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Annexe 1

Description of a sibling species of the cosmopolitan *Porcellionides pruinosus* (Crustacea, Isopoda, Oniscidea): Biological, behavioural and morphological approaches

(soumis à Zoological Journal of the Linnean Society)

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**Running title:** Evidence for a new terrestrial crustacean species

**Abstract:**

*Porcellionides pruinosus* is one of the most widespread woodlice species, mainly as a result of human introductions. Recent investigations of the cosmopolitan *Porcellionides pruinosus* have suggested that some populations, although morphologically closely related, may consist in fact of separate species. In this study, six populations from distant localities were investigated. Laboratory crossings confirmed the reproductive isolation among assorted populations; two distinct population groups were identified. Subsequently, behavioral tests were used to detect a pre-zygotic isolation through mating choice in the absence of chemical recognition between sexual partners from the two groups. These data give convergent results confirming the existence of two well-defined species. In the absence of meristic characters, it is suggested that subtle differences in morphology may be used to discriminate between *P. pruinosus* (as formerly defined), and its sibling species, *Porcellionides percanus* (sp. n.).

**Key words**
Morphology- *Porcellionides percanus* sp. n. - Reproductive isolation – Taxonomy - Woodlice.
Introduction

The terrestrial crustacean Porcellionides (=Metoponorthus) pruinosus (Brandt, 1833) has a confusing taxonomic history (Vandel, 1962). It belongs to the Porcellionidae family (Brandt & Ratzeburg, 1833), in the sub-order Oniscidea (Latreille, 1829). According to Vandel, P. pruinosus originates from the oriental Mediterranean region (Asia Minor), and has spread throughout the world as the result of human activity. This synanthropic species is commonly viewed as the most widely distributed terrestrial Isopod species (Dollfus 1897; Vandel 1960, 1962). Specimens identified as this species have been reported in Europe, Africa, America, Asia and many islands of the Pacific, Atlantic, Austral and Indian oceans (Garthwaite & Sassaman, 1985, and references therein). In fact, it seems that only Polar Regions have not been colonised by P. pruinosus (Vandel, 1962). This last author also noted a large polymorphism in P. pruinosus, with nearly 20 subspecies recognised throughout the world. Nevertheless, the validity of these subspecific statuses is solely based on morphological criteria, and can reasonably be questioned (also see Böhme, 1978).

In 1985, Juchault, Mocquard and Kouigan met with difficulties when attempting to cross specimens from France and Togo. In the same year, Garthwaite and Sassaman used laboratory crossings and morphological characters to identify a new species, which they named Porcellionides floriana. They suggested, as Racovitza had many years before (1908), that P. pruinosus (Brandt, 1833) might consist of a number of distinct and localised species rather than one cosmopolitan species. Recently, genetic results generated by mitochondrial RFLP analysis have revealed distinct mitotypes between French and Tunisian, Greek, and Réunion Island populations (Marcadé et al., 1999). Results of crossings have also suggested that the French populations on one hand (group 1), and the other three populations on the other hand (group 2), must be attributed a species status.

In the present study, an attempt was made to re-examine the taxonomic status of these two groups. Additional populations were examined as they could represent geographical clines. Laboratory crossings were performed and compared to those previously obtained by Marcadé et al. (1999). Since recognition between sexual partners is assumed to be based on chemical cues (Legrand, 1958; Mead, 1973; Ducruet, 1976), the hypothesis according to which a reproductive barrier occurs before mating was tested (pre-zygotic isolation). To this end, multiple encounters between males and females from the different populations were analysed. In addition, morphological observations were made of specimens from both groups to determine the relevant characters that could be used to discriminate between the two species.

Material and methods

Porcellionides pruinosus is a rather slender species, light grey in colour with whitish legs, and a characteristic surface bloom like that of a fresh plum. Specimens were either collected by hand under objects on the damp ground or were extracted from compost heaps where they are particularly abundant. Experiments were performed on six populations from Tunisia (Tunis), Spain (Manzanares), the Réunion Island (Saint Paul), Israel (Haifa), Greece (Athens) and France (Nevers) (Table I). Populations were previously bred in laboratory by intra-population crossings. As soon as possible (near the fourth moult), young were sexed, and then reared separately in order to experiment on virgin animals.

Laboratory crossings

Multiple crossings between sexual partners were attempted for four of the six populations available (Table I). A series of single-pair crossings was performed using sexually mature animals (one year old). For each of the four populations, 20 females were coupled with males of the same origin, and with males from the three others regions (n = 4x4 = 16 pairs). Thus, four intra-population (n = 16 pairs) and 12 inter-population crossings (n = 48 pairs) were performed (Table II). Pairs were placed in cylindrical boxes (diameter = 8 cm; area = 50 cm²) under laboratory conditions (20°C, LD 18:6). The rearing boxes were checked regularly for the presence of young throughout the three months experimental period.

Based on these results, an isolation coefficient was calculated as described by Malogolowkin, Solima and Levene (1965): proportion of within group mating minus the proportion of between group mating.
Behavioral analysis

Behavioral tests known as “male choice” were performed on males from five of the populations (Table I). Male sexual activity towards females of the same origin (five intra-population tests) or towards females from the other four origins (20 inter-population tests) was tested. Since all interactive behavior in the Oniscidea begins by antennal contact (Johnson, 1985; personal observations), behavioral analysis was based on this criterion. For each male, an index of sexual activity (ISA) was established as follows:

\[
ISA = \frac{AC^*}{AC}
\]

\(AC = \text{nb. of antennal contacts with the female}
\)

\(AC^* = \text{AC leading to sexual behavior (mounting attempt, mounting or mating posture)}
\)

Observations were performed in a Petri dish (diameter = 9.5 cm, height = 2 cm, area = 71 cm²), lined with moist filter paper, and topped with a transparent glass lid in order to limit air disturbance. The environmental conditions were: temperature = 20°C; light intensity = 150 lux; relative humidity = 90 %. The female was placed in the dish and the t0 time was associated with the introduction of the male. Sexual partners having adopted mating posture were immediately removed to avoid lengthy copulation. Male sexual behavior was followed over a fifteen minute period.

Indexes corresponding to the 25 combinations were compiled in a matrix table. A two-means cluster analysis (STATISTICA 4.5, StatSoft Inc. 1993) was performed to separate indexes into two groups according to the highest variance (“reversed ANOVA”). Euclidean distances between the five populations were also calculated in order to generate a tree diagram (hierarchical cluster analysis according to the “nearest neighbour” method).

Taxonomy

Literature on the genus Porcellionides was reviewed to identify all referenced names (varieties, races, subspecies, species) that could have been assigned to specimens from either group. A morphological analysis was performed on males and females from both groups, and particular attention was paid to characters traditionally used in the systematics of the Oniscidea (antennae, pereiopods 7, pleopods 1, uropods, pigmentation) (Vandel, 1960). Scanning microscopy of the cuticular surface was also performed to determine dorsal tubercule and tricorn arrangements.

Results

Laboratory crossings

Due to the high mortality rate in the rearing boxes, and since partial data were already available, results of crossings were compiled with those previously published by Marcadé et al. (1999) (Table II). With the exception of a single crossing, in which a French female produced one puli when paired with a Tunisian male, results demonstrated a reproductive isolation between populations from France and those from Tunisia and the Réunion Island (group 1 and group 2, as defined by Marcadé et al., 1999). In addition, specimens from Spain were inter-fertile with those from France but never with those from Tunisia or the Réunion Island, and were therefore subsequently assigned to the first group. The coefficient of isolation between these two defined groups (France and Spain on one hand, Tunisia and the Réunion Island on the other hand) was very close to a value of one: (50-1)/51 = + 0.96.

Behavioral analysis

Indexes of sexual male activity (ISA) towards females from the different populations are given in Table III. A two-means cluster analysis separated these indexes into two well-defined clusters \((F_{[1,23]} = 114.10; p < 0.001)\). The cluster with the highest scores (mean ISA ± SD = 0.2438 ± 0.0774) corresponds to combinations
with high probability of crossings; it contains the five intra-populationnal encounters as well as the France vs Israel, France vs Spain, Israel vs Spain and Tunisia vs Réunion Island crossings. In contrast, the second cluster (mean \( ISA \pm SD = 0.0097 \pm 0.0161 \)) includes all combinations with low probability of crossings: France vs Réunion Island, France vs Tunisia, Israel vs Réunion Island, Israel vs Tunisia, Spain vs Réunion Island and Spain vs Tunisia. For all of the encounters present within this second cluster, the first steps of sexual behavior were sometimes initiated but, contrary to what was observed for the others combinations, no mating posture occurred.

The five populations were organised in a tree diagram by performing a hierarchical cluster analysis on the matrix of indexes (Fig. 1). Two clades were defined: populations from France, Spain and Israel in one side (group 1), and populations from Tunisia and Réunion Island in the other side (group 2).

**Taxonomy**

The species *P. pruinosus* was first described in Europe by Brandt (1833), and then by many others naturalists throughout the continent (Kinahan, 1857; Bate & Westwood, 1868; Sars, 1899; Vandel, 1962). Thus, with respect to their origins, specimens from group 1 (France, Spain, Israel) would correspond to *P. pruinosus* as formerly defined. This supposition was confirmed by the examination of specimens from both groups. In specimens from group 1, the uropods and the pleon epimera are a shade of orange, whereas in group 2 they are generally grey. According to Schmalfuss & Ferrara (1978), this marginal orange band is characteristic of *P. pruinosus* as formerly defined. Therefore, specimens from group 1 should be assigned to *P. pruinosus* (Brandt, 1833). The taxonomic status of the second group clearly requires further considerations. One of the major problems in taxonomic investigations consists in avoiding synonymy. As has already been mentioned, *P. pruinosus* is a cosmopolitan species with more than 20 recognized subspecies throughout the world (Vandel, 1962). Authors have used geographical variability in morphology to differentiate subspecies, varieties or races. This has resulted in a confusing taxonomic history for this species (Garthwaite & Sassaman, 1985). In Greece, for example, specimens from the same locality as was used in the present study (Athens) have been reported by Strouhal (1936) to be *P. pruinosus* (var. *pruinosus*, Brandt), and by Beier (1936) to be *P. pruinosus* (var. *epirotes* Verhoeff). Thus, bearing in mind the large-scale distribution of collected samples (Tunisia, the Réunion Island, Greece), there is no doubt that specimens from group 2 have been previously assigned to various subtaxa of *P. pruinosus* (Brandt, 1833). Moreover, such an obvious synonymy at the infraspecific level cannot exclude that specimens from group 2 may also have been described as different species. In the literature, a long list of Porcellionides species is available (Kensley, 1999). However, a published description of key characters that would permit a differentiation among them is often lacking. Vandel (1962) pointed out that somatic characters are powerless to discriminate between species among the genus Porcellionides, and rather stressed the value of tegumental characteristics. Thus, Garthwaite & Sassaman (1985) used cuticulaire structures (tricorns or dorsal tubercles) and the ratio between flagellar articles to distinguish their specimens of *P. floria* (Southern and western United States) from those of *P. pruinosus*. In the present study, observations of the mid-dorsal area of the cuticle revealed no noticeable differences in the arrangement or shape of the scales (Fig 2). In both groups, specimens present the same density of scales, which are regularly positioned, giving an alignment of tricorns of approximately equal length branches. The second antennae are large, multarticulate, filiform structures composed of a basal peduncle of five articles and a distal flagellum consisting of a variable number of small articles, two in the Porcellionidae. No statistical difference in the lengths of the distal and proximal flagellar articles of the second antennae was detected when comparing specimens from France to those from the Réunion Island. It was not possible to assign the Réunion Island specimens (group 2) to *P. floria*. Also, Dollfus (1895) reported two Porcellionides species from the Réunion Island: *P. pruinosus* (Brandt) and *Porcellionides dimorphus* (1895, sp. n.). Specimens from group 2 cannot be assigned to *P. dimorphus* (Dollfus), however, as females never presented dorsal furrows from segments 2 to 4, as described by Dollfus. It would therefore appear that specimens from the second group have never been assigned to any species. Below is the description of this new species:

**Genus Porcellionides Miers, 1877**

*Porcellionides percanus* sp. n. (Fig 3)

*Holotype* ♂, GREECE, Athens, University campus, 9 December 1996, Sfenthourakis (by hand), (MNHN-Paris).
Paratypes  Same data as for holotype, 4 ♂♂ 6 ♀♀ (MNHN-Paris).
Other material  GREECE, Athens, University campus, 9 December 1996, Sfenthourakis (by hand), 2 ♂♂ 2 ♀♀, (Poitiers laboratory collection); FRANCE, Réunion Island, Saint Paul, compost, 1 ♂ 1 ♀, 20 August 1996, Juchault & Caillon (by hand), (Poitiers laboratory collection).

Etymology  The name refers to the general greyish coloration of the antennae, uropods and lateral margins of the pleon.

Diagnosis  No meristic character has been identified to indubitably discriminate between specimens of P. percanus (sp. n.) and those of P. pruinosus (Brandt, 1833). Subtle differences in morphology or coloration may be used in congruence to distinguish this new species. These include: 1- the absence of distinctive white spots on antennae in the distal extremity of the second and third articles of the endopodite (Fig 3) (note: this single character is not absolutely diagnostic, since Garthwaite & Sassaman (1985) reported that P. pruinosus from England (Elsworth) include some specimens that present white bands and others that do not); 2- the frontal line which is more sinuous in P. percanus in relation to more pronounced angles between the median and lateral lobes (Fig 3); 3- the inferior margin of the pleonite 7 which shows, in P. percanus, 2 slightly lateral convexities where appear the epimera of the pleonite 3 (in consequence, the inferior margin of the pleonite 7 is not semi-circular, contrary to what is observed in P. pruinosus) (Fig 3). Among others differences, P. percanus tends to be larger, particularly in two or three year-old animals. They also show weaker dorsal tuberculations, especially for the pleonites 6, 7 that appear totally smooth. These nuances are sometimes difficult to appreciate and some misclassifications cannot be excluded. Nevertheless, they are of great value as they are not sexual (observed in both sexes).

Description  Male (holotype). Total body length (from the median lobe of the cephalon to the tip of the telson) 8.34 mm, total body weight (alive) 35 mg, cephalon 0.80 mm x 1.92 (length x largest width), pereon 5.52 mm x 3.03 mm (length x largest width), pleon 2.02 mm x 2.23 mm (length x largest width).

Discussion  Laboratory crossings revealed two population groups that are non-interfertile; on one hand, populations from France and Spain, and on the other hand, populations from Tunisia and the Réunion Island. Molecular analysis carried out on mitochondrial DNA (mt LSU rDNA) has also confirmed the existence of two distinct population groups, which differ by a mean divergence of 20.8% (Michel-Salzat & Bouchon, 2001). Phylogenetic reconstruction identified two groups, a result confirmed by the high coefficient of isolation (0.98) observed between the French, Spanish and Israel populations and those from Tunisia, Greece and the Réunion Island.

Moreover, bearing in mind that isolation tends to be higher in nature than under laboratory conditions (Dobzhansky, 1970), there is no doubt that the two population groups clearly correspond to two well-defined species. In fact, a single interspecific crossing between a French female and a Tunisian male produced young. However, only one pulli was produced in this particular crossing, whereas intraspecific crossings (French females with French males or Tunisian females with Tunisian males) produced on average 15 pulli. This result is consistent with previous observations made by Marcadé et al. (1999) who reported fertilization and subsequent egg abortion for the same crossing combination. Such inviability among hybrid generations may be the result of a post-zygotic mechanism of reproductive isolation (Dobzhansky, 1970). Mate choice results, however, showed that the reproductive isolation observed is above all related to behavioral isolation in the absence of recognition between sexual partners. There exists strong evidence to impute this pre-zygotic isolation to chemical differences in female cuticular compounds. Indeed, male sexual behavior systematically requires a first phase involving recognition by means of antennal contacts, and antennae have been shown to be the main sensory organs, possessing at their tips a number of chemoreceptors (Mead, Gabouriaut & Corbière-Tichané, 1976; Hoese, 1989). Among the Oniscidea, chemical communication is strongly suggested in many species (Legrand, 1958; Mead, 1973; Ducruet, 1976), and is particularly evident in the monogamous Hemilepistus reaumuri, for which closed family communities require individual recognition (Linsenmair, 1984). In the case of P. pruinosus, specific compounds responsible for aggregation behavior have already been identified (aggregation pheromone) (Takeda, 1984). Thus, the involvement of chemical molecules in sexual communication is undeniable, and even if sex pheromones remain poorly documented in the Oniscidea (Lefebvre et al., in preparation), their involvement in the example of reproductive isolation described here
appears evident. Among Arthropods, such behavioral isolation is known to be one of the most widespread mechanisms of reproductive isolation (Dobzhansky, 1970; Mayr, 1974).

The different experimental approaches applied in this study (interfertility, behavioral and morphological analysis) give congruent results that lead to the same conclusion. What has been previously considered to be a single species, namely *P. pruinosus*, consists, in fact, of two groups that are reproductively isolated. One of these groups (populations from France, Spain and Israel) clearly corresponds to the species *P. pruinosus* that was first described in Europe by Brandt (1833). In contrast, populations of the second group (the Réunion Island, Tunisia and Greece) correspond to another taxon that must be raised to a species status.

Examination of specimens from both groups revealed slight differences in both coloration (antennae, uropods and epimera) and shape (frontal line and inferior margin of the pereonite 7) (Fig. 3). However, in the absence of meristic characters, the observed differences are not absolutely discriminant and need to be used in congruence to discriminate between the two species (Fig. 3). According to Mayr (1942), natural populations that differ only subtly in morphology, but are reproductively isolated, must be designated as sibling species. We propose here to name this sibling species *P. percanus*, in reference to the general grey coloration of antennae, uropods and pleonite (percanus meaning totally or uniformly greyish).

The map provided in Fig. 4 designates the different sampling locations and assigns each population to its corresponding sibling species. According to Vandel (1962), the genus Porcellionides originated from the Mediterranean region (Asia Minor). Molecular data suggest that the divergence may have occurred during the Miocene (10 Myr ago). However, due to their well-recognised synanthropy (Dollfus, 1897; Vandel, 1960; Schmalfuss & Ferrara, 1978), phylogeographic considerations of their present day distribution remain obviously uncertain. Nevertheless, it is interesting to note that, in spite of their insularity, specimens from the Réunion Island remain interfertile with those of Tunisia. This suggests a recent introduction of specimens from the Mediterranean zone. Such an introduction may have occurred by means of natural expansion due to, for example, their facility for rafting on floating vegetation (Taiti, Ferrara and Kwon, 1992), but is most probably of anthropogenic origin following the colonisation of the island during and after the 16th century.

To date, it is thought that the suborder Oniscidea contains approximately 4000 species throughout the world, and thus constitutes (one of) the largest groups among the Isopoda. While a large number of species remain to be discovered, particularly in tropical areas (Kensley, 1999), thorough investigations over the last decades have fragmented a number of formerly defined species into a complex of closely related species, either by the description of new species (*Oritoniscus remyi* by Dalens, 1964; *P. floria*, by, Garthwaite & Sassaman, 1985; *Oniscus ancarensis* by Bilton, 1992) or by a change in the sub-specific status (complex *Oritoniscus* by Dalens, Rousset & Fournier, 1996; *Oniscus galicianus* by Bilton, 1997). There is no doubt that the systematics of this group requires further consideration, not only through the use of morphological criteria, but also by applying multiple approaches that primarily address the biological concept of interfertility. The result of the present study, with the description of the new species *P. percanus*, is certainly not an isolated case. Due to close similarities in morphology, specimens collected from various localities throughout the world have been first assigned to the cosmopolitan *P. pruinosus* (Brandt, 1833). The possibility of two distinct species was first proposed based on mitochondrial DNA variability (Marcadé, 1998). The confirmed existence of two sibling species, however, was only possible by testing their reproductive isolation (interfertility and/or behavioral experiments). In the absence of such complementary approaches, one can reasonably assume that the number of sibling species is obviously underestimated.

**Acknowledgments**

We would like to thank P. Juchault, M. Caillon, C. Souty-Grosset, and S. Sfenthourakis for help in sample collection and M. Johnson for proofreading the English.
References

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Annexe 1  Evidence for a new terrestrial crustacean species


### Table I.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Coordinates</th>
<th>Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nevers (F)</td>
<td>47°00'N; 3°09'E</td>
<td>* §</td>
</tr>
<tr>
<td>Manzanares (S)</td>
<td>40°00'N; 4°00'W</td>
<td>* §</td>
</tr>
<tr>
<td>Athens (G)</td>
<td>37°56'N; 23°36'E</td>
<td></td>
</tr>
<tr>
<td>Tunis (T)</td>
<td>36°50'N; 10°15'E</td>
<td>* §</td>
</tr>
<tr>
<td>Haifa (I)</td>
<td>32°50'N; 35°00'E</td>
<td>§</td>
</tr>
<tr>
<td>St Paul, Réunion Island (F)</td>
<td>21°00'N; 55°17'E</td>
<td>* §</td>
</tr>
</tbody>
</table>

### Table II.
Results of single pair crossings between sexual partners from the different populations. Data correspond to the number of successful crossings (assessed by the presence of young). The number of live pairs at the end of the 3 month experiment is given in brackets.

<table>
<thead>
<tr>
<th>♂</th>
<th>♀</th>
<th>France</th>
<th>Spain</th>
<th>Tunisia</th>
<th>Réunion Island</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>8 (8)</td>
<td>1 (1)</td>
<td>0 (11)</td>
<td>0 (8)</td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>0 (1)</td>
<td>0 (2)</td>
<td></td>
</tr>
<tr>
<td>Tunisia</td>
<td>1 (9)</td>
<td>0 (0)</td>
<td>11 (11)</td>
<td>8 (9)</td>
<td></td>
</tr>
<tr>
<td>Réunion Island</td>
<td>0 (9)</td>
<td>0 (1)</td>
<td>11 (11)</td>
<td>9 (10)</td>
<td></td>
</tr>
</tbody>
</table>

### Table III.
Results of single pair encounters between sexual partners from the different populations (‘male choice’). Data correspond to the mean Index of Sexual Activity (amount of sexual male activity towards a given female). The number of encounters performed in each combination is given in brackets.

<table>
<thead>
<tr>
<th>♂</th>
<th>♀</th>
<th>France</th>
<th>Israel</th>
<th>Spain</th>
<th>Tunisia</th>
<th>Réunion Island</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>0.142 (20)</td>
<td>0.321 (7)</td>
<td>0.224 (6)</td>
<td>0.054 (20)</td>
<td>0.000 (6)</td>
<td></td>
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<tr>
<td>Israel</td>
<td>0.368 (6)</td>
<td>0.139 (7)</td>
<td>0.021 (6)</td>
<td>0.000 (9)</td>
<td>0.000 (9)</td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>0.346 (6)</td>
<td>0.292 (7)</td>
<td>0.291 (6)</td>
<td>0.0000 (9)</td>
<td>0.016 (9)</td>
<td></td>
</tr>
<tr>
<td>Tunisia</td>
<td>0.003 (20)</td>
<td>0.000 (7)</td>
<td>0.000 (6)</td>
<td>0.199 (35)</td>
<td>0.208 (22)</td>
<td></td>
</tr>
<tr>
<td>Réunion Island</td>
<td>0.026 (6)</td>
<td>0.006 (7)</td>
<td>0.000 (6)</td>
<td>0.187 (20)</td>
<td>0.209 (22)</td>
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</table>
Fig. 1. Tree diagram (hierarchical cluster analysis according to the “nearest neighbours” method) based on Euclidean distance calculated from the matrix shown in Table III.

Fig. 2. Electron micrographs of the dorsal surface of Porcellionides pruinosus from France (A) and P. percanus from the Réunion Island (B). Scale bar = 0.1 mm. P, pereionite.
Fig. 3. A, C, E, G. *P. pruinosus*; B, D, F, G. *P. percanus*, most reliable characters for discriminating between the two species. A, B Second antennae. Scale bar = 0.5 mm. Particular attention should be given to: the coloration of the third article (carpopodite), (C, D) the shape of the frontal line (cephalon x3), and (E, F) the inferior margin of the pereon (pereonite 7 x3). Drawing modified from Oliver & Meechan (1993), with permission from The Linnean Society of London. G, H Posterior part of pereon, pleon and uropods. Scale bar = 0.1 mm.
Fig. 4. World distribution of Porcellionides pruinosus (stippled design) and localisation of the populations studied. The countries where Porcellionides pruinosus (Brandt, 1833) were found are hachured; countries where Porcellionides percanus (sp. n.) were found are squared.
Sex pheromones in terrestrial Isopods: Evidence for chemical communication and for cuticular ecdysone

(à re-soumettre à Journal of Chemical Ecology)

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Abstract:

We first give a review on sex communication in crustaceans. Authors have long stand propose ecdysone or its derivatives as sex-attractants, or at least as components of the pheromonal complex. Nevertheless, there are also strong evidences to consider vitellogenesis process in seeking the likely source of sex pheromones. In woodlice (terrestrial crustaceans), the subject remains poorly documented, even if chemical cues are supposed in many aspects of their behaviours. By an ethological approach, we clearly demonstrate that males are able to discriminate between reproductive and non-reproductive females by the only means of cuticular compounds. We give then an appropriate method that permit to extract cuticular compounds from few animals. In thin layer chromatography, such extracts reveal the presence of esters, hydrocarbons and sterols. In gas chromatography, first attempts show that extracts from females are mainly characterised by a great peak at the fiftieth minutes, which was then identified as 20-hydroxyecdysone. Nevertheless, males also present ecdysones on their cuticle. Further investigations on sex pheromones need quantitative and qualitative analysis, which can be performed by fine comparison between sexes and by coupling gas chromatography with mass spectrometry.

Key words:
Sex pheromone, reproduction, Crustacea, Armadillidium vulgare, cuticle, gas chromatography, ecdysone

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Introduction

In 1966, Ryan first demonstrated the existence of sex pheromone in Crustacea. Since then, sex-attractants have been reported, or at least seems likely, in many aquatic species, including decapods, copepods, stomatopods and amphipods (see Bauchau, 1986; Dunham, 1978, 1988 for reviews). For most species examined, biological activity has been found in the urine released by females (Ryan, 1966; Bamber and Naylor, 1997; Eales, 1974; Mac Leese, 1977; Gleeson, 1980; Bushmann and Atema, 1994). In any case, the sex signal is mediated by water and acts at distance on male antennules (Gleeson, 1982; Ameyaw-Akumfi and Hazlett, 1975; Bamber and Naylor, 1996).

The chemical nature of crustacean sex pheromones has not yet been characterised. The frequent synchronisation between moulting and mating led some investigators to propose ecdysone or its derivatives as sex-attractants, or at least as components of a pheromonal complex (Kittredge et al., 1971; Hammoud et al., 1975). According to Kittredge et al., crustecdysones effectively induce male sexual behaviour in the crabs *Pachygrapsus crassipes*, *Cancer antennarius* and *Cancer anthonyi*. The authors report that after exposure to dilute solutions of crustecdysones (β-ecdysone, 20R-hydroxyecdysone, ecdysterone, isoinokosterone) males attempt to seize other crabs, male or female, and pull them into a precopulatory position. These observations clearly demonstrate a lack of specificity and lead to the conclusions that the moulting hormone is either one component of the chemical signals or sufficiently similar in molecular structure to the natural pheromones to mimic them. Anyway, males undergo presumably identical biochemical changes prior to moulting, and fail to evoke, in natural conditions, sexual responses from other males. This evidence, therefore, strongly suggest to consider vitellogenesis process in seeking the likely source of sex pheromones. This seems the case in *Homarus americanus* and the spider crabs, for which authors conclude that the sex pheromone might originate in the ovary (Mac Leese et al., 1977; Jones and Hartnoll, 1997).

In woodlice (terrestrial crustaceans which have evolved from aquatic ancestors), authors have long stand supposed the existence of chemical cues in many aspects of their behaviours (Legrand, 1958; Mead, 1973; Ducruet, 1976). This is, among other thing, supported by the fact that, contrary to other aquatic crustaceans, visual acuity in terrestrial forms appears extremely reduced. The same conclusions also derived from behavioral observations: any interactive behaviours always begin after previous antennal contact. Among woodlice, chemical communication certainly raises a maximum of complexity in the subsocial species *Hemilepistus reaumuri*, in which closed family communities requires interindividual recognition (Linsenmair, 1984). In some species (*Armadillidium vulgare*, *Ligia exotica* and *Porcellionides pruinosus*), an aggregative pheromone has already been demonstrated from faecal pellets (Takeda, 1980, 1984). With regard to chemical structure of the cuticular compounds, some interesting results have been done with studies on resistance to transpiration (Hadley and Warburg, 1986). Authors concluded all the examined species (*Hemilepistus reaumuri, Armadillo officinalis, Armadillo albomarginatus* and *Ligia oceanica*) revealed the presence of free lipids in which they discriminate hydrocarbons, free fatty acids, acylglycerols, cholesterol, and several unidentified pigments. Nevertheless, up to day, any studies have investigated the implication of such cuticular compounds in the sexual behaviour.

In this work, our first purpose is to provide some proofs about the implication of cuticular compounds as sex attractants in the pillbug *Armadillidium vulgare*. We give then an appropriate methodology to extract and analyse the chemical nature of the sex pheromone.

Material & Methods

*Armadillidium vulgare* derived from a population collected in Greece (Héraklion, 1989), and maintained in laboratory since then. Animals used in this work were all born during the previous spring and bred at 20°C, under natural photoperiod (Poitiers 46°40’N). As soon as possible (near the fourth moult) young of each progeny were sexed, then males and females were reared separately. The rearing boxes (26 x 13 x 8 cm) were filled with moist earth, previously dried in the open air during 1 month. Food (dead leaves and slices of fresh carrots) was provided *ad libitum*.


**Choice’s tests**

Choice’s tests were performed on males (n = 19) during the reproductive season (May). Males were tested in «open-field» within a Petri dish (area: 64 cm²; moistened paper covering the bottom; 90% HR). At to, the tested male is introduced in a central area, regarded as a neutral zone (area N = 7 cm²). During a fifteen minutes period, the male has a free choice to visit the N zone, like four adjacent zones also proposed (area = 14.25 cm²). Each one contains a “target” individual, beforehand killed by freezing. By this way, any behaviour of the “targets” cannot interfere in the choice. The “targets” were a male (M) and three females ranged according to their state of moulting (see Drach and Tchernigovtzeff, 1967 for nomenclature): C period (C), beginning of D period (D₀₋₁), end of D period (D₁₋₄). After experiments, dissections were systematically set about on “targets” females in order to determine their state of ovarian maturation (see Besse, 1976). Times spent in each zone were compared with those expected in random choice by Mann & Whitney test.

**Chemical analysis**

Immediately after sampling from the rearing boxes, animals are frozen at -80°C during 5 minutes. They are later conserved since extraction at 4°C. Morphological measurements (length (l), width (w), height (h)) are performed on each animal in order to determine an approximate cuticular surface (S=[πl((π(8h²+2w²))+4w)]/16). Whole animals are immersed in hot chloroform-methanol mixture (2:1 v/v; 50°C; 20 ml) for 9 minutes at room temperature. The extraction process is achieved by 1 minute under ultrasonic waves. The solution was then filtered and evaporated to dryness under nitrogen flux. Just before analysis, the dried extract is dissolved in 20µl chloroform, of which 1µl is injected.

In addition, solution of 20-hydroxyecdysone was prepared by diluting 1mg of crystal (extracted from the fern *Polypodium vulgare*) in 300 µl chloroform-methanol mixture (2:1 v/v), of which 1µl is injected (3.33 µg). Thin layer chromatography was performed on Alugram Sil G/UV 254 plate from Macherex-Nagel. Diethyl acetate/cyclohexane (8:92 v/v) was used as mobile phase and 5% H₃Mo₁₂O₄₀P in ethanol as staining mixture. Gas chromatographic separations were performed on a Shimadzu GC-14A, equipped with a CP-SIL 8CB capillary column (50 m length, 0.25 mm inner diameter, 0.12 µm film thickness). The gases used were H₂ (0.55 kg/cm²), synthetic air (0.45 kg/cm²) for FID and He (2.5 kg/cm²) as carrier gas. The detector was set at 300°C and the injector at 250°C. The column heating program was 5 min at 60°C, 5°C/min from 60 to 300°C, and then 20 min at 300°C. Signal treatment was accomplished with a C-R4A integrator.

**Results & Discussion**

**Choice’s tests**

We present results from choice’s test in the figure I. Males show a preference (in term of time investigated in the corresponding zones) for females in D-period. Nevertheless, only the durations for the D₁₋₄ zone significantly differ from those expected in a random choice (n=19, U=95, p=0.013). Dissections have revealed that C females were in previtellogenesis or primary vitellogenesis whereas all D females were in secondary vitellogenesis. This last state of ovarian maturation corresponds to the single period during which mating can occur (Mead, 1976; unpublished results). Since target animals have been previously killed, any behaviour has interfered in the male’s choice; therefore the discrimination was only based on chemical cues.

**Chemical analysis**

In this study, gas chromatography has been performed from extract of 5 attractive females *Armadillidium vulgare*. We estimate the total cuticular surface to 960 mm² (860 mg). The chromatogram is presented on the figure IIb. Cuticular compounds are characterised by a retention time comprised between 25 and 60 minutes. Five main peaks account for 49% of the total area, with a greatest one at the fiftieth minutes (26%). In addition, we have reported gas chromatography result from moulting hormone solution on the figure IIa (20-hydroxyecdysone, and probably some derivative compounds, at 3.33µg/µl). The moulting hormones are
revealed around the fiftieth minutes, in exact correspondence with the greatest peak observed in cuticular extracts. The presence of sterols in cuticular extracts was confirmed by thin layer chromatography (Rf = 0.1). According to our knowledge, this represent the first evidence for the presence of moulting hormone on the cuticle of woodlice. Nevertheless, such a result was quite expected. Indeed, the moulting hormone is known to block some physiological processes when present in haemolymph at critical stages, so it has to be regularly degraded and/or released outside. For the normal ecdysis to occur, the hormone level should be very low or absent at the end of the intermoult. In Armadillidium vulgare, injection of 20-hydroxyecdysone 1-2 days before the posterior ecdysis blocks the shedding of the exuvia (Maissiat and Graf, 1973; Madhavan, 1981). Moreover, in ovigerous females, applications of 20-hydroxyecdysone can stop embryonic development or induce eggs abortion (Madhavan, 1981).

In some crabs and amphipods, moulting hormones are known to induce sexual male behaviour (Kittredge et al., 1971; Hammoud et al., 1975). However, ecdysones are more generally viewed as components of a pheromonal complex. In our terrestrial forms, the problem is certainly similar since ecdysones are also detected in male extracts like for other woodlice species (unpublished result). The identification of woodlice sex pheromones needs further investigations. In insects, for which cuticular compounds are abundant, sex pheromones were generally separated and identified by coupling gas chromatography with mass spectrometry (Tamaki, 1985). In woodlice, referring to previous investigations (Hadley and Warburg, 1986), it seems that cuticular compounds are present at rather low concentrations. In Porcellio laevis, authors estimate the lipids to nearly 0.17 mg per gram of isopod. The extraction process described in this work should nevertheless permit us to collect enough materials to couple mass spectrometry with gas chromatography. Moreover, by increasing the concentration and/or the volume injected into the column, it even becomes possible to analyse individual profiles.
References


Figure I.
Times (sec.) spent by the male in each zone. N: introduction; M: male; C: female in C-period; D\(_{[0-1]}\): female at the beginning of the D-period; D\(_{[1-4]}\): female at the end of the D-period.

Figure II.
Chemical analysis by gas chromatography. a: 1µl injection of 20-hydroxyecdysone solution (1mg in 300 µl chloroform-methanol). b: 1µl injection of cuticular compound solution (dry extract from 5 attractive females Armadillidium vulgare redissolved in 20 µl chloroform-methanol).
Antennal sexual dimorphism in terrestrial isopods: A result of male contest or scramble competition?


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Abstract:
In some Oniscidea (terrestrial crustaceans) males were shown to exhibit longer antennae than females. This sexual dimorphism may result from a variety of selection pressures. However, there are some species well known for their males highly aggressive, using antennae as weapons. We test here the hypothesis according to which longer antennae in males have been selected for by means of antennal contests. For this, morphological analysis on antennae and behavioral analysis on male dyads were performed in parallel on 7 species. We demonstrate significant antennal dimorphism in 6 of the 7 species, and various form of male aggressiveness according to the species. We have to reject our first hypothesis because we found a negative correlation between the use of antennae in contests and the magnitude of the dimorphism. Furthermore, some species are sexually dimorphic although their males never compete by means of antennae. We then propose and argue that scramble competition to first find receptive females could explain why males have longer chemoreceptive antennae.

Résumé :
Chez certains Oniscidea (crustacés terrestres), il a été montré que les mâles présentent des antennes plus longues que les femelles. Un tel dimorphisme sexuel peut résulter d’une multitude de pression de sélection. Cependant, chez certaines espèces les mâles sont connus pour être très agressifs, et utilisent leurs antennes comme armes de combat. Nous nous proposons ici de tester l’hypothèse selon laquelle les antennes des mâles ont évolué sous la pression des combats antennaires. Pour cela, une analyse morphométrique des antennes, et une analyse comportementale de rencontre mâle:mâle ont été menées en parallèle sur 7 espèces. Nous montrons qu’il existe un dimorphisme antennaire significatif pour 6 des 7 espèces étudiées, et que d’autre part l’aggressivité des mâles peut se manifester sous différentes formes selon les espèces. Nous devons ensuite rejeter notre première hypothèse car nous trouvons une corrélation négative entre l’utilisation des antennes au combat et l’importance du dimorphisme observé. De plus, certaines espèces sont sexuellement dimorphique bien que leurs mâles ne combattent pas par l’intermédiaire des antennes. Nous proposons alors, en argumentant, que la compétition par scramble pour la recherche active de femelles réceptives peut très bien expliquer pourquoi les mâles présentent de plus longs organes chémorécepteurs.

Key words:
Oniscidea, terrestrial crustaceans, antennae, sexual dimorphism, male contest, sexual selection, mating strategies, scramble.
Introduction

Sexual dimorphism is one of the most striking traits that led Darwin to put forth his theory of sexual selection (1871). Body size is certainly the most conspicuous difference between males and females, but it may also concern coloration or many other ornaments. In literature on Oniscidea (terrestrial crustaceans), antennal sexual dimorphism in favor of male is reported in some families such as Trichoniscidae (Hylonuscus, Trichoniscus), Oniscidae (Ctenosia), and among the genus Porcellio, but without any explanation for the reason (Vandel 1960). This obviously may result from a variety of selection pressures, but the exact knowledge to this question would be very helpful for whom want to investigate mating strategies in Oniscidea.

We know that sexual selection theory is successful in explaining why some organs used as weapons are more developed in the fighting sex (Darwin 1871; Andersson 1994). Among Insects, male dimorphic weapons (enlarged horns, mandibles or other hornlike structures) occur in beetles, bees, flies, pentatomid bugs, earwigs, thrips (Brown et al. 1985; Conner 1989; Crespi 1986; Eberhard and Gutiérrez 1991; Hamilton 1979; Thornill and Alcock 1983; Wirtz et al. 1988). In Crustacea, fiddler crabs and some crayfishes are well known for their males having one or the two claws greatly enlarged, using it as a signal and weapons in contests over males (Crane 1975; Stein 1976).

Until to now, male contests in Oniscidea were only observed in few species that belong to various families: in the Ligidae Ligia exotica (Farr 1978), in the Armadillidae Venezillo evergladensis (Johnson 1985), in the Trachelipidae Hemilepistus reaumuri (Linsenmair 1984), in the Porcellionidae Porcellionides (Metoponorthus) sexfasciatus (Mead 1973), and in some north African Porcellio species (Linsenmair 1984). What is consistent among all these species is that antennae are systematically involved in the agonistic behavior, firstly in the initial phase for recognition and later as the weapons of contests. In Venezillo evergladensis, when two males encountered each other, Johnson (1985) reported that “their cephalon came very close to actual contact and active antennal tapping began (…) and continued 15-20 sec”.

Generally, three main kinds of resources may be coveted: food, territory and sexual partner. In contrast to fruit-eating species or flesh-eating species, competition for food is rarely observed in species like woodlice, which mainly breed on vegetal detritus (Sutton 1980). This theoretical point of view is supported by Mead (1973) who concludes that the observed aggressiveness in the species Porcellionides sexfasciatus is irrelevant to alimentary competition. On the other hand, defense territory may be suspected since suitable shelters offering both humidity and obscurity are generally patchy distributed (Schmalfuss 1998). Nevertheless, aggressiveness in Oniscidea was mainly observed between males, which strongly suggests a competition for access to reproductive female.

We propose here to test the hypothesis according to which male antennae in Oniscidea have evolved by means of antennal contests. For this, we have undertaken in parallel a morphological analysis on antennae and a behavioral analysis of male dyads. Seven species belonging to 3 different families were investigated in order to apply comparative approach on mating strategies. Other selection pressures that may act on male antennae were also discussed in the last part of this paper.

Material and methods

Animals used in this work belong to three families of Oniscidea. We have first investigated among the Porcellionidae family, for which both cases of male contest and antennal dimorphism were already known (in Porcellionides sexfasciatus and some Porcellio species). Two species belonging to the genus Porcellionides, P. pruinosus and P. cingendus, and two others belonging to the genus Porcellio, P. scaber and P. dilatatus were investigated. In addition, two Armadillidiidae species, Armadillidium vulgare and Armadillidium nasatum, and one Oniscidae, Oniscus asellus, were also studied. All males and females used in this study derived from a one-year-old cohort.

Morphological analysis: measure of antennal dimorphism

In woodlice, the second antenna consists of a pentamous peduncle, which carries a flagellum of varying segments (Wägele 1983). The distal segment of the flagellum bears the apical organ that consists of
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chemoreceptive setae (Mead et al. 1976). The first two visible articles of the peduncle (coxa and basis) derive from the protopod, whereas the 3 following articles and the flagellar segments belong to the endopod (Hoese 1989). The whole antenna was removed and fixed on a slide with silicon grease. In practice, the coxa is systematically damaged when antenna is removed. In this study, the following morphological data were considered: length and width of basis (BA and ba), length of the following 3 articles (ART1, ART2 and ART3), length of the 2 or 3 segments of the flagellum (F1, F2, and F3 in Oniscus asellus), and the total length from the basis to the tip of the flagellum. Measurements were made on the ventral face to the nearest 0.03 mm using an eyepiece micrometer. The position of these measurements is indicated on the Fig. 1. For more precision, a mean between the direct length and a cumulated length (sum of the different parts) was calculated to give a mean length of antennae (A2). Because of Mocquard (1971), in Porcellio dilatatus and Oniscus asellus, has never observed asymmetry concerning the length of different appendix (pereopods, antennae), only one antenna (left) was investigated in this study.

In Oniscidea, females are generally larger than males (Vandel 1960). For this reason, and because of additional within sex variability, antennal lengths need to be weighted with a reference dimension. Total body length is an easy and meaningful reference, but is rarely used particularly due to the imprecision of its measurements. The basis of pereopod (leg in Oniscidea) is often reported as a suitable reference dimension (Charniaux-Cotton 1957; Tessier 1955), since this article is assumed to exhibit no sexual differentiation (Mocquard 1971, in Oniscus asellus). In this study, we used a mean between the 2 basis of the third pereopods (left and right) as the reference dimension (REF). Measurements were made on the external face (Fig. 1) with the methodology applied for antenna.

Morphological analysis was then performed on the logarithm (ln) of these values. In a first step, we need to test the validity of our reference dimension. To see if basis length (REF) is effectively independent to sexual influence, we have to perform Pearson correlation tests between A2 and REF for each sex, and then to test the homogeneity between the 2 resulting coefficients. Under these assumptions, statistical comparisons between sex were performed by analysis of covariance with REF as regressor. The sexual dimorphism was expressed in term of percentage and µm in favor of male. Finally, discriminant analysis was performed in order to point out what antennal articles are involved in the observed dimorphism. The sample size was constant throughout the morphological analysis: 15 males and 15 females by studied species.

**Behavioral analysis: measure of male aggressiveness**

Because of lucifugous habits (Cloudsley-Thompson 1956; Mead 1968), the activity of woodlice is mainly concentrated during the scotophase or under suitable shelters (stone, dead wood, leaf-litter, etc.). So, standardized behavioral studies in the field cannot be easily performed. In consequence, observations in this work were made in laboratory under controlled conditions: reduced lighting intensity (200-250 lx), constant temperature (20°C) and high humidity (80-90 % H.R.). All observations were performed in a Petri dish (Ø = 9.5 cm, area = 71 cm²), lined with moist filter paper and topped with a transparent glass slide in order to limit air disturbance. Previous investigations have shown that male aggressiveness can be observed in absence of female (Mead 1973). This characteristic allows us to check the complete sequence of aggressive behavior (in absence of precopulatory behavior and mating posture) that sometimes leads to hierarchical relationship between males. In addition, particular conditions have been applied in order to give rise to male aggressiveness. Firstly, tests were practiced on sexually mature males during the reproductive period (May-June). Secondly, equal size males were confronted in order to limit asymmetry between opponents. Thirdly, since social isolation in crustaceans, like in many others animals, is known to increase agonistic behavior (Lorenz 1966), males were previously isolated before the encounter, during at least 5 days. In the same time, this isolation permits to minimize any impact of previous agonistic contests on the aggressiveness exhibited during the current encounter.

Males were confronted in dyad over a 20 minutes period. The t0 time was associated with the simultaneous introduction of the 2 males. For all species, a typical encounter was recorded and then visualized by several persons in order to limit subjectivity of such behavioral studies.
Preliminary observations have shown that the interactive behavior of a given male always begins by brief antennal contacts for recognition. Based on this information, either the male passes on or exhibits aggressive behavior. In consequence, we have characterized 2 types of interactive behavior:
recognition behavior: brief antennal contacts immediately followed by separation
aggressive behavior: brief antennal contact immediately followed by marked behavior using antennae or any other body parts (i.e. faster oscillatory movements, antennal tapping, pushings, mountings, chases, bites…)
The duration (in second) and the number of occurrences of these items were noted for each male, overall the observation’s period. In order to quantify male aggressiveness, an individual index (IAg) was calculated as follows:

\[ IAg = \frac{\text{aggressive behavior (duration x nb. occurrence)}}{\text{interactive behavior (duration x nb. occurrence)}} \]

The product “duration x nb. occurrence” was used as it translated at best the amount of aggressiveness really exhibits by each male during the encounter. Such an index permits to compare male aggressiveness within species and between species, in taking relative general activity into account (here, general activity is assimilated to interactive behavior). For example, an index equal to 1 means that all interactions with the other male have led to aggressive behavior.

**Comparative analysis**

To check the assumption that antennal dimorphism is correlated with the use of antennae in contest, a comparative analysis was performed. Ordinary statistic cannot be validly applied since our variables (observations of species) are not independent (Harvey and Pagel 1991). Indeed, closely related species tend to show similarities because of shared inheritance (common ancestry or convergent evolution), rather than through independent adaptation. Therefore, comparisons between species should be based on the number of times relations have evolved independently. We have first to generate statistically independent linear contrasts between taxa (CAIC computer package: Purvis and Rambaut 1995). The phylogeny applied here is given by recent molecular investigations based on 16S RNA sequences (Michel-Salzat and Bouchon, submitted). In absence of unequivocal units of evolutionary change, the assumption that branch lengths are equal (punctuated evolution model) has proved the most robust option in simulation studies (Purvis et al. 1994) and was used here. Then, the relationships between dimorphism in length (% in favor of male) and IAg, and dimorphism in width (% in favor of male) and IAg, were tested by regression through the origin. The expected value of the slope equals the true relation between variables in the absence of phylogenetic effects (Pagel, 1993). Only the species that use antennae as weapons in contest were taken into account in these analyses.

Behavioral and morphological results were also integrated in a general overview on the 7 species by using a cluster analysis. It was performed on the 2 following variables: dimorphic value in length (% in favor of male) and intensity of male competition (IAg). Species were ranged according to the “nearest neighbors” method (STATISTICA). Such a method would permit to class the different species in relation to the mating strategies they adopt.

**Results**

**Morphological analysis**

For each of the 7 investigated species, the statistic homogeneity of the 2 correlation coefficients (non significant \( p \) value) from the relationship between A2 and REF in each sex, revealed the independence of the reference dimension (REF) to sexual influence. Under this assumption, the antennal sexual dimorphism can be thoroughly investigated. We found that antennae are longer in males than in females for all the species (from 2.6% to +15.2% according to the species) (Table 1). The dimorphism is highly significant (\( F_{[1,27]} = 14.62 \) (lowest value); \( p < 0.001 \), except for the species *Porcellionides cingendus* (\( F_{[1,27]} = 0.02; p = 0.89 \)). For the 2 *Armadillidium* species and for *Oniscus asellus*, discriminant analysis revealed that the dimorphism mainly results from longer distal flagellar segments (F2 or F3) (Table 1). For the 2 *Porcellio* species, the
sexual difference is rather explains by basal antennal segments (BA and/or ART3), whereas all segments are involved in the dimorphism in Porcellionides pruinosus. Particular attention on basal segment of the antennae (basis) has revealed that males have larger basis in the following species: Porcellio scaber ($F_{[1,27]} = 28.25; p < 0.001$), Porcellio dilatatus ($F_{[1,27]} = 33.58; p < 0.001$) and Armadillidium vulgare ($F_{[1,27]} = 5.98; p < 0.05$) (Table 2).

**Behavioral analysis**

Males of Porcellionides pruinosus and P. cingendus showed the highest indexes of aggressiveness (IAg); respectively 0.402 and 0.660 (Table 3). The pattern of aggressiveness in these two species reminds that was described by Mead (1973) in Porcellionides sexfasciatus. When one male encounters the other one, it first scrutinizes and tastes the tergal surface by means of antennae (chemical visa detection). Generally, this recognition phase is made simultaneously by the two males. At this point, either males pass on and pursue the exploration of the box, either one, or the two, exhibit antennal tapping. When the two males do so, this can lead to intense struggles (mountings, bites).

In the species Porcellio scaber, antennal tapping sometimes occur but struggles (as described above) were never observed. This partly explains why the levels of aggressiveness is lower in this species (IAg = 0.212). In contrast, males in Porcellio dilatatus, Armadillidium vulgare, A. nasatum and Oniscus asellus seem poorly aggressive. Some traits have nevertheless been interpreted as aggressive behavior: frontal pushing in Armadillidium vulgare (0.087), mountings in Armadillidium nasatum (0.068), antennal tapping in Porcellio dilatatus (0.060) and Oniscus asellus (0.007). Such behavior exhibited by one male never induced an aggressive response from the other; in consequence, no struggle has been observed in these species. Moreover, keeping in mind that potential aggressiveness has been raised by previous social isolation, one can reasonably expected that male contests in these species are unusual under natural conditions.

**Comparative analysis**

When corrected values of the antennal sexual dimorphism in length (% in favor of male) are plotted against corrected IAg, we found a negative correlation ($n = 4; r = -0.90; p < 0.05$) (Fig. 2). This would indicate that that longer antennae in male have not been selected for by means of antennal contest. In the same way, dimorphism in width is no more associated with IAg ($n = 4; r = -0.72; p = 0.168$). These 2 congruent data clearly lead to the conclusion that antennal contests are irrelevant to explain the observed dimorphism.

A general analysis based on behavioral and morphological results (IAg, dimorphic value) clearly separates the 7 investigated species into 2 clades (Fig. 3). One of them (clade 2) regroups the poorly dimorphic species that intensively compete (Porcellionides cingendus and P. pruinosus). Due to high dimorphic value, Porcellio scaber is connected to the clade 1, that contains the others species (Porcellio dilatatus, Oniscus asellus, and the 2 Armadillidium species).

**Discussion**

Compared to aquatic Isopods, from which they have evolved, Oniscidea show relatively long antennae (Hoese, 1989). With the reduction of antennule (first antenna) and visual acuity, antennae were assumed to have evolved under natural selection to become the main sensorial appendages. In spite of this selection pressure that acts on both sex, we found longer male antennae in the 7 species investigated in this study. The antennal sexual dimorphism in favor of males is highly significant ($p < 0.001$), except for Porcellionides cingendus (+2.6 %; $F_{[1,27]} = 0.02; p = 0.89$). In literature on Oniscidea, longer antennae in males were already signaled for some Trichoniscidae (Hylöniscus, Trichoniscus), one Oniscidae (Ctenosia) and among the genus Porcellio (Vandel 1960). So, one can reasonably assume that antennal length is a secondary sex trait in most Oniscidea.

Since some species are known to use antennae in aggressive behavior, we have first thought that the sexual dimorphism could be related to selection pressure on male antennae by means of contests. Indeed, in many Arthropods, sexual selection can explain why some organs used as weapons are more developed in the fighting sex (Brown et al. 1985; Conner 1989; Crespi 1986; Crane 1975; Eberhard and Gutiérrez 1991;
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Antennal sexual dimorphism

Hamilton 1979; Stein 1976; Thornill and Alcock 1983; Wirtz et al. 1988). This hypothesis is here unconfirmed. We found negative correlations between the magnitude of the dimorphism (both in length and in width) and the intensity of antennal contests (IAg). Furthermore, even if antennal contests may partly explain the longer and larger male antennae in some Porcellionidae (Porcellio scaber, P. dilatatus), it totally failed to explain the observed dimorphism in those other species that neither use antennae in contests (i.e. Armadillidium vulgare, A. nasatum).

In an integrative approach, two other alternative hypotheses can be reasonably advanced to explain the longer antennae in males. The first one is that longer antennae may have been selected for in males via female resistance (/ choice) during the course of the sexual sequence. Indeed, in number of arthropods species, antennae are used by males for mating, either in clasping (Legrand and Juchault 1994, in crustaceans) or stimulating females (McLain 1981; Mason 1972, in some beetles). Nevertheless, when antennae are used in clasping and guarding females (in Anostraca and Ostracoda for example) these organs present fangs, spines or other structures that assure the same role (Belk 1984; Legrand and Juchault 1994). Male antennae in Oniscidea never present such structures. In fact, this function is accomplished by anterior pereopods and seventh one, which are highly dimorphic with many bristles in males (Legrand and Juchault 1994; Vandel 1960). Anyway, there are some antennal dimorphic species (Porcellionides pruinosis or Oniscus asellus) for which males never used antennae during mating, simply because they are carried rearwards (personal observations). So, neither stimulations nor clasplings by antennae cannot fully explain the sexual dimorphism in favor of males.

Advantages for males of having longer antennae rather suggest a function in chemoreception. This idea is reinforced by the fact that the distal segments of the antennae (F2 in the 2 Armadillidium species, or F3 in Oniscus asellus) are among the discriminant variables that explain the antennal sexual dimorphism. In fact, we have now a wide range of converging data to assume that males in many Oniscidea intensively compete in searching and locating receptive females. This indirect form of competition over mate is called scramble-competition polygyny (Alcock 1980). Scrambles to first find a sexual partner are often important (but yet not clearly demonstrated), and may partly explain why males in many arthropods have larger eyes, chemoreceiving antennae or locomotory organs than females (Andersson 1994; Mason 1980; Thornhill and Alcock 1983).

In theory, male scramble behavior might be developed in those species that practice promiscuity (or polygynandrous species). In literature available on Oniscidea, we dispose of some interesting works on sperm precedence or multiple paternity that clearly indicate that females are polyandrous (Johnson 1982; Sassaman 1978; Lueken 1963; 1968). By another way, it was shown that one male can fertilize multiple mates within the reproductive period (Moreau and Rigaud, in preparation, in Armadillidium vulgare, A. nasatum and Oniscus asellus; Linsenmair 1984 in Hemilepistus reaumuri). So, given these data, it appears that Oniscidea effectively practice promiscuity, a mating system in which males are assumed to intensively compete by scramble.

In addition, it was shown that the cost of reproduction, in term of energetic investment and predation risk, is greater in males than in females (Erhard 1992; Zimmer and Brauckmann 1997). These authors assume this must be related to a higher male activity during the reproductive period. This is also congruent with recent studies on operational sex-ratio that indicate that males are in a so competitive environment that the time spent in detection and clasping attempts are of little consequence as compared to the risk of missing a chance to fertilize a receptive female (Lefebvre and Caubet 1999; Moreau and Rigaud, in preparation).

This scheme of scramble strategy is particularly adapted to the dimorphic species that are poorly or not aggressive (clade 1, in Fig. 3). Indeed, in such a mating system males were assumed to simply search for receptive females and avoid any form of aggressiveness (Alcock 1980). Therefore, we assume that longer male antennae in Armadillidium vulgare, A. nasatum, Oniscus asellus, Porcellio scaber and P. dilatatus may have been selected for by means of scramble competition. For the 2 Porcellionides species (clade 2) that are highly aggressive and poorly sexually dimorphic, the problem is obviously more complex (mixed of different mating strategies?), and needs further investigations.

Acknowledgements

We are very grateful to Thierry Rigaud, Frédéric Grandjean, Jérôme Moreau and Jean-Pierre Mocquard for their painstaking comments.
References


Annexe 3  Antennal sexual dimorphism


Annexe 3  Antennal sexual dimorphism

Table 1. Antennal dimorphism in length.

<table>
<thead>
<tr>
<th>Species</th>
<th>A2 ♂</th>
<th>A2 ♀</th>
<th>♂ vs ♀</th>
<th>discr. art.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. scaber</em></td>
<td>8.969±0.0063</td>
<td>8.8275±0.0063</td>
<td>+15.2% (1040); $F_{[1,127]}= 221.97; p &lt; 0.001$</td>
<td>BA**</td>
</tr>
<tr>
<td><em>P. dilatatus</em></td>
<td>8.8277±0.0073</td>
<td>8.6907±0.0073</td>
<td>+14.7% (873); $F_{[1,127]}= 160.04; p &lt; 0.001$</td>
<td>ART3*; BA*</td>
</tr>
<tr>
<td><em>A. vulgare</em></td>
<td>8.5653±0.0066</td>
<td>8.4383±0.0066</td>
<td>+13.5% (626); $F_{[1,127]}= 152.46; p &lt; 0.001$</td>
<td>F2**; BA*</td>
</tr>
<tr>
<td><em>O. asellus</em></td>
<td>8.9899±0.0078</td>
<td>8.8781±0.0078</td>
<td>+11.8% (848); $F_{[1,127]}= 66.10; p &lt; 0.001$</td>
<td>*</td>
</tr>
<tr>
<td><em>A. nasatum</em></td>
<td>8.6206±0.0089</td>
<td>8.5228±0.0089</td>
<td>+10.3% (517); $F_{[1,127]}= 37.21; p &lt; 0.001$</td>
<td>F2**; BA*</td>
</tr>
<tr>
<td><em>P. pruinosus</em></td>
<td>8.8118±0.0076</td>
<td>8.7681±0.0076</td>
<td>+4.5% (287); $F_{[1,127]}= 14.62; p &lt; 0.001$</td>
<td>_</td>
</tr>
<tr>
<td><em>P. cingendus</em></td>
<td>7.9008±0.0095</td>
<td>7.8753±0.0095</td>
<td>+2.6% (68); $F_{[1,127]}= 0.02; p = 0.89$</td>
<td>_</td>
</tr>
</tbody>
</table>

Note: In the first 2 columns, relative length of antennae in both sex. Results are expressed in respect to the reference dimension (basis length of the third pereopod), after logarithmic transformation (Least Mean Square ± SE). In the last column, highly discriminant article(s) that explain the sexual dimorphism (*: $p < 0.05$; **: $p < 0.001$). Species are ordered according to the decreased value of the dimorphism.

Table 2. Antennal dimorphism in width.

<table>
<thead>
<tr>
<th>Species</th>
<th>ba ♂</th>
<th>ba ♀</th>
<th>ba ♂ vs ♀</th>
<th>discr. art.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. dilatatus</em></td>
<td>6.6940±0.0184</td>
<td>6.5351±0.0184</td>
<td>+17.2% (119); $F_{[1,27]}= 33.58; p &lt; 0.001$</td>
<td>_</td>
</tr>
<tr>
<td><em>P. scaber</em></td>
<td>6.7093±0.0182</td>
<td>6.5626±0.0182</td>
<td>+14.7% (104); $F_{[1,27]}= 28.25; p &lt; 0.001$</td>
<td>_</td>
</tr>
<tr>
<td><em>A. nasatum</em></td>
<td>6.1655±0.0266</td>
<td>6.0884±0.0266</td>
<td>+7.9% (35); $F_{[1,27]}= 2.56; p = 0.121$</td>
<td>_</td>
</tr>
<tr>
<td><em>A. vulgare</em></td>
<td>6.1451±0.0147</td>
<td>6.0890±0.0147</td>
<td>+5.8% (25); $F_{[1,27]}= 5.98; p &lt; 0.05$</td>
<td>_</td>
</tr>
<tr>
<td><em>O. asellus</em></td>
<td>6.4077±0.0218</td>
<td>6.3878±0.0218</td>
<td>+2.0% (12); $F_{[1,27]}= 0.04; p = 0.849$</td>
<td>_</td>
</tr>
<tr>
<td><em>P. pruinosus</em></td>
<td>6.2003±0.0181</td>
<td>6.2182±0.0181</td>
<td>-1.8% (9); $F_{[1,27]}= 0.48; p = 0.493$</td>
<td>_</td>
</tr>
</tbody>
</table>

Note: In the first 2 columns, relative width of antennae in both sex. Results are expressed in respect to the reference dimension (basis length of the third pereopod), after logarithmic transformation (Least Mean Square ± SE). In the last column, the sexual dimorphism is expressed in term of percentage (and µm) in favor of male, then studied by analysis of covariance. Species are ordered according to the decreased value of the dimorphism.

Table 3. Behavioral analysis of male dyads.

<table>
<thead>
<tr>
<th>Species</th>
<th>aggressive behavior</th>
<th>interactive behavior</th>
<th>IAg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>duration</td>
<td>occurrence</td>
<td>duration</td>
</tr>
<tr>
<td><em>P. cingendus</em></td>
<td>(n = 20)</td>
<td>29.5</td>
<td>6.0</td>
</tr>
<tr>
<td><em>P. pruinosus</em></td>
<td>(n = 30)</td>
<td>57.0</td>
<td>11.0</td>
</tr>
<tr>
<td><em>P. scaber</em></td>
<td>(n = 18)</td>
<td>37.0</td>
<td>8.0</td>
</tr>
<tr>
<td><em>A. vulgare</em></td>
<td>(n = 20)</td>
<td>41.0</td>
<td>5.0</td>
</tr>
<tr>
<td><em>A. nasatum</em></td>
<td>(n = 26)</td>
<td>24.0</td>
<td>4.0</td>
</tr>
<tr>
<td><em>P. dilatatus</em></td>
<td>(n = 20)</td>
<td>26.5</td>
<td>3.5</td>
</tr>
<tr>
<td><em>O. asellus</em></td>
<td>(n = 20)</td>
<td>3.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Note: Partial results (duration in second, number of occurrence) and indexes of aggressiveness (IAg) correspond to median scores. Species are ordered according to the decreased value of IAg. * indicate those species that use antennae as weapons.
Figure 1. A: Antenna (A2) and landmarks for the different measurements (ba: width of the basis; BA: length of the basis; ART1, ART2, ART3: lengths of the corresponding articles; F1 and F2: lengths of the 2 segments of the flagellum). B: Third pereopod and landmark for the measurement of the basis (REF).

Figure 2. Correlation between antennal dimorphism in length (% in favor of male) and intensity of antennal contests (IAg), after correction for phylogeny.

Figure 3. Cluster analysis (“nearest neighbors” method) based on the 2 variables: dimorphic value in length (% in favor of male) and intensity of male competition (IAg). Along the Y axe, species are designated by the initials of their Latin name.
Mating patterns in *Armadillidium vulgare* (Crustacea, Isopoda), a species with a large size range of potential mates

(soumis à *Behavioural Ecology*)

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Introduction

In the terrestrial crustacean Armadillidium vulgare, there is a large size range among which males and females may potentially pair and mate. Because of sexual size dimorphism and continuous growth far beyond sexual maturity, the largest individuals thus can be nearly ten times the live mass of the smallest sexually matures ones. In this study, we explored the influence of male and female body size on the mating outcome. We thoroughly detailed the sexual sequence in laboratory experiments, but under conditions that reproduce the natural field situation at the early breeding season (i.e., operational sex ratio close to unity, large size range of individuals, female-biased size dimorphism). We found that the pairing probability was constant whatever the male and female size. However, as females systematically resist male mating attempts, the probability of mating decreased with female size while remaining constant for males. In consequence, the sexual size dimorphism was reversed at mating. We also showed that the time and sperm investment made by males increased with the female size. In the discussion, we propose physical constraints and female resistance as possible mechanisms to explain the size assortment observed at pairing and mating (respectively $r = .25$ and $r = .50$). We highlighted the difficulties of large females to encounter sufficiently large males to overcome their mating resistance. We argue that female spermathecae may have constituted a prerequisite for the evolution of the female resistance behavior and for the evolution of large female in this group. Key words: sexual behavior, sexual size dimorphism, assortative pairing and mating, mate assessment, sperm investment, Armadillidium vulgare, isopod.

Because males were often found to fight or actively search for females, they have been assumed to play the major role in the mating decision. Most studies have thus concentrated on the mating patterns from the perspective of males. However, earlier investigations have no doubt underestimated the importance of female behavior, and there is now increasing evidence for an active role of the female on the mating outcome (Ahnesjö et al., 1993; Birkhead and Møller, 1993; Eberhard, 1996; Rosenqvist and Berglund, 1992; West-Eberhard et al., 1987). Because male and female reproductive interests may differ substantially (concerning e.g. occurrence of mating, number or quality of mates, mating duration), this inevitably leads to inter-sexual conflicts (Parker, 1979).

The relative body size of sexual partners is a crucial key in the resolution of these mating conflicts (Pomianowski and Møller, 1995; Wilcockson et al., 1995). In the case of males being larger than females, males may use their size advantage to harass or maintain females and to force copulation (Clutton-Brock and Parker, 1995; Smuts and Smuts, 1993). Conversely, when females are larger than males, they may successfully resist male copulation attempts, select their mating partner, and to some extent govern the mating process (Eberhard, 1996; Elgar, 1991).

In a given population, the difference in size between potential sexual partners may have two distinct origins. One sex may be larger than the other because i) all members of this sex are on average larger than all members of the other sex (i.e. sexual size dimorphism, SSD), ii) members of one or two sexes continue to grow beyond their sexual maturity, which generates a large size range of potential mates. When both conditions are verified, the resolution of inter-sexual conflicts is not only fixed by the SSD exhibited by the species (species history), but also depends on the age of the potential mates (individual history).

Continuous growth in conjunction with SSD is particularly common among crustaceans. Within this group, Gammarus amphipods have been the subject of numerous experimental and theoretical investigations concerning the pre-copulatory guarding decisions and size assortative pairings (see Jormalainen, 1998; and references therein). However, in Gammarus species, the largest females often remain smaller than the smallest males. Overlapping of individual size range with the extent of the SSD was more often encountered among isopods.

The present study will be based on the Armadillidium vulgare model (Isopoda, Oniscidea), probably the most thoroughly studied terrestrial isopod. In this species, the SSD is nearly 20% in favor of the female (for mass), though mean values strongly varied across populations and within season (Vandel, 1962). A. vulgare is a long-lived iteroparous species exhibiting an indeterminate growth and seasonal breeding events throughout its reproductive lifespan (Dangerfield and Hassall, 1992; Lawlor, 1976). In natural populations, there may coexist reproductive animals that belong to different cohorts (more or less synchronously born broods in the population). The largest animals can be nearly ten times the live mass of the smallest sexually mature ones (Brody et al., 1983). As a result, there is a large size range among which males and females may potentially
pair and mate. This gives a unique opportunity to study the effect of body size on mating outcomes, from either the male or the female perspective.

In this study, we reported empirical data on the sexual interactions between males and females belonging to different cohorts. We investigated pairing and mating probabilities according to male and female size. We analyzed SSD and size assortment throughout the sexual sequence. We also explored the role of partners’ size (and other parameters such as female proximity to the parturial molt) on the time and sperm investment. The mating pattern of *Armadillidium vulgare* will be detailed and re-interpreted in the light of previous knowledge and current findings. The influence of body size on mating strategies will be discussed from the perspective of both male and female.

**Material and Methods**

Because of lucifugous habits, and short sexual sequences, behavioral observations in terrestrial isopods cannot be easily performed in the field (Mead, 1973). All experiments in this study were thus carried out under laboratory conditions.

**Research organisms**

*Armadillidium vulgare* (Latreille, 1804) were derived from a strain collected in France (Nice) in 1967 which has been maintained in laboratory conditions ever since (20°C, natural photoperiod of Poitiers, 46°40’N). To dispose of virgin animals, some gravid females were isolated each spring, and the offspring were sexed before sexual maturity. Young males and females were then bred separately in rearing boxes (26 x 13 x 8 cm, area = 340 cm²) filled with moist earth. Food (dead leaves and slices of fresh carrots) was provided *ad libitum*. For the experiment, different cohorts were used to span the range of male and female sizes found in the field during the early breeding season (see Sutton et al., 1984, and references therein). At the time of experiment (winter/spring 1999/2000), the oldest animals completed their third years (birth in spring 1997), whereas the youngest ones completed their first year (birth in spring 1999). For the youngest animals, sexual maturity was assumed for a body length > 7.0 mm (see Paris and Pitelka, 1962).

At least 5 days before the experiment, males were individually isolated in small boxes (Ø = 8 cm, h = 5 cm, area = 50 cm², same rearing conditions as above) in order to give rise to sexual motivation (Lefebvre et al., 2000). At the start of the experiment, males were checked for the integrity of their external copulatory organs (endopodites of the first and second pleopods), and for their presence in the active phase of the molting stage (C period, see Drach, 1939). Similarly, only females “ready to mate” were used. In this species, the sexually receptive period occurs during the secondary vitellogenesis (Lefebvre and Caubet, 1999), a physiological stage that can be externally assessed by the shape of the ventral white plates (D period of a pre-parturial intermolt, Moreau and Rigaud, 2002). At the time of the experiment, all animals were thus in *time in* (see Kvarnemo and Ahnesjö, 1996).

Males and females were measured (extended body length to the nearest 0.25 mm from the anterior margin of the cephalon to the tip of the telson) and weighted (to the nearest mg), just before the experiment. Due to the constraints of the experimental design (size measurements during the experiment), the number of animals was restricted to 10 males and 10 females, but experiment were multiply replicated. In each replicate, males and females were selected along a large size range, with a maximum number of animals belonging to the medium size classes (to verify normality assumption).

At any instant, the operational sex ratio was maintained at unity, i.e. equal number of males and females available for mating. This corresponds approximately to conditions observed in the field. Indeed, though the primary sex ratio is strongly female-biased in natural populations (Juchault et al. 1993), the restriction in the female receptive period (vitellogenesis and incubation processes), and the high male potential reproductive rate both lead to a nearly equal number of males and females ready to mate (Moreau and Rigaud, 2000).

**The experimental protocol**

The observation was performed in a transparent plastic box (17 x 14 x 5 cm, area = 238 cm²) filled with moist soil, and topped with a transparent glass slide in order to limit air disturbance. Physical parameters were
Annexe 4  Mating patterns in *Armadillidium vulgare*

maintained at the following values: 80-90 % in relative humidity, reduced lighting intensity of nearly 50 lux, temperature at 20°C. The 10 males were placed in the middle of the observation box. The t₀ time was associated with the simultaneous introduction of the 10 females. The observation was conducted over one hour, but animals engaged in pairing or mating were left in the box until the completion of the sexual sequence. The following behavioral states have been identified and defined:

• **Pairing**: any male mounting onto the back of a female, in a parallel -superposed- position (to exclude non-sexual mounting that may occur during locomotion activities). The duration of this pairing phase until mating or until the split of the pair was referred to as the pairing time.

• **Mating**: the male, coiled up on a female side, exhibits up and down movement with its pleon (see Mead, 1973). During mating the female is motionless and incompletely curled into a ball. The duration of mating was referred to as the copulation time, with the only condition that the dissection (at the end of the experiment) revealed traces of sperm in the corresponding oviduct (females possess 2 symmetrical genital orifices and 2 genital tracts).

When pairing ended with the split of the sexual partners, it was referred to as pairing from aborted sexual sequence. When pairing continued to mating, the male normally successively inseminates the two genital tracts, and thus the sexual sequence may be described as follow: pairing 1, copulation 1, pairing 2, copulation 2. In some cases however, partners may prolong the sexual sequence (here referred as supernumerary pairings and matings).

When sexual partners split after a single pairing, both the male and the female were temporarily removed in order to record their length and mass. They were subsequently replaced in the middle of the observation box. When sexual partners split after a mating, male and female were definitively removed from the experiment. Their lengths and masses were recorded and the female was later dissected to check for the presence of sperm in her oviducts. At the same time, 5 oocyte diameters were randomly measured alongside the ovaries using an eyepiece micrometer (nearest µm). This provided a mean oocyte diameter, later used to assess female proximity to the parturial molt. The removal of the mating male and female was assumed to cause no serious bias in the experimental design, since the complete sexual sequence is too long to allow the sexual partners to remate within the observation time.

In order to assess the relationship between the copulation time and the quantity of transferred sperm, a DAPI count of spermatozoids was performed on a sub-sample of inseminated females (details on the methods are provided in Moreau *et al.*, 2001).

**Statistical analyses**

Replicates of the experiment described above were conducted between December 1999 and March 2000. One-way ANOVAs were performed on male and female size (length and mass) to check for no significant differences between replicates ($p > .05$). The homogeneity between replicates concerning the relative size of males and females was checked by a Levene’s test ($p > .05$) performed on the size difference (female size minus male size). The choice of the size variable (length or mass) to use in the further analyses will be based on a maximization of the sexual dimorphism (higher % in favor of the female).

Probabilities of pairing and mating according to male and female size were modeled by logistic regressions. For pairing the analysis was conducted separately for males and females, each individuals being coded as 0 = unpaired, 1 = paired. For mating, the same procedure was applied (0 = unmated, 1 = mated), but estimated probabilities were also calculated in a model that both included male and female sizes.

The significance of the SSD among tested animals was checked using independent t-tests, whereas the size difference between the sexual partners at pairing and at mating were checked by dependent t-tests. Size comparisons between groups (unpaired vs. paired, unmated vs. mated) were performed separately for males and females by independent t-tests. The magnitude of the relationship between male and female size at pairing and at mating was given by the r values of linear regressions.

The effects of male and female size on the duration of the different phases of sexual sequence were explored within a generalized linear model framework (GLM). Durations were fitted by a normal distribution after
neperian log-transformation, and an identity-link function was used. The goodness of fit of the GLMs was checked by the ratio of the model deviance to its degree of freedom (deviance/df < 2). The contribution of model terms and their significance were checked by the Chi-square values ($\chi^2$) of Likelihood Type 3 tests (STATISTICA 5.5).

To account for observed variation in the duration of overall pairing phases (including pairings from aborted sexual sequences), the male and female size were entered as continuous predictors. For pairing phases preceding copulations (pairing 1), the model also included mean oocyte diameter as an additional continuous predictor. To account for variation in the duration of the copulation 1, the oocyte diameter and the duration of the pairing 1 were entered as additional continuous predictors 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 the copulation 2 included: female size, male size, oocyte diameter, duration of the pairing 1, copulation 1 and pairing 2. In such models with more than two terms, a simplification procedure was applied before checking for the individual term contribution. This was performed on the basis of the lowest AIC’s score, a method that select the model providing the best description of the data using a minimum of parameters (Burnham and Anderson, 1998).

Other analyses were performed by non-parametric tests because of the high variability routinely observed around mean duration values. Comparisons between groups were checked by Mann-Whitney $U$ tests, and correlations by Spearman rank tests ($r_s$).

**Results**

A total of 23 replicates were conducted between December 1999 and March 2000 (in 4 of them only 9 males and 9 females were tested, because of the difficulty to find *time-in* females in the rearing). There was no difference in length nor in mass between replicates in both females and males (female mass: $F_{22,203} = .37; p = .99$; female length: $F_{22,203} = .41; p = .99$; male mass: $F_{22,203} = 1.00; p = .47$; male length: $F_{22,203} = .68; p = .85$). The variance in the mean size difference between males and females was statistically similar between replicates (Levene’s test: $p > .05$ for mass and length). All replicates were thus pooled as a single data set ($n$ male = 226 and $n$ female = 226).

In females, the mean mass was 106.21 ± 42.44 mg and the mean length was 12.01 ± 1.75 mm. In males, the mean mass was 89.81 ± 33.96 mg, and the mean length was 11.72 ± 1.62. The female biased SSD was thus more pronounced for mass (+18%; independent $t$-test: $t = 4.54$, $p < .001$) than for length (+2%; independent $t$-test: $t = 1.77$, $p = 0.08$). Mass more effectively reflects the broadening in the female body shape than does any linear dimension. In the further analyses, body size will refer to mass measurement (except where specifically mentioned).

During the course of overall observations, no take-overs (or attempted take-overs) of paired or mated females by single males were observed. A total of 73 females and 69 males engaged in the pairing posture, with some of them pairing multiply. A total of 89 pairing events were observed, of which 20 led to mating postures (22.5%). Among mating postures, 16 were associated with sperm transfer (80.0%), and 4 with no sperm transfer (i.e. pseudo-copulation, see Lefebvre and Caubet, 1999). For these pseudo-copulations, the pairing time alone was used in the analyses.

**Pairing and mating probabilities according to male and female size**

There was no difference in mass between unpaired and paired females (105.72 ± 42.28 mg vs. 107.25 ± 43.05 mg; independent $t$-test: $t = -.25$, $p = .80$). The probability of pairing (73/226 = 32%) was constant throughout the female mass range (logistic regression: $\chi^2 = .06$, $p = .80$).

There was no statistic difference in mass between unpaired and paired males (88.06 ± 34.61 mg vs. 93.81 ± 32.33 mg; independent $t$-test: $t = -1.17$, $p = .24$). As for females, the probability of pairing for males (69/226 = 31%) was not related to their mass (logistic regression: $\chi^2 = 1.36$, $p = .24$).

Among females that paired, unmated females were heavier than mated ones (109.70 ± 41.88 mg vs. 76.56 ± 27.11 mg; independent $t$-test: $t = 3.02$, $p < .01$). The probability of mating for females (16/89 = 18%) significantly decreased with their mass (logistic regression: $p = 1.0449 – 0.0282\times$female mass; $\chi^2 = 10.56$, $p < .01$).
Among males that paired, no difference was observed between unmated and mated ones (92.32 ± 30.44 mg vs. 91.94 ± 38.45 mg; independent t-test: t = .04, p = .97). The probability of mating for males (the same as for females, i.e. 18%) was steadily constant whatever their mass (logistic regression: $\chi^2 = .00, p = .97$). In fact, as shown in Figure 1, the probability of mating increased with the male mass but decreased with the female mass (logistic regression: $p = 0.4297 - 0.0327*female\ mass + 0.0110*male\ mass; \chi^2 = 11.77, p < .01$). Of particular interest was the fact that the largest females exhibited the smallest likelihood of mating.

### SSD and size assortment at pairing and mating

As shown above, the SSD was significantly female-biased among tested animals (+16.40 mg, +18%). Among overall pairings ($n = 89$), the female was heavier than the male (mean difference raw by raw: +11.49 mg, +12%), and the difference was still significant (dependent t-test: $t = 2.37, p < .05$). The exact inverse was true for matings ($n = 16$). The mean mass of mating females was smaller than the mean mass of their male partners (difference raw by raw: -15.38 mg, -20%), though the difference was not significant (dependent t-test: $t = -1.79, p = .09$). Note that SSD at mating was significant when considering length (10.76 ± 1.27 mm vs. 12.13 ± 1.48 mm, difference raw by raw: -1.34 mm, -13%; dependent t-test: $t = -3.94, p < .001$).

As shown in Figure 2, the size difference at pairing reflected the general SSD exhibited by the species, as judged by the SSD among tested animals (+11.49 mg vs. +16.40 mg; independent t-test: $t = .79, p = .43$). At mating, the size trend was completely reversed, with females an average of 20% smaller than the mating males. The difference in size between pairing and mating partners was significant (+11.49 mg vs. -15.38 mg; independent t-test: $t = 2.25, p < .05$). The male mass was steadily constant at each step of the sexual sequence, indicating this way that the reversion of the SSD was primarily due to change in female mass.

Among overall pairings, there was a positive significant relationship between female mass and male mass (female mass = 73.18 + 0.33*male mass; $r = .25, F_{1,87} = 5.98, p < .016$) (Figure 3). Considering matings only, there was a higher positive correlation coefficient between female mass and male mass (female mass = 44.35 + 0.35*male mass, $r = .50, F_{1,14} = 4.59, p = .0503$) (Figure 3).

### Time and sperm investments during the sexual sequence

The pairing phase showed high variability around the mean duration (291.49 ± 378.29; $n = 89$), particularly in the case of aborted sexual sequences (245.16 ± 339.47; $n = 73$). It was significantly longer when followed by successful matings (502.88 ± 477.78; $n = 16$) ($U = 275.00, p < .001$). None of the partners’ size parameters significantly accounted for the variations in the pairing phase duration when considering all pairs ($n = 89$) (female mass: $\chi^2 = .06, p = .81$, male mass: $\chi^2 = .37, p = .55$).

Models and results for the different phases of the complete sexual sequence ($n = 16$) are summarized in Table 1. The oocyte diameter was systematically entered as additional predictor (from 344 to 471 µm; mean: 418.10 ± 36.57; $n = 196$ because 1 female exhibited degenerated oocytes) and then checked for its supplied contribution to the model. The AIC simplification procedure never retained this variable in the final model. The AIC simplification procedure never retained this variable in the final model.

The oocyte development (i.e. the proximity of the parturial molt) was thus assumed to have little if any contribution to the model. The AIC simplification procedure never retained this variable in the final model. The female mass had a significant effect on the duration of the pairing phase 1 ($\chi^2 = 9.79, p < .01$). The duration of copulation 2 was strongly influenced by copulation 1 ($\chi^2 = 12.17, p < .001$) and pairing phase 2 ($\chi^2 = 8.18, p < .01$). The oocyte development (i.e. the proximity of the parturial molt) was thus assumed to have little if any influence on the duration of the different phases of the sexual sequence. In contrast, the female mass is the major contributing variable in explaining durations of the different phases. The female mass had a significant effect on the duration of the pairing phase 1 ($\chi^2 = 12.17, p < .001$) and pairing phase 2 ($\chi^2 = 8.18, p < .01$). The heaviest the female the longer were the pre-copulatory pairing phases (pairing 1: $r_s = .62, n = 16, p < .05$; pairing 2: $r_s = .45, n = 15, p = .09$; all pairing phases including supernumerary ones: $r_s = .59, n = 16, p < .05$) (see Figure 4a).

For copulation 1, none of the model terms significantly accounted for observed variation in its duration. The duration of copulation 2 was strongly influenced by copulation 1 ($\chi^2 = 9.79, p < .01$). The longer the first copulation, the more extended was the second one ($r_s = .73, n = 15, p < .01$). However, when pooling all copulation phase durations (including supernumerary ones), only female mass had any significant effect ($\chi^2 = 9.43, p < .01$). The heavier the female, the longer was the time spent in copulation ($r_s = 0.37, n = 16, p = .16$) (see Figure 4b). Female mass thus regulated the time investment in copulation of both male and female. There was a positive relationship between the duration of copulation and the inseminated « sperm number ».

Results were highly significant for the first inseminated oviduct ($r_s = .88, n = 9, p < .01$), not significant for the second ($r_s = .29, n = 8, p = .49$), and highly significant when considering total copulation time ($r_s = .85, n = 9, p < .01$). So the duration of copulation was a suitable estimator of the amount of inseminated sperm.
**DISCUSSION**

**The mating pattern**

In *Armadillidium vulgare* there is some evidence for a male scramble competition (Lefebvre et al., 2000). Males actively search for females and compete for the first access to receptive females. The decision to initiate pairing is under the male initiative only. It is the male that engages the sexual sequence by mounting and gripping onto the female’s back. We showed that the probability to engage in pairing is constant whatever the male and female size. Of particular interest, the mass difference between pairing partners (+12% in favor of female) closely reflects the SSD exhibited by the species (+18% among tested animals, and nearly +20% according to literature). In other terms, this means that all animals in *time in* may engage in pre-copula. However, within this populational given, males and females did not pair randomly with regard to body size. There was a clear trend for small males to pair with small females and large males to pair with large females ($r = .25$).

During the pairing phase, the male attempts to maintain the female and adopts a stereotyped behavior. Its pereiopods cling on the female body shape and its second antennae are curved forward and seem to stimulate the anterior body part of the female (also see Mead, 1973). This step shows high variability in length and its duration is shortened in cases of aborted sexual sequence. Females may refuse to complete the sexual sequence by actively walking, or more typically, by exhibiting body manoeuvres (jerky oscillatory -left/right-movements) that inevitably split the pairs. It thus seems that the decision for separation is under the female initiative, though we have no conclusive evidence (interpretation of observational data).

In a normal case, the female responds to male mount by a rolling up and the male attempts to manipulate her to engage a mating posture. The probability in engaging mating strongly decreased with female size. Large females may therefore have difficulties in encountering sufficiently large males able to maintain them in pre-copula and mating posture. This is evident in the present experiment, where the 56 biggest females (from 132 to 251 mg) never mated, while among the 56 smallest females (from 42 to 65 mg), 7 of them have mated (44% of the overall copulation). Females in mating were thus smaller than females found in pairing, while the male size did not change. As a result, the size ratio at mating was reversed, the mated female being systematically smaller than her sexual partner (-20%).

Within the limits of size allowing mating (herein, from 43 to 133 mg for females, and from 54 to 218 mg for males), males and females did not associate randomly, and there was a clear trend for positive size assortment ($r = .50$). The biggest females only mated with the biggest males and the smallest females mated with the smallest males.

**The causes of size assortment**

Positive size assortative mating has been found in almost all animal groups (Crespi, 1989; Ridley, 1983) and is particularly well documented in amphipods and isopods (Adams and Greenwood, 1987; Jormalainen and Merila, 1993; Nilsson, 1977; Shuster, 1981; Veuille, 1980; Jormalainen, 1998, and others references therein). Among the sets of hypotheses that are routinely invoked to interpret assortative mating by size (Crespi, 1989), two of them may be applied 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 (Clark, 1977; Juliano, 1985; Price and Willson, 1976). The effectiveness of male courtship may thus be reduced by a large relative size difference. For example, Pinto and Mayor (1986) showed that in a species of meloid beetle, mismatched males and females had difficulties in mating because “the lack of coincidence of anatomical parts prevents normal courtship delivery”. No doubt that such physical constraints may generate non-random assortment in *A. vulgare* when considering the large size range between potential mating partners. In the present experiment, the male size ranged between 34 to 221 mg and the female size from 42 to 251 mg. This means that the smallest males may encounter 7 fold heavier females, with which any mating posture is possible (herein, the maximum body size difference for successful copulation was +30 mg in favor of the female).
Female resistance to male attempts may be an additional cause of size assortment. Under this hypothesis, large males are presumed to have higher vigor to overcome the higher resistance to mating of large females. Such a mechanism has already been suggested to operate in a brentid beetle (Mc Cauley and Wade, 1978) and in *Gammarus pulex* (Ward, 1984), but the relationship between female resistance and size assortment has never been clearly demonstrated. In the case of *A. vulgare*, the pre-copulatory (pairing) phase may be viewed as a female resistance period during which the male forces the female to engage the mating posture. The fact that pre-copulatory phase duration increases with female size may be another indication for female resistance. Nevertheless, the only way to test the effectiveness of the female resistance hypothesis would be to treat some females by a neuromuscular blocking agent. If female resistance really occurs, the pre-copulatory phase duration and the mating size correlation coefficient would be smaller in the experimental group than in a control group of similar size animals. Using the above procedure, Jormalainen and Merilaita (1995) have successfully shown that inter-sexual conflicts in the marine isopod *Idotea baltica* were resolved in accordance with female interest.

**Male and female mating strategies**

Males were shown to invest more in time and sperm towards large females. In *A. vulgare*, as a general rule in dioecious organisms, the female’s fecundity increases with size (Sutton et al., 1984) in such a way that the fitness benefit conferred by large females are obvious from a male’s mating perspective. Both field and simulations data highlight the reproductive importance of large females (3 years old and more), by showing that, though relatively rare (15-25% of the total population), they produce as many offspring than all others females (Caubet, 1998).

Females were systematically observed to resist male attempts, the resistance time strongly increasing with female size. What may be the adaptive significance of this behavior? Female resistance behavior to mating seems common in isopods (see Jormalainen, 1998, for a review of authors and species). Authors generally argue that females resist males in order to assess potential mates (Dick and Elwood, 1989; Elwood et al., 1987; Jormalainen and Merilaita, 1993; Ridley and Thompson, 1979; Sparkes et al., 2000; Ward, 1984). We are in agreement with this hypothesis under which female resistance may be considered as a mate choice strategy. By resisting and by adopting mating posture only with the males able to maintain them, females thus select larger males 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 (large amount of sperm, shorter pairing/mating durations) (Balmford and Read, 1991; Maynard Smith, 1991).

However, by resisting males indiscriminately, large females strongly reduce their probability of mating. This clearly reminds the fitness cost of large females (because of their reduced chance to be inseminated) highlighted by Hatcher and Dunn (1997) in *Gammarus duebeni*. However, in terrestrial isopods there is an anatomical particularity to consider which concerns the existence of sperm storage organs, also called spermathecae. Females can store the sperm of one or several males in their genital tracts over several years (Schöbl, 1880; Vandel, 1941). The validity of lengthy sperm storage, as well as the conservation of the fertilization power, was experimentally verified in *A. vulgare* (Lueken, 1963, 1968) and in other terrestrial isopods (Moreau, 2001). Therefore, large females in natural populations have probably already been inseminated when young, in such a way that they can yield several broods and fertilize their eggs without any further copulations. Such a particularity may have constituted a prerequisite to the evolution of large females in this group.

**Acknowledgements**

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References


Table 1.
Models terms and their contribution to observed variation in the duration of the different phases of the sexual sequence.

<table>
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<th>Model terms</th>
<th>contribution</th>
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<tr>
<td>Pairing 1 (n = 16)</td>
<td>Female mass</td>
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<tr>
<td>502.88 ± 477.78 s</td>
<td>$\chi^2 = 12.17$, $p &lt; .001$</td>
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<td>Male mass</td>
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<td>$\chi^2 = .18$, $p = .28$</td>
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<td>Copulation 1 (n = 16)</td>
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<td>1677.88 ± 780.19 s</td>
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<td>Male mass</td>
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<td>Copulation 1</td>
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<tr>
<td>Total pairings (n = 16)</td>
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<td>Total copulations (n = 16)</td>
<td>Female mass</td>
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<td>3086.50 ± 1194.50 s</td>
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Mean durations are given ± SD. Contribution of model terms and their significance are checked by likelihood Type III test after AIC simplification procedure.
Figure 1.
Estimated probabilities of mating according to male and female mass (plane). Open circles correspond to the observed values of mass for male and female in pairs. Open circles with a bar below indicate pairs that have led to copulations ($n = 16$), and the others indicate pairs that have not led to copulations ($n = 73$).

Figure 2.
Mean mass (± SD) of male and female among tested animals, at pairing, and at mating.
Figure 3.
Relationship between male and female mass at pairing \((n = 89)\), and at mating \((n = 16)\).
Figure 4.
Pairing duration (a), and copulation duration (b), in relation to male and female size. Open circles correspond to observed values. In (a), the pairing duration was best fitted by the following plane equation: $z = 1.6775 + (0.000038 \times \text{female mass}^3) - (0.000005 \times \text{male mass}^3)$ ($r^2 = .92$, $n = 16$). In (b), the copulation duration was best fitted by the following plane equation: $z = 31.9384 + (0.000018 \times \text{female mass}^3) + (0.090983 \times \text{male mass})$ ($r^2 = .53$, $n = 16$).
On the male-effect in the terrestrial Crustacean
Armadillidium vulgare (Latreille, 1804)


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Summary
In the terrestrial Crustacean Armadillidium vulgare, the onset of female reproduction can be sped up by a male-induced stimulation. This male-effect is mainly characterised by a shortening of the vitellogenesis period, which occurs during the preparturial intermoult. The determinism of this phenomenon, for the first time reported by Jassem in 1982, was here investigated by both experimental and ethological approaches. It was shown that a male deprived of its copulatory organs is significantly less stimulating than an integrated one. On the other hand, a paired female with obturated genital apertures is significantly less stimulated. According to literature, mating takes place only when vitellogenesis is nearly over and therefore cannot be related to the male-effect. Nevertheless, the ethological approach has revealed that females are early attractive for males, and that mating postures can be observed during the whole preparturial intermoult. In fact, insemination can happen as early as the initiation of the secondary vitellogenesis. Before this stage, short mating postures are still observed but no sperm was found in the female genital ducts (pseudocopulation). However, spermatozoa and other seminal substances are not implicated in this phenomenon since a male unable to ejaculate is as efficient as a normal one. Therefore, it is strongly assumed that the male-effect results from mating postures during which male copulatory organs act on mechanoreceptors located in the female genital apparatus.

Key words:
Crustacea, Armadillidium vulgare, reproduction, male-effect, vitellogenesis, mating behaviour
Introduction

Among terrestrial Crustacean (Oniscidea), as throughout the animal kingdom, most species show a seasonal reproduction which allows them to breed and release the young in the best environmental conditions, and consequently increase the success of offspring. In Oniscidea, the seasonal breeding observed in nature is the result of a synchronization of all females by environmental factors, since in constant experimental conditions, females always undergo periods of sexual activity and sexual rest, but never at the same time (Mocquard et al., 1989). The main abiotic factors regulating the onset of female reproduction are now well known: photoperiod (Mocquard et al., 1980; McQueen and Steel, 1980), temperature (Mocquard et al., 1989), and latitudinal origin (Souty-Grosset et al., 1988).

In the pill bug *Armadillidium vulgare*, in spring under natural conditions of Poitiers, all females of reproductive age (even virgins) spontaneously begin ovarian maturation. The vitellogenesis-inhibiting hormone (V.I.H.) (Gohar et al., 1984), that blocks oocytes in primary vitellogenesis during sexual rest is no longer synthesized, and secondary vitellogenesis can begin. This ovarian maturation, due to an accumulation of vitellogenin (Picaud et al., 1989), takes place in the course of a single intermoult, known as preparturial intermoult (PPI), during which mating can occur. Sexual behaviour in *Armadillidium vulgare* has been largely described by Patane (1959) and Mead (1973). It always begins by a male antennal contact on the body of the female. Then, if the female is sufficiently attractive, the male grips her and climbs onto her dorsal face (mating posture). Both sexes in most Crustaceans have two genital organs located, in *Armadillidium vulgare*, on the second pair of pleopods for the male, and at the base of the fifth pair of pereopods for the female. Males generally perform two successively crossed inseminations; the right copulatory organ into the left genital aperture (or the contrary), and then the reverse order. Whether the oocytes are fertilized or not, they always be laid and incubated in a marsupium (brood pouch formed by the differentiation of the first five oostegites) which appears at the parturial moult (PM).

In 1982, Jassem et al. showed the influence of a biotic factor on the initiation of the seasonal breeding. Indeed, they signalled the onset of female reproduction is clearly sped up by the presence of a male. Male-induced stimulation of this kind has been largely described, in particular in domestic mammals, and was called male-effect. In *Armadillidium vulgare*, the male effect is, among others, characterized by a shortening of the PPI in relation to an acceleration of the secondary vitellogenesis period (Caubet et al., 1998). According to literature, mating takes place only a few days before the parturial moult and therefore cannot be related to the male-effect. Nevertheless, direct body contact between the two sexes is necessary to induce the stimulation (Jassem et al., 1991). This phenomenon was shown to be species-specific and dependent on the androgenic hormone (Jassem et al., 1991). One male is able to stimulate at least four females, and an intermittent contact (once every three days or once a week) is as efficient as a permanent one (Caubet et al., 1998). Moreover, it seems that there is a sensible period to the stimulation around the fourth week of the PPI (Caubet et al., 1998). Authors then suggested that the stimulation performed by the male is related to tactile (or chemical) stimuli acting on accurate sensorial receptors on the dorsal face of the female.

On the basis of these results, further experiments were carried out to find out more about the determinism of the male-induced stimulation. Experiments (ablation, varnishing) were performed either on males or females to prevent the emission or the reception of male stimuli. A new approach to the problem was also investigated by studying one-day and one-hour encounters between males and females.

Materials and Methods

*Armadillidium vulgare* were derived from a strain collected in Greece (Heraklion) and maintained in laboratory since 1989. Animals used in these experiments were born during the previous spring and bred at 20°C under the natural photoperiod of Poitiers (46°40’N). Near the fourth moult they were sexed, then males and females were reared separately in boxes until sexual maturity. The rearing boxes (26 x 13 x 8 cm) were filled with moist earth, and each one contained about fifty individuals. Food (dead leaves and slices of fresh carrots) was provided *ad libitum*. The following spring all these virgin animals were puberal and experiments were carried out.
**Experimental approach**

After a normal moult (growth ecysis), females, isolated or coupled with a male according to the experiments, were placed in a cylindrical box (Ø=8 cm, h=5 cm, area=50 cm²) under the preceding conditions. The weight was noted (m in mg±SD), and females were observed until the next moult, which was either a normal moult (NM) or a parturial moult (PM). After experiments, females were dissected in order to see whether there was any sperm in the genital ducts. For each set of experiments, two criteria were studied: the percentage of females undergoing a parturial moult (%PM), and the duration of the preparturial intermoult (ØPPI in days±SD).

**Controls groups (1 and 2)**

Coupled female: 1a February 1996 (n = 20); 1b March 1996 (n = 20); 1c February 1997 (n = 24).

Isolated female: 2a February 1996 (n = 24); 2b March 1996 (n = 10); 2c February 1997 (n = 28).

**Experimental groups acting on female (3 to 8)**

3: Female with tergites overlaid by varnish (nail varnish, no 53, Bourjois) + male (n = 21) (February-March 1996)

4: Female without the first and second pair of antennae + male (n = 18) (February-March 1996)

5: Female without the first pair of pereopod + male (n = 17) (February-March 1996)

6: Female with the two genital apertures obturated (by varnish) + male (n = 7) (February 1997)

7: Female with one genital aperture obturated + male (n = 5) (February 1997)

8: Isolated female with the two genital apertures obturated (n = 8) (February 1997)

**Experimental groups acting on male (9 to 11)**

9: Female + male with varnished tergites (n = 19) (February-March 1996)

10: Female + male without the endopodites of the first two pairs of pleopods (copulatory and associated organs) (n = 19) (February-March 1996)

11: Female + male with the two spermiducts cut out between the seminal vesicle and the copulatory organ (n = 16) (February 1997)

**Ethological approach**

**One-day encounter**

In order to determine the minimal state of ovarian maturation allowing insemination, one-day encounters were practised (n = 36). One female and one male were placed together in a cylindrical box (Ø = 8 cm, h = 5 cm, area = 50 cm²), under the conditions as described before. In reproductive females the ovarian development (see Besse, 1976, in *Porcellio dilatatus*, for the description of the vitellogenesis stages) and the moulting cycle (see Drach, 1939; Maissiat, 1978 for the nomenclature of the moulting periods) are closely related. Previtellogenesis (oocyte diameter (OD) < 110 μm) takes place during the A-B period and the beginning of the C period; primary vitellogenesis (110 μm < OD < 240 μm) during a part of the C period, and secondary vitellogenesis (OD > 240 μm) during the end of the C period and the D period. Since the different stages of the moulting cycle can be externally approximated by some criteria (soft cuticle in the A-B period, white plates on the first fourth sternites during D period), females have been chosen in order to represent every moulting stages (so every vitellogenesis stages). At the end of the one-day encounter, each female was dissected, and the two genital tracts were placed on a slide in order to determine an exact OD (in μm) using an eyepiece micrometer. At the same time, the genital ducts were scanned for the presence or absence of sperm.

**One-hour encounter**

The co-action male-female was also studied by direct observations of one-hour encounters between one male and one female. It was performed in a Petri dish lined with moist paper (Ø = 9.5 cm, h = 2 cm, area = 71 cm²), and under the following conditions: temperature = 20° C, light = 100 lux, relative humidity = 90 %. The male behaviour was tested towards females at different stages of the moulting cycle (A-B, C, D period) (n = 42). A list of items was drawn up in order to take both male and female behaviour into account (see
Finally, all these items were grouped into the following behavioural classes: no interactive male behaviours (immobility/locomotion) and interactive male behaviours (no sexual interaction/sexual interaction). The criteria studied were the duration (s) and the number of occurrences of these behavioural classes. When observation was over, each female was dissected, and the OD was measured (in µm).

**Statistical analysis**

The present paper is a summary of two successive years of research. Each experimental group was compared with the control groups of the same year. The relation between the weight of the female and its δPPI was analyzed using the Pearson correlation test. The variance of weight between all groups was studied by ANOVA. The experimental percentages of parturial moult (% PM) were compared by the Yates corrected $X^2$ text. With regard to δPPI, results were analyzed by ANOVA or ANCOVA in order to take the female weight into account. Ethological profiles were compared using the Mann & Whitney test since the data do not meet the assumptions necessary for the use of parametric tests.

**Results**

**Influence of weight (Fig. 1).**

Although all animals were born during the year preceding the study, differences in weight were observed, partly due to unequal density in the rearing boxes. In order to test the influence of weight on the δPPI, a correlation test was carried out (Fig. 1). There was a positive correlation between the weight of isolated females (114.09 ± 21.32 mg) and their δPPI (52.84 ± 13.31 day) : the heavier they were, the longer the intermoult (n = 45; $r = 0.17$; $p > 0.05$). A global analysis of variance revealed significant differences in weight according to the groups ($df = 13$, $F = 1.91$; $p < 0.05$). This result indicates that female weight must be taken into account when comparing the different experiments; this is why analysis of covariance will be used.

**Male-induced stimulation : comparison between isolated and coupled females (Table 1)**

The male presence slightly increased the number of females that performed a parturial moult; pooled results from the two successive years shows a percentage of 95.3 % for coupled females and 87.1 % for isolated ones. These percentages are not significantly different (1a-b-c vs 2a-b-c : $df = 1$, $X^2_c = 1.74$, $p = 0.19$) and remain high, which would indicate that experiments were carried out when the only environmental factors were sufficient to initiate female reproduction.

The male-effect is particularly evident with respect to the duration of the preparturial intermoult (1a-b-c vs 2a-b-c : $F = 131.65$, $p < 0.001$). For an isolated female, 53.81 ± 14.07 days are necessary to undergo a parturial moult, whereas a coupled female needs only 31.54 ± 5.32 days, i. e. 22 days less (41%).

Moreover, the comparison between the two control groups of paired females during the first year shows that the δPPI is shorter in February than in March (respectively, 29.95 ± 4.42 and 35.70 ± 5.18; 1a vs 1b : $F = 10.16$, $p < 0.01$). By another way, no difference appears between the two groups of isolated females of the same year (δ PPI in February = 58.18 ± 15.32, in March = 58.67 ± 17.49 ; 2a vs 2b : $F = 0.005$, $p = 0.94$). In others words, the more advanced the seasonal reproduction period, the less the male-induced stimulation.

**Experiments acting on females (Table 2)**

**Ablations**

Removing the two pairs of antennae in coupled females (group 4) significantly reduced the number of females which performed a parturial moult (% PM = 50) in comparison with intact coupled females (% PM = 100) (4 vs 1a-b : $df = 1$, $X^2_c = 20.01$, $p < 0.001$). Such a reduction is due to the bilateral restriction of antennae because the privation of others organs, like the first pair of pereopod (group 5), is not so disturbing (% PM = 82.4) (5 vs 1a-b : $df = 1$, $X^2_c = 4.33$, $p = 0.04$). Therefore, the nature of the next moult appears to be greatly influenced by whether or not these two main sensorial appendages are present or not.
On the other hand, the 50% of females undergoing reproduction reacted in the same way as the intact females regarding the $\delta$PPI ($4 \text{ vs } 1\text{a-b}: F = 1.25, p = 0.27$). It even seemed the ablation slightly reduced the intermoult ($\delta$ PPI 4 = 30.33 ± 2.83, $\delta$ PPI 1a-b = 32.82 ± 5.57). However, this is a common occurrence with Crustaceans, and it was confirmed with group 5 ($\delta$PPI = 32.36 ± 7.89) that the ablation of any organs (first pair of pereopods for instance) had the same intrinsic effect as the antennary ablation ($4 \text{ vs } 5 : F = 0.64, p = 0.43$). Thus, the male stimuli, which accelerate the ovarian maturation, appear not be perceived by the two pairs of antennae, and other sense organs elsewhere on the female body must be investigated.

**Cuticular varnishing**

The coupled females with varnished tergites (group 3) reacted in the same way as non-varnished females ($1\text{a-b vs } 3 : F = 0.01, p = 0.90$) and no disturbance of the % PPI was observed ($df = 1, X^2c = 1.51, p = 0.22$). In a previous study, it was shown that artificial stimulation of the dorsal face has no stimulating effect (Caubet at al., 1998). It seems therefore that the perception of male stimuli does not take place on the back of the female.

**Genital obturation**

In coupled females with the two genital apertures obturated (group 6), the % PM was not affected in comparison with the control group ($6 \text{ vs } 1\text{c} : df = 1, X^2c = 0.07, p = 0.80$), but, on the other hand, the $\delta$PPI was significantly increased ($6 \text{ vs } 1\text{c} : F = 5.72, p < 0.05$). It was then confirmed that the varnish did not repulse the male. Indeed, females with only one genital aperture varnished (group 7) are all fertilized on the other free side and normally stimulated ($7 \text{ vs } 1\text{c} : F = 0.33, p = 0.57$). A possible intrinsic effect of varnish was also studied by comparing the $\delta$PPI of unvarnished (group 2c: 47.74 ± 8.63) and varnished (group 8: 43.75 ± 4.68) isolated females. The statistical analysis revealed no significant difference between these two groups of females ($8 \text{ vs } 2\text{c} : F = 1.04, p = 0.32$). Moreover, varnished isolated females never reacted in the same way as coupled ones ($8 \text{ vs } 1\text{c} : F = 64.25, p < 0.001$), which again confirms that varnish has no stimulating effect. Therefore, it appears that an obturation of the female genital apertures reduces the male-induced stimulation.

In these three experiments (groups 6, 7 and 8), the obturation of genital apertures by varnish was not permanent throughout the intermoult because of some ungluing of varnish. As a result, sperm was found in the genital ducts of many females of the initial sample. These females cannot be taken into account and this is why the resulting size of the sample appears sometimes very small (i.e., group 6, initial size: $n = 20$, resultant size $n = 7$). Moreover, even if no sperm was detected among resting females, some intromissions may nevertheless have occurred during the preparturial intermoult. This could partly explain why the comparison between both side varnished females and those varnished only on one side shows no significant difference ($6 \text{ vs } 7 : F = 0.82, p = 0.39$).

**Experiments acting on males (Table 2)**

The males with varnished tergites (group 9) were as efficient as unvarnished males (group 1a-b) ($9 \text{ vs } 1\text{a-b} : F = 2.43, p = 0.12$). Therefore, it seems that a male stimulant pheromone released by the dorsal face is not implicated in this phenomenon.

The females paired with males which were deprived of their copulatory organs (group 10) were significantly less stimulated than females paired with intact males (group 1a-b) ($10 \text{ vs } 1\text{a-b} : F = 19.99, p < 0.001$). In previous works it has been shown that the ablation of important organs (antennae) did not alter the sexual male activity (Jassem et al., 1991). These two results suggest that the male copulatory organs are involved in the male effect.

There was no significant difference regarding to the stimulation between males with spermiducts cut out (group 11) and intact males (group 1c) ($11 \text{ vs } 1\text{c} : F = 1.50, p = 0.23$). It was then verified that such males were effectively unable to ejaculate since there were no embryos in the marsupium and no sperm in seminal receptacles of the female. Thus, it seems that spermatozoa or other seminal substances are not implicated in the ovarian stimulation.

**One-day encounter (Fig. 2)**
It was confirmed that insemination (and particularly double insemination of the two genital tracts) generally occurs when vitellogenesis is nearly over (D-stage: 13 females inseminated / 15 females in total = 86.67%). In contrast, females in previtellogenesis and primary vitellogenesis are never inseminated (no sperm detected in their genital ducts). Nevertheless, the crucial point demonstrated here is that females can be inseminated as early as the initiation of secondary vitellogenesis; that is to say when the oocyte diameter reaches nearly 240 µm.

One-hour encounter (Table 3 & 4)

The no-interactive male behaviours (immobility and locomotion) are at their height towards females in pre- and primary vitellogenesis (OD < 240 µm = 2894 s; 80 % of the observation time), and at their lowest towards females at the end of secondary vitellogenesis (OD > 500 µm = 2243 s; 62 % of the observation time). These scores are mainly explained by differences in immobility (respectively, 1706 s and 1272 s). In contrast, the interactive male behaviours, and particularly the sexual interactions (male and female in mating posture), were mainly observed with females at the end of secondary vitellogenesis (OD > 500 µm = 813 s; 240 < OD < 500 µm = 396 s; OD < 240 µm = 65 s). However that may be, males in contact with females in pre- and primary vitellogenesis still showed sexual interactions. Particular attention of this last item (Table 4) even reveals that the higher percentage of encounter with mating posture was obtained with females in pre- and primary vitellogenesis (OD < 240 µm = 75 %; 240 µm < OD < 500 µm = 52 %; OD > 500 µm = 67 %). Nevertheless, the mean duration per occurrence appeared here very short (86 s), compared with to those observed with females in secondary vitellogenesis (240 µm < OD < 500 µm = 550 s; OD > 500 µm = 860 s). There was effectively a significant difference in the duration of the mating posture, depending on whether the male was tested with females in pre- and primary vitellogenesis or with other females in secondary vitellogenesis (U = 23, p = 0.02).

Discussion

The male-effect in Armadillidium vulgare species was reported for the first time by Jassem et al., in 1982. In the present study, it was found that isolated females needed about 54 days to undergo the preparturial intermoult, whereas only 32 days were necessary for coupled ones; this means that the onset of female reproduction is sped up by the male’s presence to a level of nearly 40 %. Nevertheless, this result can also be expressed from a female point of view, as a delay in ovarian maturation among the unpaired. However that may be, isolation is an unusual and unfavourable situation for such gregarious animals (Friedlander, 1964). It has been shown in A. vulgare that the duration of the preparturial intermoult is a little longer for isolated females than for females paired with another one (Jassem et al., 1982, 1991). Moreover, females deprived of co-specific stimuli (by isolation or by ablation of antennae) are also affected in the percentage of parturial moult in the sense that more females remain in sexual rest under these circumstances (Jassem et al., 1991). This means that a female reared with a co-specific individual, whatever the sex, never reacts like an isolated one. Thus, in further studies, the control group should consist of 2 females, in order to test the only specific male stimuli.

In this study, male-induced stimulation was improved in a Greek population (Heraklion), whereas previous works have all been carried out on French populations (Jassem et al., 1982: Caen; Jassem et al., 1991: Niort; Caubet et al., 1998: Celles). It has already been shown that females originating from low latitudes start reproduction more precociously than those from high latitudes, and that they retain this characteristic after several generations in laboratory (Souty-Grosset et al., 1994). Thus, in spite of their capacity to breed earlier, this study shows that the Greek females can still be subject to the male stimulation. Moreover, since the male-effect is observed for all studied populations, it appears henceforward evident that this phenomenon is not only a populational characteristic but also a general characteristic of the species.

With regard to the determinism of this male-effect, preceding authors assimilated stimulation to tactile stimuli acting on the dorsal face of females, as observed during the courtship of Jaera albifrons (Solignac, 1972). If the male stimuli effectively acted on the back of females, dorsal varnishing would have prevented the male effect, which is not the case here. Moreover, it has been previously shown that artificial stimulation of the
tergits has no effect on the δPPI (Caubet, unpublished data). So, this long-standing hypothesis must be now questioned. In this study, it was shown that males without copulatory organs are less stimulating, and that females with obturated genital apertures are less stimulated. On the basis of these results, it may be suggested that the stimulation occurs when male copulatory organs come into contact with the female genital apparatus. Moreover, it appears that results from simple physical stimulation, since males unable to ejaculate seminal substances (by section of their spermiducts) keep their stimulating power. This is consistent with previous experiments in which females implanted with androgenic glands (which synthesize the male hormone) are as stimulating as males; although such females are unfertile, they exhibit a male phenotype with a sexual male behaviour (Jassem et al., 1991). Thus, unlike what was described in other models, especially in Diptera and Coleoptera species (Huignard et al., 1977; Boulétreau, 1974), the stimulation observed in *A. vulgare* cannot be related to a putative stimulant component transmitted during insemination, like the « sex-peptide » (Chen and Diem, 1961).

At this point, the hypothesis put forward is this mechanical stimulation occur during copulation, but it could not account for the observed shortening of the PPI (40%), if copulation takes place only at the end of the intermoult, as described in the literature. The dissection of females in period C, placed with a male for 24 h, has revealed the presence of sperm in their genital ducts. That is to say, insemination can occur earlier than expected, since an oocytes diameter of approximately 240 µm. According to Besse (1976) who has worked on the Isopod *Porcellio dilatatus*, this stage of ovarian maturation corresponds to the initiation of the secondary vitellogenesis. This level is reached during the fourth week of the PPI for a coupled female, and during the fifth week for an isolated one (Caubet et al., 1998). Since differences in time are already observed as soon as the onset of the secondary vitellogenesis, this means therefore that the male-induced stimulation even begins before this stage.

The ethological approach gives new evidences to support this pattern of stimulation by precocious sexual male behaviour. Indeed, direct observations of male-female encounter over an hour have revealed that mating postures can be observed during the whole preparturial intermoult. Nevertheless, toward females in pre- and primary vitellogenesis (OD < 240 µm), these mating postures appear too short (mean duration per occurrence = 86 s) to be assimilated with effective copulations (10-18 min according to Mead, 1973). The dissection of such females effectively revealed no trace of sperm in their genital ducts. Thus, such mating postures, without insemination, must be named pseudocopulations. Therefore, since it was shown that sperm is irrelevant with the male-effect, it is assumed that these pseudocopulations, by only physical contact with the female genital apparatus, could stimulate the first stages of vitellogenesis.

Among Insects, many works have shown that mating can modify the female sexual activity, facilitate laying and stimulate oogenesis (Engelmann, 1960, 1970). The stimuli act generally on chemoreceptors located in the genital ducts (Merle, 1970; Brousse-Gaury, 1974), but also on mecanoreceptors located on or close to genital apparatus (Quo, 1959; Roth, 1970; Brousse-Gaury, 1971). The influence of only mechanical mating stimuli on oogenesis has effectively been reported in *Drosophila melanogaster* (Boulétreau, 1974). In cockroaches, authors have even shown that a female must go through a precopulatory period with a male before she becomes receptive and mates (Roth, 1970). In *Armadillidium vulgare*, the mating postures observed throughout the preparturial intermoult, with or without insemination, must be henceforward considered as the determining factor for the ovarian stimulation. In woodlice, it has been demonstrated that oviducts are abundantly innervated (Besse, 1976, in *Porcellio dilatatus*). Thus, the mechanical stimuli would be transmitted towards the protocerebral neurosecretory cells which control the synthesis of V.I.H. (Besse, 1971; Juchault et al., 1989). The synthesis breakdown of the neurohormone, which occurs spontaneously when photophase increases in spring, is then accelerated, allowing a speedy synthesis of Vitellogenin (Picaud et al., 1989), and consequently a shortening of the vitellogenesis period.

In certain woodlice species, like *Porcellio dilatatus* and *Armadillo officinalis*, the initiation of the secondary vitellogenesis is strictly dependent on copulation, and isolated virgins females cannot undergo parturial moult (Legrand, 1958b). In spite of this difference with *A. vulgare*, the intermoult of fecundated females is also clearly accelerated in comparison with the intermoult of virgins (Legrand, 1958b). Nevertheless, the releaser in these species was shown to be chemical and would be contained in the mucous substance secreted with the sperm (Besse, 1976). Another point is that isolated females of *P. dilatatus* can however undergo a parturial
moult spontaneously, but only after 2 or 3 years of sexual rest. According to Besse, at that time, seasonal factors become preponderant and sufficient to initiate reproduction, as observed for the most Oniscidea and Crustacea species. Thus, the *A. vulgare* case appears to be an intermediate between species where reproduction is only dependent on seasonal factors and those where female reproduction breaks away from these external factors, depending only on copulation.

In *A. vulgare*, whereas external seasonal factors synchronize sexual activity within the population, the onset of female reproduction can be modulated by speeding up or delaying oogenesis, depending on whether males are present or not. Such a mechanism of regulation is a real advantage for females since it tends to restrict the emission of non-fertilized oocytes, and consequently optimizes the energy allocated in reproduction. Furthermore, it is easier to understand the male-effect in *A. vulgare* as in many natural populations, the chance of encounters between sexual partners must be rather low. Indeed, epigenetic sex determinants (*Wolbachia* bacteria, *f* factor) are known to reverse genetic males into functional phenotypic females, which frequently induces a high deficit in males (40 % to 20 %) (Juchault and Legrand, 1981; Juchault et al., 1993).

**Acknowledgements**

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Table 1.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>% PM</th>
<th>δ PPI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paired female:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>20</td>
<td>100</td>
<td>29.95 ± 4.42</td>
</tr>
<tr>
<td>1b</td>
<td>20</td>
<td>100</td>
<td>35.70 ± 5.18</td>
</tr>
<tr>
<td>1a-b</td>
<td>40</td>
<td>100</td>
<td>32.82 ± 5.57</td>
</tr>
<tr>
<td>1c</td>
<td>24</td>
<td>87.5</td>
<td>29.10 ± 3.86</td>
</tr>
<tr>
<td>1a-b-c</td>
<td>64</td>
<td>95.3</td>
<td>31.54 ± 5.32</td>
</tr>
<tr>
<td>Isolated female:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>24</td>
<td>92</td>
<td>58.20 ± 15.32</td>
</tr>
<tr>
<td>2b</td>
<td>10</td>
<td>90</td>
<td>58.70 ± 17.49</td>
</tr>
<tr>
<td>2a-b</td>
<td>34</td>
<td>91</td>
<td>58.32 ± 15.68</td>
</tr>
<tr>
<td>2c</td>
<td>28</td>
<td>82.1</td>
<td>47.74 ± 8.63</td>
</tr>
<tr>
<td>2a-b-c</td>
<td>62</td>
<td>87.1</td>
<td>53.81 ± 14.07</td>
</tr>
</tbody>
</table>

Table 2.
Effect of experiments acting either on female or male (δ PPI in day). 3: female with tergites overlaid by varnish + male; 4: female without the first and second pair of antennae + male; 5: female without the first pair of pereopod + male; 6: female with the two genital apertures obturated + male; 7: female with one genital aperture obturated + male; 8: isolated female with the two genital apertures obturated; 9: female + male with varnished tergites; 10: female + male without copulatory organs; 11: female + male with the two spermiducts cut out.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>% PM</th>
<th>δ PPI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>21</td>
<td>90.5</td>
<td>34.84 ± 13.63</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>50</td>
<td>30.33 ± 2.83</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>82.4</td>
<td>32.36 ± 7.89</td>
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<td>7</td>
<td>100</td>
<td>35.29 ± 6.42</td>
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<tr>
<td>7</td>
<td>5</td>
<td>100</td>
<td>30.4 ± 6.98</td>
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<tr>
<td>8</td>
<td>8</td>
<td>100</td>
<td>43.75 ± 4.68</td>
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<td>9</td>
<td>19</td>
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<td>11</td>
<td>16</td>
<td>93.8</td>
<td>31.53 ± 3.80</td>
</tr>
</tbody>
</table>
Table 3.
Mean duration of male behaviours (s), in respect to the oocyte diameter of the female proposed (µm), during a one-hour encounter.

<table>
<thead>
<tr>
<th>Oocyte diameter</th>
<th>No interactive male behavior</th>
<th>Interactive male behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immobility</td>
<td>Locomotion</td>
</tr>
<tr>
<td>&lt; 240</td>
<td>1706</td>
<td>1188</td>
</tr>
<tr>
<td>[240 ; 500]</td>
<td>1325</td>
<td>1294</td>
</tr>
<tr>
<td>&gt; 500</td>
<td>1272</td>
<td>971</td>
</tr>
</tbody>
</table>

Table 4.
Characteristics of sexual interactions (mating postures) in respect to the oocyte diameter of the female proposed (µm).

<table>
<thead>
<tr>
<th>Oocyte diameter</th>
<th>n</th>
<th>Encounters with MP</th>
<th>Mean duration /occurrence(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 240</td>
<td>8</td>
<td>6 (75 %)</td>
<td>86 s (1mn 26s)</td>
</tr>
<tr>
<td>[240 ; 500]</td>
<td>21</td>
<td>11 (52.4 %)</td>
<td>550 s (5mn 10s)</td>
</tr>
<tr>
<td>&gt; 500</td>
<td>15</td>
<td>10 (66.7 %)</td>
<td>860 s (14mn 20s)</td>
</tr>
</tbody>
</table>

Appendix.
Items used for the behavioural analysis of one-hour encounter, between one male and one female.

<table>
<thead>
<tr>
<th>No interactive male behaviour</th>
<th>Interactive male behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobility during</td>
<td>Locomotion during</td>
</tr>
<tr>
<td>Female locomotion</td>
<td>Female locomotion</td>
</tr>
<tr>
<td>Female immobility</td>
<td>Female immobility</td>
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<tr>
<td></td>
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</table>
Figure 1.
Correlation between the weight (in mg) and the δ PPI (in day), for isolated female.

![Graph showing the correlation between weight and δ PPI](image)

Figure 2.
Relation between the female physiological stage (moulting cycle and reproductive cycle) and the number of inseminated seminal receptacle(s), after a one-day encounter with a male.

![Graph showing the relation between physiological stage and inseminated oocytes](image)