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1 **Male *Gammarus roeseli* provide smaller ejaculates to females infected**  
2 **with vertically transmitted microsporidian parasites**

3

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20

21 **Abstract**

22 The effects of parasites on the reproduction of their hosts are widespread, but studies  
23 investigating the effect of female parasitic status on sperm allocation in the males, a  
24 form of post-copulatory mate choice, remain scarce. Because males are often sperm-  
25 limited, strategic sperm investment, in which females of low reproductive value  
26 receive fewer sperm, is predicted to occur so as to maximise long-term male  
27 reproductive success. In this study based on pairs collected *in natura*, we  
28 investigated how *Gammarus roeseli* (Crustacea: Amphipoda) males allocated sperm  
29 when paired with females infected with the vertically-transmitted, sex ratio distorting,  
30 microsporidian parasites, *Nosema granulosis* or *Dictyocoela* sp. Since infected  
31 females had similar fecundity than uninfected ones, and offspring of females infected  
32 with *N. granulosis* showed a higher survival rate, we predicted equivalent or even  
33 larger sperm investment from males paired with infected females. Contrary to our  
34 predictions, males paired with infected females had lower sperm reserves prior to  
35 ejaculation and provided smaller size ejaculates compared to those paired with  
36 uninfected females. This pattern suggests either a strategic sperm investment as a  
37 function of the female parasitic status, or that males in good condition managed to  
38 pair with uninfected females with a higher probability than those in bad condition.

39

40 **Keywords:** mate choice, mate guarding, sperm investment, *Nosema granulosis*,  
41 *Dictyocoela*

42

43

44 The effects of parasites on the reproduction of their hosts are widespread and  
45 diverse (Poulin 2007). In particular, the seminal paper by Hamilton & Zuk (1982) drew  
46 attention to the fact that parasite infections could be a major evolutionary force  
47 driving host's sexual selection and sexual behaviour. Among this broad area of  
48 research, studies on effects of parasites on male sperm production and investment  
49 are scarce. Most of them have investigated the direct effect of the parasitic status of  
50 the male on its sperm production, with evidence of either negative effects (e.g. Yan &  
51 Stevens 1995; Galipaud, Dechaume-Moncharmont, Oughadou & Bollache, 2011) or  
52 positive effects (reflecting an increased investment to compensate negative effects of  
53 parasitism on other life-history traits) (Figenschou et al. 2013; Haeussler, Schmera &  
54 Baur, 2014). Only a few studies have investigated the effect of female parasitic status  
55 on sperm allocation in the male (Edward & Chapman 2011). Due to intense male-  
56 male competition and because males are sperm-limited, strategic sperm investment  
57 is predicted to occur at each mating event to maximise the overall fertilisation  
58 success (Wedell, Gage & Parker, 2002; Jarrige, Riemann, Goubault & Schmoll,  
59 2015; but see Arundell, Wedell & Dunn, 2014). In particular, males may provide  
60 females of low reproductive value with fewer sperm (Reinhold, Kurtz & Engqvist,  
61 2002). Parasitic infections decrease host fitness, and are therefore a source of  
62 variation in the quality of potential mates. Female parasitic status could therefore  
63 promote strategic sperm allocation by males, with greater allocation of sperm to high  
64 quality (uninfected) females, and prudent sperm allocation to females of low quality.  
65 This is especially true in the eventuality of numerous potential future mates, because  
66 these future mates could be of higher quality (Reinhold, Kurtz & Engqvist, 2002,  
67 Edward & Chapman 2011).

68           When parasites are vertically transmitted (from mother to offspring via the  
69   eggs), they are under strong evolutionary pressure to distort the primary sex ratio of  
70   their hosts, through male killing or feminisation (Bandi, Dunn, Hurst & Rigaud 2001).  
71   By reversing genetic males into phenotypic females, feminising microbes increase  
72   their transmission efficiency by increasing the frequency of the transmitting sex  
73   (females). For instance, feminisation is induced by the bacterium *Wolbachia*  
74   (Bouchon, Rigaud & Juchault 1998; Kageyama, Nishimura, Hoshizaki & Ishikawa  
75   2002) and by parasites from the eukaryotic phylum Microspora (Terry et al. 2004).  
76   Because they often result in female-biased populations, parasitic sex ratio distorters  
77   are a selective force in the evolution of mating behaviour (Charlat, Hurst & Mercot  
78   2003) and mate choice (Jiggins, Hurst & Majerus 2000; Moreau, Bertin, Caubet &  
79   Rigaud 2001). In the crustacean isopod *Armadillidium vulgare*, not only males prefer  
80   to mate with uninfected (real) females vs. infected ones (genetic males reversed by  
81   the feminising bacterium *Wolbachia*) (Moreau, Bertin, Caubet & Rigaud 2001), but  
82   they also allocate smaller size ejaculates to infected females, resulting in a decrease  
83   in fertility (Rigaud & Moreau 2004). Because on average infected and uninfected  
84   females had the same fecundity, this lesser allocation to infected females has been  
85   attributed to the abnormal (incomplete) behaviour expressed by the reversed-females  
86   during courtship and copulation (Moreau & Rigaud 2001, Rigaud & Moreau 2004).  
87   The crustacean amphipod *Gammarus duebeni* has been found to be infected with  
88   sex ratio distorting parasites: the microsporidian parasites *Nosema granulosis* and  
89   *Dictyocoela duebenum* (Terry, Smith, & Dunn 1998; Ironside & Alexander 2015).  
90   Dunn et al. (2006) showed that males provide smaller size ejaculates to females  
91   infected with *N. granulosis*, relative to uninfected females. As infected females  
92   produce fewer eggs than uninfected ones and males are sperm-limited, the smaller

93 ejaculate size has been interpreted as a strategic sperm allocation; the males saving  
94 most of their sperm reserve for females of high quality. Assessment of female quality  
95 could be favoured in amphipods because of a long-lasting phase of precopulatory  
96 mate guarding involving close proximity with the female for up to three weeks  
97 (Galipaud, Bollache, Oughadou & Dechaume-Moncharmont 2015).

98         In most freshwater crustaceans, including *Gammarus roeseli*, reproduction is  
99 characterized by a precopulatory mate guarding phase during which males guard a  
100 potential mate by carrying a female beneath their ventral surface for several days  
101 before copulation. This behaviour is tightly linked to females' moulting cycle. While  
102 males are considered available for mating during most of their moult cycle (Sutcliffe  
103 1992), females are only receptive to copulation shortly after moulting and just for a  
104 few hours. After copulation, they begin a new moulting cycle, which can last from  
105 several days up to several weeks depending on the species (Jormalainen 1998).  
106 *Gammarus roeseli* belong to the group of multivoltine iteroparous annuals (lifetime  
107 12-24 months) and females moult six to eight times, thus potentially producing six to  
108 eight broods (Pöckl 1993).

109         Because females' moulting cycles, and hence receptivity to copulation, are  
110 asynchronous, the operational sex ratio is predicted to be strongly biased towards  
111 males. Precopulatory mate guarding behaviour is thought to have evolved as a male  
112 competitive strategy in response to the brief period of female receptivity (Parker  
113 1974; Grafen & Ridley 1983). Males are in competition for access to females, in  
114 particular to 'high-quality' females. From a male perspective, the female's quality as a  
115 mate strongly depends on fecundity, and the male pairing decision to engage mate  
116 guarding is known to be negatively influenced by female parasitism status (Bollache  
117 et al. 2002).

118 Most populations of the amphipod *G. roeseli* are infected with microsporidian  
119 parasites in the rivers of Western Europe (Haine et al. 2004; Gismondi, Rigaud,  
120 Beisel & Cossu-Leguille 2012; Grabner et al. 2015). Three of these parasite species  
121 (*Nosema granulosis*, *Dictyocoela muelleri* and *Dictyocoela sp. (roeselum)*) have been  
122 shown to be vertically-transmitted, and associated with female-biased sex ratios  
123 (Haine et al. 2004; Haine, Motreuil & Rigaud 2007). In contrast with the parasites  
124 infecting *G. duebeni*, the microsporidia in *G. roeseli* are not associated with a  
125 decrease in fecundity (Haine et al. 2004) and the microsporidia of the genus  
126 *Dictyocoela* had only a slight impact on the host's physiology in absence of other  
127 stresses (Gismondi, Rigaud, Beisel & Cossu-Leguille 2012). *Nosema granulosis* even  
128 provides its hosts an advantage in survival relative to uninfected females (Haine,  
129 Motreuil & Rigaud 2007). Here, we tested the hypothesis of an effect of females'  
130 parasitic status on males' reproductive strategy. Male gammarids are sperm-limited,  
131 and long lasting precopulatory mate guarding leaves opportunities for an accurate  
132 assessment of female quality. We thus predicted that *G. roeseli* males allocate more  
133 sperm to females infected with *N. granulosis*, but show no difference in sperm  
134 allocation between uninfected females and females infected with parasites of the  
135 genus *Dictyocoela*.

136

## 137 **Methods**

138

### 139 *Ethical note*

140 This work followed the ABS/ASAB guidelines for the treatment of animals in  
141 behavioural research. Information about individuals' origin, and housing conditions  
142 are described below. Transport between sampling site and laboratory, housing

143 conditions, handling, as well as experimental monitoring, have been conducted in  
144 order to reduce stress and maximise animals' welfare. We complied with the French  
145 regulations for experiments on invertebrates.

146

147 *Animal collection, maintenance and general procedures*

148 Animals were collected in the river Ouches in Dijon (47°17'51.6"N; 5°02'33.3"E),  
149 using kick sampling method with a hand net. Gammarids exhibit a precopulatory  
150 mate guarding behaviour (they grasp females several days before egg laying) in  
151 order to ensure the reproduction of the future batch of eggs (Sutcliffe 1992).  
152 Immediately upon sampling, couples were isolated in individual plastic tubes, and  
153 were brought back to the laboratory. Each pair was then housed individually under a  
154 12:12 light:dark cycle regime, at 15°C ( $\pm 1$ ), in boxes (h=7cm;  $\varnothing$ =9cm) filled with  
155 water from the river mixed with tap water previously dechlorinated, UV-treated and  
156 oxygenated.

157 Couples were then randomly assigned to one of the two following experiments. The  
158 first experiment aimed at understanding the effect of female infection status on male  
159 ejaculate size. Fertilisation is semi-external in gammarids: after the female has laid  
160 eggs, the male deposits sperm in her ventral incubating 'pouch'. As water can flow in  
161 the pouch, it is impossible to collect male ejaculates. Sperm investment is therefore  
162 estimated by comparing sperm reserves in testes between two treatment groups;  
163 males before copulation (during mate guarding), and males after a copulation event  
164 (not holding the female anymore, the female having released eggs) (Dunn et al.  
165 2006; Lemaître, Rigaud, Cornet & Bollache 2009). Males were anaesthetised using  
166 carbonised water and dissected to estimate their sperm reserves. In the first group (n  
167 = 64), males were dissected before insemination (within two days after their arrival in



168 the lab). In the second group (n = 61), sperm remaining in testes were counted after  
169 copulation, on the same day. Females were also anaesthetised and dissected. The  
170 eggs were flushed out from the incubating pouch and counted. To assess the  
171 female's infection status by microsporidia, the gonads were dissected and stored in  
172 pure ethanol until molecular analysis. Prior to dissection, the size of each animal was  
173 estimated by measuring the height of the fourth coxal plate, using a Nikon SMZ 1500  
174 stereoscopic microscope and the Lucia G 4.81 software.

175 The second experiment aimed at assessing sperm replenishment kinetics after  
176 copulation. Sperm were therefore counted in males 2, 4, 8 and 12 days after the  
177 insemination (n = 21, 22, 20, 20, respectively). The second group of the first  
178 experiment served as reference for sperm reserves just after insemination (0 days  
179 after insemination, n = 64). At the end of the experiments, all non-dissected  
180 individuals were released back into the river they came from.

181

### 182 *Sperm counting*

183 Sperm was counted as described in Lemaître, Rigaud, Cornet & Bollache (2009).  
184 One testis per individual was isolated in a watch glass, in 1000 µL of demineralised  
185 water. After isolation, the gonad was dissected under a binocular microscope. The  
186 fragments of gonad were submitted to 10 s of ultra-wave treatment to separate the  
187 membranes from the sperm (Ultra-waves tank, Branson 2200 Branson Cleaning  
188 Equipment Company, Shelton, Co, U.S.A.) and homogenised. For each male, four 10  
189 µL samples were placed on a slide and dried for 10 min at 37°C. All sperm in each  
190 drop were counted under an optic microscope Nikon Eclipse E600 (magnification  
191 x100). Statistical analyses were carried out using the sum of the number sperms in

192 the four drops, since the counting appeared repeatable between drops ( $R = 0.964$ ,  
193 95% CI = [0.953; 0.975]).

194

#### 195 *Infection status*

196 Microsporidia detection and identification were performed using a PCR-RFLP  
197 method, following Haine et al. (2004). After DNA extraction from the female's gonads,  
198 a PCR test was conducted with the primers V1f and 530r, amplifying a fragment of  
199 the microsporidian 16S ribosomal gene only in case of parasite infection (Haine et al.  
200 2004). The size of the amplification product allowed to discriminate between *Nosema*  
201 *granulosis* and *Dictyocoela* parasites, and the use of restriction enzymes *Vspl* and  
202 *Bst1107I* (MBI fermentas) allowed to discriminate between the *D. muelleri* and the *D.*  
203 *sp. (roeselum)* sequences, respectively (Haine et al. 2004). *Vspl* enzyme revealed  
204 only females infected with *Dictyocoela sp. (roeselum)* in this study, hereafter referred  
205 as "*Dictyocoela*". Males were not tested for the presence of parasites since  
206 prevalence in males in this population is close to zero (Haine et al. 2004).

207

#### 208 *Statistical analyses*

209 Female fecundity was analysed with a general linear model including the following  
210 factors: female size, insemination status of males (before or after insemination), and  
211 infection status of females (uninfected, infected with *N. granulosis*, infected with  
212 *Dictyocoela*) and their two-order interactions. The repeatability of sperm count was  
213 estimated using 'rptR' packages (Stoffel, Nakagawa & Schielzeth 2017). Sperm  
214 reserves in males were analysed using a general linear model including the following  
215 factors: male size, insemination status (before or after insemination), and infection  
216 status of females (uninfected, infected with *N. granulosis*, infected with *Dictyocoela*)

217 and their two-order interactions. Sperm replenishment data were analysed using a  
218 general linear model including the following factors: male size, day after insemination  
219 (treated as categories, 0, 2, 4, 8 and 12 days after insemination) and their interaction.  
220 We reported Cohen's *d* with their bootstrapped 95% confidence intervals (Nakagawa  
221 & Cuthill 2007) as measures of effect size for the comparison between mean sperm  
222 reserves before and after copulation (Garamszegi 2009). We reported standardised  
223 slopes as a measure of effect size for the relationship between female body length  
224 and fecundity (Schielzeth 2010). Non-parametric post-hoc comparisons after Kruskal-  
225 Wallis' tests were performed using Conover's-test implemented in PMCMR packages  
226 (Pohlert 2014). The tests were performed using *R* 3.2.5 (R core team 2016) or JMP  
227 10.0 (SAS institute).

228

## 229 **Results**

230

### 231 *Animal size and female fecundity*

232 A total of 108 pairs (*n* = 53 before ejaculation, and *n* = 55 after ejaculation) were  
233 measured. Thirty-seven females were infected with *N. granulosis*, 23 were infected  
234 with *Dictyocoela*, and 48 were uninfected (control). Body length did not differ as a  
235 function of the female's parasitic status in either males ( $F_{2,105} = 0.43$ , *p* = 0.65,  
236 supplementary Fig. S1a) or females ( $F_{1,106} = 0.03$ , *p* = 0.97, supplementary Fig. S1b).  
237 There was no difference in body length between the two groups (before and after  
238 ejaculation) in males ( $F_{1,106} = 0.42$ , *p* = 0.52, Cohen's *d* = -0.13, 95% CI = [-0.53;  
239 0.22], supplementary Fig. S1c) or in females ( $F_{1,106} = 0.18$ , *p* = 0.68, Cohen's *d* = -  
240 0.08, 95% CI = [-0.48; 0.30], supplementary Fig. S1d). The mean body size was 3.00  
241 mm (95%IC = [2.94; 3.06]) for males, and 2.70 mm (95% CI = [2.64;2.75]) for

242 females. There was a significant positive relationship between female size and  
243 fecundity ( $F_{1,106} = 22.3$ ,  $p < 10^{-5}$ , standardised slope = 0.42, 95%CI = [0.24; 0.59],  
244 Fig.1), but there was no effect of the parasitic status of the female ( $F_{2,104} = 0.18$ ,  $p =$   
245 0.82) or the insemination status of the male ( $F_{2,104} = 0.0002$ ,  $p = 0.99$ ) on female size.  
246

#### 247 *Sperm reserves in males before and after insemination*

248 There was no effect of male body size on sperm reserves before ( $F_{1,51} = 1.89$ ,  $p =$   
249 0.18) or after ejaculation ( $F_{1,53} = 0.68$ ,  $p = 0.41$ ). Sperm reserves were significantly  
250 affected by the interaction between the insemination status and the parasitic status  
251 ( $F_{2,104} = 3.32$ ,  $p = 0.040$ , Fig.2A). The sperm count was significantly larger for males  
252 in amplexus (i.e. before copulation) than for males after copulation in each treatment  
253 groups: *N. granulosis* infected females (Cohen's  $d = 1.37$  with 95% CI = [0.92;1.95],  
254  $F_{1,35} = 17.14$ ,  $p = 0.00021$ ), *Dictyocoela* infected females (Cohen's  $d = 1.04$  with 95%  
255 CI = [0.39;1.84],  $F_{1,21} = 6.07$ ,  $p = 0.022$ ) and uninfected females (control) (Cohen's  $d$   
256 = 2.78 with 95% CI = [2.029;4.13],  $F_{1,46} = 92.078$   $p < 10^{-5}$ ). Sperm reserves before  
257 ejaculation were significantly different among males, depending on the parasitic  
258 status of the female (Kruskal-Wallis test  $\chi^2 = 6.7824$ ,  $df = 2$ ,  $p = 0.034$ ). Males paired  
259 with a female infected with *N. granulosis* (Conover's post-hoc test,  $p = 0.048$ ) or  
260 *Dictyocoela* ( $p = 0.016$ ) had significantly lower sperm reserves than males from the  
261 control group. After ejaculation, sperm reserves were not significantly different among  
262 males, whether paired with infected females or uninfected females (Kruskal-Wallis  
263 test  $\chi^2 = 1.77$ ,  $df = 2$ ,  $p$ -value = 0.41). The comparison of sperm investment as a  
264 function of the parasitic status of females was based on the effect size (Cohen's  $d$ ) of  
265 the average difference in sperm count before and after ejaculation. For each  
266 treatment, the value of the effect size was large (sensu Cohen 1988) but a direct

267 comparison of the 95% CIs (Cumming & Finch 2005, Krzywinski & Altman 2013)  
268 revealed a significantly larger sperm investment (twice larger) for the uninfected  
269 group than for the two infected groups (Fig. 2B).

270 We estimated the average sperm number in the ejaculate as the difference between  
271 the mean sperm reserves before and after ejaculation (+/-95% confidence interval  
272 based on 10 000 bootstraps):  $n = 532$  (95% CI = [415; 639]) for the uninfected  
273 females,  $n = 383$  (95% CI = [212; 565]) for females infected with *N. granulosis*, and  $n$   
274 = 234 (95% CI = [49; 427]) for females infected with *Dictyocoela*. Because we  
275 counted the sperm number in 40  $\mu\text{L}$  among the 1000  $\mu\text{L}$  in which one testis was  
276 dissected, we can estimate the total ejaculate size as  $((532*1000)/40)*2 = 26600$   
277 sperm for uninfected females (CI = [20750; 31950]), 19150 for females infected with  
278 *N. granulosis* (CI = [10600; 28250]), and 11700 sperm for females infected with  
279 *Dictyocoela* (CI = [2450; 21350]).

280

### 281 *Sperm replenishment*

282

283 Sperm reserves increased significantly in males with time spent since copulation  
284 ( $F_{4,138} = 47.19$ ,  $P < 10^{-5}$ ) but was not influenced by male size ( $F_{1,137} = 2.36$ ,  $P =$   
285 0.140) nor by the interaction between male size and time since copulation ( $F_{4,133} =$   
286 0.767,  $P = 0.548$ ). Post hoc comparisons showed no difference in sperm reserves  
287 between freshly-mated males and males two days after a copulation event (Fig. 3).  
288 Eight and 12 days after a copulation event, males had more sperm than other males,  
289 without any significant difference between them (Fig. 3).

290

### 291 **Discussion**

292 We observed a negative effect of females' infection status on males paired with  
293 them, in terms of both sperm reserves before ejaculation and ejaculate size. Indeed,  
294 males paired with infected females had significantly lower initial sperm reserves than  
295 males paired with uninfected females, whereas their final sperm reserves after  
296 insemination did not differ, reflecting a lower number of sperm provided during  
297 insemination. This could be explained by three non-exclusive hypotheses. First,  
298 based on our screening of sperm replenishment dynamics, males paired with infected  
299 females could be those that had less time to replenish their reserves between two  
300 copulations. Such a pattern could occur if infected females moulted more rapidly than  
301 uninfected ones, which would reduce amplexus duration before copulation.  
302 Alternatively, males paired with uninfected females could be in pair for longer prior  
303 collection. However, since the dynamics of sperm replenishment is quite rapid  
304 (around 8 days), this possible difference was not huge. If such a pairing pattern was  
305 true, this would mean that males engaging pairing with uninfected females would be  
306 those able to monopolize females earlier. These hypotheses remain to be tested.  
307 Second, when paired with infected females, males could strategically allocate less  
308 energy in the production of gametes. As first argued by Dewsbury (1982), a single  
309 male gamete may be cheap, but as males transfer large numbers of sperm, gamete  
310 production could be energetically expensive. Thus, males could modulate their  
311 allocation in sperm production according to female quality by allocating less energy in  
312 gamete production when paired with infected females (Reinhold, Kurtz & Engqvist,  
313 2002). Third, males paired with infected females could differ in competitiveness or  
314 body condition from males paired with uninfected females, as suggested by the fact  
315 that unpaired males have generally lower sperm reserves than paired males  
316 (Lemaître, Rigaud, Cornet & Bollache 2009). Consistent with the major role of male-

317 male competition for access to receptive females in gammarids (Ward & Porter 1993,  
318 Dick & Elwood 1996, Bollache & Cézilly, 2004), it is possible that those in the best  
319 condition have a higher access to uninfected females. If males in poorer condition  
320 were less competitive and less efficient in finding or monopolising uninfected  
321 females, they might have to accept the second-choice females remaining in the  
322 population. Furthermore, the decision to enter into precopula might not solely be  
323 under male control, and females could resist male attempts to grasp them  
324 (Jormalainen & Merilaita 1995, Jormalainen 1998). If uninfected females were more  
325 able to resist attempts from males than infected ones, only the most competitive and  
326 in best condition males would be able to pair with them.

327 Our results are consistent with previous observations in *G. roeseli* reporting that the  
328 parasitic status of females does not appear to affect their fitness, neither in terms of  
329 fecundity nor body length (Haine et al. 2004). This pattern is in contrast with the  
330 situation in *G. duebeni*, where infection confers a fitness cost in terms of fecundity  
331 (e.g. Dunn et al. 2006), and therefore, makes it difficult to invoke for *G. roeseli* the  
332 adaptive strategic male sperm allocation based on differences in female fecundity  
333 proposed in *G. duebeni* (Dunn et al. 2006). Differences in sperm reserves and  
334 ejaculate size may therefore result from differences in cues used by males to identify  
335 infected females. As precopulatory mate guarding is a long-lasting and intimate  
336 interaction between the partners, we may propose that the male relies on behavioural  
337 cues to assess whether its partner is a genuine female or a feminised male. *Nosema*  
338 *granulosis* and *Dictyocoela* sp. have been shown to be sex ratio distorting parasites  
339 in gammarids, reversing male hosts into phenotypic females (Terry et al. 2004;  
340 Rodgers-Gray et al. 2004; Haine, Motreuil & Rigaud 2007; Ironside & Alexander  
341 2015). A number of infected females are therefore genetic males reversed by

342 parasites. In terrestrial isopod crustaceans infected with the feminising *Wolbachia*  
343 bacteria, such 'false females' do not behave entirely as normal females during the  
344 copulation sequence, resulting in higher rates of copulation failure and, in case of  
345 successful copulations, lower sperm investment in infected females compared to  
346 uninfected ones (Moreau, Bertin, Caubet & Rigaud 2001, Rigaud & Moreau 2004).  
347 Since mate recognition in crustaceans is based on contact pheromones present on  
348 the cuticle (Caskey, Hasenstein & Bauer 2009; Zhang et al. 2011), differences in  
349 cuticular compounds between infected and uninfected females may explain the  
350 observed pattern. Investigating the behaviour and cuticle compounds of infected vs.  
351 uninfected *G. roeseli* females during mate guarding would therefore be helpful to  
352 further understand the sperm investment pattern observed in this study. Another  
353 explanation, which does not involve a directional preference for uninfected female,  
354 could be that infected females show less resistance to pairing attempts (Jormalainen  
355 & Merilaita 1995). Thus, only the most competitive males (with the largest reserves of  
356 sperm) could pair with more resistant uninfected females, the less competitive males  
357 only being able to pair with the less resistant infected females.

358 *Gammarus roeseli* males showed large sperm investment at each insemination  
359 event (between c.a. 50% and 75% of their sperm reserves constitute the ejaculate).  
360 Such a sperm depletion is consistent with the values reported in *Gammarus pulex*  
361 (Lemaître, Rigaud, Cornet & Bollache 2009), and confirms that the sperm investment  
362 is substantial in *Gammarus*. However, as also noted by Lemaître, Rigaud, Cornet &  
363 Bollache (2009), sperm replenishment was achieved within less than a dozen days.  
364 Initial sperm reserves in *G. roeseli* were double those in *G. pulex*: around 10  
365 sperm/ $\mu$ L in *G. pulex* before copulation, and less than 2.5 sperm/ $\mu$ L after copulation,  
366 while in *G. roeseli* they were around 22 sperm/ $\mu$ L before copulation and more than 5



367 sperm/ $\mu$ L after copulation. As observed in terrestrial isopods (Moreau & Rigaud  
368 2003), we may propose that these higher sperm reserves could be due to a selection  
369 pressure induced by the excess of females in populations of *G. roeseli* (Haine et al.  
370 2004).

371 Future studies should carefully assess the influence of parasites on male mate  
372 choice and female behaviour in order to understand pairing processes leading to  
373 these mating patterns, and the link between male sperm reserves and female  
374 infection status.

375

376

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380

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530

531

532 **Caption for figure**

533

534 **Fig. 1.** Female fecundity as a function of female body size (estimated from the height  
535 of the fourth coxal plate, in millimetres) and female parasitic status: uninfected  
536 females, females infected with *N. granulosis*, females infected with *Dictyocoela*. The  
537 regression line is given for each treatment group.

538

539 **Fig. 2.** (A) Mean sperm number (+/- 95% confidence interval) as a function of the  
540 male status (solid circle: before copulation, open circle: after copulation) and the  
541 treatment group (the male is either paired with an uninfected female, a female  
542 infected with *N. granulosis*, or a female infected with *Dictyocoela*). (B) Effect size of  
543 the sperm investment (Cohen's d) with bootstrapped 95% CI as a function of the  
544 treatment group. Different letters indicate statistically significant differences based on  
545 comparison of the 95% CIs.

546

547 **Figure 3.** Mean sperm number (+/-95% confidence interval) as a function of the time  
548 since copulation. The first points correspond to the mean sperm number measured in  
549 the testes a few hours after copulation. Different letters indicate statistically significant  
550 differences (Tukey post-hoc comparisons).

551

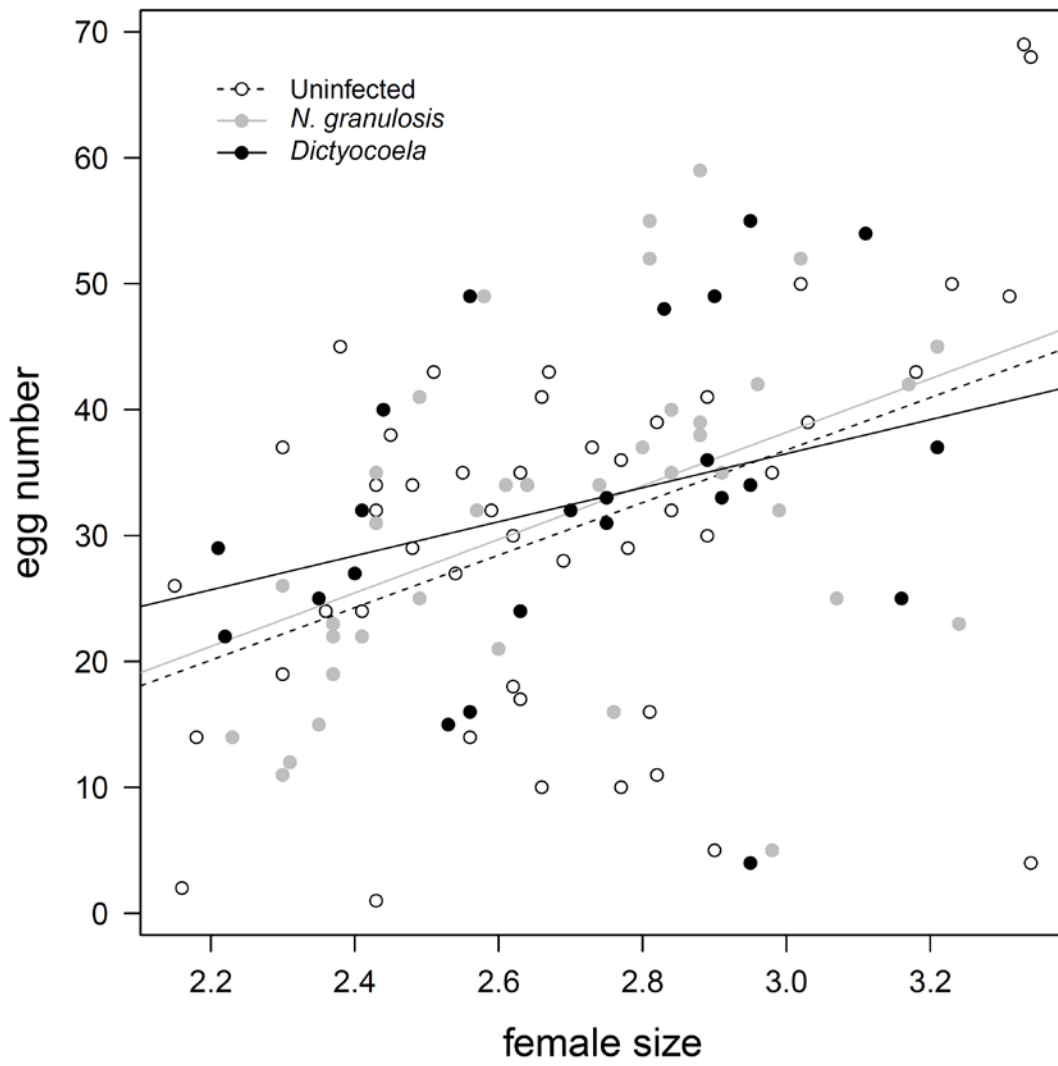
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554 **Fig. 1**

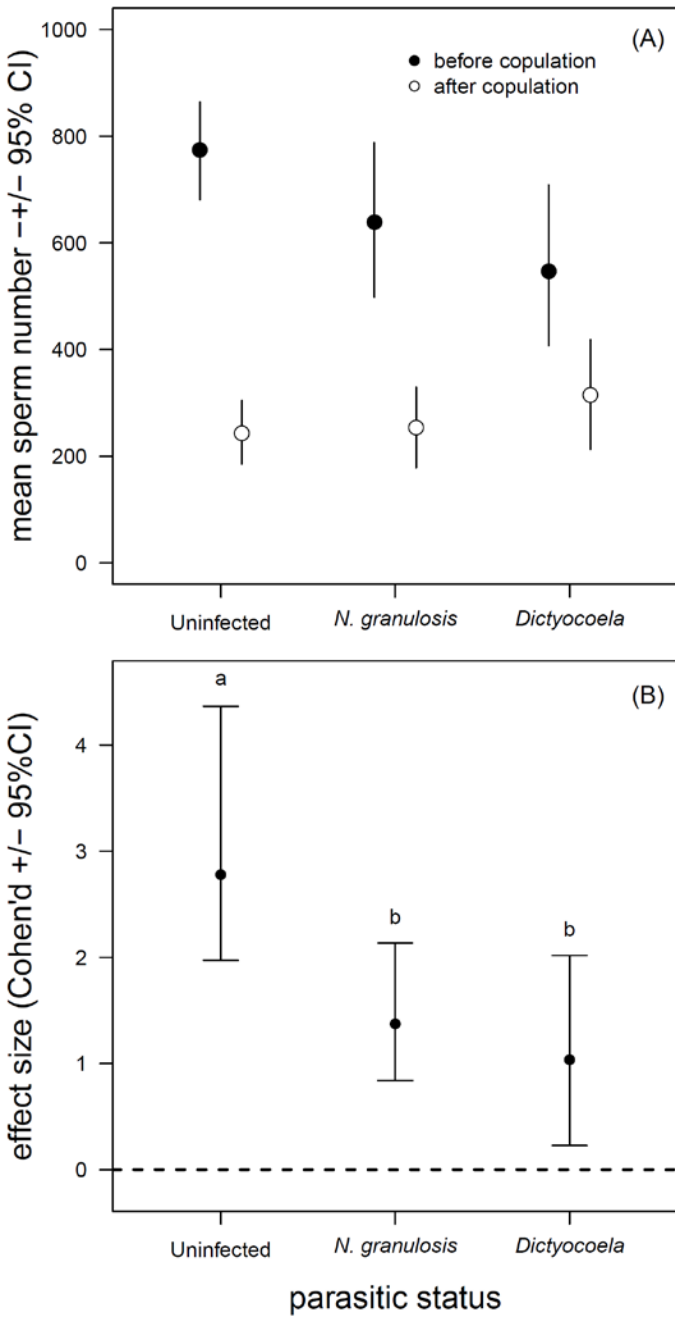
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Fig. 2



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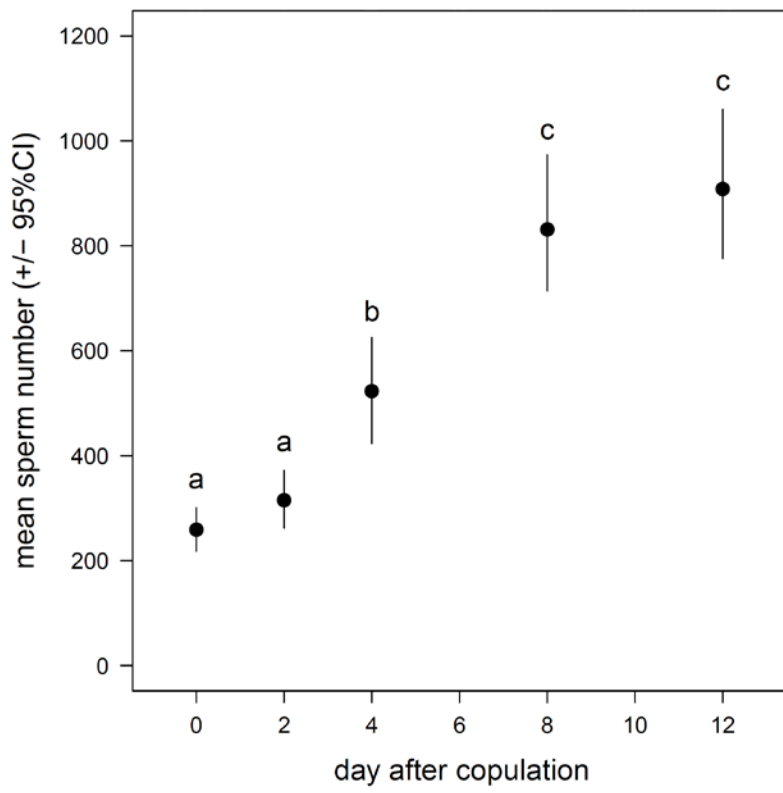
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563 **Fig. 3**

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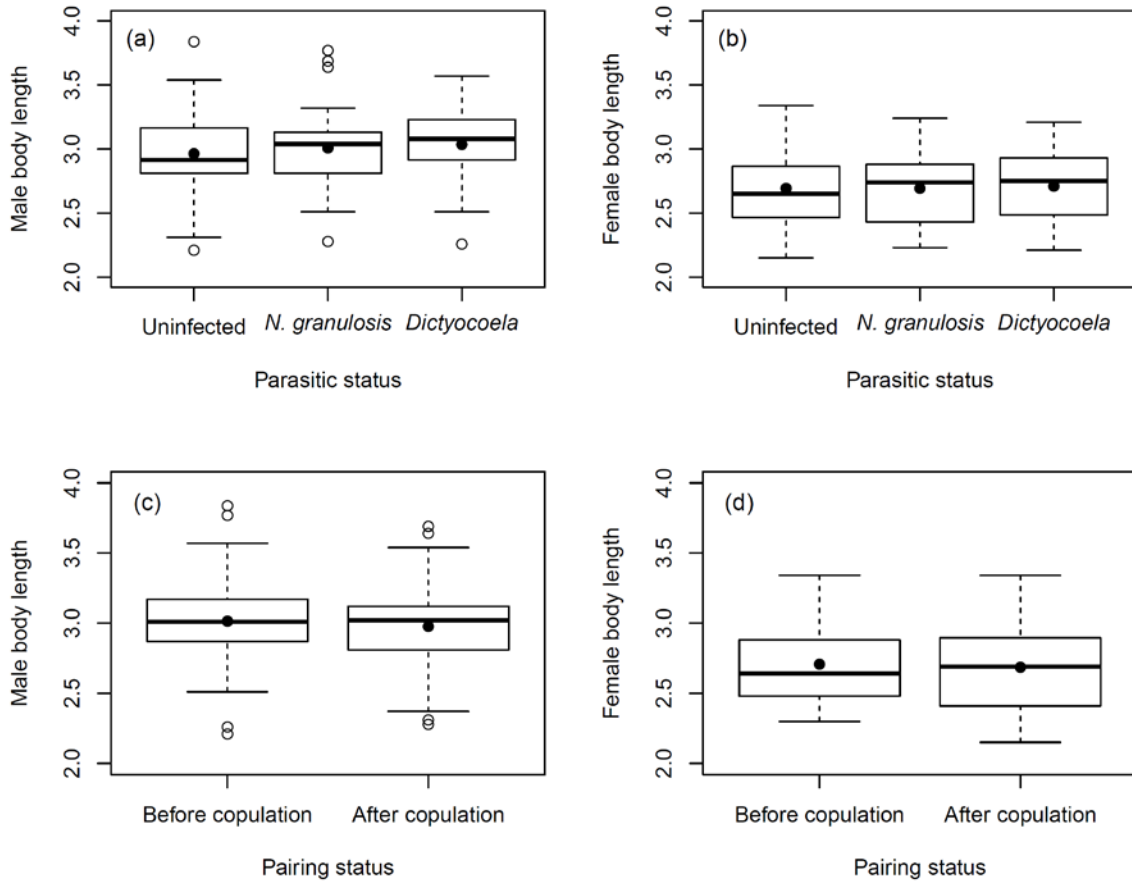
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569 **Supplementary figure**

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572

573 **Fig S1.** Boxplot of body length (estimated from the height of the fourth coxal plate, in  
574 millimetres) as a function of treatment group (parasitic status of the female) for males  
575 (a) and females (b), and as a function of pairing status (before or after copulation) for  
576 males (c) and females (d). For each group, the box figures the interval interquartile  
577 (IIQ), the dark line the median value, the solid circle the mean value, and the open  
578 circles the outliers.

579