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► **To cite this version:**

Sylvine Durand, Christine Braquart-Varnier, Sophie Beltran-Bech. Promiscuity and sex ratio in the terrestrial isopod *Armadillidium vulgare* and consequences on genetic diversity. *Behavioural Processes*, 2020, 171, pp.104030. 10.1016/j.beproc.2019.104030 . hal-03111010

HAL Id: hal-03111010

<https://hal.science/hal-03111010>

Submitted on 21 Jul 2022

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1 **Promiscuity and sex-ratio in the terrestrial isopod *Armadillidium vulgare* and consequences on**
2 **genetic diversity**

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11

12 **Abstract:**

13 In promiscuous mating systems, both males and females mate with several partners. While the benefits
14 of multiple mating are well recognized for males, there are several non-mutually exclusive hypotheses
15 to explain multiple mating for females. Promiscuity is widespread in terrestrial isopods. Here, we
16 placed experimental populations of the terrestrial isopod *Armadillidium vulgare* under varying sex-
17 ratios to manipulate the number of available partners, and better characterise the mating system in this
18 species by performing paternity tests using microsatellite markers. We observed that females usually
19 mate multiply with up to 5 males in a single event of reproduction. A higher number of fathers in
20 broods did not increase brood size nor heterozygosity, but increased allelic richness. Promiscuity
21 seems to be asymmetric in this species, with females being less affected by changes in sex-ratio than
22 were males and would be an adaptive mating system maximising the offspring genetic diversity.

23

24

25 **Keywords:** crustaceans, multiple paternity, promiscuity, sex-ratio bias

26

27

28 **Introduction**

29 Unlike polygamy, where only one of the two sexes mates with several partners (the female in the case
30 of polyandry and the male in polygyny), promiscuity, also called polygynandry, is a mating system in
31 which individuals of both sexes mate with several partners during the same breeding period. The
32 question of the benefits of multiple mating for both sexes has been studied (Reynolds 1996; Jennions
33 and Petrie 2000). For males, these advantages are obvious, since the number of offspring increases
34 with mating success and therefore with the number of partners (Bateman 1948). For females, however,
35 the benefits of multiple mating remain uncertain, as a single male is usually sufficient to fertilize all
36 oocytes. Two types of advantages resulting from multiple mating have been proposed for females: (i)
37 direct benefits resulting from resources provided by males to females or offspring (Arnqvist and
38 Nilsson 2000), such as food or protection, and (ii) indirect benefits, also known as genetic benefits,
39 leading to better genetic quality in offspring (Yasui 1998; Jennions and Petrie 2000), such as
40 inbreeding avoidance or increased allelic diversity in broods.

41 In addition to these advantages, multiple mating increases costs associated with mating
42 (Pomiankowski 1987), such as the risk of predation during partner search and mating (Rowe 1994),
43 the risk of injury during mating (Chapman et al. 1995) and the risk of contracting a sexually
44 transmitted disease (Thrall et al. 2000). Despite these costs, the observation of multiple paternity in
45 broods in many vertebrate species (fish (Coleman and Jones 2011), amphibians (Adams et al. 2005),
46 reptiles (Uller and Olsson 2008), birds (Griffith et al. 2002), mammals (Avise et al. 2011)) but also in
47 invertebrates (insects (Simmons et al. 2007), molluscs (Wacker et al. 2018), crustaceans (Walker et al.
48 2002; Yue and Chang 2010; Jossart et al. 2014)) reveals that multiple mating in females is quite
49 widespread.

50 When it comes to mating systems in terrestrial isopods, monogamy is rare. A few cases have been
51 recorded within the genus *Hemilepistus* (Trachelipodidae) (Lefebvre 2002), this mating system
52 resulting from a need for biparental care (burrow digging, offspring care) in relation to the arid and
53 desert environmental conditions under which these organisms live. An intermediate step towards the
54 evolution of a monogamous system would also be reported for species of another family
55 (Porcellionidae, genus *Porcellio*) even if the majority of terrestrial isopods are promiscuous (Lefebvre

56 2002). Indeed, in many species, both males and females are able to mate with several species
57 (Lefebvre 2002). And in these cases, as expected, parental care is provided exclusively by the female
58 (Krebs and Davies 1997).

59 In *Armadillidium vulgare* (as in terrestrial isopods in general), multiple paternity is facilitated by to the
60 presence of a seminal receptacle in females that can store sperm after mating (Suzuki and Ziegler
61 2005; Ziegler and Suzuki 2011). Stored sperm can be used to produce offspring even after females
62 have mated with and received sperm from another male (Moreau et al. 2002). However, because of
63 this sperm storage capacity, a single mating is sufficient to fertilize all the oocytes produced for a year
64 (Howard 1943) and even throughout the life of a given female (Beltran-Bech S., personal
65 observation), which raises the question of the evolution of promiscuity in this species and the direct or
66 indirect benefits that this promiscuity mating system can bring to reproductive partners.

67 In this species, multiple paternity has been observed in laboratory experiments (Moreau et al. 2002;
68 Verne et al. 2007; Durand et al. 2017; Bech et al. 2017) when two males were proposed to a same
69 female (simultaneously or sequentially), and in a natural population in which all of the 23 tested
70 broods presented multiple paternity, with an average of 4.48 ± 1.24 fathers per brood (Valette et al.
71 2017). This clearly shows that experimental observations of multiple paternity in the laboratory are not
72 an artefact and that in this species, the mating strategy is promiscuity. However, the study on a natural
73 population did not allow to access to the number of males encountered by females, or to the timing in
74 which mating took place (in a single or in multiple breeding events, *A. vulgare* females usually
75 producing two to three broods per year between April and October (Vandel 1962)). In particular, it is
76 currently unknown how the availability of sexual partners (e.g. sex ratio) affects the propensity of
77 females to mate.

78 Wishing both to simulate more natural conditions in our experiments and to test the effect of sex ratio
79 on the intensity of multiple paternity in *A. vulgare*, we placed experimental populations under three
80 different sex ratio conditions to measure the effect of the number of present reproductive partners on
81 the number of fathers per brood during the same reproductive event, using microsatellite markers to
82 perform paternity tests. These markers also allowed us to estimate the level of heterozygosity and
83 allelic richness of broods as a function of the number of fathers in these broods.

84 **Material and methods**

85 **1. Ethical statement**

86 The European Directive 2010/63/EU and the French decree n°2013-118 regulating animal research do
87 not require ethical evaluation prior to research on arthropods. However, we complied with the 3R
88 ethical rules: even though replacement was not possible, sample size was not disproportionate and we
89 took care in providing our animals with food and suitable living conditions during the whole duration
90 of the experiment. Tissue sampling for adult genotyping was not lethal, and offspring that had to be
91 killed were frozen before DNA extraction. After the end of the experiment, surviving animals were
92 placed together in large boxes under standard rearing conditions.

93 **2. Experimental setup**

94 We used 120 one-year-old, virgin, sexually mature individuals in our experiment (48 males and 72
95 females) belonging to a laboratory lineage descending from animals collected in 1982 in Helsingør,
96 Denmark. **This lineage is free from the endosymbiont *Wolbachia*, known to affect *A. vulgare* biology,**
97 **including sexual behavior (Moreau et al. 2001).** A leg was collected from each individual using thin
98 tweezers right before the experiment to perform subsequent genetic analyses.

99 Animals were gathered into twelve experimental populations of ten animals, distributed into four sex-
100 ratio conditions each with three replicates: (i) male-biased (2 females and 8 males, i.e. 20% of females,
101 hereafter 20%_F), (ii) balanced (5 females and 5 males, 50%_F), (iii) female-biased (8 females and 2
102 males, 80%_F) and (iv) female-biased with 9 females and 1 male (90%_F) as a control to determine how
103 many females a single male could inseminate within the duration of the experiment in the absence of
104 male-male competition. We did not place siblings or cousins in the same population to avoid close
105 inbreeding avoidance or kin selection mechanisms. Animals were placed in boxes filled with humid
106 loam, with dried linden leaves and carrot slices for food, and placed under a stimulating photoperiod
107 (18L: 6D) to enhance reproduction.

108 Once a week after the beginning of the experiment, boxes were searched for all animals to monitor
109 death and female reproductive status: after mating, females lay fertilized eggs in the *marsupium*, a

110 ventral pouch which appears after moulting. Fully developed offspring leave the *marsupium* after one
111 month (Surbida and Wright 2001). All females (except one) had undergone a parturial moult after
112 three weeks of experiment, so males were removed to avoid disturbing females during incubation.
113 Three weeks after the first parturial moult within a population (i.e. five or six weeks after the
114 beginning of the experiment), all females were individualized then controlled daily, and all maternal
115 siblings were counted on their day of birth or the following day. The number of offspring collected for
116 molecular analyses depended on sex-ratio conditions: the more males are present in a population, the
117 more fathers are expected to share paternity in a brood, and the more analyzed offspring are needed to
118 increase our power to detect all fathers. We thus collected 40 offspring for 20%_F, 30 offspring for
119 50%_F and 20 offspring for 80%_F populations. For 90%_F populations, no paternity analyses were
120 required with only one possible father, but 15 offspring were collected from three randomly selected
121 broods in each population (hence nine 90%_F broods collected) for estimation of brood genetic diversity
122 under this control condition.

123 **3. Genotyping**

124 DNA was extracted individually from legs of all 120 adult using a phenol-chloroform extraction
125 (Kocher et al. 1989; Durand et al. 2015) and from 1145 whole offspring using a Chelex (Sigma-
126 Aldrich) extraction (Durand et al. 2017). Eleven microsatellite markers (Av1, Av2, Av3, Av4, Av5,
127 Av6, Av9 (Verne et al. 2006); Av18, Av32 Av56, Av63 (Giraud et al. 2013)) were gathered into three
128 multiplexes and amplified in the same conditions as in Durand et al. (2015) with fluorescein-marked
129 forward primers. PCR products separation was performed by GenoScreen (Lille, France) on an
130 automated sequencer. Resulting electropherograms were analyzed with the software GeneMapper
131 version 3.7 (Applied Biosystems) to score microsatellite alleles.

132 **4. Paternity analyses**

133 We used the software COLONY v.2.0.6.3 to identify the parents of each offspring. Analyses were
134 performed using a genotyping error rate of 0.01 for all loci, including only offspring for which at least
135 three loci were genotyped (N = 1078 offspring). Genotypes of all offspring in a given brood were

136 entered into the software, as well as genotypes of all candidate mothers and fathers for the considered
137 brood (i.e. all adults within the population), and we specified that all offspring shared the same mother
138 (as well as the same father for 90%_F broods). We decided to consider only offspring whose father was
139 identified with a probability higher than 0.75 (N = 958 offspring, i.e. 88.86% of genotyped offspring),
140 as in other studies (Coetzer et al. 2017).

141 For each offspring, we computed individual genetic diversity using the function GenHet (Coulon
142 2010) in the software R (R Development Core Team 2013). As some individuals presented missing
143 genotypes for some loci (mean \pm standard deviation = 9.38 ± 2.70 genotyped loci per individual), we
144 computed the multilocus standardized heterozygosity H_s (Coltman et al. 1999) (hereafter named
145 “heterozygosity”) which divides the proportion of typed heterozygous loci by the mean observed
146 heterozygosity for typed loci. For each brood, allelic richness was computed using the software
147 FSTAT (Goudet 1995).

148 **5. Statistical analyses**

149 **5.1. Preliminary adjustments and investigations**

150 Two males from different populations died within the first week of experiment. Their death was
151 attested by the observation of remains for both individuals, confirming that we did not just simply fail
152 to detect a living animal when searching loam. In each of those two populations, all males except one
153 produced offspring with at least one female. It is reasonable to conjecture that the two males died
154 before having any opportunity to mate and that sex-ratio was modified at the beginning of the
155 experiment. Therefore, we adapted our analyses to the death of those two males. (i) **One of them**
156 **belonged to a population with 50%_F. This population was analysed normally alongside with the two**
157 **other 50%_F populations (creating a new category with 55.6% females would not make sense with a**
158 **single replicate). (ii) The second male belonged to a population with 80%_F, leading to a population**
159 **with a single male. This population was thus treated as a fourth 90%_F (control) population, leaving**
160 **only two 80%_F populations**

161 **5.2. Promiscuity description**

162 Mann-Whitney tests were used to compare the number of reproductive partners between males and
163 females according to the rarity of the opposite sex (i.e. in excess, balanced or in deficit). **To compare**
164 **the number of reproductive partners between the different sex-ratio conditions within each sex, we**
165 **performed two Kruskal-Wallis tests (for males and for females) followed by Dunn test with Holm-**
166 **Šidák adjustment for pairwise comparisons.**

167 **5.3. Brood characteristics**

168 We modeled three brood characteristics as a function of the number of fathers in the brood: (i) brood
169 size, using a GLMM with a log link and a variance given by a negative binomial distribution; (ii)
170 mean brood heterozygosity and (iii) brood allelic richness using LMMs. In all cases, population
171 identity nested within sex-ratio was included as a random effect. For the model with brood size as a
172 function of the number of fathers, all females in 90%_F populations were included, as genotyping the
173 offspring was not necessary to obtain the number of fathers (always 1 in this case). Significance of
174 variables was tested using type II Wald χ^2 -tests.

175 The software R v.3.4.1 (R Development Core Team 2013) was used to perform statistical analyses.
176 LMMs and GLMMs were realized with the functions “lmer” and “glmer” from the package “lme4”
177 (Bates et al. 2014), excepted for models with brood size as a response variable, which were realized
178 with the function “glmer.nb” of the same package. Type II Wald χ^2 -tests were performed with
179 “Anova” from the “car” package (Fox et al. 2017). **Dunn tests were performed with the “dunn.test”**
180 **package (Dinno 2016).** Significance threshold was set to 0.05 and means are given \pm standard error
181 (se).

182

183 Results

184 1. General overview

185 Out of the 72 females used, 6 died without having offspring (1 in 80%_F and 5 in 90%_F populations), 1
186 never presented a *marsupium* (in 80%_F) and 2 produced **developed embryos that were dead before**
187 **birth (stillborn broods, in 80%_F and 90%_F)**. Three females produced a low number of offspring (6
188 offspring for 2 females in 50%_F and 1 female in 80%_F) which is way below the usual brood size (Table
189 1) and were discarded. The remaining 60 females produced a brood (6 females in 20%_F, 13 in 50%_F,
190 12 in 80%_F and 29 in 90%_F), from which a total of 1145 offspring were genotyped (15 to 40 offspring
191 per brood from 47 analyzed broods, as 13 90%_F broods were not genotyped), among which 958 were
192 assigned a father with a probability higher than 0.75.

193 2. Mating system

194 All surviving females in 90%_F populations produced a brood (**or stillborn brood**), confirming that
195 males are not limited by their capacity to inseminate all available females (up to 8 within a replicate).
196 Moreover, up to 5 fathers were assigned for a single brood in a 20%_F population, confirming the
197 propensity of females to mate with several males during only one breeding event (**Supplementary**
198 **Material 1**).

199 All males in 80%_F populations reproduced at least once (Table 1), and a single male did not reproduce
200 in a 50%_F population. However, under male-biased conditions (20%_F), only 62.5% of males produced
201 offspring (Table 1, **Supplementary Material 2**).

202 Multiple mating was observed for both sexes, with an intensity varying according to both sex and sex-
203 ratio (Figure 1). **The number of reproductive partners depended on sex-ratio in both females (Kruskal-**
204 **Wallis KW = 15.59, df = 2, p < 0.001) and males (Kruskal-Wallis, KW = 21.96, df = 2, p < 0.001),**
205 **with fewer partners when the opposite sex was in deficit than in excess or under balanced conditions,**
206 **but no difference between balanced and excess conditions (number of partners for females, Dunn test:**
207 **deficit – balanced p = 0.0012, deficit – excess p = 0.001, balanced – excess p = 0.209; number of**
208 **partners for males, Dunn test: deficit – balanced p < 0.001, deficit – excess p < 0.001, balanced –**

209 **excess $p = 0.054$**). While males and females did not differ significantly in the number of mates when
210 the opposite sex was in excess ($U = 6$, $p = 0.21$, $n = 10$) or balanced ($U = 94.5$, $p = 0.88$, $n = 27$),
211 females had more mates than did males when the opposite sex was rare ($U = 231$, $p = 0.014$, $n = 36$).

212

213

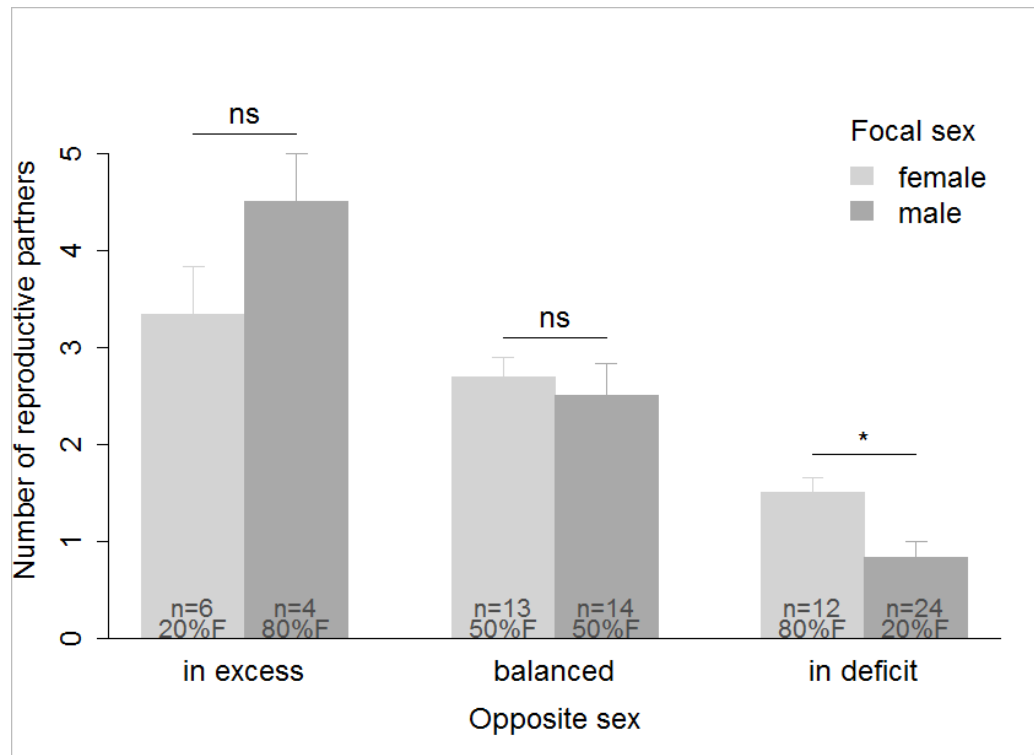
214 **Table 1** Summary of mean adult genetic characteristics and reproduction-related variables for each sex-ratio condition.

Sex-ratio	% of ♂ reproducing at least once	% of broods fathered per ♂^a	% of offspring sired within each brood per ♂^a	Brood size^b	Brood allelic richness	Mean brood heterozygosity
20% _F (N=3)	62.5 ± 10.10	41.67 ± 7.77	30.00 ± 3.72	158.33 ± 14.63	1.5 ± 0.01	1.07 ± 0.07
50% _F (N=3)	92.86 ± 7.14	58.21 ± 7.41	37.14 ± 3.00	124.69 ± 9.88	1.45 ± 0.01	0.94 ± 0.03
80% _F (N=2)	100 ± 0	75.00 ± 8.33	66.67 ± 8.64	123.67 ± 13.42	1.4 ± 0.02	0.97 ± 0.07
90% _F (N=4)	100 ± 0	100 ± 0	100 ± 0	126.88 ± 11.72	1.4 ± 0.02	0.96 ± 0.04
overall (N=12)	78.26 ± 6.15	54.67 ± 5.35	52.81 ± 3.52	129.47 ± 6.24	1.43 ± 0.01	0.97 ± 0.03

215 ^a Values have been calculated only on reproducing males, which is why the value sometimes exceeds 50%.

216 ^b When including 13 additional broods in 90%_F populations that were not genotyped, 90%_F mean brood size is 129.07 ± 9.81, and overall mean brood size is 129.97 ± 6.04.

217



218

219 **Figure 1** Mean number of reproductive partners for each sex according to sex-ratio condition. Error bars represent standard errors. Population sex-ratio is indicated in each

220 bar. n = sample size for the focal sex. ns = non-significant difference, * = significant difference.

221

222 **3. Brood characteristics**

223

224 For all populations (including 13 90%_F broods which were not genotyped), the number of offspring

225 was not significantly affected by the number of fathers ($\chi^2=0.280$, $df=1$, $p=0.597$, $n=60$ females).

226 While mean brood heterozygosity did not depend on the number of fathers ($\chi^2=0.202$, $df=1$, $p=0.653$,

227 $n=47$ females), a high number of fathers lead to a higher allelic richness ($\chi^2=5.756$, $df=1$, $p=0.016$,

228 $n=47$ females, $\beta = 0.026 \pm 0.011$).

229

230 Discussion

231 In this study, we placed experimental populations of ten *Armadillidium vulgare* individuals under
232 different sex-ratio conditions to estimate rates of multiple mating for both sexes and better describe the
233 promiscuous mating system in this species.

234 All surviving females hosted with a single male became gravid and produced a brood (or much less
235 frequently stillborn brood), which confirms that males are polygynous and not limited by their ability
236 to inseminate up to 8 females in a single reproductive event. However, multiple mating may still be
237 costly for males, as sperm depletion has been observed in this species (Rigaud and Moreau 2004). This
238 depletion has been shown to decrease female fertility when females were infected by the
239 endosymbiont *Wolbachia*. Under female-biased and balanced sex-ratio conditions, all males but one
240 reproduced at least once, whereas only 62.5% of males did so under male-biased conditions: this
241 suggests that males are limited in their access to females when these are rare. It then appears that sex-
242 ratio affects the intensity of multiple paternity: all males reproduce but paternity is shared between few
243 fathers within each brood when sex-ratio is female-biased, while not all males reproduce but more
244 fathers share paternity in the opposite situation. Thus, female choice for male characteristics and/or
245 competition between males seem to take place under male-biased sex-ratio. Lefebvre et al. (2000)
246 have shown that in *A. vulgare*, the long antennae present in males are not used in male contests as in
247 other terrestrial isopod species. Male-male competition seems to be based on their ability to
248 successfully locate females (scramble competition) rather than monopolizing mates or excluding other
249 males from reproduction. This suggests that the lower access to paternity under male-biased conditions
250 is more the result of a female refractory behaviour than direct contest competition between males.

251 On the female side, polyandry was largely observed during a single reproductive event with up to 5
252 fathers per brood. A study on a natural population revealed an average of 4.48 fathers per brood with
253 up to 7 fathers (Valette et al. 2017), in line with the multiple breeding events in the wild, the
254 possibility for females to store sperm from previously encountered males (Howard 1943; Verne et al.
255 2007), and a strong copulatory capacity for females. The number of fathers was predictably higher
256 under male-biased or balanced conditions than under female biased conditions, with a trend for more

257 fathers when males were in excess than when sex-ratio was balanced. Broods of more polyandrous
258 females were more polymorphic but did not present more offspring, in line with previous observations
259 (Durand et al. 2017). Interestingly, in a recent study, females hosted under different sex-ratio
260 conditions did not differ in the number of offspring they produced per month over six months, but a
261 lower female survival was observed under male-biased sex-ratio (Fortin et al. 2019). This suggests the
262 existence of female costs associated with polyandry, potentially as a result of male harassment, and
263 raises the question of the evolution of polyandry in this species. Anyhow, by allowing more males to
264 get access to reproduction, polyandry contributes to the persistence of genetic diversity in populations
265 (Sugg and Chesser 1994).

266 Our study highlighted an asymmetric promiscuous mating system in *A. vulgare*, with males being
267 more impacted than females by sex-ratio variations. Indeed, they were more affected by a restriction in
268 sexual partners, with significantly fewer mates per male than per female, and some males not even
269 accessing to reproduction. This is expected since sexual selection is usually stronger in males than in
270 females in most species. In wild populations, sex-ratio varies widely (38 to 81% females, (Rigaud et
271 al. 1997); 26 to 86% females, (Verne et al. 2012). This is due to the presence of diverse sex-
272 determining factors (Cordaux et al. 2011), like the feminizing endosymbiont *Wolbachia* (Bouchon et
273 al. 1998) and nuclear element (Leclercq et al. 2016), as well as the masculinizing M gene (Rigaud
274 and Juchault 1993). This suggests that the intensity of sexual selection acting upon males also varies
275 as a function of the prevalence of these factors.

276

277 **Acknowledgements**

278 We thank Alexandra Lafitte for technical assistance with live animals. This work has been funded by
279 the 2015-2020 State-Region Planning Contracts (CPER), the European Regional Development Fund
280 (FEDER), the partnership arrangements in ecology and the environment (DIPEE) and intramural funds
281 from the Centre National de la Recherche Scientifique, and the University of Poitiers.

282

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