Experimental Ecology and Geobotany

Sexual selection, sperm competition and sperm expenditure in 
a freshwater crayfish species

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Sperm competition has generated a variety of behavioural adaptations enhancing the competitive advantage of a male’s sperm or countering the sperm of rivals, as for example the strategic allocation of sperm to multiple copulation and the displacement or removal of sperm previously deposited by rivals. Sperm allocation strategies and sperm competition mechanisms in many taxa with different mating systems have been described, but few similar studies have been ever conducted on crustacean decapods. Astacids offer a good opportunity to study the effects of sperm competition on copulatory behaviour of both sexes and sperm allocation by males, due to their promiscuous breeding system, which includes multiple mating, the absence of mate guarding, an external fertilization and a peculiar modality of sperm competition, involving sperm removal by rivals. Thus, in the present paper, the results of four experiments conducted on a freshwater crayfish species (Austropotamobius italicus) are summarized. The aim of these experiments have been to analyze i) the variation of male sperm expenditure and behaviour of both sexes in relation to female status; ii) the variation of ejaculate size among successive inseminations; iii) the variation of male sperm expenditure and mating behaviour of both sexes in relation to different level of sperm competition risk; iv) the variation of sperm expenditure and sperm removal by last-mating males in relation to copulation behaviour and male secondary sexual traits.

1 Introduction

In many sexually reproducing animals, an important fraction of individual breeding success is determined by post-copulatory processes. With the advance in molecular techniques, it has been shown that the classical modes of sexual selection [1], intermale competition and mate choice by females, continue in the form of sperm competition and of cryptic female choice after copulation has occurred.

Sperm competition, the post-copulatory competition between the sperm of more males to fertilize female eggs during a breeding season [2-4], has generated a variety of behavioural adaptations enhancing the competitive advantage of a male’s sperm or countering the sperm of rivals [5]. Among these adaptations, there are the strategic allocation of sperm to multiple copulation [4], and the displacement or removal of sperm previously deposited by rivals [6]. The energetic costs associated to production of millions of sperm lead to the prediction that males should be selected to modulate their sperm expenditure [7, 8] in relation to: 1) male quality, 2) female fecundity, 3) future mating opportunity, and 4) risk and intensity of sperm competition.

Information on relationships between ejaculate structure and male quality as revealed by secondary sexual traits (SSCs) are scanty. Sheldon’s [9] phenotype-linked fertility insurance hypothesis suggests that females benefit from choosing males with elaborated ornaments if they maximize the chances of mating with fertile males. According to this hypothesis, SSCs would honestly advertise male fertility, and more attractive males should deliver higher-quality ejaculates. Some studies attempted to test this hypothesis in birds and fishes, yielding contradictory results: in some cases, a positive relationship between male phenotype and ejaculate quality has been found [10-14], but in others, no relationship or even a negative correlation emerged [15-17]. In fact, males may face a trade off between investments in sperm production (required for fertilization) and expression of SSCs (required for mate acquisition), since both processes are competitively costly [18].
Concerning female quality, several studies in insects, molluscs and fishes provided evidence that males prefer larger or younger mates, and allocate larger ejaculates to heavier females, which are often more fecund [e.g. 19-21]. Recent models suggest also that males should increase their ejaculate expenditure when experiencing a high risk of sperm competition [8, 22]. For example, the mating status (virgin or mated) of a polyandrous female may alter her reproductive value to a male, and males should be able to assess it and vary their reproductive behaviour accordingly, e.g. by increasing sperm number or displacing rival sperm when mating with a mated female.

Basically, there are two main ESS models to predict optimal sperm expenditure under varying condition of sperm competition [4]. In the “risk” model, a copulating male is predicted to increase his ejaculate expenditure in proportion to the probability that sperm competition occurs with at least one other male [23]. In the “intensity” model, a copulating male is predicted to decrease his ejaculate expenditure as the number of males that he is competing with increases above two, because the expected gain from each unit of ejaculate expenditure declines as the number of competitors rises above two [24]. These models accurately describe sperm allocation strategies in many taxa with different mating systems and sperm competition mechanisms [8], but few similar studies have been ever conducted on crustacean decapods [26-29]. Astacids offer a good opportunity to study the effects of sperm competition on copulatory behaviour of both sexes and sperm allocation by males, due to their promiscuous breeding system, which includes multiple mating, the absence of mate guarding, an external fertilization and a peculiar modality of sperm competition, involving sperm removal by rivals [29].

In *A. italicus* (Faxon, 1914) an astacid crayfish species native to Italy [30], who reproduce once a year in October-November [31], males are larger and possess larger chelae than females, using them to threaten and fight with rivals during male-male conflicts. Also, they use their chelae to secure female position prior to and during copulation. For these reasons male body and chelae size can be the target of sexual selection [32]. Mating consists of brief tactile and olfactory exchanges between partners, after which the male grasps the female by her claws, forcing her to turn on the back. Copulating males release spermatophores, which are attached to the thoracic sternites of females, mainly on a specialised external receptor, the spermatophoric plate [33]. Fertilisation is therefore external, and spawning occurs within days to weeks after mating [34]. This time-lag leaves ample opportunities for multiple mating by both sexes and hence for sperm competition. In fact, *A. italicus* males remove and feed on all or most spermatophores previously deposited on the female spermatophoric plate by other males before releasing their own sperm [29]. However, despite they are able to counter the sperm of rivals prior to transferring their own sperm, males of this species cannot avoid competition with ejaculates of rivals mating subsequently, since they do not have any postcopulatory sperm competition avoidance mechanisms such as mate-guarding, repellents or copulatory plugs.

Previous studies on this crayfish evidenced that female favour small-sized, large-clawed males by spawning larger eggs for them [34]; on the other hand, males are sensitive to female size and produce larger ejaculates when mating with larger females, which are also more fecund [35]. Moreover, ejaculate size increases during the breeding season, which suggests an increased risk of sperm competition or lower chances to obtain further matings at that time [35]. However, ejaculate size covary negatively with male body size (and hence with age), suggesting that large males may experience senescence of their reproductive performance. How males may face sperm competition under this scenario still remains to be clarified, as well as whether male SSCs may honestly reveal their sexual competence.

This extended abstract reports the results of four experimental studies (already published or in press [see references 36-39].

Crayfishes were collected from northern Apennines streams and then held and tested in laboratory in semi-natural conditions in 2005 and 2006. For a detailed description of methods and experimental designs see references [36-39]. The four experiments were aimed at answering the following questions:

Does sperm expenditure by males and behaviour of both sexes vary according to female mating status? [36]
I paired receptive females to two virgin males in sequence to assess: a) whether mated females refuse or resist longer to the second partner and b) if males vary their copulation behaviour and sperm expenditure when mating with a mated female. If multiple mating is an adaptive female strategy and previous mating does not render females refractory to mating, I expected no differences in female resistance between two successive matings. Concerning the second question, since males remove sperm of rivals, I could expect either no differences in sperm expenditure between first and second males, or that second-mating males ejaculate more sperm when mating with a mated female, due the perceived increased risk of sperm competition or the greater probability to sire most offspring in the resulting clutch.

Do males vary their ejaculate size among successive inseminations? [37]

A previous study showed that ejaculate size decreased with increasing male body size while simultaneously increasing with female body size [35]. Thus, large old crayfishes males could be senescent individuals, experiencing a functional decline of their reproductive system or, as an alternative possible explanation, these males may tailor their sperm expenditure to fertilize a greater number of partners than small males, since they are able to gain more mating opportunities in male-male contests. Therefore, I allowed males of varying body size to mate with an ad libitum number of females at 24-h time lag, recording the number of successful matings performed by each male. If sperm reserves were limited, and males could not refill their sperm stores within 24-h, I expected ejaculate size to decrease with order of fertilization. In addition, if large males are either more sperm-limited or do not adopt more efficient sperm allocation tactics than small males, I could also expect ejaculate size either to decrease more rapidly or to vary to the same extent with successive fertilizations among large compared with small males.

Do male sperm expenditure and mating behaviour of both sexes vary with increasing risk of sperm competition? [38]

Since males remove the sperm of previous males before copulating with mated females, it could be hypothesized that first-mating males progressively decrease their sperm expenditure or defer copulation with an increasing number of nearby competitors (or potential partners, by a female point of view). Thus, in this experiment, I paired a male and a female under different levels of sperm competition risk (no male, one male and three males , which were unable to directly interfere with copulation, Fig. 4), in order to assess: a) whether the probability to attain copulation varied with increasing immediate risk of sperm competition; b) if males tailor the size of ejaculates according to the perceived risk; c) whether males or females vary their copulation behaviour in relation to the number of competitors/potential partners. My main predictions were that males should decrease their level of sexual activity in response to an increased immediate risk of sperm competition either by forgo copulation or by decreasing sperm allocation with increasing number of competitors, since first-mating males cannot prevent successive sperm removal.

Do sperm expenditure and sperm removal by last-mating males vary in relation to copulation behaviour and male SSCs? [39]

In this experiment I investigated the variation in sperm removal ability of second-mating males, in order to assess whether it was affected by their copulation behaviour and phenotypic traits. Since most males (70%) do not completely remove previous rivals’ sperm, I hypothesize that males removing only parts of pre-existing spermatophores experience more difficulties during mating, due for example to small or asymmetric claws. Chela asymmetry can result from autotomy, which in this species and in other crustacean decapods is a common anti-predatory tactic that enhance survival chances of individuals [40]. On the other hand, chela asymmetry can negatively affect males’ copulatory ability, since chelae are used by males to position and secure females before and during copulation. Clearly, chela asymmetry may have consequences for sperm removal ability as well. Thus, in this experiment, females were mated to two males in sequence, in order to assess: a) if second-mating males adjusted sperm removal and ejaculate size in relation to first-mating male ejaculate size; b) if copulation behaviour of second-mating males affected their sperm removal ability; c) whether second-mating male traits (body size, chela length and chela asymmetry) co-vary with copulation behaviour, sperm removal and ejaculate size.
2 Results

Does sperm expenditure by males and behaviour of both sexes vary according to female mating status? [36]

Female resistance did not differ between the first and the second mating, nor males refused or took more time to mount a mated female. However, when mating with a mated female, males reached an effective copulation position significantly later. This occurred because second-mating males removed, by eating, all or most spermatophores previously deposited by first males. As removal was often incomplete (on average 77% of previous sperm is removed, Fig. 1), this resulted in a larger amount of sperm being deposited on female ventral parts after the second mating, although second males did not allocate more sperm to mated females than first males did.

Do males vary their ejaculate size among successive fertilizations? [37]

Males fertilized between 0 and 4 females, but most (42.5%) fertilized a single female (Fig. 2). The ejaculate sizes decreased markedly (Fig. 3) with consecutive fertilizations irrespectively of male size, but increased according to female body size. The overall number of females fertilized as well as the total ejaculate size over successive fertilizations decreased with increasing male body size.

Do male sperm expenditure and mating behaviour of both sexes vary with increasing risk of sperm competition? [38]

Male mating behaviour varied significantly with increasing number of rivals, because interactions between focal males and competitors, as well as male refusals to copulate, increased with number of rivals. As a consequence, the probability to reach an effective copulation decreased with increasing number of competitors. However, males released similar amounts of sperm independently of the number of surrounding competitors.

Do sperm expenditure and sperm removal of last-mating males vary in relation to copulation behaviour and male SSC? [39]

Second-mating males did not adjust their ejaculate size in relation to first-mating male ejaculate, nor to the first-mating male’s sperm removed. Moreover, the amount of sperm removed by second-mating males increased with increasing first-mating males ejaculate size, and first-mating male sperm remaining after removal did not correlate with its original ejaculate size. Interestingly, the amount of sperm removed by second-mating males decreased with increasing relative chelae asymmetry, while increasing with male body size. However, second-mating (but not first-mating) asymmetric-clawed males produced larger ejaculates than symmetric-clawed ones (fig. 5). Importantly, the proportion of second-mating male sperm remaining after the two matings did not vary with relative chelae asymmetry nor with body size of second-mating males.

3 Conclusion

*Austropotamobius italicus* has proved to be an excellent model to investigate sexual selection strategies hinged on its peculiar sperm removal strategy.

Males do not increase their sperm expenditure when mating with already mated females, accordingly to the theoretical model provided by Parker [4] to explain the case of equivalent sperm release by two males competing for fertilizing a female. This outcome is predicted to occur when: a) there is a loaded raffle (i.e. a paternity bias toward the sperm-removing male), b) males are aware of their role in the raffle (mating order), c) males are randomly either in the favoured or disfavoured role. Actually, *A. italicus* appears to match all these conditions. Thus, from the male point of view, the best mating strategy to win sperm competition should be not to copulate with a virgin female or increase sperm expenditure when mating with a mated female, but rather to copulate with mated females as near to egg spawning as possible, since males do not attempt to mate with spawning females anymore [41], and to remove as much previously deposited sperm as possible. Importantly, female mated twice received a 30% greater sperm load than females mating only once. If females crayfish are sperm limited, polyandrous females may greatly benefit from second matings.
in terms of egg fertilization success. In fact, my results showed that either sperm production or release involves non-trivial costs in these crayfishes, and suggests that large/old males may face greater difficulties in gamete release than small/young males, as shown by the lower number of females fertilized by large compared with small males, which may reflect the ongoing senescence of their reproductive performance.

Moreover, *A. italicus* males seem unable to tune their ejaculate size in response to short-term variation in the immediate risk of sperm competition, probably either because males spend more time interacting with rivals, or because they actively defer copulation and wait for more rewarding mating opportunities, which occur when the number of competitors decline.

However, when copulating with mated females, *A. italicus* males are able to modulate their ejaculate size according to their sperm removal ability, which covary negatively with chelae asymmetry and positively with body size in this crayfish species. Indeed, small and asymmetric males showed an inferior sperm removal ability compared with large and symmetric ones but, interestingly, they appeared to cope with their handicap by increasing ejaculate size compared to symmetric ones. This could be interpreted as a direct response to sperm competition risk by asymmetric-clawed males, which, in fact, did not increase their ejaculate size compared to symmetric ones when copulating with virgin females. This compensation tactic is likely to confer to these individuals the same success in sperm competition as symmetric-clawed males, thus contributing to the evolutionary maintenance of strategic chelae autotomy.
**Fig. 2:** Frequency (%) of the overall number of fertilizations performed by 40 freshwater crayfish males in successive days (0 = copulation without fertilization).

**Fig. 3:** Variation in ejaculate size (sperm area) among successive fertilizations in freshwater crayfish males (n = 94 trials of 40 individual males) tested on consecutive days. Sperm area of copulations that did not result in sperm transfer was scored as 0, as well as mating trials after the initial one that did not result in copulation. Values are median (bold horizontal lines), interquartile range (boxes) and minimum–maximum values (whiskers). The dot indicates an outlier (value >1.5 times the box length). Numbers inside or above boxes represent the number of trials for each fertilization order.
Fig. 4: Schematic view of the experimental jars used to manipulate crayfish sperm competition risk.

Fig. 5: Relationships between ejaculate size and relative chelae asymmetry by second-mating (filled circles, n=29) or first-mating (open circles, n=65) males. Fitted simple linear regressions are shown (second-mating males, continuous line: sperm area\(=(0.005\pm0.002)\times\text{relative chelae asymmetry}+(0.28\pm0.04), t_{27}=2.49, P=0.019, R^2=0.19\); first-mating males, broken line: sperm area\(=(0.002\pm0.001)\times\text{relative chelae asymmetry}+(0.29\pm0.03), t_{63}=1.52, P=0.13, R^2=0.04\)). The difference between the slopes was statistically significant in a mixed model analysis taking into account repeated matings by the same females.
Acknowledgements  I’m very grateful to all the people who helped me during my PhD years, in particular Paolo Galeotti, Roberto Sacchi, Diego Rubolini, Mauro Fasola and Anna Maria Picco.

References