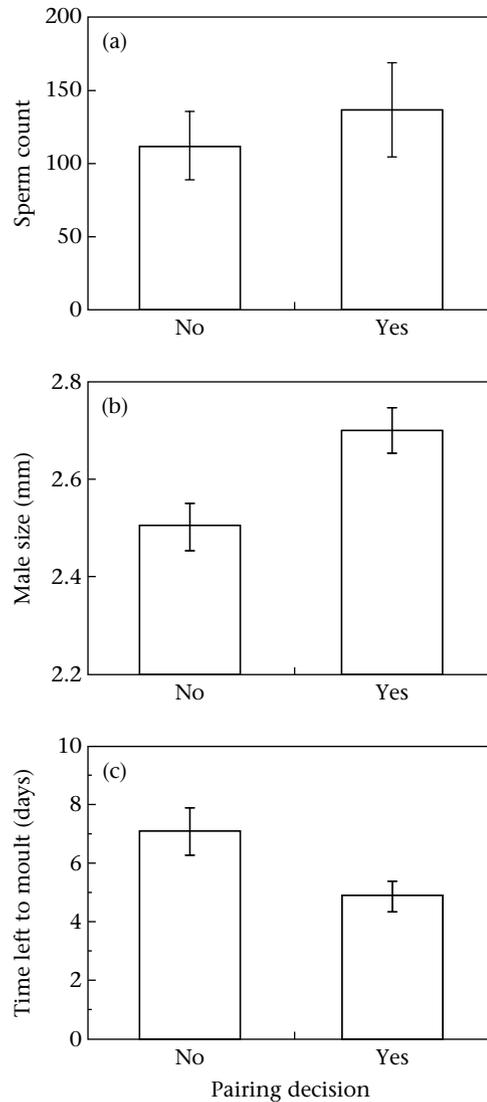


Figure 3. Comparison of (a) sperm count, (b) male size and (c) female time to moult between pairs where the male initiated a re-pairing or not, 4 h after a previous mating (male pairing inclination experiment). Means are shown \pm SD.

(Parker et al. 1997; Parker 1998). This picture is surprising for a species where precopulatory mate guarding is thought to have evolved to limit postfertilization competition between males (Jormalainen 1998; Zimmer 2001), but more investigations are needed to assess the possibility that, after one mating event and the separation of mating partners, a second male can mate with the female. An apparent contradiction to the high relative sperm investment is the limited number of sperm a *G. pulex* male has in its gonads. From our counting, a rough estimate of the absolute sperm number gives average values around 10 000 gametes per testis (average counting in paired males is around 400 sperm for 40 μ l sampled in a solution of 1000 μ l; Fig. 1). This value of 'total sperm reserve' in *G. pulex* is in the same range as that described by Dunn et al. (2006) in *G. duebeni*. These estimates are between 5- and 15-fold lower than the ejaculate of *Armadillidium vulgare* (values from Rigaud & Moreau 2004), a crustacean isopod with internal fertilization where sperm competition is present (Moreau et al. 2002). Clearly, more investigations are needed to understand the evolutionary pattern that led to a generally low sperm production associated with a high investment per copulation in gammarids. These investigations should also consider the fluctuation in the rate of spermatogenesis which can occur during the course of the *G. pulex* reproductive season. Indeed,

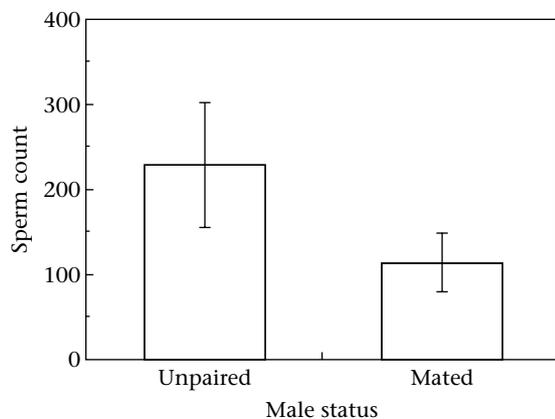


Figure 4. Mean \pm SD sperm numbers in unpaired and mated males during the male competition experiment. For each group $N = 14$.

it is well established that in aquatic species spermatogenesis increases with photoperiod and water temperature (Borg 1982; Fraïlle et al. 1994).

Having established this pattern of sperm depletion, we wanted to test its impact on pairing ability and the potential consequences for the OSR. Despite the substantial investment of sperm by males in each reproductive event, we found no effect of sperm depletion on the male's pairing decision. Consistent with some previous observations, our results suggest that *G. pulex* males take their pairing decision on the basis of their own size and time left to the female's moult (Dick & Elwood 1989, 1990), rather than on the basis of their own sperm level. A probable explanation of the absence of any sperm depletion effect is to be found in the dynamics of sperm replenishment. Indeed, our results revealed that 6 days after fertilization, males reacquire a level of sperm similar to that of males found in amplexus in the field. Because mean precopula duration in wild populations or laboratory experiments exceeds 6 days (14.1 days: Elwood et al. 1987; 12.2 days: Elwood & Dick 1990), even if males engage in a new amplexus after a first copulation, they therefore have enough time to recover their sperm level before a second copulation.

Consequently sperm depletion has no effect on the reproductive 'time-out' of *G. pulex* males. Another aim of our study was to investigate the consequence of sperm depletion for male competitive abilities. Our results did not support the hypothesis that sperm level may explain the outcome of male competition for pairing. This conclusion confirms our previous result that sperm depletion has no effect on mating inclination. In addition, higher levels of sugar or lipid reserves did not explain the success of male–male competition for pairing. We even found that the males that won in pairing competitions were those with lower lipid contents. This contrasts somewhat with the results of Plaistow et al. (2003), who showed that males paired in the field on average have higher lipid and glycogen levels than unpaired ones. Perhaps the difference between these results comes from the fact that we analysed only the pairing decision in the present study. It is possible that, in the wild, only males with high energy reserves can sustain the amplexus for a time sufficient to remain paired until copulation, explaining the discrepancies between our results and those of Plaistow et al. (2003).

The only parameter that seemed to confer an advantage in terms of pairing success was the males' immediate previous mating experience, since we found that previously mated males had a higher pairing success than previously unpaired males. We can formulate two hypotheses to explain this result. First, according to Bollache & Cézilly (2004b), unpaired males used in our experiment could be close to the moult, a short period of the male reproductive cycle where a male is unable to copulate and could therefore refrain

from engaging in a precopula. Nevertheless, no male moult was observed during our experiment, which tends to suggest that this explanation is unlikely. Second, as neither sperm levels nor energy reserves are parameters conferring an advantage in competition, there could be differences in female resistance according to male quality (Jormalainen 1998). Nevertheless, if female resistance occurs, it can modulate male mate choice; our results suggest that this assessment of male quality is not based on sperm level, male size or energy reserves (as suggested in Sparkes et al. 2002) but may be based on inherited (unidentified) male qualities which could give males an advantage in terms of pairing success (e.g. good genes hypothesis, Arnold 1983). Clearly, further studies should investigate which factors affect male pairing and mating success, because of its importance for the OSR and male–male competition. If some males of 'high quality' (whatever the criterion for quality) have a competitive advantage in pairing, as suggested by our experimental results, this will generate a skew in male mating success. The apparent OSR may therefore be much less male biased than previously thought because only this set of 'high-quality' males may be able to compete for reproduction.

In addition, mating costs for males and females increase with the sperm depletion intensity and may interfere with male mate choice in *G. pulex*. Since sperm depletion can be costly because of decreases in female fertility (Dunn et al. 2006), males will not benefit from competing for a large fecund female if they are not able to fertilize all of her eggs. Mating costs are an important parameter which can drive male mate choice (Kokko et al. 2003). For example, differential mate choice between resource-depleted males and nonresource-depleted males is supported by some empirical studies such as that of Byrne & Rice (2006), where resource-depleted male *Drosophila melanogaster* were more stringent in mate choice (based on female size) than males that were nonresource depleted (see also Wong & Jennions 2003).

In natural populations of *G. pulex*, since large males have several advantages compared to smaller ones (Adams & Greenwood 1983; Ward 1984; Dick & Elwood 1989), and since our results suggest that the better competitors when it comes to pairing are most often the same males, strategic sperm allocation across multiple matings should be favoured. For example, to avoid complete sperm depletion, recently mated males or males with high mating rates should invest their sperm reserves more carefully than recently unmated males or males with low mating rates (Wedell et al. 2002; Sato & Goshima 2007). Further investigations should be concentrated on the identification of factors responsible for such a putative variation in sperm allocation.

Acknowledgments

We thank Sébastien Motreuil and Matthias Galipaud for technical assistance. We are grateful to Dr Paula Stockley and Dr Steven Ramm who by their comments improved the quality of the manuscript. We acknowledge Dr Jaco Greeff and two anonymous referees for their helpful comments.

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