

Identifying a key host in an acanthocephalan-amphipod system.

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3	Identifying a key host in an acanthocephalan-amphipod system
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12 Summary

13

14 Trophically transmitted parasites may use multiple intermediate hosts, some of which may be 'key-hosts', 15 i.e. contributing significantly more to the completion of the parasite life cycle, while others may be 'sink 16 hosts' with a poor contribution to parasite transmission. Gammarus fossarum and Gammarus roeseli are 17 sympatric crustaceans used as intermediate hosts by the acanthocephalan Pomphorhynchus laevis. 18 Gammarus roeseli suffers higher field prevalence and is less sensitive to parasite behavioural manipulation 19 and to predation by definitive hosts. However, no data are available on between-host differences in 20 susceptibility to P. laevis infection, making it difficult to untangle the relative contributions of these hosts to 21 parasite transmission. Based on results from estimates of prevalence in gammarids exposed or protected 22 from predation and laboratory infections, G. fossarum specimens were found to be more susceptible to P. 23 laevis infection. As it is more susceptible to both parasite infection and manipulation, G. fossarum is 24 therefore a key host for P. laevis transmission. 25 26 27 28 Key words 29 30 multi-host parasites; prevalence; host specificity; host quality; transmission; infectivity 31

32 Key findings

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- 34 The manipulative *Pomphorhynchus laevis* has both *Gammarus roeseli* and *G. fossarum* as intermediate
- 35 hosts.
- 36 Infected *G. fossarum* are relatively more predated, explaining higher field prevalence in *G. roeseli*.
- 37 However, differences in susceptibility to *P. laevis* infection between the two host species are unknown.
- 38 Field experiments and laboratory infections indicate that *G. roeseli* is less susceptible to infection.
- 39 *Gammarus fossarum* is confirmed as a key host for *P. laevis*.

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41 Introduction

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43 While the majority of parasites are known to exploit multiple host species, either sequentially or because 44 they have a range of suitable hosts for the same stage of their cycle (Ruiz-González et al., 2012), host-45 parasite interactions are usually studied in simplified one-to-one relations, disconnected from the real-life 46 complex systems (Rigaud *et al.*, 2010). Multi-host parasites may use host species differing in abundance, 47 exposure and susceptibility, and thus unlikely to contribute equally to parasite transmission and fitness. 48 The 'key hosts' are those contributing significantly more to the completion of the parasite life cycle 49 (Streicker et al., 2013). Three non-exclusive processes serve to identify a host as a key species, contributing 50 disproportionately to parasite transmission: high host abundance, high exposure and/or susceptibility to 51 infection, and/or large number of infective stages produced per infected individual (Streicker et al., 2013). 52 Parasites with complex life cycles are, by definition, multi-host parasites because they require at least two 53 successive host species to achieve their development. However, they may also use several different host 54 species at any stage of their cycle. Such parasites may show weak specificity when infecting the 55 intermediate host, or sometimes even the definitive host, although there is great interspecific variation in 56 these traits (Combes, 2001). Numerous parasites with a complex life cycle have evolved the ability to 57 modify several aspects of the phenotype of their intermediate hosts, concomitantly increasing the 58 probability of transmission to their definitive hosts (reviewed in Poulin, 2010). Many trophically transmitted 59 parasites can even modify certain behaviours of their intermediate hosts (Thomas et al., 2005; Perrot-60 Minnot et al., 2014). Modification of a number of anti-predatory behaviours is directly linked to the 61 modulation of predation rates in intermediate hosts, either increasing for infected versus non-infected 62 hosts (Kaldonski et al., 2007; Lagrue et al., 2007), or decreasing when the parasites are not yet infective for 63 the definitive host (Dianne et al., 2011; Weinreich et al., 2013). These behavioural changes have been 64 referred to as 'host manipulation' because parasites alter the phenotype of their hosts in ways that 65 enhance their own fitness at the expense of that of infected hosts (Thomas et al., 2005; Cézilly et al., 2010). 66 For these parasites, the sensitivity of the host to manipulation should be included to determine key host 67 species, because of its implication in parasite transmission. 68 Acanthocephala are trophically transmitted parasites for which the ability to modify host phenotype is

69 ubiquitous, possibly having evolved in the common ancestor of the group (Moore, 1984). They all use at

70 least two hosts to complete their cycle, whether for intermediate, definitive or paratenic hosts, with

71 different degrees of fitness depending on the hosts and/or spatial distribution of these hosts (see Kennedy

72 2006 for an overview). *Pomphorhynchus laevis* have been extensively studied in the contexts of host

manipulation and ecology (Kennedy, 2006). They infect several freshwater gammarid amphipod species as

- 74 intermediate hosts, and several freshwater fish species as definitive or paratenic hosts (Kennedy, 2006;
- 75 Médoc et al., 2011). In central and eastern France, the cryptic Gammarus pulex and Gammarus fossarum
- species (Lagrue *et al.*, 2014) are resident intermediate host species, while *Gammarus roeseli* is a relatively

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77 recent colonizer from Southern Central Europe (Jazdzewski, 1980). These gammarids are often found in 78 sympatry (Chovet and Lécureuil, 1994) and infected by P. laevis in these sympatric sites (e.g. Bauer et al., 79 2000; Rigaud and Moret, 2003; Lagrue et al., 2007). Prevalence and infection intensity are usually higher in 80 G. roeseli than in G. pulex (Lagrue et al., 2007, Lagrue, unpublished data), despite the fact that the latter is 81 generally more abundant than the former when present in sympatry (e.g. Lagrue et al., 2007). It would 82 therefore seem logical for P. laevis to rely more on G. roeseli than on G. pulex for its transmission. However, 83 several elements indicate that exactly the opposite situation could be the rule. Crude prevalence is not an 84 accurate measure to quantify the abundance of a manipulative parasite, since observed prevalence 85 diminishes as infected intermediate hosts are preferentially preyed upon by the next host(s), rather than 86 uninfected hosts (Lafferty, 1992; Rousset et al., 1996). Lagrue et al. (2007) showed that the prevalence of P. 87 laevis in G. pulex was low in the river benthos but high in the definitive host's stomach, whereas prevalence 88 in G. roeseli was higher in the field and lower in the stomach of the definitive host. In addition, by analysing 89 the distribution of parasite intensity, they showed that parasites accumulate in older G. roeseli, but not in 90 older G. pulex, confirming a higher death rate of infected G. pulex compared to infected G. roeseli. This 91 result is consistent with the fact that infected *G. roeseli* is known to be less strongly manipulated than *G.* 92 pulex by P. laevis (Bauer et al., 2000). Furthermore, uninfected G. roeseli has been found to be less sensitive 93 to predation by trout (Bollache et al., 2006) or bullhead (Kaldonski et al., 2008) than uninfected G. pulex, 94 because of more efficient anti-predatory defences. The combination of all these factors provides 95 reasonable evidence of a predation differential between infected animals of each species, and so G. roeseli 96 can reasonably be considered a lower quality host for *P. laevis* transmission. 97 However, the relative susceptibility of the two amphipod species to infection by *P. laevis* remains 98 undetermined. Yet this information is crucial to assess the relative importance of the two concurrent hosts 99 in the P. laevis life cycle. If G. pulex is more susceptible to infection than G. roeseli, then both susceptibility 100 and behavioural manipulation would act in synergy, making this host a true key host for transmission. If, 101 conversely, G. roeseli is more susceptible than G. pulex, then P. laevis transmission would be 'diluted' by 102 the presence of this host, because of its inefficiency in transmitting the parasite, and could potentially 103 impact the epidemiology of the infection (see Hall et al., 2009; Johnson et al., 2009, for examples). We 104 conducted a laboratory infection experiment by submitting both species to the same dose of P. laevis eggs 105 to measure the susceptibility of these sympatric gammarid species to P. laevis. To assess the impact of 106 predation, we compared prevalence in two contrasted amphipod collections from the field: animals directly 107 collected from rivers (i.e. previously exposed to natural predation), and animals collected from the same 108 rivers, but then maintained for several weeks in the laboratory (i.e. in the absence of any fish predation

109 pressure).

110 Methods

111 Amphipod collection and prevalence in the field

- 112 Since field prevalence may be variable between populations, two rivers were chosen, where *G. fossarum*
- and G. roeseli live in sympatry and are naturally infected by P. laevis. Amphipods from the Albane River, in
- 114 Trochères (47°20'34"N, 5°18'21.8"E), and the Meuzin River, near Villy-le-Moutier (47°2'7.71"N,
- 115 4°59'53.87"E), were sampled between September and October 2013.
- 116 Amphipods (G. roeseli and G. fossarum) were captured using kick nets. All potential habitats present at
- each site were sampled, and the collected animals were randomly divided into three groups, each
- 118 maintained in a container with aerated water from the river.
- 119 The first group was used to estimate the 'field/direct' prevalence. Animals from this group were kept in
- 120 well aerated aquaria at 15 °C and all checked for parasite presence within two days after capture. Infected
- 121 individuals were dissected to confirm parasite species. Larval parasites can be detected through the host
- 122 cuticle, either at the late acanthella stage of their development (translucent light orange, shapeless larval
- 123 stage) or at cystacanth stage (bright yellow-orange, spherical larval stage). Earlier acanthella stages (where
- 124 parasites are small and translucent) can only be detected after dissection. Preliminary investigation showed
- 125 that acanthella detection could only be certified after 40 days (without microscope and staining), so that all
- 126 prevalence reported in the following experiments is prevalence for *P. laevis* of more than 40 days old
- 127 (Labaude et al., submitted).
- 128 Gammarids from the second group were kept individually in the laboratory, in cups of c.a. 50 mL at 15 °C. 129 for 96 days. All gammarids where infection was detectable by eye were removed from this group so that, at 130 the beginning of this survey, the remaining animals were classified as "uninfected". However, as previously 131 stated, younger acanthella stages are too small to be detected through host cuticule, so some of these 132 isolated gammarids may have already been infected in the field in the days preceding their capture. It is the 133 prevalence of these undetected infections that was recorded during this survey. Animals dying during this 134 period were dissected the day after their death, and all living animals were checked and dissected 96 days 135 post isolation, a delay long enough to ensure that all parasites could be detected. This survey therefore 136 allowed prevalence to be estimated in gammarids not exposed to predation during parasite development 137 (hereafter called 'field/protected' prevalence). All infected G. fossarum were kept in ethanol for genetic 138 analysis (see above).
- 139 A third group of gammarids was used for experimental infections (see below).

140 Experimental infection

- 141 Before being isolated for the experiment, all gammarids were inspected under a dissecting microscope to
- 142 remove naturally infected animals. The remaining gammarids were kept in quarantine for 30 days, to

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distinguish any further natural infection (by parasites too young to be detected) from experimental
infection. Some additional *G. pulex* were also collected in a small tributary of the Suzon River at Val-Suzon
(47°4'12.6"N; 4°52'58.2"E). Given that the *G. pulex* from Val-Suzon are particularly sensitive to
experimental infection by *P. laevis* (Franceschi *et al.*, 2010), they were used to confirm the success and
timing of experimental infection.

148 Gravid P. laevis females were collected from the intestines of chubs (Leuciscus cephalus), from naturally 149 infected fish caught in September 2013 in the Vouge River (Burgundy, Eastern France: 47°9'34.36"N; 150 5°9'2.50"E). A foreign parasite population was chosen to avoid potential local adaptation in our two 151 gammarid populations (Franceschi et al., 2010), so that it was possible to estimate gammarid sensitivity to 152 parasite strains with which they had not evolved. Molecular identification of parasites and exposure of 153 gammarids to parasite eggs followed the procedure described in Franceschi et al. (2008). Gammarus, in 154 cups filled with c.a. 50 mL of aerated water, were allowed to feed for 48 hours on a 1 cm² piece of elm leaf, 155 on which a suspension of 100 mature eggs per gammarid had been deposited (see detailed procedure in 156 Franceschi et al., 2008). Food was then removed, and gammarids were maintained at 15 °C for 3 months. 157 The field/protected group described above was used as control. Individuals from this group were treated 158 and maintained under the same conditions as exposed gammarids but were unexposed to parasite eggs. A 159 total of 615 G. fossarum (162 males and 109 females from Albane, 214 males and 130 females from 160 Meuzin) and 440 G. roeseli (157 males and 102 females from Albane, 121 males and 60 females from 161 Meuzin) were exposed to parasite eggs, as were the G. pulex (155 males from Val-Suzon). 308 G. fossarum 162 (104 males and 61 females from Albane, 89 males and 54 females from Meuzin) and 324 G. roeseli (102 163 males and 67 females from Albane, 104 males and 51 females from Meuzin) were used as control 164 individuals. All infected G. fossarum, along with 100 individuals from the control group, were kept in 165 ethanol for genetic investigation (see below).

166 The water of each dish was completely renewed every two weeks with aerated water from the river, and 167 water levels were restored to original levels twice a week. The amphipods were fed ad libitum with elm 168 leaves, and their diet was enriched with a chironomid larva twice a month. A daily mortality survey was 169 carried out, and animals were dissected the day after their death to detect young acanthella stages. From 170 the sixth week post-exposure, living gammarids were inspected every week under a dissecting microscope 171 to detect the presence of parasites. Infected animals were examined every two days after detection to 172 estimate the date when the cystacanth stage was reached. Gammarids from Val-Suzon (where *P. laevis* is 173 absent) were a control group for the timing and success of experimental infection. Previous studies 174 revealed that P. laevis reaches cystacanth stage in about 80-120 days in laboratory conditions (Franceschi 175 et al., 2008, 2010). In gammarids from the Meuzin and Albane rivers, even after a guarantine of 30 days 176 before exposure, parasites from the wild can develop. Therefore, if *P. laevis* were detected before the first 177 signs of infection in animals from Val-Suzon, individuals were removed from the analysis to avoid any 178 potential confounding effect.

179 Gammarid genotyping

180 Because of the recently discovered cryptic genetic diversity within the *Gammarus fossarum-pulex* species 181 complexes (e.g. Lagrue et al., 2014), there is a need to examine patterns of infection in the light of this 182 diversity (see Westram et al., 2011). Such a study is not necessary for G. roeseli because no cryptic diversity 183 has been detected in Western and Central Europe (Moret et al., 2007). Genetic diversity was assessed in 184 these two rivers using the amplification of part of the mtDNA cytochrome c oxidase subunit 1 (CO1) by 185 polymerase chain reactions (PCR) and a subsequent RFLP procedure (Lagrue et al., 2014). Only G. fossarum 186 belonging to one group were known to occur at the Meuzin site (Gfl, see Lagrue *et al.*, 2014), while genetic 187 diversity for the Albane River had not previously been estimated. All infected G. fossarum and G. pulex 188 from each river were preserved in pure ethanol after death, for subsequent DNA extraction. In addition, 189 100 uninfected animals randomly sampled from each site were also preserved. Gammarid DNA was 190 extracted from two pereopods ('walking legs' in amphipods), following the standard chelex method (Lagrue 191 et al., 2014). The DNA was then amplified for CO1 using universal primers (LCO1490 and HCO2198; Folmer 192 et al., 1994). The PCR were performed using Qiagen Multiplex DNA polymerase kits (Qiagen Inc, Düsseldorf, 193 Germany), as in Lagrue et al. (2014). The PCR-amplified DNA products were then digested overnight using 194 the appropriate reaction buffer and restriction endonuclease(s), following manufacturer's instructions 195 (New England Biolabs, Ipswich, Massachusetts, USA). The resulting fragments were separated by gel 196 electrophoresis in a 1.5 % agarose gel. Restriction enzyme profiles were used to assign each individual 197 amphipod to its respective genetic group (see Lagrue et al., (2014) for the detailed procedure and the 198 specific digestion enzymes for each gammarid genetic group).

199

200 Statistical analyses

All statistical analyses were performed using R software (R Development Core Team, 2013) or JMP software
 (version 10.0.0).

203 For natural infections, a binomial logistic regression was performed to analyse prevalence, with the

following potential explanatory factors: site (Albane River vs. Meuzin River), Gammarus species (G. roeseli

vs. G. fossarum), Gammarus sex (males vs. females), experiment (field/direct: natural infection from the

field sample vs. field/protected: natural infection after maintenance in the laboratory), and their second-

207 order interactions.

208 For experimental infections, a binomial logistic regression was performed to analyse prevalence, with site,

209 species and sex, and their second-order interactions, as potential explanatory factors.

- 210 All possible models were compared using the Akaike Information Criterion (AICc). The models presented
- 211 are those minimizing the AICc.
- 212

213 **Results**

214 Genetic diversity among G. fossarum-like gammarids

- For the gammarids from the Albane River, PCR-RFLP revealed 87 % of *G. fossarum* and 13 % of *G. pulex* in
- the 50 randomly sampled, uninfected animals, with 82 % of *G. fossarum* and 18 % *G. pulex* in the 68
- 217 infected animals. The species ratios in infected and uninfected groups were not significantly different (χ^2 =
- 218 0.2438, P = 0.6215). As we detected no difference in sensitivity to infection between *G. pulex* and *G*.
- fossarum, and since the majority of the gammarids, even at the Albane site, are G. fossarum, this term is
- used to encompass all *G. fossarum*-like gammarids.
- 221

222 Natural infection: direct field prevalence vs. field prevalence protected from

223 predation

- 224 Prevalence of *P. laevis* was higher in *G. roeseli* than in *G. fossarum* in direct field prevalence, at both sites,
- 225 whereas reverse relative prevalence was observed when measured after keeping putative uninfected
- animals in the laboratory, where they were preserved from predation (Table 1, Figure 1).

227

228 Experimental infection

- 229 The first observations of acanthellae through the host cuticle occurred 60 days post-exposure for the
- 230 control Val-Suzon gammarids, as was the case for gammarids of both species from the Albane and Meuzin
- rivers. The cystacanth stage was achieved 82 ± 10 days post-exposure of the control Val-Suzon group, after
- 232 80 \pm 6 days for *G. fossarum*, and after 83 \pm 3 days for *G. roeseli*.
- 233 We found a strong effect of river origin on infection (Table 2, Figure 1), with gammarids from the Albane
- River being three times more sensitive to infection. The difference in prevalence between species, with *G*.
- 235 *fossarum* being approximately twice as infected as *G. roeseli*, was nevertheless not strong enough to be
- 236 fully supported statistically (Table 2, Figure 1).

237

238 **Discussion**

239

240 Our data initially showed that the crude P. laevis prevalence is higher in G. roeseli than in G. fossarum, 241 confirming results of Lagrue et al. (2007) for another site. In the 'field/protected' experiment, the 242 prevalence was reversed, and was higher in G. fossarum for the two populations investigated. In addition, 243 prevalence in G. fossarum was approximately twice that in G. roeseli in both populations after 244 experimental infection by a non-coevolved parasite population, even though this result was not fully 245 supported statistically (probably due to the stronger population effect). Prevalence observed in the field is 246 therefore not a reliable measure of the actual parasite burden for this manipulative trophically transmitted 247 parasite. Differences in the duration of parasite development could possibly have explained the differences 248 in prevalence observed between the two *Gammarus* species. However, parasite growth was synchronous 249 for all hosts during the laboratory infection experiment.

250

251 As the two hosts have similar lifespans, parasites developing in G. roeseli have a lower probability of 252 completing their life cycle, due both to reduced natural predation by fish compared to G. pulex (Bollache et 253 al., 2006; Kaldonski et al., 2008) and lower manipulation levels for infected individuals (Bauer et al., 2000). 254 Therefore, G. roeseli seems to 'dilute' P. laevis transmission when this host is sympatric with G. fossarum. 255 However, as shown here, G. roeseli is not more susceptible than G. fossarum to infection by P. laevis, so the 256 dilution effect is not as strong as previously thought when natural prevalence alone was considered. Lower 257 infection success in G. roeseli counterbalances the low predation rate, limiting the 'sink effect' for the 258 parasite. As G. fossarum is first more susceptible to infection and then more predated, our data confirm 259 this species as a key host for *P. laevis*.

260

Our results also have implications in explaining the role of parasites in the success of biological invasions. 261 Gammarus roeseli is a species that colonised Western Europe during the 20th century (Chovet and 262 263 Lécureuil, 1994). Parasitism may play a role in the coexistence of native and introduced (or invasive) host 264 species. Some studies support the 'enemy release' hypothesis, in which invaders are no longer exposed to 265 their original parasites, but also less susceptible to infection by native parasites, providing invasive hosts 266 with a competitive advantage (Dunn and Dick, 1998; Kopp and Jokela, 2007). In contrast, other studies 267 show a decrease in prevalence in native species by the dilution effect, both experimentally (Kopp and 268 Jokela, 2007) and in natura (Telfer et al., 2005). The invader acts in that case as a dead-end sink for the 269 parasite. G. roeseli being less susceptible to both infection (this study) and to behavioural changes induced 270 by P. laevis (Bauer et al., 2000; Moret et al., 2007), our results are in line with the ennemy realese 271 hypothesis.

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273 Our results complete and strengthen the hypothesis that sympatric G. roeseli and G. fossarum are not hosts 274 of the same quality for acanthocephalan parasites. Should this assumption be extended to all gammarid 275 hosts of freshwater acanthocephalans? Because of the high level of cryptic speciation in the Gammarus 276 pulex/fossarum group (e.g. Westram et al., 2011b; Lagrue et al., 2014), the situation will probably be quite 277 complex to study. Westram et al. (2011a), coupling natural prevalence estimations and field infection 278 experiments, also showed differences in susceptibility between Gammarus species to infection by the 279 acanthocephalan Pomphorhynchus tereticollis, with G. pulex being less infected than G. fossarum. 280 Differences within the G. fossarum group, while less marked, were also detected. However, in Switzerland, 281 where the study was carried out, different species (and/or cryptic species) are rarely found in sympatry, 282 each stream or river harbouring a single gammarid species, so there is confusion between host species and 283 the sites where the host-parasite couple is living, with the potential for local adaptation confounding the 284 results of host specificity (Franceschi et al., 2010). Apart from our case-study of the G. roeseli / G. fossarum 285 system, no clear data are available yet on infectivity and behavioural changes induced by the same local 286 parasite strains on two sympatric species. In the present study, we found no significant difference in 287 prevalence between sympatric G. pulex and G. fossarum from the Albane River. However, this result should 288 be replicated in other rivers, with more individuals and more species tested. Behavioural modifications 289 should also be measured to confirm this apparent lack of specificity.

290

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292

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296

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301

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- 1 Table 1. Logistic regression testing for the effects of site (river), *Gammarus* species and experiment (direct
- 2 field prevalence or protected field prevalence) on the field prevalence of *P. laevis*. The model initially
- 3 included sex of gammarids, and other interactions. After removing these non-significant factors, the model
- 4 presented now minimizes the AICc.

5

Source of variation	d.f.	LR χ2	Р
Site	1	1.2999	0.2542
Species	1	0.8110	0.3678
Experiment	1	0.1877	0.6648
Species*experiment	1	7.7271	0.0054
Site*experiment	1	2.3673	0.1239

6 Global model: LR $\chi 2 = 15.4448$, 5 d.f., P = 0.0086; n = 1787

- 1 Table 2. Logistic regression testing for the effects of site (river) and *Gammarus* species on the prevalence of
- 2 *P. laevis* after experimental infection by parasites from the Ouche River. The model initially included sex of
- 3 gammarids and interactions. After removing these non-significant factors, the model presented now
- 4 minimizes the AICc.
- 5

Source of variation	d.f.	LR χ2	Р
Site	1	16.9051	< 0.0001
Species	1	3.3303	0.0680

6 Global model: LR $\chi 2$ = 19.9606, 2 d.f., P < 0.0001; n = 807





106x81mm (300 x 300 DPI)