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MALE MATING SUCCESS DURING PARTURIAL INTERMOULTS IN THE TERRESTRIAL ISOPOD *ARMADILLIDIUM VULGARE* REVEALED BY THE USE OF A MICROSATELLITE LOCUS

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ABSTRACT

We investigated the value of microsatellite DNA markers to improve our knowledge of mating strategy with inference to sperm competition in particular, in the woodlouse *Armadillidium vulgare*. In terrestrial isopods, mature females develop a brood pouch or *marsupium* before egg laying, the pouch being formed by overlapping oöstegites during a special moult called parturial moult. Under laboratory conditions, we show that *Armadillidium vulgare* females are able to mate during parturial intermoults, even in the presence of a physical barrier such as that represented by the ventral marsupial plates. Our results reveal that the contribution of a second male mating with a female between two parturial moults could represent up to 50% of the paternal alleles in the brood (mean = 28% ± 15). This contrasts with data reported in the literature concerning closely related woodlice species, where males are suggested not to be able to mate with the female during the parturial intermoult period.

INTRODUCTION

In terrestrial isopods, the reproductive cycle is closely associated with the female moulting cycle (Mead, 1973, 1976). During the sexual rest period, females undergo a normal moult and grow. With the increase in photoperiod and temperature, ovarian maturation begins (Mocquard et al., 1989). Female receptivity in woodlice is higher during the stage where oöcyte maturation is nearly over (Moreau and Rigaud, 2000, 2002), whereas males can mate at any time except during moulting (Moreau and Rigaud, 2000). Fertilisation is internal and isopod females possess two genital apertures each independently linked to one ovary by an oviduct. Males have to perform one insemination in each oviduct in order to fertilize the eggs originating from both ovaries. Sperm is deposited within the oviduct in the form of a large white ball. Sperm is not mobile in woodlice (Hollande and Fain-Maurel, 1965) and is stored in the oviduct until oöcytes are deposited. As far as is known, oöcytes are fertilised when passing through the balls of sperm. After laying, remaining spermatozoon are stored in the spermatheca at the junction between the oviduct and the ovary and will be used to fertilise the next broods (Warburg, 1993). Eggs and embryos are incubated in a marsupium (ventral pouch limited by lamellar structures) that differentiates during the special moult called "parturial moult". Manca emerge from the pouch about five weeks after laying. The marsupium remains until the next moult. This moult generally occurs approximately two weeks later. During this stage, most females cannot mate due to the physical barrier of ventral marsupial plates (Moreau and Rigaud, 2000). The moult following the release of young can be a second parturial moult (eggs will be fertilized by sperm reserve), or a normal moult (growth moult), depending on environmental conditions, such as photoperiod and temperature (Mocquard et al., 1989).

The recent development of very polymorphic molecular markers such as microsatellites in *A. vulgare* (Verne et al., 2006) has provided the opportunity to determine if males are able to mate with females between two parturial moults, and if their sperm can fertilize the eggs. Microsatellites are codominant markers, highly reproductible, and inherited in a Mendelian fashion, which makes them ideal tools to understand mating strategies and to test hypotheses regarding mating behaviour and reproductive systems (Queller et al., 1993).

MATERIALS AND METHODS

Strain Maintenance

The *A. vulgare* individuals used were derived from strains that have been maintained under the same laboratory conditions for many years on moistened soil at 20°C and at the natural photoperiod of Poitiers (latitude 46°40′N). See Moreau et al. (2001) for routine procedure for strain maintenance. For each generation, males and females from the same brood are sorted by sex before they reach sexual maturity. They are then reared separately, thus ensuring that all females are virgin.

Breeding Procedures

At the time of the experiment, females and males were one-year old. To obtain virgin females receptive to mating, females were reared under LD 18:6 photoperiod, which stimulates the onset of reproduction (Mocquard et al., 1989). Female receptivity was assessed by checking the shape of the white plates of calcium carbonate on the ventral surface that differentiate a few days before moulting. Receptive females show incomplete plates whereas their non-receptive counterparts exhibit complete plates (Moreau and Rigaud, 2002). Only females receptive to mating were used.

Each of 15 receptive females was coupled with one male (Origin from Nice, South of France) of approximately equal size (random asymmetry) to avoid the occurrence of reproductive physical incompatibility, and each

Table 1. Parental Allele composition using microsatellite locus Av7 are shown. The allele sizes are given in bp for the locus. For maternal and paternal alleles in offsprings, the numbers of observations are given in parentheses.

Crossing	Mother	Father 1	Father 2	Offsprings
1	286 288	290 290	304 318	286 (9), 288 (11), 290 (10), 304 (4), 318 (6)
2	288 298	290 290	304 318	288 (8), 298 (12), 290 (19), 318 (1)
3	288 298	290 290	304 318	288 (9), 298 (11), 290 (13), 304 (4), 318 (3)
4	286 288	290 290	304 318	286 (12), 288 (8), 290 (15), 304 (2), 318 (3)
5	286 288	290 290	258 304	286 (10), 288 (10), 290 (17), 258 (2), 304 (1)
6	286 298	290 292	304 304	286 (9), 288 (11), 290 (11), 292 (9), 304 (4)
7	288 288	290 290	304 318	288 (20), 290 (16), 304 (2), 318 (2)
8	286 288	290 290	318 318	286 (11), 288 (9), 290 (12), 318 (8)

mating couple was placed in large a box (25 cm) filled with moistened soil at 20°C under natural photoperiodic conditions. Once females had begun to undergo a parturial moult, the male was removed. Females were isolated immediately after their parturial moult in small boxes and maintained under the stimulating photoperiod LD 18:6, again to maintain female reproduction. During this intermoult period, just after the release of offspring, females were coupled with a second male of different origin from that of the first male (Poitiers, West of France), as previously described. The females that underwent a normal moult (as assessed by checking calcium carbonate plates, Moreau and Rigaud, 2002) were removed from the experiment. Females undergoing a second parturial moult were immediately transferred to small boxes in order to isolate and subsequently dissect the young to determine paternity.

Genetic Analysis

Twenty individuals from each brood resulting from the second parturial moult were analysed. DNA was obtained by standard phenol extraction and ethanol precipitation (Kocher et al., 1989) from the muscles, nerve tissue, and gonads of each individual.

The most polymorphic microsatellite, Av7, recently identified by Verne et al. (2006) in *A. vulgare*, was used in the present study to assess paternity. Amplification conditions are detailed in Verne et al. (2006). The genotype of each of the 20 offspring per brood was compared to both the mother's and both putative father's genotypes to identify the maternal and paternal alleles for each locus. The contribution of each father in the siring of offspring was subsequently determined.

RESULTS

Among the 15 females used in this experiment, 8 females underwent a second parturial moult. 5 females underwent a normal moult and were discarded. 2 females died during the experiment. Table 1 gives the allelic composition of both each parent and each offspring.

Of the 8 females that underwent two parturial moults, the second male always participated in the production of offspring. Table 2 gives the relative contribution of each father to the offspring. The contribution of the second male to the offspring, expressed as a percentage, ranges from 5% to 50% (mean = 28% ± 15).

DISCUSSION

In this study, we successfully used a polymorphic microsatellite marker to study the existence of multiple paternity in *A. vulgare*.

Table 2. Male contribution to offspring born from the second parturial moult, expressed as a percentage. Results obtained by genotyping with the microsatellite locus Av7.

	Number of juveniles	Male 1	Male 2
Female 1	20	50	50
Female 2	20	95	5
Female 3	20	65	35
Female 4	20	75	25
Female 5	20	65	35
Female 6	20	85	15
Female 7	20	80	20
Female 8	20	60	40
Mean		72 ± 15	28 ± 15

Firstly, the results reveal that, in *A. vulgare*, males are able to mate with females during parturial intermoults (when oöstegites mask the genital apertures). This confirms the results obtained in the closely related species, *A. nasatum* (Moreau and Rigaud, 2000). This mating possibility is not universal among woodlice due to the presence of a marsupium, which acts as physical barrier to reproduction. Indeed, in other species (*Oniscus asellus*, *Philoscia muscorum*) receptive females with an empty marsupium (after the release of young) are not able to copulate during parturial intermoult (Moreau and Rigaud, 2000).

Secondly, both stored sperm (from the first male) and freshly ejaculated sperm (from the second male) were used by the females to sire their offspring, although the two males did not exhibit the same fertilization success. This confirms previous findings obtained using an albino mutation in this species (Moreau et al., 2002). In this last study, the first male (stored sperm) sired a mean of 72% (± 15) of the female offspring. This fertilization success, however, was not proportional to the number of sperm present. Indeed, the counting of sperm stored in the reserve revealed the presence of very few sperm in comparison with the number observed in fresh ejaculates (Moreau et al., 2002). It is suggested here that sperm position may play an important role in sperm precedence. If this hypothesis is correct, and if indeed fertilization occurs when oöcytes pass through the sperm, then the sperm encountered first may gain a slight advantage. This hypothesis could explain the 'competitiveness' of the stored sperm despite their low numbers, as stored sperm will be encountered by the eggs first during egg laying. Interestingly, the value of sperm precedence observed in the present study differs slightly from that obtained previously (70% here *versus* 50%, Moreau et al., 2002). However, the protocol used in both studies differed slightly. In Moreau et al. (2002), the competition between freshly ejaculated and stored sperm was the same, but the stored sperm was obtained after a period of sexual rest, i.e., remating occurred during two different receptive intermoults. Therefore, the stored sperm was 4 months old. In our study, the stored sperm was younger (only 1 month old) since it was obtained after two successive parturial moults, i.e., remating occurred during the same receptive intermoult. It can therefore be suggested that the duration of storage can affect the viability of spermatozoa under controlled conditions. Further experiments are needed to confirm this hypothesis.

An alternative explanation concerning the difference in pattern of sperm precedence is that the second male encounters difficulties when attempting to inseminate females with an empty marsupium. Indeed, the presence of oöstegites in front of the genital apertures in receptive females makes mating more difficult. As a result, the second male may deposit less sperm in the genital duct than a male mating with a female without marsupium. A sperm count is necessary to answer this question.

In this study, 100% of the males inseminated a female. This result is higher than has been observed in *Armadillidium nasatum*, where only 41% of males inseminated females with an empty marsupium while 90% of males inseminate a female without a marsupium.

This work reveals that the microsatellites isolated from *A. vulgare* may reveal themselves to be very useful in the study of sexual selection intensity and sperm competition in this species. This species harbours an alpha-proteobacteria, *Wolbachia*, which induces the feminization of genetic males, termed neo-females. Recently, Moreau et al. (2001) reported that males had a preference for real females during mating. Further investigations will be undertaken to investigate paternity patterns in both the real and neo-females.

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