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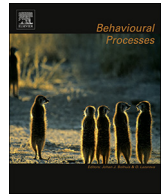
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# Mass drives mating success in *Armadillidium vulgare* (Crustacea, Oniscidea)

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## ABSTRACT

In the terrestrial crustacean *Armadillidium vulgare*, a large size range exists in natural populations within which males and females could potentially mate. Because of continuous growth far beyond sexual maturity, the largest individuals can be nearly ten times the live mass of the smallest sexually mature individuals. In this study, we explored the influence of male and female body mass on the mating behaviour and success. Starting with a representative panel of males and females in which females are significantly larger than males in average, we followed the sexual behaviour of 23 groups of 20 mixed-sex virgin animals under conditions comparable with natural field situation during the early breeding season. We found a correlation between paired individuals showing an assortative pairing. During pairing male stimulates female and duration of stimulation is determinant for pairing follow-up: efficient stimulation is correlated with female size and not with male size. In consequence, pairs in mating show a reversed size dimorphism between male and female where female are about 20% smaller. Largest females were not mated. During copulation behaviour, the quantity of sperm transferred is positively correlated with copulation duration. Stored sperm can be used for immediate breeding by the female and stored in the spermatheca for future breeding. The last option allows to largest females in the field to continue breeding without additional mating, avoiding the lack of availability of large males able to stimulate them efficiently.

## 1. Introduction

Mate choice is now widely accepted to be a fundamental selective force in driving the evolution of sexual characters in the opposite sex, but, in many species, the factors contributing to individual variation in the partner choice decision are still poorly understood (Andersson, 1994; Arnqvist and Rowe, 2005). Theoretical framework shows that the relative body size of a mate is a crucial component in the resolution of these mating conflicts (Pomiankowski and Møller, 1995; Wilcockson et al., 1995; Dillen et al., 2010). Males that are larger than females may use their size advantage to harass or hold females and to force copulation (Smuts and Smuts, 1993; Clutton-Brock and Parker, 1995). Conversely, in arthropods when females are larger than males, they may successfully resist male copulation attempts and, to some extent, govern the mating process (Elgar, 1991; Eberhard, 1996; Gavrilets et al., 2001).

Continuous growth in conjunction with sexual size dimorphism (SSD) is particularly common among entomostracan crustaceans.

Within this group, gammarids have been the subject of numerous experimental and theoretical investigations concerning pre-copulatory guarding decisions and size-assortative pairings (Jormalainen, 1998; Bollache and Cézilly, 2004; Franceschi et al., 2010). However, in gammarid species, the largest females often remain smaller than the smallest males due to pre-copulatory guarding behaviour of males. An overlap of individual size range with the extent of the SSD is more often encountered among isopods (Jormalainen, 1998). Size difference between males and females of the same age in terrestrial isopods is the result of resource allocation during reproduction impacting female growth (Caubet, 1998). In the terrestrial isopod *Oniscus asellus*, size affects the probability of a female being courted, but her mating success is independent of female and male size (Stange et al., 2008).

In the present study, we focused on the mate choice of *Armadillidium vulgare* (Latreille, 1804) (Isopoda, Oniscidea). In natural population of this species, female size is nearly 20% higher than male size, though mean values strongly vary across populations and within seasons (Vandel, 1962). Both males and females continue to grow through

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moulting cycles, even after sexual maturity (which may take place within the year of birth), with a close link between female moulting and reproductive cycles (Steel, 1980). Breeding events occur for both males and females of this iteroparous species throughout their entire reproductive lifespan during up to four years (see Lawlor, 1976; Stearns, 1989; Dangerfield and Hassal, 1992; Caubet, 1998), including one or more broods within a season (Hornung, 2011). In terrestrial isopods, the existence of sperm storage organs, in the spermathecae, allow females to keep the sperm of previous copulations in their genital tracts and to use the sperm months, or even years, later to fertilize their oocytes (see Zimmer, 2001, for a review). In natural populations, reproductive animals thus belong to different cohorts (i.e., more or less synchronously born broods), and the largest animals can be nearly ten times of the size of the smallest sexually mature ones (Brody et al., 1983). Thus, a large size variation exists in males and females that may potentially pair and mate. We predicted positive assortative mating since body size in oniscideans closely correlates with the estimates of reproductive value in both males and females (Lawlor, 1976; Sutton et al., 1984).

In *A. vulgare*, body size is expected to be a key determinant in the mating outcome. In females, egg production increases almost linearly with body size, and large size implies a high fertility potential (Sutton et al., 1984; Caubet, 1998; Warburg, 2012). In males, size steadily increases with age (no parental care as observed in females); therefore, large size means at least high survival potential (Lawlor, 1976). This species thus provides an interesting model to investigate the respective influence of male and female sizes on mating probabilities. Moreover, females can roll up into an almost perfect ball (i.e., perform volvation, during which genital apertures are physically obstructed), to eventually exert control over the mating decision, as a means of direct rejection (see Mead, 1973).

Crustacean females possess two genital apertures (each associated with a corresponding oviduct and ovary), and during the most typical sexual sequence, two mating events (i.e., one for each genital aperture) are separated by a short pairing posture (Suzuki and Ziegler, 2014). During mating, the female is motionless and incompletely rolled up (acceptance position, allowing the intromission of male's copulatory organ into female's genital apertures during two successive hemi-copulations (Mead, 1973): when the male is on the upper left side of the female, insemination occurs at the right female genital aperture and reciprocally). Alternatively, the sexual sequence described above may be stopped at almost any time, most often by the female. The female may indeed react to any male sexual initiative with a variety of typical rejection behaviours (complete rolling, running escapement or jerky body movements; see Mead, 1973; Lefebvre and Caubet, 1999, 2010; Moreau et al., 2001).

In this study, we report observational data on the sexual interactions between individuals belonging to different cohorts (individuals born at the same period) before investigating (through behaviour) the relative importance of male and female size on mating patterns. In particular, we i) investigated pairing and mating probabilities according to male and female size; ii) explored the role of partners' size to test assortative mating, among other key reproductive parameters, such as time and sperm investment; and iii) analysed the impact of pairing duration between individuals engaged in pairing and then in mating to test female resistance.

As fecundity is higher for large females, we expect males to prefer and invest more sperm in larger females and as a consequence to observe assortative mating in population with individual size variation. By comparing pairing duration followed or not by mating we will evaluate gender resistance behaviour and identify the choosy sex. We expect older and larger females to be more choosy as under natural condition they are more likely to be previously inseminated. So we expect to observe a positive correlation between pairing duration followed by mating when female size increases.

## 2. Methods

### 2.1. Animals and general experimental conditions

Specimens of *A. vulgare* derived from a strain collected in France (Nice) and were maintained under laboratory conditions (moistened soil with dry leaves and fresh carrots provided *ad libitum*, a temperature of 20 °C, the natural photoperiod of Poitiers, at a latitude of 46°40'N). The strain proved to be free of *Wolbachia*, an endosymbiotic bacterium that could eventually interfere with the individual mating decision of both males and females (Bouchon et al., 2008; Moreau et al., 2001; Richard, 2017; Beltran-Bech and Richard, 2014). Each spring, as a routine laboratory procedure, randomly chosen adult females were mated with one male, and the mated individuals were kept together until the release of offspring. The offspring were immediately separated from their parents at that time. Young males and females were then reared separately in unisex boxes (l = 26 cm, w = 13 cm, h = 8 cm) until reaching sexual maturity. This experiment used different clutches to span the range of male and female sizes found in the field during the early breeding season (see Caubet, 1998; Sutton et al., 1984; and references therein). The oldest experimental animals had completed their third year, whereas the youngest ones were still in their first year. For the youngest, sexual maturity was assumed for a body length over 7.0 mm and an age of 4 months (see Paris and Pitelka, 1962). Thus, all the animals used in this study were virgin, sexually mature and shared the same mating history, independently of their clutch of origin.

At least 5 days before the experiment, the test males were individually isolated in new small boxes ( $\emptyset$  = 8 cm, h = 5 cm, area = 50 cm<sup>2</sup>, same rearing conditions as above) to increase sexual motivation (Lefebvre et al., 2000). At the start of the experiment, males were checked to ensure the integrity of their external copulatory organs (endopodites of the first and second pleopods) and females were checked for sexual receptivity (active phase of the intermoult ie C period) (see Drach, 1939; Suzuki and Ziegler, 2014). The relationship between the moulting status and mating capacity in *A. vulgare* showed that the sexually receptive period occurs during the secondary vitellogenesis (Caubet et al., 1998; Lefebvre and Caubet, 1999, 2010; Verne et al., 2007; Beauché and Richard, 2013). At the time of the experiment, all the animals were thus in "time-in" (Clutton-Brock and Parker, 1992; Kvarnemo and Ahnesjö, 1996).

The males and females were weighed (extended body mass to the nearest mg), just before the experiment. Mass quite closely reflects the structural body size of animals and shows a strong correlation with female fecundity in terrestrial isopods (see Sutton et al., 1984).

### 2.2. Experimental protocol

Observations were performed in a transparent plastic box (l = 17 cm, w = 14 cm, h = 5 cm) filled with moistened soil and topped with a transparent glass slide to limit air disturbance. To mimic natural conditions, the physical parameters were 80–90% relative humidity, a constant light intensity of 50 lx, and a temperature of 20 °C (see Mead, 1973; Moreau et al., 2001).

In the behavioural encounter tests, the operational sex ratio was set at unity (i.e., an equal number of 'time-in' males and females), which approximately corresponds to the situation observed in natural populations for this species (see Moreau and Rigaud, 2000; Lefebvre and Caubet, 2010). Due to a limited number of time-in individuals at any given moment in the rearing boxes, the encounter tests were performed with 10 males and 10 females and repeated 23 times, with the replacement of all individuals each time. In each replicate, individuals were selected along a large mass range of potential mates, with nearly half the number of males and females belonging to the medium mass classes (to verify the normality assumption).

Ten males were placed in the middle of the arena, immediately followed by the introduction of 10 females, which constituted the  $t_0$

time of the behavioural test. Observations were conducted over one hour, but the animals engaged in pairing or mating were followed until the completion of the sexual sequence and their duration were recorded. These sexual behaviours were categorized and defined as follows:

- i) pairing: interactive sequence starting when a male mounts on a female's dorsal surface and adopts a stereotyped behaviour: its pereopods grasp on the female, and its second antennae curve forward and stimulate the anterior body part of the female (also see Mead, 1973) and finishing either via the assumption of a mating posture by the animals (see below) or via their separation. This pairing behaviour contributes to female stimulation. The total duration spent in the pairing posture(s) is hereafter referred to as the pairing duration.
- ii) mating: interactive sequence starting with a posture in which the male and the female are physically imbricated with their ventral body surfaces in contact, partially overlapping to allow the penetration of male copulatory organ into one of the female genital apertures, while the male performs up and down movements (true copulation process with insemination, see Mead, 1973). The mating sequence finishes either with another pairing posture (see above) or with the separation of the animals.

The complete sexual sequence between paired sexual partners may be described as follows: pairing #1, mating #1, pairing #2, mating #2 and the duration of each part of the sequence will be recorded and analysed separately. The sexual sequence described above may be stopped at almost any time, most often by the female. The total duration in the mating posture(s) is hereafter referred to as the mating time. Mating will be then confirmed if female dissections (at the end of the experiment) revealed traces of sperm in the corresponding oviduct(s) and/or spermatheca(s) (true copulation and not pseudo-copulation without the transfer of sperm).

When paired sexual partners separated before mating, both the male and the female were weighted and immediately returned to the middle of their observation box. When sexual partners separated after mating (either mating #1 or #2), the male and the female were removed from the experiment and weighted. The female was later dissected to check for the presence of sperm in its oviducts. On this occasion, the diameter of 5 mature oocytes was measured using an eyepiece micrometre (extended diameter to the nearest  $\mu\text{m}$ ), which provided a mean oocyte diameter for later use to assess female closeness to the parturial moult, during which eggs are laid in the marsupium (Moreau and Rigaud, 2002). Removal of the mating male and female was assumed to cause no serious bias in the experimental design since the completion of the full sexual sequence is normally too long (approximately 1 h) to allow sexual partners to re-mate within the observation time (see Mead, 1973; Lefebvre and Caubet, 2010).

To assess the relationship between the mating time and the amount of transferred sperm, the number of spermatozoa was assessed for a sub-sample of inseminated females ( $N = 9$  after mating #1 and  $N = 8$  after mating #2). For that assessment, a direct-count technique was applied using a DAPI stain and an epifluorescent microscope (see Porter and Feig, 1980, for details on the general method, and Moreau et al., 2001, for application in Oniscidea).

Although 23 replicates were conducted for the behavioural encounter tests, due to difficulty finding 'time-in' females in the rearing boxes, certain replicates were smaller than planned (19 with 10 females and 10 males each, as planned, but 4 with only 9 females and 9 males each, providing  $N = 226$  per sex). In any case, the number of potential partners during the observation time is not the limiting factor in our experiment.

### 2.3. Statistical analyses

Body size variation was measured between the sex among the 452

animals used in the experiments: the mean mass + SD was  $106.21 + 42.44$  mg in females ( $N = 256$ ) compared to  $89.81 + 33.96$  mg in males ( $N = 256$ ). The significance of mean size difference between males and females was checked using a Student *t*-test when examining the mass of all the females and males in the experiment (Welch two-sample *t* test:  $t_{429,35} = 4.535$ ,  $P < 0.00001$ ). Once the replicates were composed, one-way ANOVA was performed to check for any significant differences in mass between replicates. Mass was significantly related to sex (ANOVA:  $F_1 = 19.77$ ,  $P < 0.0001$ ) but not to replicates (ANOVA:  $F_{22} = 0.69$ ,  $P = 0.8507$ ). To consider each replicate independently of the others and to avoid an influence of absolute values, we used a standardised value (std-mass), dividing the absolute mass by the mean of the individuals of the same sex in the same replicate. A std-mass below 1 indicates that an individual is lighter than the group average for the same sex, and a std-mass above 1 indicates that an individual is heavier than that average. We confirmed that the homogeneity of variance values in the std-mass comparison among females and among males were not significantly different between replicates (Levene's test:  $F_{45} = 0.9559$ ,  $P = 0.5564$ ).

The mass difference between the sexual partners was checked by dependent *t*-tests (matched-pair tests) using the std-mass value. Mass comparisons between groups (unpaired vs. paired, unmated vs. mated) were performed separately for females and males by *t*-tests.

The probabilities of pairing and mating according to female and male std-mass values were modelled by linear regressions. For pairing, the analysis was conducted separately for females and males, with each individual coded as 0 = unpaired or 1 = paired. We used one-way ANOVA followed by a general linear hypothesis test with multiple comparisons of means by the Tukey contrast test. For mating, the same procedure was applied (0 = unmated, 1 = mated), but the estimated probabilities were also calculated in a model that included both female and male std-mass values.

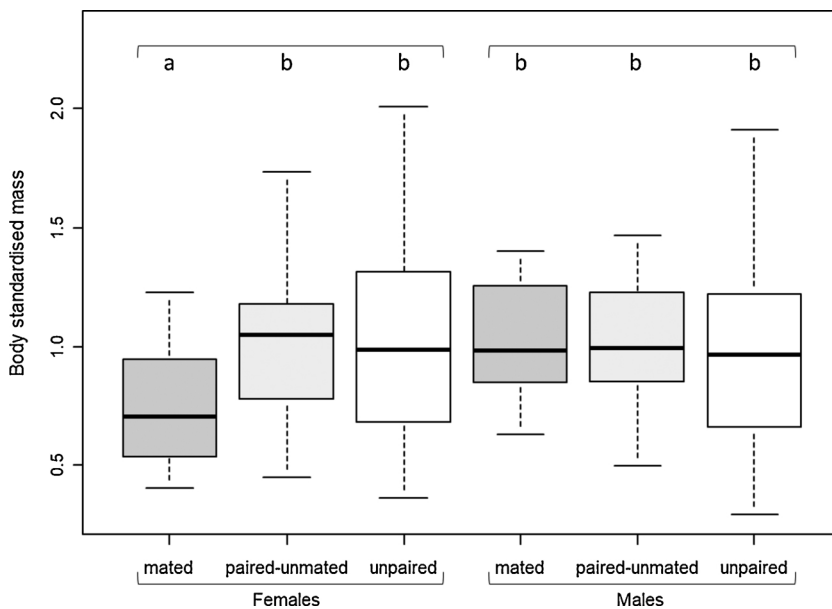
The effects of female and male std-mass values on the duration of the different phases of the sexual sequence were explored using linear models. The durations fit a normal distribution after Napierian log transformation. To account for the observed variation in the duration of pairing #1 (including pairings from interrupted sexual sequences), the male std-mass, the female std-mass and the mean oocyte diameter were entered as continuous predictors. For the duration of mating #1, the duration of pairing #1 was entered as an additional continuous predictor in the model. At each step of the sexual sequence, the duration of the previous phase was added in the model. For instance, the model for the duration of mating #2 included female std-mass, male std-mass, oocyte diameter and the duration of pairing #1, mating #1 and pairing #2.

Correlations were performed using Spearman or Pearson correlation tests after Shapiro-Wilk normality tests.

The statistical treatments were performed using R software (R Core Team, version 3.4.2). Central tendencies were expressed as the mean + SD (standard deviation) or median + SIQR (semi-interquartile range) according to the sample size. All the tests were two-tailed, and a *P* value below 5% ( $P < 0.05$ ) was considered significant.

### 3. Results

During the course of overall observations, no displacement (or attempted displacement) of paired or mating males by single males was observed. Overall, 73 females and 69 males engaged in pairing (32% and 30.5%, respectively), with some of them pairing up to three times ( $N = 9$ , later called "additional pairing" followed by mating 3 times) within the one-hour observation period (two times for 7 females, two times for 6 males, and three times for a single male). A total of 89 pairings were observed, of which 20 led to mating postures (22.5%). Among those mating postures, 16 were associated with sperm transfer (80%) (mating #1 for only 1 individual as well as mating #1 and mating #2 for 15 individuals), with 4 showing no sperm transfer (i.e.,



**Fig. 1.** Box plots (box delimited by lower and upper quartiles, median symbolised by bold line, dispersion limits are quartiles  $\pm 1.5 \times$  interquartile range) for the standardised mass of females and males among tested individuals and their interaction status. The significance of mean comparisons is separately denoted for each sex by different letters above the boxes (ANOVA followed by Tukey's post hoc test, with  $P < 0.05$ ). Group size: males mated ( $N = 16$ ); unpaired ( $N = 157$ ); paired unmated ( $N = 57$ ); females mated ( $N = 16$ ); unpaired ( $N = 153$ ); paired unmated ( $N = 57$ ).

pseudo-copulation; see Lefebvre and Caubet, 1999).

### 3.1. Pairing and mating probabilities according to respective female and male mass

In the following results, mass is expressed as a standardized value (std-mass). The proportion achieving the end of the sexual sequence (UP: unpaired, P-UM: paired-unmated, and M: mated) was similar between females and males (Pearson's chi-square test:  $\chi^2_2 = 0.1971$ ,  $P = 0.9062$ ), with approximately 68%, 25% and 7%, respectively, in both sexes. For males, the std-mass did not influence the end of the sequence (one-way ANOVA:  $F_{2,223} = 0.495$ ,  $P = 0.61$ ), and the mean std-mass values  $\pm$  SD were comparable (Fig. 1) among unpaired paired-unmated and mated males. However, the female mass significantly affected the end of the sequence (one-way ANOVA:  $F_{2,223} = 4.402$ ,  $P = 0.0133$ ). The std-mass was similar between unpaired and paired but unmated females (Tukey post hoc test:  $P = 0.632$ , Fig. 1). However, both categories of unmated females were significantly larger than that of mated ones ( $P = 0.009$  for P-UM vs M and  $P = 0.022$  for UP vs M; Fig. 1).

Considering the overall influence of std-mass and the probability of pairing and mating, for males, no significant correlation existed concerning either pairing (Pearson's correlation:  $r = 0.060$ ,  $t_{224} = 0.904$ ,  $P = 0.3671$ ) or mating (Pearson's correlation:  $r = 0.051$ ,  $t_{224} = 0.758$ ,  $P = 0.4492$ ). Similarly, for females, the std-mass was not significantly correlated with pairing probability (Pearson's correlation:  $r = -0.0202$ ,  $t_{224} = -0.303$ ,  $P = 0.7622$ ). However, a significant negative correlation existed between the std-mass and the probability of mating, with smaller females showing a higher mating rate (Pearson's correlation:  $r = -0.1858$ ;  $t_{224} = -2.8295$ ,  $P = 0.0051$ ).

### 3.2. Mass assortment at pairing and mating

We compared the std-mass among overall pairings ( $N = 89$ ), and no significant difference was observed between the sexes for the paired individuals (std-mass  $\pm$  SD for females:  $0.98 \pm 0.38$ ,  $N = 73$ ; and for males:  $1.03 \pm 0.29$ ,  $N = 69$ ; paired  $t$ -test:  $t_{88} = -1.170$ ,  $P = 0.2451$ ). Moreover, we observed a significant positive correlation between the std-mass values of partners, indicating size assortment in pairing partners (Pearson's correlation:  $r = 0.2842$ ;  $t_{87} = 2.766$ ,  $P = 0.0035$ ; Fig. 2).

A different pattern was revealed for mating individuals ( $N = 16$ ). The mean std-mass of mating females was significantly smaller than

that of their male partners (paired  $t$ -test:  $t_{15} = -4.062$ ,  $P = 0.0010$ ; Fig. 1). Moreover, we found a positive correlation between the standardized mass values of mated pairs (Pearson's correlation:  $r = 0.4517$ ,  $t_{14} = 1.8946$ ,  $P = 0.0395$ ; Fig. 2). The results also showed that males mate with females lighter than themselves.

#### 3.2.1. Determination of pairing phase duration

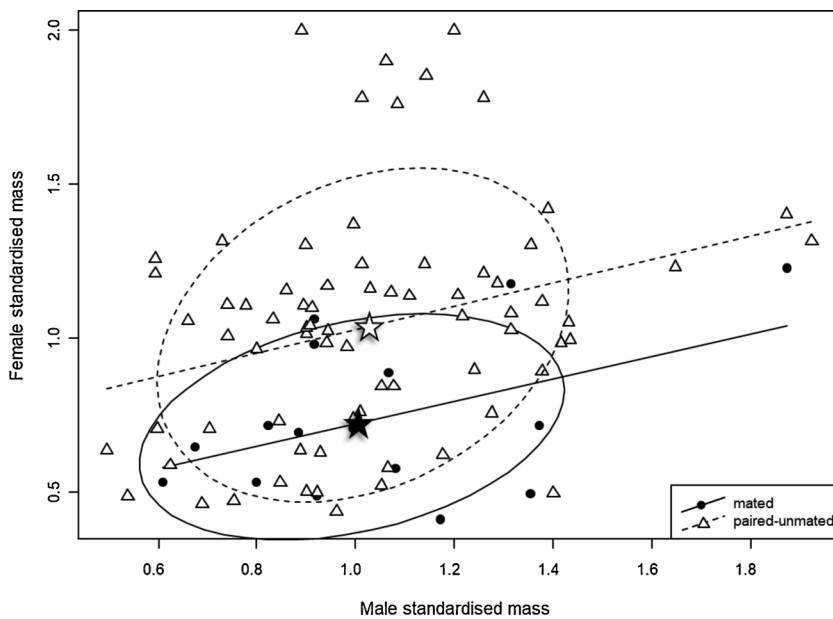
The total time spent in pairing showed high variability (mean  $\pm$  SD:  $449.74 \pm 805.51$  s,  $N = 89$ ). We addressed separately the first pairing phase (pairing #1) (mean  $\pm$  SD:  $291.49 \pm 378.29$  s,  $N = 89$ ) and the second one (pairing #2), occurring only when the first pairing was followed by a copulation (or pseudo-copulation) sequence (mean  $\pm$  SD:  $741.26 \pm 1180.02$  s,  $N = 19$ ). Due to the high variability, the absolute duration was normalized using Napierian log transformation.

The initial pairing phase (pairing #1) showed high variability, particularly in the case of aborted sexual sequences (pairing not followed by mating) (mean  $\pm$  SD:  $245.16 \pm 399.47$  s,  $N = 73$ ) compared to successful pairing (i.e., followed by mating) (mean  $\pm$  SD:  $502.88 \pm 477.78$  s,  $N = 16$ ), which was significantly longer (one-way ANOVA:  $F_{1,87} = 11.62$ ,  $P = 0.0010$ ).

A specific linear model (Table 1: Model 1) was designed with pairing #1 duration as the response variable and three variables as predictors: the std-mass and std-mass ratio of partners and their pairing success (i.e., followed by mating or not). The model showed a single significant influence on pairing #1 duration due to pairing success. The std-mass of partners had no significant influence on the duration of pairing #1, either for the std-mass difference between partners, the male's std-mass or the female's std-mass.

However, the relationship between the std-mass of partner bodies and the first stimulation duration (pairing #1) differed with sex and the result of the pairing phase (i.e., followed by mating or not). For males, we found a significant negative correlation when pairing did not lead to mating (Spearman's rank correlation:  $r_s = -0.2347$ ,  $N = 73$ ,  $P = 0.0456$ ; Fig. 3a) but no significant correlation when pairing was followed by mating (Pearson's correlation:  $r = 0.1875$ ,  $t_{14} = 0.7142$ ,  $P = 0.4868$ ; Fig. 3a). For females, we found an inverse pattern: no correlation when pairing did not lead to mating (Spearman rank correlation:  $r_s = 0.0508$ ,  $N = 73$ ,  $P = 0.6697$ ; Fig. 3b); however, a significant positive correlation was found for pairing followed by mating (Pearson's correlation:  $r = 0.5861$ ,  $t_{14} = 2.7069$ ,  $P = 0.0170$ ; Fig. 3b).

In the successful sequences (i.e., those followed by mating behaviour), pairing #2 duration was correlated with pairing #1 duration

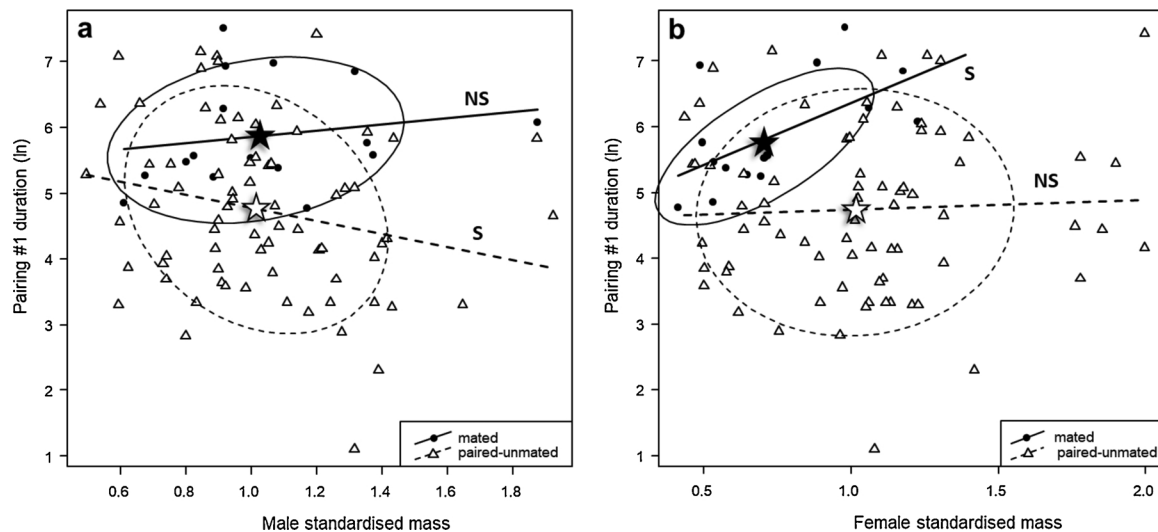


**Fig. 2.** Relationship between the standardised mass values of males and females engaged in pairing only ( $N = 89$ , empty triangles) and those engaged in pairing followed by mating ( $N = 16$ , black dots). The regression lines indicate a significant relationship (Pearson's product-moment correlation) between female and male masses involved in pairing (dotted line:  $t_{87} = 2.76$ ,  $P = 0.0035$ ) and in mating (solid line:  $t_{14} = 1.89$ ,  $P = 0.0395$ ). Ellipses show the confidence interval for the 0.75 quartile (dotted line: paired-unmated; plain line: mated).

**Table 1**

Linear models outputs addressing effects of various predictors (such as partners' size, oocytes diameter, duration of previous steps) on duration and efficiency of behavioural steps of the mating sequences (significant effects are highlighted in bold with \*).

Model 1: Initial pairing step (leading or not leading to mating)					
Response variable	Predictors				
Pairing#1 duration	Female std-mass	Male std-mass	Partners std-mass ratio (M/F)	Pairing success	
$R^2 = 0.1622$					
$F_{4,84} = 4.066$	$P = 0.4154$	$P = 0.1945$	$P = 0.8705$	$P < 0.0001$	*
$P = 0.0046$					
Model 2: Total stimulation duration in successful sequence (leading to mating)					
Response variable	Predictors				
Total pairing duration	Female std-mass	Male std-mass	Partners std-mass ratio (M/F)	Oocytes diameter	
$R^2 = 0.2749$					
$F_{4,33} = 4.506$	$P = 0.0063$	$P = 0.0247$	$P = 0.0530$	$P = 0.7361$	
$P = 0.0051$					
Model 3: Total copulation duration					
Response variable	Predictors				
Copulation duration (mating)	Female std-mass	Male std-mass	Partners std-mass ratio (M/F)	Oocytes diameter	Stimulation duration (pairing)
$R^2 = 0.0275$					
$F_{5,26} = 1.175$	$P = 0.0913$	$P = 0.1049$	$P = 0.1569$	$P = 0.6881$	$P = 0.0370$
$P = 0.3479$					*
Model 4: Sperm investment					
Response variable	Predictors				
Sperm quantity	Female std-mass	Male std-mass	Partners std-mass ratio (M/F)	Stimulation duration (pairing)	Copulation duration (mating)
$R^2 = 0.7693$					
$F_{6,8} = 8.780$	$P = 0.0295$	$P = 0.0465$	$P = 0.0197$	$P = 0.7396$	$P = 0.0003$
$P = 0.0036$					*



**Fig. 3.** Relationship between male (a) and female (b) standardised mass on pairing #1 duration (ln transformed) for pairing only ( $N = 73$ , triangle, dotted lines) and for pairing followed by mating ( $N = 16$ , dot, continuous lines). The regression lines indicate correlation between pairing #1 duration and the individual standardised mass. Letters indicate significant (S) and non-significant (NS) correlation (see text for the significance values of tests). Ellipses show 0.75 quartile confidence interval. Stars indicate group means.

(Pearson's correlation:  $r = 0.4989$ ,  $t_{14} = 2.154$ ,  $P = 0.0492$ ). Concerning partners' body size, pairing #2 duration was correlated neither with the ratio of the partners' body sizes ( $r = -0.234$ ,  $t_{14} = -0.9004$ ,  $P = 0.3059$ ) nor with the male's std-mass ( $r = 0.181$ ,  $t_{14} = 0.689$ ,  $P = 0.5022$ ). However, pairing #2 duration was significantly positively correlated with the female's std-mass ( $r = 0.513$ ,  $t_{14} = 2.235$ ,  $P = 0.0423$ ).

The occurrence and duration of potential additional pairings ( $N = 9$  among 16 behavioural sequences) were addressed using a linear model predicting additional-pairing duration with both partners' std-mass values as predictors, without any significant effect ( $R^2 = -0.1905$ ,  $F_{3,5} = 0.5732$ ,  $P = 0.6569$ ). However, a linear model using total-previous-pairing durations (pairing #1 and pairing #2) and total-previous-mating durations (mating #1 and mating #2) as predictors showed a significant effect of previous-phase durations ( $R^2 = 0.9662$ ,  $F_{4,3} = 50.98$ ,  $P = 0.0043$ ). The duration of additional pairings was significantly related to both pairing #1 duration ( $P = 0.0221$ ) and mating #2 duration ( $P = 0.0165$ ). Longer durations for pairing #1 associated with longer durations for additional pairing (Pearson's correlation:  $r = 0.792$ ,  $t_7 = 3.435$ ,  $P = 0.0109$ ), but longer durations for mating #2 associated with shorter durations for additional pairing ( $r = -0.874$ ,  $t_6 = -4.412$ ,  $P = 0.0045$ ).

### 3.2.2. Determination of mating sequences

A dataset of 34 complete sequences (pairing followed by mating) was analysed using linear models to check the relationship between sequence phases. We used the body size of partners (standardised mass of both female and male) and the ratio of these sizes as predictors of the pairing duration. The mature oocyte diameter (range from 344 to 471  $\mu\text{m}$ , mean + SD = 418.10 + 36.57  $\mu\text{m}$ ,  $N = 15$ ) was entered as an additional predictor in the models. We added pairing phase as an additional predictor in the model focused on the mating phase.

Concerning the duration of stimulation, the model (Table 1: Model 2) revealed a significant influence of partner body mass ( $R^2 = 0.2749$ ,  $F_{4,33} = 4.506$ ,  $P = 0.0051$ ; female std-mass:  $P = 0.0063$ ; male std-mass:  $P = 0.0247$ ; ratio between male and female std-mass:  $P = 0.0530$ ). However, oocyte diameter did not affect the total pairing duration ( $P = 0.7361$ ).

Concerning the mating phase duration, the model (Table 1: Model 3) revealed a significant influence of the total duration of stimulation but not the partner body mass. Finally, oocyte diameter did not interact

with the duration of the mating phase. The mating duration was significantly and positively correlated with the duration of stimulation (Spearman's rank correlation:  $r_s = 0.4054$ ,  $N = 14$ ,  $P = 0.0174$ ). Stimulation lasting less than 250 s never led to mating. However, above that threshold, the probability of mating reached 57% (Fig. 4).

### 3.2.3. Sperm investment

To estimate sperm investment, we first counted DAPI-stained sperm in a sub-sample from 9 mated females representing 17 available data sources (one by inseminated oviduct).

We designed a linear model with sperm quantity as the response variable and the following as predictors: mating and pairing durations, female and male std-masses, the ratio between partner body mass and oocyte diameters. The model (Table 1: Model 4) showed a strong relationship, with a significant influence by the copulation duration but not by the stimulation duration on the amount of sperm transferred. Moreover, the model also revealed an effect with the std-mass of both females and males and the ratio between partners mass. The amount of sperm transferred was positively correlated with the copulation duration (Spearman's correlation:  $r_s = 0.7419$ ,  $N = 17$ ,  $P = 0.0007$ ; Fig. 5). However, the amount of sperm is not significantly correlated with the std-mass, neither for females ( $r_s = 0.533$ ,  $N = 17$ ,  $P = 0.1475$ ) nor males ( $r_s = 0.350$ ,  $N = 17$ ,  $P = 0.3586$ ).

## 4. Discussion

Our results showed a complex relationship between partners body size and behavioural exchange between individuals (Fig. 6). Understanding of those findings must be related to sexual selection concepts (mate choice and resistance behaviour) and populations demography (succession of cohorts, reproductive success) of the model used. We discuss our results regarding both proximate and ultimate factors in terms of males and females reproductive strategies.

### 4.1. Findings on mating pattern

In the terrestrial crustacean *Armadillidium vulgare*, some evidences exist for male scramble competition, i.e., males actively search and compete for the first access to receptive females (Lefebvre et al., 2000). Therefore, the decision to instigate pairing is under the male initiative only, and the male engages the sexual sequence by mounting and

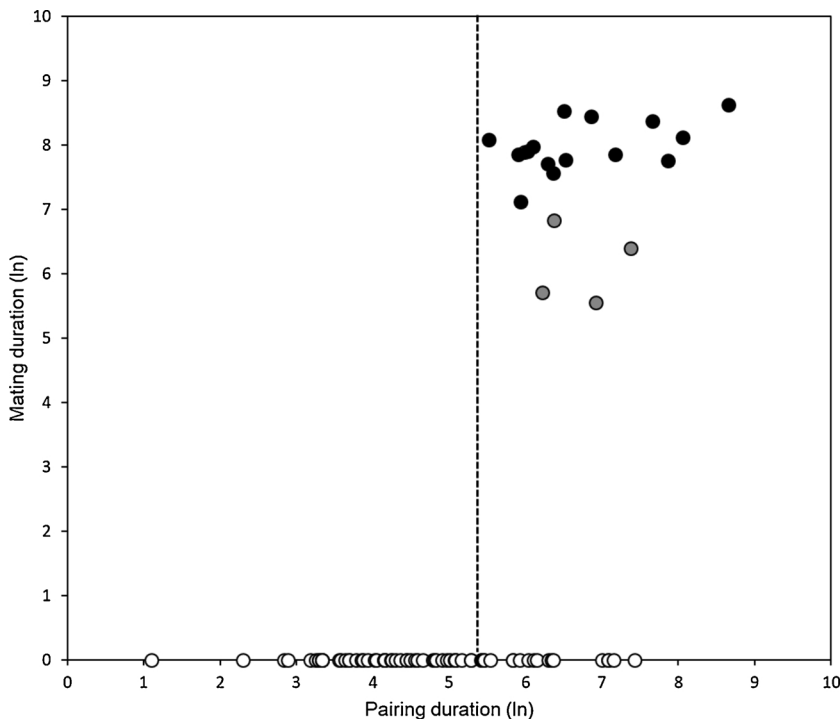


Fig. 4. Scatter plot of stimulation phases ( $N = 89$ ) with pairing and mating durations Neparian log transformed. Empty circles represent pairing sequences not followed by a mating posture ( $N = 59$ ); grey-filled circles represent pairing sequences followed by mating without sperm transfer (pseudocopulation) ( $N = 4$ ) and black-filled circles represent pairing sequences followed by a mating with insemination (true copulation) ( $N = 16$ ). The vertical dotted line shows the stimulation duration threshold potentially followed by a mating posture and corresponding to 250 s.

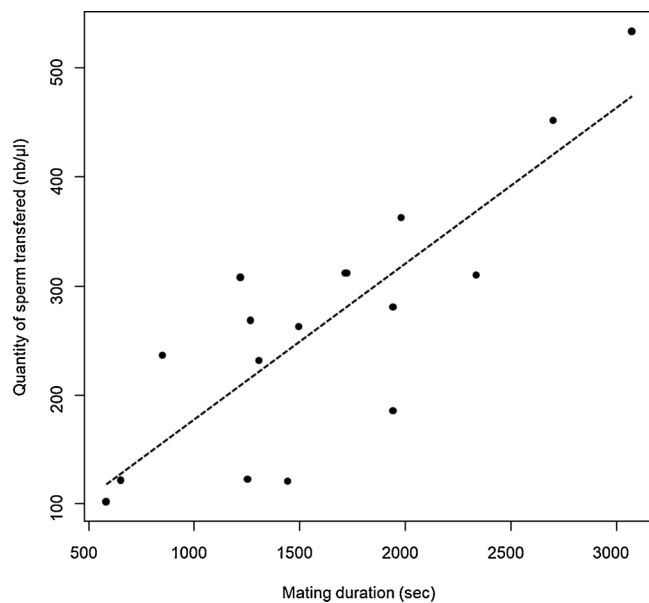


Fig. 5. Scatter plot between mating duration (log-transformed) and the quantity of sperm transferred. The regression line shows a significant positive correlation (Spearman's correlation:  $r_s = 0.7419$ ,  $N = 17$ ,  $P = 0.0007$ ).

gripping onto the female's back. We showed that the probability of engaging in pairing is constant whatever the size of female and male. Of particular interest, the mass difference between pairing partners (+12% in favour of females) closely reflects the mass variability between genders exhibited by the species (+18% among the tested animals and nearly +20% according to the literature (Vandel, 1960)). In other terms, this finding means that all animals in 'time-in' may be engaged in pairing (ie pre-copula). However, males and females did not pair randomly with regard to body mass. A clear trend occurred for small males to pair with small females and large males to pair with large females ( $r = 0.28$ ). We have a nice example of homogamy in this case, without large individuals trying to displace smaller ones,

supporting the mate choice.

The probability of engaging in mating strongly decreased with increased female mass. Females in mating were thus smaller than females found in pairing only, while the male mass did not change. As a result, the mass ratio at mating was reversed, with the mated female being systematically smaller than its sexual partner (-20%). The male mate choice, in favour of a small female, increases probability of mating. Consequently, large females may have a low chance of finding males large enough.

#### 4.2. Proximate factors: the causes of assortative mating

Within the specific mass range leading to mating in our current study (herein, from 43 to 133 mg for females and from 54 to 218 mg for males), males and females did not associate randomly, and a clear trend for positive mass assortment occurred ( $r = 0.45$ ). The biggest females only mated with the biggest males, and the smallest females mated with the smallest males.

Positive size-assortative mating has been found in almost all animal groups (Ridley, 1983; Crespi, 1989) and is particularly well documented in amphipods and isopods (Nilsson, 1977; Veuille, 1980; Shuster, 1981; Adams and Greenwood, 1987; Jormalainen, 1998; Bollache and Cézilly, 2004; Lefebvre et al., 2005). Among the sets of hypotheses routinely invoked to interpret assortative mating by size (Crespi, 1989), two of them may apply in the case of *A. vulgare*: physical constraint on mating and female resistance.

Males and females may have difficulty in pairing, mating or remaining paired when they differ sufficiently in body size (Price and Willson, 1976; Clark, 1977; Juliano, 1985). The effectiveness of male courtship may thus decline with a large relative size difference. For example, Pinto and Mayor (1986) showed that in a species of meloid beetle, size-mismatched males and females had difficulty in mating because "the lack of coincidence of anatomical parts prevents normal courtship delivery". No doubt, such physical constraints may generate non-random assortment in *A. vulgare*, considering the large size range between potential mating partners. In the present experiment, the wide ranges mean that the smallest males may encounter 7-fold heavier females (ranged from 34 to 221 mg in males and 42 to 251 mg in



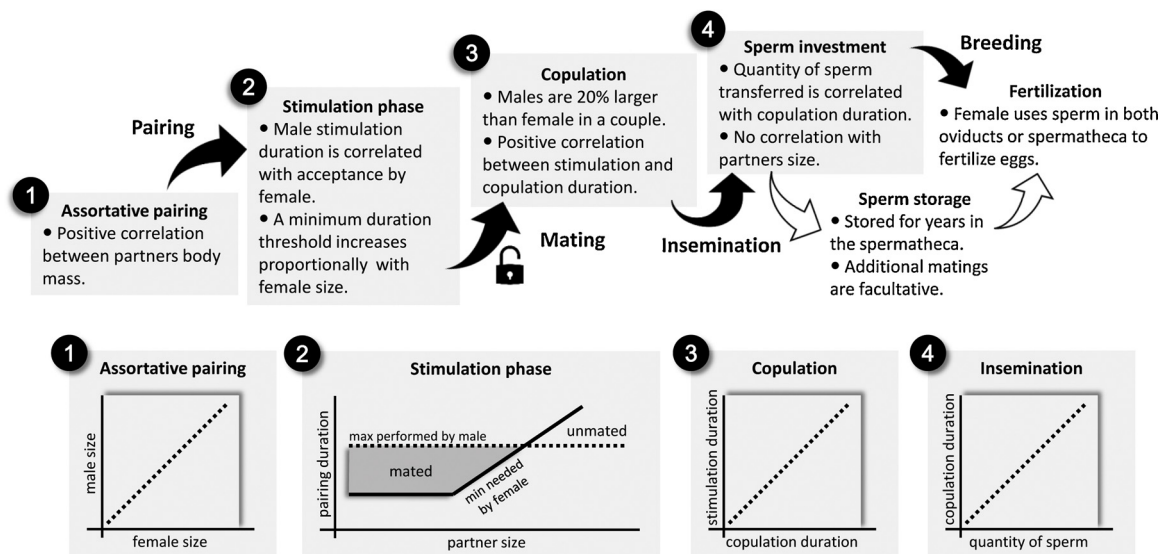


Fig. 6. Overview of *A. vulgare* mating behaviour.

females). In the present study, the maximum body mass difference for successful mating was +30 mg in favour of the female (1.4-fold) and +97 mg in favour of the male (1.8-fold).

The mean mass of males engaged at each step (pairing #1, mating #1, pairing #2, mating #2) of the sexual sequence was similar, indicating that the observed differences in mass ratio were primarily due to change in female mass. For *A. vulgare*, the pre-copulatory (pairing) phase may be viewed as a period of female stimulation during which the male tries to engage the female in a mating posture. The fact that the duration of the pre-copulatory phase increases with female size may be another indicator of increased male courtship efforts. Here, we have a correlation with female mass and the stimulation duration received before the decision to accept mating: the longest stimulations were performed on the heaviest females. In contrast, male mass did not affect the duration of pairing leading to mating. Stimulations show high variability in duration and, in most cases, this step is too short and ends with the separation of the pair without mating.

#### 4.3. Ultimate factors: male and female mating strategies

Our study showed that longer copulation leads to the transfer of more sperm into female spermathecae. However, female mass did not affect the quantity of sperm transferred. Over the years, females use the stored sperm to fertilize their oocytes (Vandel, 1941; Howard, 1943; Lueken, 1963) even after several moults (Warburg, 1993; Kight, 2009). Females maintained semen in their spermathecae from the first mating and later used it without sperm precedence to fertilize eggs of a new clutch (Moreau et al., 2002; Verne et al., 2007). Then, the presence of the spermatheca could make multiple mating unnecessary. Indeed, experienced females who had produced offspring and rested a few months refused further copulation (Fortin et al., 2018).

The pairing duration of mated females correlated with their mass, and mating duration increased when the stimulation duration increased. When mating did not follow pairing, no relationship existed between female mass and pairing #1 duration. In contrast, no correlation occurred between mass and pairing #1 duration for mated males, but a negative correlation occurred when pairing was aborted. Large unmated males have a lower stimulation duration compared to mated males of the same size. Mating resistance behaviour by females seems common in isopods (see Jormalainen, 1998, for a review of authors and species). Authors generally argue that females resist males to assess potential mates (Ridley and Thompson, 1979; Ward, 1984; Elwood et al., 1987; Dick and Elwood, 1989; Jormalainen and Merilaita, 1993;

Sparkes et al., 2000). Here, we favour the hypothesis of a threshold stimulation allowing mating and depending on female size. By resisting and adopting a mating posture only with those males able to stimulate them for long enough to trigger mating. Moreover, females accept males larger than themselves as mates. Females may effectively prefer larger males over smaller ones because size is a correlate of age and because, on average, old animals offer greater future benefits to offspring (longer survival, higher competitive abilities) or direct benefits to the female (many sperm and shorter pairing/mating durations, decreasing her exposure to predators) (Balmford and Read, 1991; Maynard Smith, 1991).

## 5. Conclusion

In *A. vulgare*, as is the general rule in dioecious organisms, female fecundity increases with size (Sutton et al., 1984), so that the fitness benefits conferred by large females are obvious from a male mating perspective. Both field and simulation data for the species highlight the reproductive importance of large females (3 years old or more) by showing that these females, though relatively rare (15–25% of the total population), contribute to half of each new generation, producing as many offspring as all the other females (Caubet, 1998). Here we showed that the stimulation threshold for the largest females to accept mating is probably too difficult to reach and strongly reduces their probability of mating. Therefore, large females in natural populations are likely to be already inseminated and still capable of yielding several broods and fertilizing their eggs without any further insemination due to sperm storage in the spermathecae. Such a particularity may have constituted a prerequisite to the evolution of female-biased size in this group. Then, the genders mass variability of this species increases the probability that males will mainly mate with small females that request less stimulation compared to large females. The probability of mating then increases for small and virgin females, which are relatively attractive (Beauché and Richard, 2013) and decreases for large previously mated females.

### Significance statement

Sexual selection is one of the key aspects for better understanding species evolution. Individuals use specific characteristics to select their partner, such as colour, size, and odour. In gregarious woodlice, the groups are heterogeneous (a mix of individuals: males, females, young, old, small, large, etc.), which creates a large set of potential mates. We

showed that successful mating happens when male reach a threshold of stimulation. Mated females were 20% lighter than males.

### Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of invertebrate animals were followed. This article does not contain any studies with human participants performed by any of the authors.

### Declaration of Competing Interest

Authors FL, FJR, JM, TB and YC declare that they have no conflict of interest.

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