Title page

Identifying a key host in an acanthocephalan-amphipod system

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Summary

Trophically transmitted parasites may use multiple intermediate hosts, some of which may be 'key-hosts', i.e. contributing significantly more to the completion of the parasite life cycle, while others may be 'sink hosts' with a poor contribution to parasite transmission. *Gammarus fossarum* and *Gammarus roeseli* are sympatric crustaceans used as intermediate hosts by the acanthocephalan *Pomphorhynchus laevis*. *Gammarus roeseli* suffers higher field prevalence and is less sensitive to parasite behavioural manipulation and to predation by definitive hosts. However, no data are available on between-host differences in susceptibility to *P. laevis* infection, making it difficult to untangle the relative contributions of these hosts to parasite transmission. Based on results from estimates of prevalence in gammarids exposed or protected from predation and laboratory infections, *G. fossarum* specimens were found to be more susceptible to *P. laevis* infection. As it is more susceptible to both parasite infection and manipulation, *G. fossarum* is therefore a key host for *P. laevis* transmission.

Key words

multi-host parasites; prevalence; host specificity; host quality; transmission; infectivity

Key findings

The manipulative *Pomphorhynchus laevis* has both *Gammarus roeseli* and *G. fossarum* as intermediate hosts.

Infected *G. fossarum* are relatively more predated, explaining higher field prevalence in *G. roeseli*.

However, differences in susceptibility to *P. laevis* infection between the two host species are unknown.

Field experiments and laboratory infections indicate that *G. roeseli* is less susceptible to infection.

Gammarus fossarum is confirmed as a key host for *P. laevis*.

Introduction

While the majority of parasites are known to exploit multiple host species, either sequentially or because they have a range of suitable hosts for the same stage of their cycle (Ruiz-González *et al.*, 2012), host-parasite interactions are usually studied in simplified one-to-one relations, disconnected from the real-life complex systems (Rigaud *et al.*, 2010). Multi-host parasites may use host species differing in abundance, exposure and susceptibility, and thus unlikely to contribute equally to parasite transmission and fitness. The ‘key hosts’ are those contributing significantly more to the completion of the parasite life cycle (Streicker *et al.*, 2013). Three non-exclusive processes serve to identify a host as a key species, contributing disproportionately to parasite transmission: high host abundance, high exposure and/or susceptibility to infection, and/or large number of infective stages produced per infected individual (Streicker *et al.*, 2013).

Parasites with complex life cycles are, by definition, multi-host parasites because they require at least two successive host species to achieve their development. However, they may also use several different host species at any stage of their cycle. Such parasites may show weak specificity when infecting the intermediate host, or sometimes even the definitive host, although there is great interspecific variation in these traits (Combes, 2001). Numerous parasites with a complex life cycle have evolved the ability to modify several aspects of the phenotype of their intermediate hosts, concomitantly increasing the probability of transmission to their definitive hosts (reviewed in Poulin, 2010). Many trophically transmitted parasites can even modify certain behaviours of their intermediate hosts (Thomas *et al.*, 2005; Perrot-Minnot *et al.*, 2014). Modification of a number of anti-predatory behaviours is directly linked to the modulation of predation rates in intermediate hosts, either increasing for infected versus non-infected hosts (Kaldonski *et al.*, 2007; Lagrue *et al.*, 2007), or decreasing when the parasites are not yet infective for the definitive host (Dianne *et al.*, 2011; Weinreich *et al.*, 2013). These behavioural changes have been referred to as ‘host manipulation’ because parasites alter the phenotype of their hosts in ways that enhance their own fitness at the expense of that of infected hosts (Thomas *et al.*, 2005; Cézilly *et al.*, 2010). For these parasites, the sensitivity of the host to manipulation should be included to determine key host species, because of its implication in parasite transmission.

Acanthocephala are trophically transmitted parasites for which the ability to modify host phenotype is ubiquitous, possibly having evolved in the common ancestor of the group (Moore, 1984). They all use at least two hosts to complete their cycle, whether for intermediate, definitive or paratenic hosts, with different degrees of fitness depending on the hosts and/or spatial distribution of these hosts (see Kennedy 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 intermediate hosts, and several freshwater fish species as definitive or paratenic hosts (Kennedy, 2006; 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

recent colonizer from Southern Central Europe (Jazdzewski, 1980). These gammarids are often found in sympatry (Chovet and Lécureuil, 1994) and infected by *P. laevis* in these sympatric sites (e.g. Bauer *et al.*, 2000; Rigaud and Moret, 2003; Lagrue *et al.*, 2007). Prevalence and infection intensity are usually higher in *G. roeseli* than in *G. pulex* (Lagrue *et al.*, 2007, Lagrue, unpublished data), despite the fact that the latter is generally more abundant than the former when present in sympatry (e.g. Lagrue *et al.*, 2007). It would therefore seem logical for *P. laevis* to rely more on *G. roeseli* than on *G. pulex* for its transmission. However, several elements indicate that exactly the opposite situation could be the rule. Crude prevalence is not an accurate measure to quantify the abundance of a manipulative parasite, since observed prevalence diminishes as infected intermediate hosts are preferentially preyed upon by the next host(s), rather than uninfected hosts (Lafferty, 1992; Rousset *et al.*, 1996). Lagrue *et al.* (2007) showed that the prevalence of *P. laevis* in *G. pulex* was low in the river benthos but high in the definitive host's stomach, whereas prevalence in *G. roeseli* was higher in the field and lower in the stomach of the definitive host. In addition, by analysing the distribution of parasite intensity, they showed that parasites accumulate in older *G. roeseli*, but not in older *G. pulex*, confirming a higher death rate of infected *G. pulex* compared to infected *G. roeseli*. This result is consistent with the fact that infected *G. roeseli* is known to be less strongly manipulated than *G. pulex* by *P. laevis* (Bauer *et al.*, 2000). Furthermore, uninfected *G. roeseli* has been found to be less sensitive to predation by trout (Bollache *et al.*, 2006) or bullhead (Kaldonski *et al.*, 2008) than uninfected *G. pulex*, because of more efficient anti-predatory defences. The combination of all these factors provides reasonable evidence of a predation differential between infected animals of each species, and so *G. roeseli* can reasonably be considered a lower quality host for *P. laevis* transmission.

However, the relative susceptibility of the two amphipod species to infection by *P. laevis* remains undetermined. Yet this information is crucial to assess the relative importance of the two concurrent hosts in the *P. laevis* life cycle. If *G. pulex* is more susceptible to infection than *G. roeseli*, then both susceptibility and behavioural manipulation would act in synergy, making this host a true key host for transmission. If, conversely, *G. roeseli* is more susceptible than *G. pulex*, then *P. laevis* transmission would be 'diluted' by the presence of this host, because of its inefficiency in transmitting the parasite, and could potentially impact the epidemiology of the infection (see Hall *et al.*, 2009; Johnson *et al.*, 2009, for examples). We conducted a laboratory infection experiment by submitting both species to the same dose of *P. laevis* eggs to measure the susceptibility of these sympatric gammarid species to *P. laevis*. To assess the impact of predation, we compared prevalence in two contrasted amphipod collections from the field: animals directly collected from rivers (i.e. previously exposed to natural predation), and animals collected from the same rivers, but then maintained for several weeks in the laboratory (i.e. in the absence of any fish predation pressure).

Methods

Amphipod collection and prevalence in the field

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 Trochères (47°20'34"N, 5°18'21.8"E), and the Meuzin River, near Villy-le-Moutier (47°2'7.71"N, 4°59'53.87"E), were sampled between September and October 2013.

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 maintained in a container with aerated water from the river.

The first group was used to estimate the 'field/direct' prevalence. Animals from this group were kept in well aerated aquaria at 15 °C and all checked for parasite presence within two days after capture. Infected individuals were dissected to confirm parasite species. Larval parasites can be detected through the host cuticle, either at the late acanthella stage of their development (translucent light orange, shapeless larval stage) or at cystacanth stage (bright yellow-orange, spherical larval stage). Earlier acanthella stages (where parasites are small and translucent) can only be detected after dissection. Preliminary investigation showed that acanthella detection could only be certified after 40 days (without microscope and staining), so that all prevalence reported in the following experiments is prevalence for *P. laevis* of more than 40 days old (Labaude et al., submitted).

Gammarids from the second group were kept individually in the laboratory, in cups of c.a. 50 mL at 15 °C for 96 days. All gammarids where infection was detectable by eye were removed from this group so that, at the beginning of this survey, the remaining animals were classified as "uninfected". However, as previously stated, younger acanthella stages are too small to be detected through host cuticle, so some of these isolated gammarids may have already been infected in the field in the days preceding their capture. It is the prevalence of these undetected infections that was recorded during this survey. Animals dying during this period were dissected the day after their death, and all living animals were checked and dissected 96 days post isolation, a delay long enough to ensure that all parasites could be detected. This survey therefore allowed prevalence to be estimated in gammarids not exposed to predation during parasite development (hereafter called 'field/protected' prevalence). All infected *G. fossarum* were kept in ethanol for genetic analysis (see above).

A third group of gammarids was used for experimental infections (see below).

Experimental infection

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

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.

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

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

Gammarid genotyping

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

Statistical analyses

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

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-order interactions.

For experimental infections, a binomial logistic regression was performed to analyse prevalence, with site, species and sex, and their second-order interactions, as potential explanatory factors.

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

Results

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 infected animals. The species ratios in infected and uninfected groups were not significantly different ($\chi^2 = 0.2438$, $P = 0.6215$). 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.

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

Prevalence of *P. laevis* was higher in *G. roeseli* than in *G. fossarum* in direct field prevalence, at both sites, 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).

Experimental infection

The first observations of acanthellae through the host cuticle occurred 60 days post-exposure for the 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 80 ± 6 days for *G. fossarum*, and after 83 ± 3 days for *G. roeseli*.

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. fossarum* being approximately twice as infected as *G. roeseli*, was nevertheless not strong enough to be fully supported statistically (Table 2, Figure 1).

Discussion

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

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

Our results also have implications in explaining the role of parasites in the success of biological invasions. *Gammarus roeseli* is a species that colonised Western Europe during the 20th century (Chovet and Lécureuil, 1994). Parasitism may play a role in the coexistence of native and introduced (or invasive) host species. Some studies support the ‘enemy release’ hypothesis, in which invaders are no longer exposed to their original parasites, but also less susceptible to infection by native parasites, providing invasive hosts with a competitive advantage (Dunn and Dick, 1998; Kopp and Jokela, 2007). In contrast, other studies show a decrease in prevalence in native species by the dilution effect, both experimentally (Kopp and Jokela, 2007) and *in natura* (Telfer *et al.*, 2005). The invader acts in that case as a dead-end sink for the parasite. *G. roeseli* being less susceptible to both infection (this study) and to behavioural changes induced by *P. laevis* (Bauer *et al.*, 2000; Moret *et al.*, 2007), our results are in line with the enemy release 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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Table 1. Logistic regression testing for the effects of site (river), *Gammarus* species and experiment (direct field prevalence or protected field prevalence) on the field prevalence of *P. laevis*. The model initially included sex of gammarids, and other interactions. After removing these non-significant factors, the model presented now minimizes the AICc.

Source of variation	d.f.	LR χ^2	P
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

Global model: LR χ^2 = 15.4448, 5 d.f., P = 0.0086; n = 1787

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

Source of variation	d.f.	LR χ^2	P
Site	1	16.9051	< 0.0001
Species	1	3.3303	0.0680

Global model: LR χ^2 = 19.9606, 2 d.f., P < 0.0001; n = 807

Figure 1: Prevalence levels for *G. fossarum* (Gf) and *G. roeseli* (Gr) in the two populations, for all experiments (field/direct: prevalence in natura; field/protected: prevalence in gammarids kept in the laboratory, i.e. protected from predation; experiment: experimental infection). Number in bars are sample size.
106x81mm (300 x 300 DPI)