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Patterns of infection in a native and an invasive crayfish across the UK

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Abstract

Invasive crayfish and the introduction of non-native diseases pose a significant risk for the conservation of endangered, white-clawed crayfish (*Austropotamobius pallipes*). Continued pollution of waterways is also of concern for native species and may be linked with crayfish disease dynamics. We explore whether crayfish species or environmental quality are predictors of infection presence and prevalence in native *A. pallipes* and invasive signal crayfish (*Pacifastacus leniusculus*). We use a seven-year dataset of histology records, and a field survey comparing the presence and prevalence of infectious agents in three isolated *A. pallipes* populations; three isolated *P. leniusculus* populations and three populations where the two species had overlapped in the past. We note a lower diversity of parasites (Simpson's Index) in *P. leniusculus* ('Pacifastacus leniusculus Bacilliform Virus' - PlBV) (n= 1 parasite) relative to native *A. pallipes* (n= 4 parasites), which host *Thelohania contejeani*, 'Austropotamobius pallipes bacilliform virus' (ApBV), *Psorospermium haeckeli* and *Branchiobdella astaci*, at the sites studied. The infectious group present in both species was an intranuclear bacilliform virus of the hepatopancreas. The prevalence of *A. astaci* in *A. pallipes* populations was higher in more polluted water bodies, which may reflect an effect of water quality, or may be due to increased chance of transmission from nearby *P. leniusculus*, a species commonly found in poor quality habitats.

1. Introduction

Conservationists have long sought to understand the traits that make invasive non-native species (INNS) successful in their introduced ranges (Van Kleunen et al., 2010; Parker et al., 2013). Understanding these attributes can help in assessing comparative risk and formulating preventative management measures. Infectious agents can play an important role in biological invasions, influencing the success of an introduced species and the resilience of native species in the introduced range; ultimately determining the outcome of the invasion (Hatcher et al., 2012; Dunn et al., 2012; Dunn and Hatcher, 2015).

We know little about the diversity and potential for parasite transmission in invasive species, particularly for the Crustacea (Bojko et al. 2020a). Those non-native species that vector infectious agents may result in parasite 'spill over' into native populations, resulting in an emerging disease (Kelly et al., 2009; Tompkins et al., 2011; Okamura and Feist, 2011; Hatcher et al., 2012). Acquisition of non-native diseases by native species depends on the number of infected individuals introduced; host-specificity of the infectious agent; the immunity of native species; and suitable environmental conditions (water chemistry, presence of secondary hosts) for the infectious agent to survive (Okamura and Feist, 2011; Hatcher et al., 2012). If the non-native species is a competent host, it may act as a reservoir for the agent, increasing its prevalence and resulting in 'spill back' into susceptible native populations (Kelly et al., 2009; Poulin et al., 2011; Strauss et al., 2012). In contrast, if the INNS is a less competent host than the native species it may act as a sink for the agent, reducing infection prevalence in native species through a dilution effect (Poulin et al., 2011).

Stochastic and selective pressures during the invasion process may result in non-native species losing their infectious agents, a concept termed 'enemy release' (Keane and Crawley, 2002). Factors surrounding enemy release depend on the size of the invading propagule; selective pressures in the introduced habitat favouring fitter (i.e. uninfected/resistant) hosts; reduced transmission opportunities in the introduced range due to low (founder) population density, or absence of an intermediate host; or, the founder population could be an uninfected life history stage (Torchin et al., 2002; Dunn and Hatcher, 2015).

At the species scale of disease introduction, biogeographical studies have reported that INNS may escape up to 75% of their native infectious agents (Torchin and Mitchell, 2004). Such studies may over-represent the effects of enemy release if they do not compare the invasive population with the specific source population from which it was founded (Colautti et al., 2004; Colautti et al., 2005) as there may be genetic heterogeneity in different native populations, which could influence their resistance to infectious agents as well as spatial heterogeneity in prevalence (MacLeod et al., 2010). Community studies comparing the diversity of infectious

agents in native and invasive conspecifics in the introduced range have often identified similar levels of infectious agents in both species (Colautti et al., 2004).

Under natural conditions, host-parasite interactions are likely to be affected by external factors in the host population's abiotic environment (Sures, 2008; Johnson and Paull, 2011). Freshwater habitats are affected by multiple environmental stressors including pollution, habitat degradation, agricultural run-off and flow modification, as well as INNS (Dudgeon et al., 2006). Environmental factors can affect the competitive ability of native and non-native hosts and their resistance to disease, as well as altering the survival and virulence of the parasite (Keane and Crawley, 2002; Prenter et al., 2004; Sures, 2008; Poulin et al., 2011). Such studies highlight the importance of considering enemy release in the context of other environmental factors (Torchin et al., 2001; Roy et al., 2011).

UK freshwater environments are increasingly imperilled by INNS (Dudgeon et al., 2006; Jackson and Grey, 2012; Gallardo and Aldridge, 2014). One of the impactful freshwater INNS is the American signal crayfish *Pacifastacus leniusculus*, which was first introduced into the UK during the 1970s for the purposes of aquaculture but subsequently escaped and dispersed forming widespread wild populations (Holdich et al., 2014). It commonly out competes native *Austropotamobius pallipes* for food and habitat (Vorburger and Ribi, 1999; Dunn et al., 2008) and is an asymptomatic carrier of the oomycete *Aphanomyces astaci* (causative agent of the disease crayfish plague) (Alderman, 1983). Crayfish plague is associated with sudden and acute mortality events in crayfish (Alderman, 1983; Longshaw, 2011) including subsequent local extinction in *A. pallipes*, and is a major contributor to its listing as an endangered species on the IUCN Red List (Füreder et al., 2010).

Although mortalities are an obvious impact of this invasion, the role that sub-lethal infectious agents play in invasion dynamics have received less attention in crayfish (Longshaw, 2011). Infectious agents in *A. pallipes* show high diversity and prevalence of infection in some cases, while similar data of non-native crayfish imports show that 66.4% of non-native crayfish were free from infection (Longshaw et al., 2012a; Longshaw et al., 2012b). Although these studies indicate that enemy release may play a role in invasion success, others suggest that the fitness of native crayfish, and prevalence of some infections in native crayfish, may be affected by the quality of the local environment (Imhoff, 2010).

We explore the pathogen profile of invasive populations of *P. leniusculus* in the UK and corelate this information with environmental measurements. We also explore the pathogen profile of UK native *A. pallipes*. In both cases we explore specimens from populations that have and have not interacted and compare diversity indices. We discuss these pathological,

geographical, and environmental data in relation to the ongoing crayfish invasion across the UK.

2. Methods

2.1 Curation of a seven-year dataset on crayfish disease

All histological surveys of *A. pallipes* (25 populations; 210 individuals) and *P. leniusculus* (44 populations; 818 individuals) conducted by the Centre for Environment, Fisheries and Aquaculture Science (Cefas) between 2007 and 2014 were combined into a single seven-year dataset (hereby "Sample A") (Table 1). Only crayfish populations subjected to full histological examinations were included in the dataset to investigate the influence of co-infections. Other *A. pallipes* populations (25 populations; 123 individuals, Sample B) that exist within a Cefas dataset exploring mortality events [Table 2 were analysed separately to investigate putative predictors of *A. astaci* prevalence, using molecular screening (PCR) instead of histology].

2.2 Specimen collection for field studies

To explore any relationships between water quality (chemical and ecological status of the catchment) and the presence/prevalence of parasites, crayfish were collected from three *A. pallipes* populations and six *P. leniusculus* populations under license from Natural England (Licence number: 20122156) and the Environment Agency (FR2 licenses), between June and October 2012 (Sample C). Efforts to locate overlapping mixed-species populations of the two crayfish species were unsuccessful, in part due to a lack of available documentation on their distribution. As a proxy for mixed populations, three single-species *P. leniusculus* populations (Bookill Gill Beck, Cawthorne Dike, and the River Ure; Table 2) where the co-occurrence of *A. pallipes* had been recorded within the previous two years were used (Dunn et al., 2008; Haddaway et al., 2012). The other six populations (three of *A. pallipes* and three of *P. leniusculus*) were selected because they had been isolated from introductions of other crayfish species in the past, according to the Rivers Trusts, local records centres, National Biodiversity Network Gateway, and ecological consultants.

2.3 Histological screening

Samples were prepared and histologically analysed in accordance with a standard crustacean disease screening protocol (Longshaw 2011; Longshaw et al. 2012a; Longshaw et al. 2012b). All crayfish were examined for external abnormalities (e.g. missing claws, damage to carapace), sexed and measured (carapace length) before being exposed to an overdose of chloroform vapours to humanely euthanize them prior to sampling for histology.

Following the standard protocol (described in Longshaw et al. 2012a), Juvenile crayfish (≤ 10mm carapace length) were euthanized and fixed whole by direct injection of Davidson's freshwater fixative. Larger animals (>10mm carapace length) were dissected and samples of the carapace, gill, gonad, gut, hepatopancreas and tail muscle were collected immediately and preserved in Davidson's freshwater fixative for 24h before being transferred to 70% industrial methylated spirits (IMS). If required, the tissue samples were decalcified in a rapid decalcification solution prior to paraffin infiltration. The tissues were processed to wax block using an automatic vacuum infiltration tissue processor (Peloris, Vision Biosystems). Sections were cut at 3 to 5 µm and routinely stained with haematoxylin and eosin (H&E) in an automatic tissue stainer (Tribune Autostainer, Surgipath). The tissues were examined on a light microscope using brightfield illumination. A record was made of any pathologies or infectious agents in organs and tissues including an indication of the infection severity. Images were captured using a LUCIA™ (Nikon, UK) screen measurement system.

2.4 PCR screening for *Aphanomyces astaci*

Biopsies were collected from the cuticle and sub-cutis of all adult crayfish and individually placed in 100% ethanol and stored at -20˚C (sample numbers are located in Table 1 and Table 2). The full methods used to extract DNA from tissues have been previously described in (Oidtmann, 2004). DNA was extracted using a DNeasy tissue kit (Qiagen) following the manufacturer's instructions. Animals were screened for *A. astaci* using the PCR protocol described in (Oidtmann et al., 2006).

2.5 Environmental parameters

The Water Framework Directive requires all European Union (EU) Member States to assess and classify the status of their river catchments according to a unified set of ecological and chemical standards (Water Framework Directive, 2012). The ecological classification ('high' to 'bad' on a five-point scale), scores each water body against a set of biological quality (abundance of fish and rooted plants), general chemical and physio-chemical (temperature and nutrient levels) water quality with respect to specific pollutants (synthetic and non-synthetic) and hydro-morphological (water flow and physical habitat) criteria (Water Framework Directive, 2012). The chemical classification ('good' or 'fail') examines the presence of polluting substances that could adversely affect the ecology of the catchment by checking whether the water meets Environmental Quality Standards (EQSs) for substances listed in Annex IX (Dangerous Substances Directive and associated daughter Directives) and Annex X (WFD Priority List Substances) (Water Framework Directive, 2012). According to the Environment Agency, a status of good means that concentrations of priority

substances and priority hazardous substances do not exceed the environmental quality standards in the EQS Directive (cite EA glossary). To ensure that the environmental parameters were policy-relevant as well as biologically robust, the chemical and ecological status of the sub-catchment of each crayfish population in the Cefas dataset was checked using the Environment Agency's Catchment Data Explorer website (environment.data.gov.uk/catchment-planning/).

2.5 Statistical analysis

Generalised linear mixed-effects models (GLMMs) were used to determine which variables were predictors of the presence/absence, prevalence, and diversity (Simpson's index) of parasites in *A. pallipes* and *P. leniusculus* populations in the UK based on the Cefas dataset. Explanatory variables included crayfish species; presence of other infectious agents in the population; prevalence of other infectious agents in the population; chemical status of the sub-catchment; and ecological status of the sub-catchment. For single-species *P. leniusculus* sites the former presence of *A. pallipes* crayfish in the catchment was added to the model. Due to the wide temporal and geographical range of the data, site and year were included as random factors in each model. All models were fitted with a binomial error distribution and a logit link function since the response variables consisted of both binary and proportion data (Crawley, 2007).

The models met the assumptions of homogeneity of variance based on visual assessment of the plots of residuals vs. fitted values. The normality of residuals was checked using quantile-quantile plots and histograms. Log-likelihood tests were used to compare simplified models to null models (random effects only) and conditional R^2 values were calculated to describe the proportion of variance explained by both the fixed and random factors (Johnson, 2014).

3. Results

3.1 Predictors of infection (Cefas dataset)

Austropotamobius pallipes were found to have a higher number of parasites than *P. leniusculus* (4:1) based on histopathology results. Histology results revealed that *A. pallipes* populations were infected with four agents: *Thelohania contejeani*, 'Austropotamobius pallipes bacilliform virus' (ApBV), *Psorospermium haeckeli* and *Branchiobdella astaci* (mean Simpson's index = 0.17), while *P. leniusculus* were infected by 'Pacifastacus leniusculus Bacilliform Virus' only (PlBV) (Fig. 1). The crayfish species was the only significant predictor of infectious agent diversity in the minimum adequate model (Simpson's index) (Table 2). Crayfish plague data were collected for *A. pallipes* using PCR and is explored in section 3.2. Individual signal crayfish were screened for crayfish plague, using PCR, by Cefas and

reported for 5 sites over 2011 and 2014 (Table 1). This constituted 34 individual signal crayfish in total, but the overall sample size was too small for reliable statistical comparison between sites.

None of the variables (ecological status, chemical status, or crayfish species) were significant predictors of the presence/absence of bacilliform viruses in crayfish populations; however, crayfish species was a significant predictor of the prevalence of infection (Table 2), with a higher prevalence observed in *A. pallipes* populations (13/25 populations infected, mean prevalence = 0.39) than *P. leniusculus* populations (11/44 populations infected, mean prevalence = 0.13).

3.2 Predictors of infection in *A. pallipes*

The presence and prevalence of *T. contejeani, P. haeckeli* and *B. astaci* were explored in the *A. pallipes* dataset (Sample B) *Thelohania contejeani* was present in 11/25 *A. pallipes* populations in the long-term dataset and the mean prevalence among infected populations was 27 % (range 9 – 37 %). *Branchiobdella astaci* and *P. haeckeli* were identified histologically (Rosewarne et al., 2012) and present in 2/25 populations each (not the same two populations) and infected populations had a mean prevalence of 19 % and 60 %, respectively. Neither the ecological nor chemical status of the catchment were significant predictors of the presence or prevalence of the three parasites (model P values >0.05). The presence of *T. contejeani* in the population was a significant predictor of ApBV prevalence (Table 2). The mean prevalence of ApBV was 58 % in *A. pallipes* populations with *T. contejeani* and 23 % in populations without *T. contejeani* infection.

Populations of *A. pallipes* that were tested positive for *A. astaci* (Sample B; Table 1) included 13 of the 25 populations in the UK. Infection prevalence in infected populations ranged from 10% to 100%. A significant relationship was found between the chemical status of the water body and the presence of crayfish plague: waterbodies with a “fail” for chemical status appeared more likely to test positive for *A. astaci*; however, none of the variables were significant predictors of the prevalence of *A. astaci*.

3.3 Field sampling to investigate enemy release

Nine sites with three different population compositions were assessed to compare enemy release: i) isolated *A. pallipes* populations; ii) isolated *P. leniusculus* populations; iii) *P. leniusculus* with recent *A. pallipes* overlap. *Austropotamobius pallipes* populations at these sites were infected with three parasites (*T. contejeani*, ApBV, *Branchiobdella astaci*), while signal crayfish populations were only infected with PlBV (Fig. 2). Crayfish population composition (single species vs. proxy mixed) was a significant predictor of bacilliform virus

prevalence (Estimate =2.62±0.89, t = 2.96, p<0.05; Model R^2= 0.21), with higher prevalence associated with single-species *A. pallipes* populations (72 %) and *P. leniusculus* populations that had recently overlapped with *A. pallipes* (20%) and low prevalence among *P. leniusculus* only populations (<1 %) (Table 2; Fig. 2).

4. Discussion

Our study identified that non-native *P. leniusculus* are hosts to a significantly lower diversity of infectious agents when compared to native *A. pallipes*, in the UK (Table 2)**.** Our results did not provide evidence that the presence and prevalence of sub-lethal crayfish infections are affected by the chemical or environmental status of their habitat; however, this data did suggest that water quality may be linked with a tentatively increased prevalence of crayfish plague. We sub-divide our discussion by parasite group to explore pathology, impacts and conservation but provide a single section to discuss environmental quality and the presence of disease.

4.1 Intranuclear bacilliform virus

Hepatopancreatic bacilliform viruses have been identified from a range of crustacean species including multiple observations among crayfish (Bateman and Stentiford, 2017; Bojko et al. 2017; Bojko and Ovcharenko, 2019). Recent work has identified that these viruses are likely novel members of the *Nudiviridae*, based on genomic and ultrastructural data (Yang et al. 2014; Holt et al. 2019; Allain et al. 2020). Their impact on crustacean hosts is sparse, including data pertaining to biological invasions. Some evidence has been gathered using amphipod models, where infection level is associated with increased activity (Bojko et al. 2018); however, this has not been explored in crayfish invasion, nor has the distribution of the two viruses in UK populations. To date, five 'bacilliform viruses' have been identified in crayfish, but genomic data is necessary to determine their taxonomy (see Table 1 of Bojko et al. 2017).

Bacilliform viruses in both *P. leniusculus* and *A. pallipes* populations in this study were most prevalent in single-species *A. pallipes* populations. A high prevalence of PlBV was also detected in *P. leniusculus* populations that had previously overlapped with *A. pallipes.* Low prevalence, or absence, was detected in *P. leniusculus* populations where no native crayfish were located. Greater genomic data is necessary to determine if these viruses are part of the same species complex or if they are different, species-specific, viruses of each crayfish species.

In previous studies, no gross pathological changes were observed in crayfish infected with bacilliform viruses, suggesting that this virus has a minimal impact on host health, and it may not be a major driver of crayfish mortality, nor a determinant of competitive success (Stentiford

et al., 2004; Longshaw, 2011; Longshaw et al., 2012a; Longshaw et al., 2012c). Interestingly, a higher prevalence of *T. contejeani* in *A. pallipes* populations was a predictor for bacilliform virus presence, suggesting the presence of shared stressors that could increase susceptibility to these two parasites. Alternatively, the virus and microsporidian may share a relationship that is yet to be discovered. The presence of microsporidian-virus coinfection was described by Bojko et al. (2019) at high prevalence in amphipods, and there may a relationship to untangle between the two during an invasion dynamic.

4.2 *Thelohania contejeani* and other Microsporidia

Crayfish have been associated with several microsporidian lineages. These include members from clades III (*T. contejeani*), IV (AM261754) and V (*Camabaraspora floridanus* and *Ovipleistophora diplostumuri*) (Bojko et al. 2020b; Bojko et al. 2020c). In the UK, aside from the detection of a *Bacillidium*-like microsporidian via PCR, this is dominated by the detection of *T. contejeani* (Dunn et al. 2008). This microsporidian is thought to be a native parasite of *A. pallipes* and results in extensive muscular degeneration, resulting in limited movement, feeding (30% reduction) and predator behaviour (Alderman and Polglase, 1988; Oidtmann et al., 1996; Longshaw et al. 2011). Sub-lethal impacts that facilitate competitive exclusion and are thought to play an important role in competitive interactions between *A. pallipes* and *P. leniusculus* (Haddaway et al., 2012).

Thelohania contejeani was common among *A. pallipes* populations (present in 46% of populations from the Cefas dataset) with a maximum prevalence of 37%, consistent with other studies (Cossins and Bowler, 1974; Dieguez-Uribeondo et al. 1997; Mori and Salvidio, 2000; Rodgers et al., 2003; Dunn et al., 2008; Quaglio et al. 2011; Longshaw et al., 2012c). Despite examining 50 populations (966 individuals) of *P. leniusculus* in the field study and Cefas datasets combined, there were not any histologically identifiable microsporidian infections found in *P. leniusculus*. The lack of detection is surprising given that *P. leniusculus* populations had previously tested positive for microsporidian infection and were re-sampled as part of this study (Dunn et al., 2008)**.** This may suggest that although *T. contejeani* is present in *P. leniusculus* using PCR (Dunn et al., 2008; Imhoff et al., 2012), it may be a less competent host, resulting in lower parasite burden and preventing an infection from reaching detectable levels using histopathology alone. Imhoff et al. (2012) found that *P. leniusculus* could become infected with *T. contejeani* by consuming infected tissue from *A. pallipes,* confirming the capability to infect; however, in the wild this may be rare.

The native range of *P. leniusculus* has been little studied for parasitic associations; however, one study isolated microsporidian parasites from this species in California (McGriff and Modin, 1983). Since this data includes morphological comparison but lacks genetic identification, it

remains speculative to suggest this is *T. contejeani*. Such information could however suggest that this parasite was introduced by the signal crayfish invasion. Our data suggest this is unlikely, because multiple white clawed crayfish populations that are not affected by the invasion exhibit this microsporidian infection, suggesting that the parasite found in California could be a different species.

4.3 *Branchiobdella astaci*

Branchiobdellids are ectobionts generally considered to be commensal. In our study we found their presence in two *A. pallipes* populations. Histology images show an association between the presence of *B. astaci* and gill melanisation, a localised immune response that may impair the gill function (Alderman and Polglase, 1988; Rosewarne et al., 2012). *Branchiobdella astaci* has not been associated with crayfish mortality (Longshaw, 2011), nor co-infection, and did not appear to cause any gross pathological signs in *A. pallipes,* suggesting that it would have minimal impact on competitive interactions between native species and INNS.

4.4 Environmental parameters

The environment of the host is an important determinant of disease dynamics (Strayer, 2010; Johnson and Paull, 2011). Here we show that this may also be the case for crayfish and some of the disease groups we explore. A relatively small-scale study of *A. pallipes* in the River Wharfe catchment in Yorkshire reported a positive correlation between the presence of *T. contejeani* and the levels of zinc, lead, and dissolved oxygen (Imhoff, 2010). Similarly, a laboratory experiment showed a trend with penaeid shrimp that revealed the prevalence of a shrimp-specific bacilliform virus increased from 23 % to 75 % after 35 days, when shrimp were exposed to aquatic pollutants (polychlorinated biphenyls) but only increased from 23 % to 46 % in the control group (Couch and Courtney, 1977). In contrast to these previous studies, we did not reveal a relationship between the presence or prevalence of either *T. contejeani* or bacilliform virus and the chemical status of the catchment.

There was a significant relationship between the chemical status of the water body and the prevalence of *A. astaci*, the causative agent of crayfish plague. The prevalence of *A. astaci* was higher in catchments that received ‘fail’ status for their chemical classification. This may be because the immunity of *A. pallipes* is reduced in more polluted river catchments, or because these catchments provide optimal conditions for oomycete growth. The motility of *A. astaci* is dependent on water temperature, while high magnesium levels and low calcium levels are considered less favourable for spores (Oidtmann, 2000). Alternatively, external factors such as pollution are considered to reduce the resilience of freshwater ecosystems to invasion (Dudgeon et al., 2006; Strayer, 2010). The correlation we observe may have been

the result of polluted catchments being more susceptible to invasion by *P. leniusculus*, an asymptomatic carrier of *A. astaci,* rather than resulting in decreased host immunity. Such a theory requires testing, but the basis is hinted at from our environmental data.

The Water Framework Directive catchment classification takes multiple environmental parameters into account to produce an overall "status", which may omit the impacts of particularly important stressors that were not measured in this study. Moreover, localised pollution events affecting acute areas of a river may have been missed at the broad spatial scale at which the catchments are assessed, and therefore recommend that more localised studies are conducted in future to explore these findings in higher resolution.

4.5 Study conclusions

The objectives of this study were to determine whether either crayfish species or environment were predictors of infection in UK habitats. This information could be highly informative for conservation efforts, providing the best chances to develop crayfish havens where water quality may avoid further disease outbreaks. We did not find a relationship between environmental quality and the presence or prevalence of sublethal infections in crayfish. Our results did suggest a link between water quality and the presence of *A. astaci*, it is difficult to disentangle the contribution of water quality from that of *P. leniusculus* presence, as the species is an asymptomatic carrier of *A. astaci* and is more tolerant of low-quality waterbodies.

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518

519 **Tables and Figures:**

520

521 Table 1: A breakdown of the crayfish datasets, including historical Cefas data (2007-2014)
522 and primary data collected for the study in 2012.

Sampling sub-set		Number of individuals (*A. pallipes*)	Number of individuals (*P. leniusculus*)	Tests conducted
A	Seven-year Cefas dataset on crayfish disease	210 individuals from (25 populations)	818 individuals from (44 populations)	Full histological examination
B	Crayfish collected by Cefas during mortality events	123 individuals from (25 populations)	37 individuals from (5 populations)	*A. astaci* presence and prevalence using PCR
C	Field-based collections specifically to inform study	68 individuals from (3 populations) (Table 2)	128 individuals from (6 populations) (Table 2)	Full histological examination

523

524 Table 2: Location and composition of crayfish populations sampled as part of the 2012 survey.
525 The results show the prevalence of the three parasites recorded during the study for
526 *Austropotamobius pallipes* and *Pacifastacus leniusculus* populations.

Species	Site (coordinates)	n	*T. contejeani*	Bacilliform virus	*B. astaci*
A. pallipes	Wyke Beck, Leeds, West Yorkshire (53.8225, -1.4819)	24	0.37	0.58	0.25
A. pallipes	Clapham Beck, Clapham, North Yorkshire (54.118116, -2.391811)	33	0.09	0.80	0.13
A. pallipes	River Kent, Kendal, Cumbria (54.341219, -2.747489)	11	0.09	0.80	0
P. leniusculus (formerly mixed species)	Bookill Gill Beck, Long Preston, North Yorkshire	28	0	0	0

	(54.022255 - 2.242651)				
P. leniusculus (formerly mixed species)	Cawthorne Dike, Cawthorne, South Yorkshire (53.575938, -1.555192)	33	0	0.6	0
P. leniusculus (formerly mixed species)	River Ure, West Tanfield, North Yorkshire (54.203132, -1.589163)	9	0	0	0
P. leniusculus	River Clyde, Elvanfoot, Scotland (55.433032, -3.649609)	22	0	0.2	0
P. leniusculus	Aske Estate, Richmond, North Yorkshire (54.424541, -1.724253)	24	0	0	0
P. leniusculus	Loch Ken, Dumfries and Galloway, Scotland. (55.0116161,-4.0593604)	32	0	0	0

Table 2: Results of mixed effects models with significant predictors of parasitism and prevalence of parasites in crayfish populations using the Cefas dataset. Predictors are the variables that remained in the minimum adequate model. Non-significant predictors are variables removed to reach the minimum adequate model. X^2 reflects the result of log likelihood test comparing minimum adequate model to null model. The term "Ecostat" is the ecological status of the sub-catchment according to the water framework directive. The term "Chemstat" is the chemical status of the sub-catchment according to the water framework directive. The terms "Ba" = *Branchiobdella astaci*, "Ph" = *Psorospermium haeckeli*, "Tc" = *Thelohania contejeani*, "BV" = bacilliform virus (putative *Nudiviridae*).

Response variable	Significant predictor(s)	Non-significant predictors	Model R^2	X^2	P value
Model 1: Parasite diversity (Simpson's Index)	Crayfish species	Ecostat Chemstat	0.98	7.50	<0.05

Model 2: Prevalence of bacilliform virus (both species)	Crayfish species	Ecostat Chemstat Presence of Ph, Ba, Tc Prevalence of Ph, Ba, Tc	0.24	11.30	<0.001
Model 3: Response: Prevalence of bacilliform virus (*A. pallipes* only).	Presence of *Tc*	Ecostat Chemstat Presence of Ph, Ba Prevalence of Ph, Ba, Tc	0.27	0.01	<0.001
Model 4: Presence of crayfish plague (*Aphanomyces astaci*) in *A. pallipes.*	Chemstat	Ecostat	0.21	4.20	<0.05

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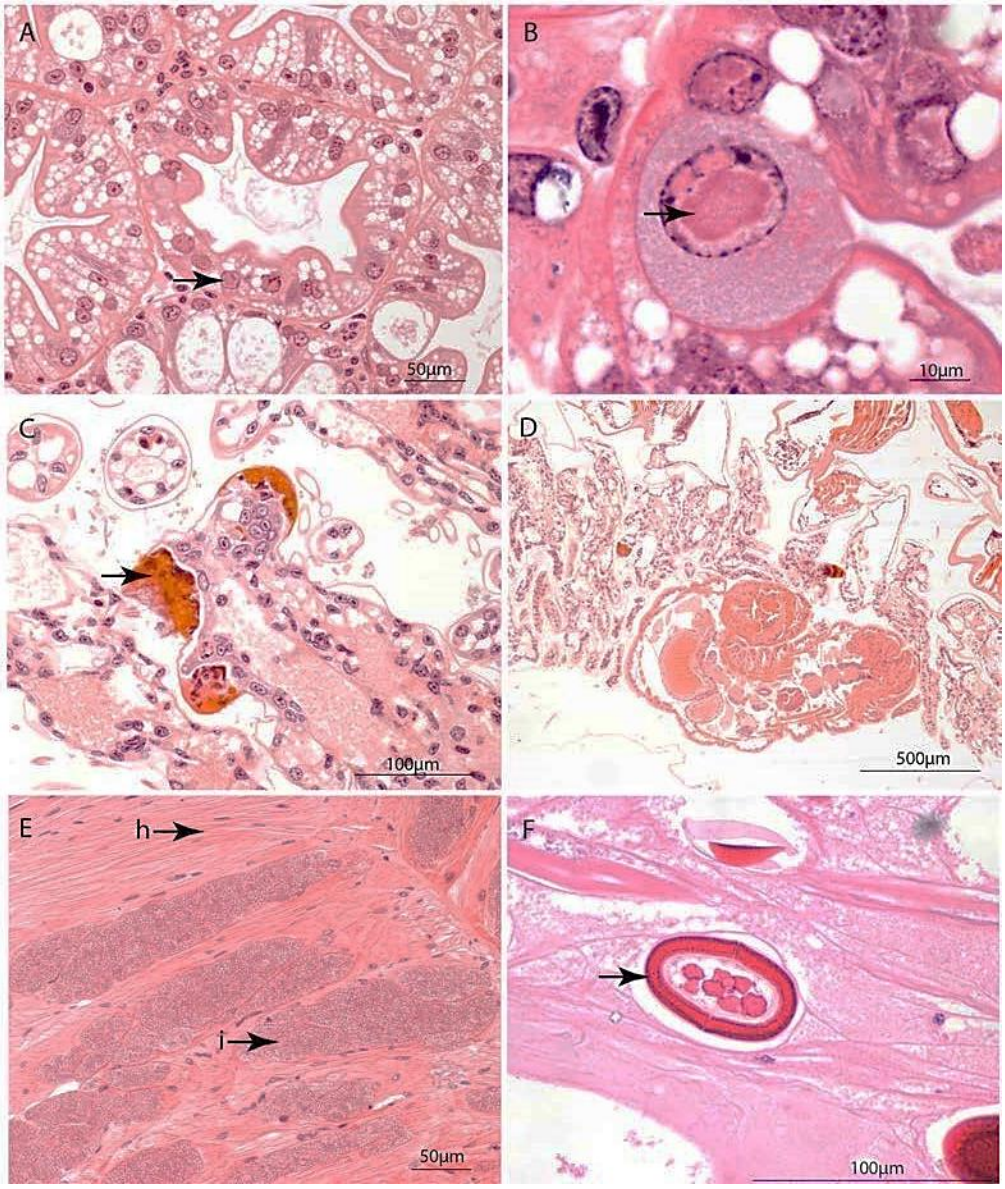

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Figure 1: Histological micrographs identifying infectious agents of crayfish. A) Low magnification view of a *Pacifastacus leniusculus* hepatopancreatic epithelial cell infected with a bacilliform virus (solid pink viroplasm in centre surrounded by cell organelles). B) High magnification view of a hepatopancreatocyte with a virally infected nucleus (arrow). C) Melanised gill tissue of an *Austropotamobius pallipes* infected with *Branchiobdella astaci*. D) Cross-section through an individual *B. astaci* parasite infecting the gill of *A. pallipes*. E) Tail muscle tissue of *A. pallipes* heavily infected with *Thelohania contejeani*. The arrow marked 'h' shows healthy, striated muscle tissue. The arrow marked 'i' shows infected muscle tissue which has been replaced with spores. F) A longitudinal section through a *Psorospermium haeckeli* sporocyst in the connective tissue of an *A. pallipes* host.

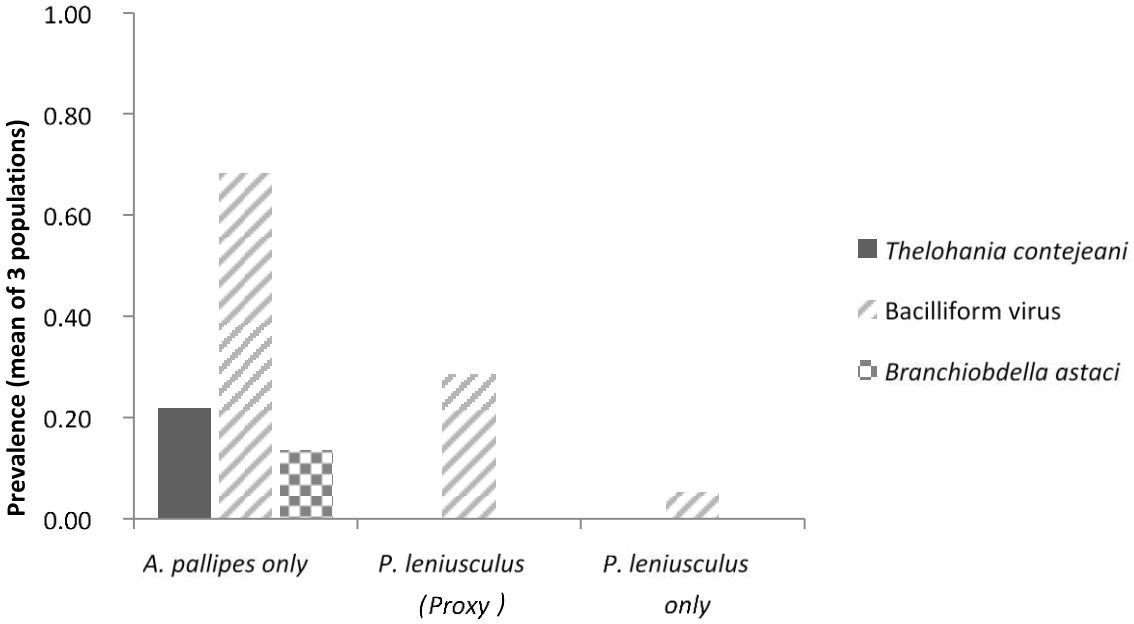

Figure 2: Presence and mean prevalence of parasites across the three population compositions. Isolated *Austropotamobius pallipes* populations (n=3 populations), isolated *Pacifastacus leniusculus* populations (n=3 populations) and *P. leniusculus* populations with recent *A. pallipes* overlap (n=3 populations). *Thelohania contejeani* (Microsporidia), Bacilliform virus (putative *Nudiviridae*) and *Branchiobdella astaci* (Annelida).