

Research Article

Cite this article: Fayard M, Cezilly F, Perrot-Minnot M-J (2019). Inter-population variation in the intensity of host manipulation by the fish acanthocephalan *Pomphorhynchus tereticollis*: are differences driven by predation risk? *Parasitology* **146**, 1296–1304. <https://doi.org/10.1017/S0031182019000520>

Received: 21 December 2018
Revised: 12 April 2019
Accepted: 12 April 2019
First published online: 28 June 2019

Key words:

Antioxidant capacity; *Gammarus fossarum*; host manipulation; phenoloxidase; *Pomphorhynchus tereticollis*; predation risk; prey–predator interaction; trophic transmission; variation

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Inter-population variation in the intensity of host manipulation by the fish acanthocephalan *Pomphorhynchus tereticollis*: are differences driven by predation risk?

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Abstract

Many trophically-transmitted parasites induce behavioural alteration in their intermediate hosts that tend to increase host vulnerability to predation. Inter-population variability in parasite-induced alterations is expected to arise from variable local opportunities for trophic transmission. Yet, this hypothesis has not been investigated so far. We addressed the issue in four populations of the fish parasite *Pomphorhynchus tereticollis* (Acanthocephala), using variable fish biomass density as a proxy for transmission opportunities. We found variation in the intensity of parasite-induced changes in phototaxis and refuge use among populations. Two of the populations with the lowest predator biomass exhibited the highest levels of behavioural manipulation and prevalence, as expected at low transmission opportunities. They also exhibited micro-habitat segregation between infected and uninfected gammarids in the field. In addition, infection had variable effects on two physiological defence systems, immunity and antioxidant capacity, and on total protein content. Overall, our study brings partial support to the prediction that host manipulation and prevalence should be higher at low predator biomass. Although stronger evidence should be sought by increasing population replicates, our study points to the importance of the ecological context, specifically transmission opportunities brought about by predation pressure, for the evolution of parasite manipulation in trophically-transmitted parasites.

Introduction

Some parasites have developed the ability to alter the phenotype of their intermediate host in ways that are supposed to increase their own fitness at the expense of that of their hosts, generally through increased parasite transmission (Thomas *et al.*, 2005). This phenomenon, known as ‘host manipulation by parasites’ (HMP), is currently regarded as one of the most compelling examples of an extended phenotype (*sensu* Dawkins, 1982). In parasites with complex life-cycles and trophic transmission, such changes are assumed to increase the vulnerability of infected intermediate hosts to predation by definitive hosts (Lafferty, 1999; Moore, 2002; Cézilly and Perrot-Minnot, 2005). For instance, the drifting behaviour of *Gammarus pulex* (Amphipoda: Crustacea) infected with *Pomphorhynchus laevis* (Acanthocephala) is increased compared to that of uninfected ones, a behavioural change supposedly contributing to the predation bias towards infected prey recorded in the field (Lagroe *et al.*, 2007).

Following a classical cost-benefit approach to understand the evolution of HMP, it has been predicted that the intensity of changes induced by parasites should vary according to transmission constraints and opportunities (Poulin, 2010; Thomas *et al.*, 2011). Such constraints may vary between host–parasite systems according to parasite transmission strategies. In trophically-transmitted parasites, for instance, the probability for a parasite to pass from an intermediate host onto an appropriate final one strongly depends upon the pattern of predation. Transmission constraints may also vary between and within populations within a given host–parasite system (Poulin, 2010; Thomas *et al.*, 2011; Hafer-Hahmann, 2019). Consequently, the pattern and magnitude of HMP are expected to vary at multiple scales, between host–parasite systems but also between and within host populations.

Environmental factors may actually play a key role in the intensity of manipulation (Thomas *et al.*, 2012). For instance, seasonal fluctuations may be responsible for variations in HMP in relation to parasite requirements (to become mature), mainly because habitat quality varies according to season (Gotthard, 2001). Spatial variability in microhabitat features, and in the diversity and abundance of definitive hosts with different foraging strategies, may also contribute to variation in HMP (Thomas *et al.*, 2012). Indeed, parasite manipulation is not expected to be strong in populations where the abundance of definitive hosts is high, as parasites are likely to get transmitted by chance (Lafferty, 1992). Conversely, high intensity of HMP is expected under low abundance of definitive hosts. However, despite the diversity of host–parasite systems, and of the abiotic and biotic factors possibly modulating HMP, only a few studies have quantified variation in manipulation by trophically-transmitted parasites (Franceschi *et al.*, 2010). Moreover, to the best of our knowledge, no study has related

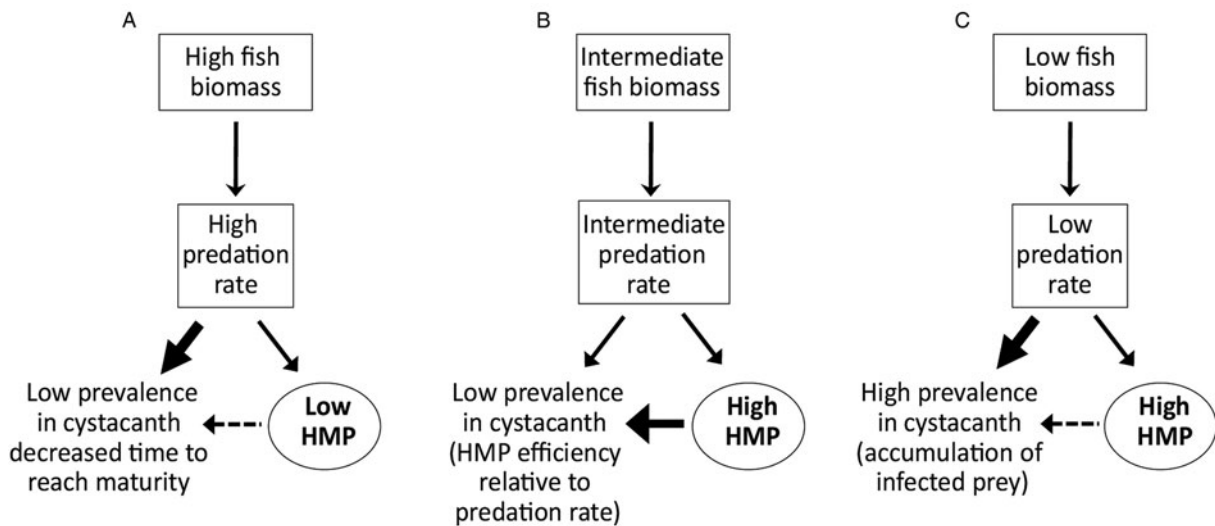


Fig. 1. Predicted consequences of local fish biomass on the level of host manipulation by parasite (HMP) through predation rate. Transmission opportunities for trophically transmitted fish parasite are assumed to increase with fish biomass when most fish predators are suitable as definitive hosts. Arrow thickness represents the impact of overall predation rate (independently of infection status) and of infected prey vulnerability to predation relative to uninfected ones (HMP); their relative impacts modulate the observed prevalence of infective parasite stages.

variability in manipulation by trophically-transmitted parasites among natural populations to environmental factors.

The aim of the present study was precisely to investigate whether spatial variation in biotic factors modulates the magnitude of intermediate host manipulation by a fish acanthocephalan parasite. Acanthocephalans are well represented in studies on HPM (Moore, 2002; Fayard *et al.*, 2019), including reported cases of inter-population variation in the intensity of manipulation. For instance, the intensity of HMP in experimentally-infected *G. pulex* has been shown to differ according to the geographical origin of *P. laevis* population (Franceschi *et al.*, 2010). Conversely, variation in parasite-induced mortality among populations of *G. fossarum* harbouring acanthocephalan parasites has been related to the differential susceptibility of host genetic lineages to HMP (Galipaud *et al.*, 2017). Here, we investigate the link between the magnitude of HMP in *G. fossarum* naturally infected with the fish acanthocephalan *P. tereticollis* and transmission opportunities to fish hosts at a local scale, using local fish biomass density as a proxy for predation pressure (Maurer *et al.*, 2014). The underlying assumption is that local fish biomass provides a proxy for transmission opportunities. This can be an approximation given that the host range of *P. tereticollis* includes both highly competent hosts and less competent ones (Perrot-Minnot *et al.*, 2019). However, we considered here the whole fish community as a proxy for predation pressure and transmission opportunities, given theoretical evidence that trophic transmission to suboptimal or non-appropriate final hosts does not impose a cost high enough to constrain the evolution of HMP (Seppälä and Jokela, 2008).

We recorded the magnitude of intermediate host manipulation by *P. tereticollis* in four populations from four rivers varying in local fish biomass. We first estimated the local prevalence of *P. tereticollis* cystacanths and the density of *G. fossarum*. We then quantified HMP on both behavioural and physiological traits. Host manipulation by acanthocephalans is known to be multidimensional (Cézilly *et al.*, 2013), notably involving traits related to taxis, protection and immune system (Fayard *et al.*, 2019). Here, we recorded phototaxis and refuge use, two behavioural traits markedly altered by *P. tereticollis* (Tain *et al.*, 2006; Perrot-Minnot *et al.*, 2007). Both traits are also altered under anxiety-like state, i.e. a state of sustained apprehension of the environment, as recently evidenced in two crustacean species,

including gammarids (Fossat *et al.*, 2014; Perrot-Minnot *et al.*, 2017). As defensive physiological traits, we estimated the level of host immunocompetence provided by the (pro) phenoloxidase system, and the level of antioxidant defences. The prophenoloxidase (proPO) cascade is involved in melanization reactions accompanying innate immune responses and is a common response to infection in arthropods (Rigaud and Moret, 2003). Suppression of the proPO system has already been reported in *Pomphorhynchus*-infected gammarids (Rigaud and Moret, 2003; Cornet *et al.*, 2009). In addition, oxidative stress has been associated with predation risk in several aquatic species (Slos and Stoks, 2008; Janssens and Stoks, 2013). We predict that the level of HMP should be lower in localities where the high fish biomass enhances transmission probability independently of manipulation (and therefore relaxes selection for HMP) (Fig. 1a). Conversely, we predict that the level of HMP should be high in localities where fish biomass is low or intermediate, in response to selection on parasite for enhancing host vulnerability to predation (Fig. 1). As a consequence, the prevalence of *P. tereticollis* cystacanths in *G. fossarum* could be either low, if strong manipulation leads to faster predation of infected individuals relative to uninfected ones (i.e. at intermediate fish biomass, Fig. 1b), or high, if low predator biomass and, hence, predation rate, leads to the accumulation of cystacanth-infected prey despite high HMP (Fig. 1c). Under the assumption that physiological changes are associated with infection, we also expect a decrease in host immunity (Cornet *et al.*, 2009; Fayard *et al.*, 2019).

Materials and methods

Biological model, field areas and maintenance

Four distinct rivers located in Bourgogne-Franche-Comté, eastern France, were prospected from mid-February to early-April 2017. We chose sampling localities based on previous records of *P. tereticollis*, and available information on fish communities, obtained through the fish-based ecological assessment achieved by the Office Français pour la Biodiversité (OFB) in the framework of EU Water Directive: Talmay on Vingeanne river, Vadans on Cuisance river, Orgeux on Norges river, and Marandeuil on Bèze river (Table 1). We retrieved information on local fish biomass density (g.100 m²; thereafter fish biomass) from the OFB database. Fish survey was based on one to

Table 1. Description of the localities with coordinates, temperature, descriptors of local fish assemblage structure, intermediate hosts (gammarid) density, and prevalence of *P. tereticollis* cystacanths in gammarids

River (locality)	Coordinates (lat; long)	Average temperature (min-max) (°C)	Fish survey (nb and time period)	Fish species richness	Total fish biomass (g. 100 m ⁻²)	Shannon Index	Main fish host biomass (%)* (g. 100 m ⁻²)	Gammarid density (ind.m ⁻²) (nb repl.)	Prevalence in gammarid hosts (%)
Bèze (Marandeuil)	47°20'52.30"N; 5°20'58.36"E	12.08 (3–18)	4x: 2008–2014	12	162.64	3.45	81.42 (50.2)	160.87 (3)	3.65
Cuisance (Vadans)	46°55'29.29"N; 5°42'10.65"E	9.50 (7–12)	4x: 2008–2014	9	401.55	2.18	138.4 (34.5)	146.85 (4)	1.67
Norges (Orgeux)	47°21'40.61"N; 5°9'30.53"E	11 (8–13)	1x: 2004	10	775.98	1.86	200.86 (25.9)	473.60 (3)	10.70
Vingeanne (Talmay)	47°20'51.51"N; 5°27'9.27"E	11.90 (2–23)	6x: 2001–2006	25	15 507.12	3.44	2645.14 (17.1)	370.73 (3)	0.90

The most competent fish species (*) are assumed to be five benthic feeders – barbel, dace, bullhead, loach and gudgeon – as reported in Perrot-Minnot *et al.* (2019). However, other species – notably the chub *Squalius cephalus*, ranging in relative biomass in these localities from 12 to 35% – also harbours gravid females of *P. tereticollis* albeit of smaller size (Perrot-Minnot *et al.*, 2019). Gammarid density is only a semi-quantitative estimate, as the standardized protocol used provides relative abundance among populations rather than absolute abundance. Sampling replicates (nb repl.) are the number of kick-sampling replicates on a fixed area of 1.5 m².

four replicates at different time periods (Table 1). At each locality, we sampled gammarids twice, at 2-week intervals. We first randomly sampled gammarids in the benthos to estimate *P. tereticollis* prevalence, gammarid individual size, and gammarid density. Two weeks later, we collected uninfected and *P. tereticollis*-infected gammarids at the same place, for behavioural and physiological assays. Gammarids were kept in the lab for no longer than 48 h (prevalence estimates) or 24 h (behavioural assays). During this time, they maintained at 16 °C under a 12:12 light regime in tanks filled with well-oxygenated water from the river, and fed with elm leaves.

Parasite prevalence and gammarid density

Both parasite prevalence and gammarid density were assessed over a 6-week period, from mid-February to late March, to allow comparison between localities, thus avoiding the potential confounding effect of seasonality. We randomly sampled gammarids from the benthos in both the bank and the bed of the river, following the kick sampling procedure (Turner and Trexler, 1997) on fixed areas. The procedure consisted of moving the river substrate (gravel, plants, rocks and sand) by kicking, before harvesting using a fine mesh net downstream. Sampling was semi-quantitative: the surface area of benthos harvesting was standardized to 5 m² per sample replicate, and three or four replicates were collected in each locality. For each sample replicate, we used a metal frame kick net (0.5 mm mesh size) to collect the benthos in three contiguous passes of 5 m in length and approximately 0.3 m in width. For each locality, the same experimenter collected at least 2000 individuals, to estimate gammarid density and *P. tereticollis* prevalence. Semi-quantitative estimates of gammarids density using this standardized protocol reflect relative rather than absolute abundance among populations (Davies, 2001). Gammarids from the bank and the bed were kept alive and brought back to the laboratory in two separate ice boxes. Assessment of prevalence, under a stereomicroscope, began the very same day the benthos was collected. Once the prevalence was assessed, individuals were pooled according to their status and the habitat they came from (bank or bed of the river), for further size measurement. Body size was measured for all infected individuals, and for a subset of 5–10% of uninfected individuals picked up at random. Body size was estimated from the height of the fourth coxal plate, following Bollache *et al.* (2002), using a Nixon SMZ 1500 stereoscopic microscope.

Pattern of HMP: behavioural assays

Behavioural assays were conducted within a short time period, from 13 of March to 19 of April 2017. We collected at least 60 infected and 60 uninfected individuals from each locality for behavioural and physiological tests. Phototaxis and refuge use were recorded by scan and time sampling, under a light intensity of 700 lux. Reaction to light was assessed by scoring the position of a single individual in a two-choice (light/dark) arena every 15 s for 5 min, following Perrot-Minnot *et al.* (2014). The two-choice (light/dark) arena consisted of a 23 cm long and 3 cm diameter closed glass tube, with half side painted in black while the other half was left translucent. A hole was made in the middle to allow the introduction of a single gammarid. Phototaxis score ranged from 0 (always in the lightened side: strongly photophilic) to 20 (always in the darkened side: strongly photophobic). Following Dianne *et al.* (2014), refuge-use was assessed using a 10.5 × 16 cm rectangular box where a refuge (half a terracotta saucer) was placed. The position of a single individual was registered every 30 s for 10 min. An individual was considered to be outside of the refuge when at least half of its body was. Refuge-use score ranged from 0 (always out of refuge) to 20 (always under the

refuge). Both phototaxis and refuge use scores were expressed as a proportion of the maximum score (20). To minimize handling stress between tests, phototaxis was scored first and then refuge use, as gammarids were more easily introduced from phototaxis tube to the refuge box than the reverse.

Just after the completion of behavioural assays, we dissected each gammarid in 100 μL PBS – 0.2% Triton X100 pH 7.7 (reagents from Sigma-Aldrich, St. Quentin Fallavier, France) in an Eppendorf cap to remove the parasite, when present. After the addition of 100 μL PBS, samples were quickly frozen in liquid nitrogen, and stored at -80°C for subsequent physiological assays. Empty tubes were individually weighed prior to dissection, and then weighed again before biochemical assays, in order to estimate the fresh weight of individual gammarids.

Pattern of HMP: physiological assays

All biochemical assays were performed on batches of 36 samples within five days, after randomizing the samples with respect to population and infection status. Upon biochemical assay, samples were thoroughly grinded using a ball mill (RETSCH MM 400 Mixer Mill) during two rounds of 2 min interspersed with 2 min on ice. The homogenate was then centrifuged at 9000 g at 4°C for 15 min. Clear supernatant was collected and kept on ice to proceed to biochemical assays right away (detailed below). All dosages were conducted using a microplate spectrophotometer (Spectramax Plus384 Absorbance Microplate Reader; Molecular Devices LLC, Sunnyvale, CA, USA).

Phenoloxydase (PO) and prophenoloxydase (PPO) dosages were performed according to Cornet *et al.* (2009), with a few modifications, using 25 μL of supernatant for each. For PO dosage, 20 μL of filtered PBS pH 7.4 0.1 M and 120 μL of L-DOPA at 10 mM were added to the supernatant. The reaction was monitored by reading the optical density (OD) at 490 nm every 15 s for 40 min, and PO enzyme activity was quantified as the slope (V_{max} value) of the curve during the linear phase of the reaction. For PPO dosage, the sample was left for 10 min in 5 μL of chymotrypsin at 5 mg mL^{-1} after addition of 20 μL of filtered PBS, prior to the addition of L-DOPA.

The antioxidant potential was measured using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay, described in Re *et al.* (1999). The antioxidant potential of a sample was estimated from its capacity to quench the free radicals of an oxidized ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] solution. The oxidized ABTS solution (ABTS+) was generated by reacting 7 mM of ABTS solution in water with 2.45 mM of potassium persulfate at obscurity and ambient temperature for at least 14 h. Just before dosage, the absorbance at 734 nm of ABTS+ solution was adjusted to 0.7 by dilution in filtered PBS. Ten microliters of supernatant or of Trolox standard solution was then mixed with 240 μL of the ABTS+ solution in microplate well, and the absorbance at 734 nm was read every minute for 20 min, at 30°C . The range of standard Trolox concentrations (from 100 to 800 μM) was prepared by diluting a 2.5 mM stock solution in PBS triton X100 pH 7.7, in order to get from 20 to 80% ABTS+ bleaching. TEAC was estimated by calculating the proportion of change in OD (debleaching) in 10 min, corrected by blanks, both in the samples and in Trolox standard, and by using the Trolox standard curve to derive the Trolox-equivalent antioxidant capacity of the sample in μM . To assess global investment in both immune system and antioxidant capacity, we corrected PPO-PO and TEAC raw values by the weight of individuals, using residuals from the linear regression between PPO-PO and TEAC raw values and the weight of individuals. All reagents for the PPO-PO and TEAC assays were purchased from Sigma-Aldrich.

Total protein concentration was estimated in 5 μL of supernatant using the DC™ Protein Assay kit (Biorad) and bovine serum albumin (BSA) as standard (from 0.15 to 0.30 mg mL^{-1}), following the manufacturer's instructions.

Analyses

All analyses were conducted using the R software (v. 3.4.3, R Development Core Team, 2018).

We first calculated effect size, in order to quantify the magnitude of behavioural alterations and physiological changes induced by parasites, and then used non-parametric Cliff's delta effect size with 95% confidence interval (CI) (*effsize* R-package; Torchiano, 2017). An effect was considered as non-significant when its 95% confidence interval crossed zero. Negligible, small, medium and large effects correspond to an absolute value lower than 0.15, 0.33, 0.47 and higher than 0.47, respectively (Romano *et al.*, 2006).

To assess the contribution of predictor variables to variation in prevalence, behavioural and physiological traits, we used the information theoretical approach based on model comparison, as an alternative to traditional null hypothesis testing (Galipaud *et al.*, 2014). The risk of multicollinearity among two of the predictor variables – fish biomass and river locality – was avoided, by using the most relevant to variation in the dependent variable (fish biomass for prevalence and behavioural traits, locality for physiological traits). We used general linear model (GLM) with binomial-logistic (logit) regression (*lme4* R-package; Bates *et al.*, 2015) to analyse variation in prevalence according to environmental variables (gammarid density and total fish biomass) and gammarid size. We used β -regressions (*beta-reg* R-package; Cribari-Neto and Zeileis, 2010) to assess the contribution of environmental variables (fish biomass and prevalence) and individual variables (physiology and infection status), to variation in phototaxis and refuge-use (Ferrari and Cribari-Neto, 2004). Behavioural scores were transformed following Smithson and Verkuilen (2006) to exclude 0 and 1. We used GLMs to assess the contribution of infection status, weight and population to variation in the levels of PO-PPO, TEAC and total proteins content. In all regressions, gammarid density, total fish biomass and PPO were log-transformed and TEAC was squared-transformed to meet normality requirements.

For all regressions, we performed model selection (*MuMIn* R-package, functions 'dredge' and 'subset'; Bartoń, 2016) based on the Akaike Information Criterion (AICc) (Akaike, 1973). There is currently no consensus about the best cut-off criterion to select models, more specifically to balance the risk of keeping spurious models with that of excluding biologically meaningful models. Here, we proceeded to model selection using as cut-off criterion an AICc weight above 0.01, or a large gap of delta AICc (at least 2) between two consecutive models, relatively to the delta AICc of lower ranking models (M. Galipaud and F.-X. Dechaume-Moncharmont, pers.comm.). Because both quantitative and qualitative predictors were used, we did not use model averaging to calculate averaged coefficients for predictors, because these coefficients would have had no meaning for qualitative predictors (F.X. Dechaume-Moncharmont, pers.comm.). However, we used the subset of 'best' models to identify predictors that more likely contribute to variation in the dependent variable, based on the above-mentioned cut-off criterion.

Results

Prevalence

Prevalence of *P. tereticollis* cystacanth was high in the Norges river (above 10%) compared to the other three localities where it

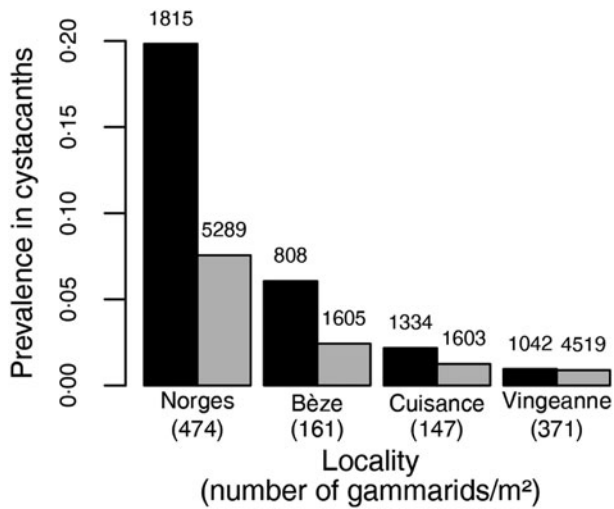


Fig. 2. Prevalence (percentage of cystacanth-infected gammarids) in the study localities from four rivers ordered from the highest to the lowest prevalence of *P. tereticollis*. Numbers above the bars correspond to the number of gammarids sampled in three (Bèze, Norges and Vingeanne) or four (Cuisance) replicates. Density is expressed as the number of gammarids per m², and is only a semi-quantitative estimate, as the standardized protocol used reflects relative abundance among populations rather than absolute abundance. Black and grey bars correspond to bank and bed prevalence, respectively.

ranged from less than 1% (Vingeanne) to almost 4% (Bèze) (Fig. 2). Significant spatial segregation of infected and uninfected gammarids was observed in river Norges and river Bèze, with a higher proportion of infected individuals found on the river bank compared to the river bed (χ^2 tests: Bèze, $\chi^2 = 17.60$, $P < 0.0001$; Cuisance, $\chi^2 = 3.15$, $P = 0.08$; Norges, $\chi^2 = 17.59$, $P < 0.0001$; Vingeanne, $\chi^2 = 0.002$, $P = 0.96$) (Fig. 2).

The initial global model to analyse variation in prevalence included total fish biomass, gammarid density, their interaction, and gammarid body size, as predictor variables. Variation in prevalence was best explained by the model including total fish biomass, gammarid density and gammarid body size (logistic regression: log-likelihood = -1152.91; D.F. = 4; AICc = 2313.80). Considering the subset of best models, local fish biomass and gammarid density appeared to play a key role in driving variation in prevalence in *P. tereticollis* cystacanth (see Appendix, Table S1).

Pattern of HMP: host behaviour

The magnitude of behavioural changes in infected individuals differed among the four localities (Fig. 3). Phototaxis and refuge use were strongly to moderately altered by infection in the Norges and Bèze rivers, while only phototaxis was altered in the Cuisance river. No parasite-induced behavioural change was evidenced in the Vingeanne river.

The global model prior to model selection included the status of individuals (infected or uninfected), total fish biomass, their interaction, physiological parameters (total PPO-PO activity and TEAC, corrected by the weight of individuals) and prevalence, as predictor variables. Variation in phototaxis was best explained by the model including infection status, total fish biomass, and their interaction (β -regression: log-likelihood = 296.12, D.F. = 6, pseudo $R^2 = 0.22$, AICc = -580) (Fig. 4; Table S2a). Parasite-induced change in phototaxis was significant in the three localities with the lowest fish biomass, but not in the locality with the highest fish biomass (Figs 3 and 4). For refuge-use, the best model included infection status, total fish biomass and their interaction (β -regression: log-likelihood = 185.27, D.F. = 5, pseudo $R^2 = 0.06$, AICc = -360.40) (Fig. 4; Table S2b), with parasite-induced change in refuge use being significant only in two of the three

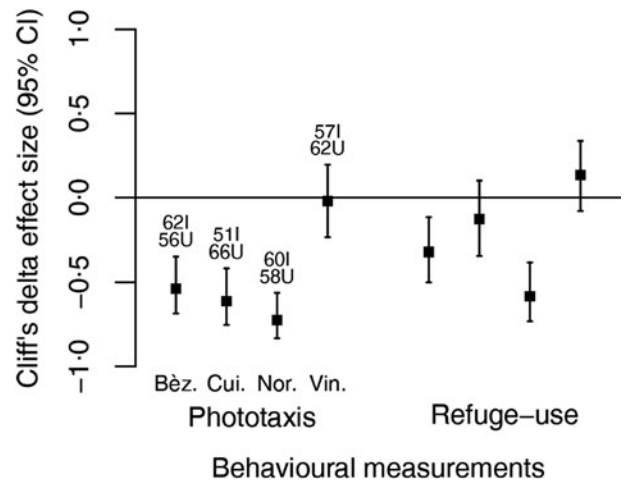


Fig. 3. Cliff's delta effect sizes (with 95% CI) for changes in behaviour induced by infection with *P. tereticollis* in *Gammarus fossarum* (phototaxis and refuge-use) for Bèze (Bèz.), Cuisance (Cui.), Norges (Nor.) and Vingeanne (Vin.) localities, respectively. The effect of infection was considered as non-significant when its 95% confidence interval crossed zero. Negligible, small, medium and large effects correspond to $|d|$ lower than 0.15, 0.33, 0.47 and higher than 0.47, respectively (Romano et al., 2006). Numbers above the error-bars correspond to the numbers of gammarids used (I, infected; U, uninfected).

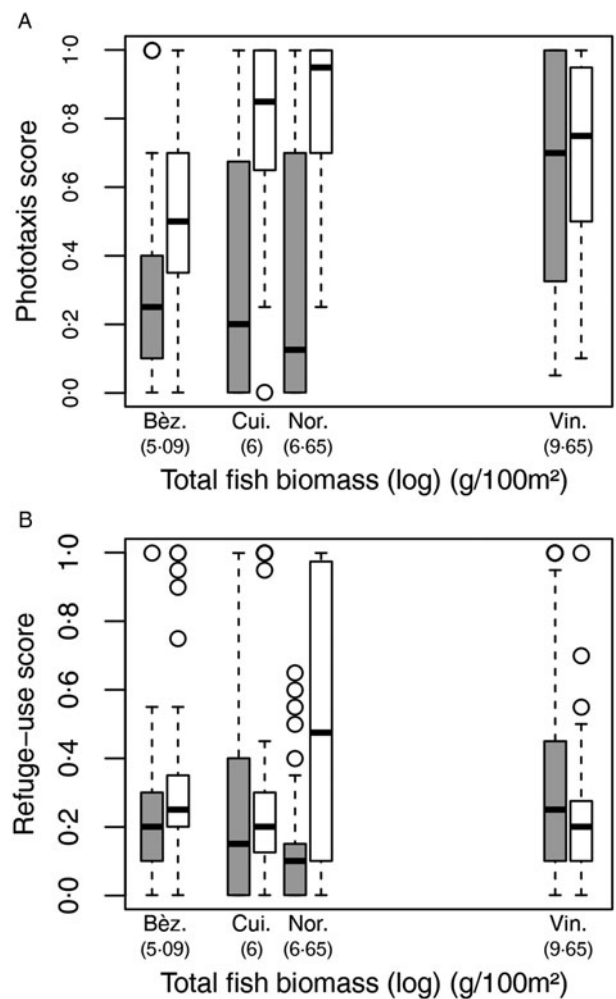


Fig. 4. Behavioural scores: (a) phototaxis and (b) refuge-use plotted against total fish biomass in interaction with the status of individuals (infected or uninfected). Phototaxis score ranges from photophilic (0) to photophobic (1), and refuge use score as always outside (0) to always under refuge (1). Grey and white boxes correspond to infected (Bèze, $N = 59$; Cuisance, $N = 51$; Norges, $N = 46$ and Vingeanne, $N = 55$) and uninfected (Bèze, $N = 52$; Cuisance, $N = 63$; Norges, $N = 44$ and Vingeanne, $N = 60$) individuals, respectively. Horizontal lines and vertical dashed lines correspond to median and interquartiles, respectively.

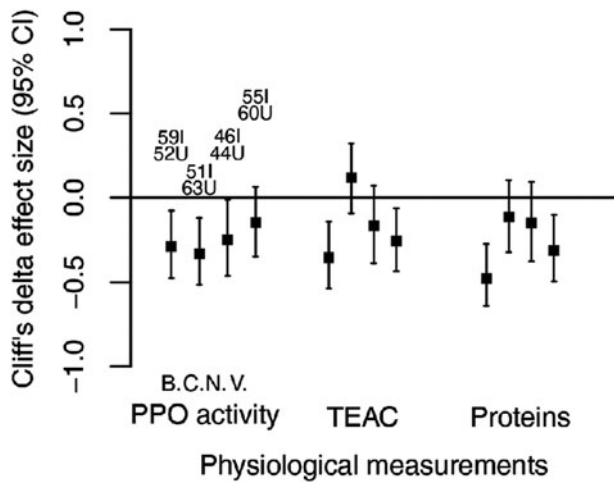


Fig. 5. Cliff's delta effect sizes (with 95% CI) for changes in physiological state (total (pro)phenoloxylase activity (PPO activity), antioxidant capacity (TEAC) and total protein content (Proteins), all corrected by the weight of individuals) for Bèze (B.), Cuisance (C.), Norges (N.) and Vingeanne (V.) localities, respectively. Negligible, small, medium and large effects correspond to $|d|$ lower than 0.15, 0.33, 0.47 and higher than 0.47, respectively (Romano *et al.*, 2006). Numbers above the error-bars correspond to the numbers of gammarids used (I = infected, U = uninfected).

localities with lower fish biomass (Fig. 4). However, effect sizes tended to be low for refuge use compared to phototaxis (Fig. 3), partly due to the overall weak use of refuge both in uninfected and infected gammarids (Fig. 4).

Pattern of HMP: host physiological state

Effect sizes of parasite-induced changes were medium to large for all three physiological parameters in river Bèze only. Depressed levels of PO-PPO activity, antioxidant capacity and protein concentration (all corrected for weight) were evidenced in infected individuals compared to uninfected ones from the Bèze river (Fig. 5). The effect of infection in Norges and Cuisance rivers was restricted to a moderate decrease in PO-PPO activity, while in Vingeanne river, infection moderately decreased antioxidant capacity and protein concentration (Fig. 5). Interestingly, these effects of infection status on PO-PPO activity and antioxidant capacity were no longer significant after correction with total protein content instead of gammarid weight, except for PO-PPO in the Cuisance river (Figs S2 and S3).

The initial global model for studying variation in physiological parameters included individual status (infected or uninfected), individual weight, their interaction and locality. The first-ranking model to explain variation in protein content included all four predictors (GLM: protein content: log-likelihood = -1044.40, D.F. = 8, $R^2 = 0.22$, AICc = 2105.10). The first-ranking model to explain variation in PPO-PO and TEAC included individual status, individual weight, and locality (log-likelihood = -369.79, D.F. = 7, $R^2 = 0.18$, AICc = 753.80; log-likelihood = -5608.67, D.F. = 7, $R^2 = 0.14$, AICc = 11 231.60, respectively) (Fig. 6; Table S3). However, the interaction between individual status and weight should not be discarded, as it appears in the subset of best models (Fig. 6; Table S3). Protein content, antioxidant potential and PPO-PO increased with gammarid weight, and were lower in infected gammarids. The difference between infected and uninfected individuals in the three physiological parameters increased with gammarid weight (Fig. 6).

Discussion

The aim of our study was to address the environmental causes of variation in prevalence and host manipulation by a trophically-

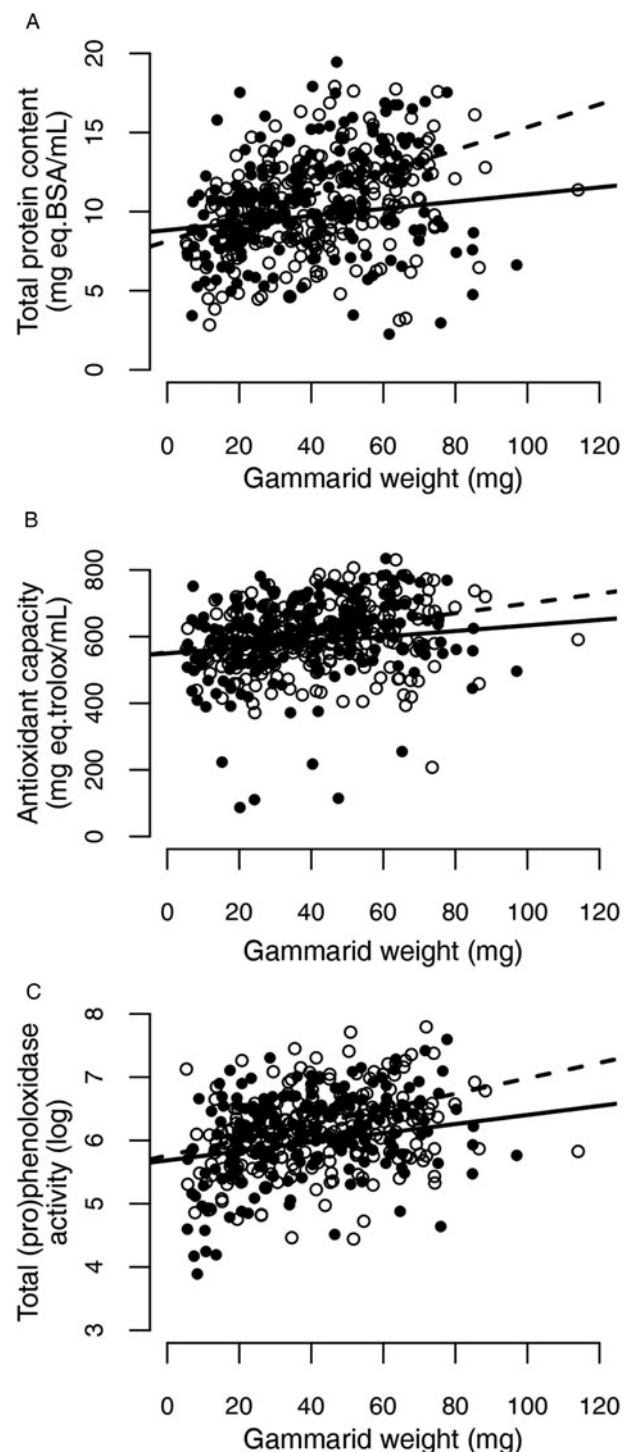


Fig. 6. Physiological parameters: (a) total protein content, (b) antioxidant capacity and (c) total (pro)phenoloxylase activity, all plotted against the weight of individuals, in interaction with the status of individuals (infected or uninfected). Solid circles and lines correspond to infected individuals and empty circles and dashed lines to uninfected ones.

transmitted fish parasite, the acanthocephalan *P. tereticollis*. More specifically, we tested the hypothesis that high predation pressure would be associated with low cystacanth prevalence and low level of manipulation in an intermediate host, by comparing four localities with contrasted fish biomass.

We first highlighted variation in both the prevalence of *P. tereticollis* cystacanth and behavioural alterations in infected gammarids among the four localities. Overall, infected individuals displayed lower photophobia and refuge use than uninfected ones,

as previously reported (see Cézilly *et al.*, 2013; Fayard *et al.*, 2019), with the exception of one locality. Interestingly, the two localities with the highest level of HMP (Norges and Bèze) also exhibited a significant spatial segregation between infected and uninfected gammarids, as previously observed in another fish acanthocephalan (MacNeil *et al.*, 2003). Our data on these two populations further suggest that lab estimates of HMP could match micro-habitat segregation of infected hosts in the field. However, since the density estimates provided with kick-sampling on fixed area are only semi-quantitative (Davies, 2001), it should be emphasized that our conclusion relies on relative abundance for comparison between habitats. Further investigation should consider quantitative methods to specifically test the link between spatial segregation between infected and uninfected gammarids, and the level of HMP.

Strong HMP was associated with high cystacanth prevalence and low fish biomass (Norges river), and conversely, low and non-significant HMP was associated with low prevalence and high fish biomass (Vingeanne river). This result is in agreement with the hypothesis that the magnitude of HMP should partly match predation pressure, and should be associated with variable cystacanth prevalence. Two processes may explain the observed pattern. First, low HMP in population with high predation pressure could be the expression of an optimal parasitic strategy balancing the costs and benefits of host manipulation, independently of cystacanth age. This optimal strategy could be shaped by plastic adjustment and/or adaptive response at a local scale. Alternatively, low HMP in naturally-infected gammarids could be due to cystacanths being too young to manipulate. Indeed, in the course of behavioural switching from 'protection' by acanthella to 'facilitation' by cystacanth (Dianne *et al.*, 2011), young cystacanth may induce a low level of manipulation as evidenced in *P. laevis* infecting *G. pulex* (Franceschi *et al.*, 2008). Under high predation pressure, cystacanth-infected gammarids could be rapidly removed from the population, such that those present would be, on average, younger than in populations with low predation pressure, where cystacanth-infected gammarids tend to accumulate and age. Under this hypothesis, gammarids from the Vingeanne river would have been infected with younger cystacanths, and therefore displayed a lower intensity of or even no parasite-induced manipulation. Because behavioural assays were performed right after sampling, it is still possible that differences in mean parasite age among populations were responsible for variable levels of HMP. Alternatively, under the first hypothesis, selection for high or low HMP would depend on whether manipulation is costly and whether such costs are compensated by the benefits of increased probability of transmission to final hosts. Since transmission probability is depending on predation pressure, predator density or biomass is likely to be a selective force shaping the pattern of HMP. Indeed, a recent model (de Vries and van Langevelde, 2018) showed that the selective advantage of two manipulative strategies – predation enhancement and predation suppression – depends on host density. Predation enhancement by mature parasites (i.e. infectious to final hosts) would be beneficial at low final host density, whereas at high host density, selection would rather favour predation suppression by immature parasites (i.e. not-yet infectious to final hosts; de Vries and van Langevelde, 2018). Apart from this ecological context, energetic costs associated with HMP could limit its evolution, although evidence for such costs is still weak (Hafer-Hahmann, 2019). We cannot disentangle the two alternative hypotheses here, because the age of field-collected infected gammarids was unknown. To address this issue, a conditioning period during which parasites can grow older to reach their 'manipulation endpoint' could be applied, before assessing the level of HMP. In addition, the evidence for a link between predation pressure, HMP and prevalence should be considered with

caution since only two populations matched the predicted pattern. The two other localities with low fish biomass presented intermediate to high levels of HMP (refuge use and phototaxis respectively) as expected, but prevalence was low (Cuisance and Bèze rivers). Therefore, stronger evidence should be sought by increasing population replicates, in order to cover a larger range of fish biomass.

One relevant question to the evolution of HMP in response to predator biomass is the mechanisms by which such fine-tuned response of parasites to variable transmission opportunities can evolve. The more parsimonious assumption would be that the increased predation risk associated with high predator biomass induces chronic stress in uninfected individuals, and thereby activate an anxiety-like state. Indeed, chronic stress might trigger a sustained apprehension of the environment and elevated vigilance, akin a 'mood-shift' towards anxiety-like state. Interestingly, phototaxis and refuge use have been recently associated with general anxiety-like state (Fossat *et al.*, 2014; Perrot-Minnot *et al.*, 2017). The parasite could thus reverse the behavioural response to predation risk by interfering with this modulatory pathway. One possible pathway involved both in anxiety-like state and parasite manipulation is the serotonergic neuromodulatory system (Tain *et al.*, 2006; Fossat *et al.*, 2014; Perrot-Minnot *et al.*, 2014). A topical injection of serotonin is indeed mimicking parasite-induced behavioural alteration of phototaxis but not that of refuge use (Perrot-Minnot *et al.*, 2014). Both lab experiments reproducing the chronic effect of parasite and predation stress, and field studies correlating the expression of anxiety-like behaviour to predator biomass across several localities, should therefore be undertaken to test these hypotheses.

We found that three populations exhibiting moderate to high HMP were also immunosuppressed. However, we did not find any effect of immunocompetence on HMP, suggesting that the intensity of HMP was independent of parasite-induced immunosuppression, in agreement with Cornet *et al.* (2009). Furthermore, two populations had lower antioxidative defences and protein concentration. Whereas a depressed protein content in the mid-gut glands of *G. pulex* infected with *P. laevis* has been previously reported (Bentley and Hurd, 1995), no evidence for an effect of infection with acanthocephalans on host antioxidant capacity has been published so far. Previous studies have focused on energetic metabolism, including lipid and carbohydrate reserves (see for instance Plaistow *et al.*, 2001; Caddigan *et al.*, 2017). Interestingly, total (pro)phenoloxidase activity and antioxidant capacity were no longer significantly affected by infection when corrected by individual total protein content instead of body weight. This suggests that immunosuppression and decreased antioxidant defences in infected gammarids are the consequences of a more general depression in protein content. Such depression of protein content and physiological defences likely contributes to the ecological and evolutionary cost of infection for the parasite (Cornet *et al.*, 2009). Alternatively, depressed protein content and physiological defences may be the consequences of resource reallocation to the growing parasite. The fact that depressed physiological defences arise from a general depression in protein content instead of a specific effect of infection should therefore be considered when interpreting the evolutionary significance of such changes. For instance, immunosuppression should not be considered as an adaptive parasitic strategy to increase survival by controlling host immune response, but rather as a side-effect of resource acquisition.

In conclusion, our data indicate that among population variation in the intensity of HMP in both behavioural traits could be related to predator biomass. We postulate that the activation of the modulatory pathway of stress and anxiety-like behaviours in prey by predation risk might be exploited by parasites. Therefore, to understand variation in HMP, variation in the

intensity of antipredatory and anxiety-like behaviours of uninfected individuals with respect to predation risk should be considered first. Overall our results suggest that considering the ecological context, particularly prey–predator interactions, might be essential for understanding the transmission strategies of trophically-transmitted parasites.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182019000520>.

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Acknowledgements. We would like to thank an anonymous referee for suggestions on a previous version of the manuscript, and Aude Balourdet and Jean-Emmanuel Rollin for help with field sampling and lab experiments.

Financial support. This study was supported by the Centre National de la Recherche Scientifique (CNRS), and the Université de Bourgogne-Franche Comté. MF was funded by a Ph.D. grant from the Ministère de l'Éducation Nationale et de la Recherche, France.

Conflict of interest. None.

Ethical standards. Not applicable.

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