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Pathogens of *Dikerogammarus haemobaphes* regulate host activity and survival, but also threaten native amphipod populations in the UK

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ABSTRACT: *Dikerogammarus haemobaphes* is a non-native amphipod in UK freshwaters. Studies have identified this species as a low-impact invader in the UK, relative to its cousin *Dikerogammarus villosus*. It has been suggested that regulation by symbionts (such as Microsporidia) could explain this difference in impact. The effect of parasitism on *D. haemobaphes* is largely unknown. This was explored herein using 2 behavioural assays measuring activity and aggregation. First, *D. haemobaphes* were screened histologically post-assay, identifying 2 novel viruses (*D. haemobaphes* bi-facies-like virus [DhbflV], *D. haemobaphes* bacilliform virus [DhBV]), *Cucumispora ornata* (Microsporidia), Apicomplexa, and Digenea, which could alter host behaviour. DhBV infection burden increased host activity, and *C. ornata* infection reduced host activity. Second, native invertebrates were collected from the invasion site at Carlton Brook, UK, and tested for the presence of *C. ornata*. PCR screening identified that *Gammarus pulex* and other native invertebrates were positive for *C. ornata*. The host range of this parasite, and its impact on host survival, was additionally explored using *D. haemobaphes*, *D. villosus*, and *G. pulex* in a laboratory trial. *D. haemobaphes* and *G. pulex* became infected by *C. ornata*, which also lowered survival rate. *D. villosus* did not become infected. A PCR protocol for DhbflV was also applied to *D. haemobaphes* after the survival trial, associating this virus with decreased host survival. In conclusion, *D. haemobaphes* has a complex relationship with parasites in the UK environment. *C. ornata* likely regulates populations by decreasing host survival and activity, but despite this benefit, the parasite threatens susceptible native wildlife.

KEY WORDS: *Cucumispora* · Population regulation · Behaviour · Pathogen profile · Virus · Wildlife pathogen · Emerging disease

1. INTRODUCTION

Invasive species impact negatively on the environments they encounter, causing damage to biodiversity (Molnar et al. 2008) and ecosystem services (Dukes & Mooney 2004). An often-overlooked concept in invasion biology, particularly in behavioural assessment, is the complex relationships that invasive alien species share with their parasites and pathogens (Vilcinskas 2015). In some invasions, symbionts are left

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behind in the native range (parasite release), increasing the fitness of an invasive population (Torchin et al. 2003, Lee & Klasing 2004, Heger & Jeschke 2014). Alternatively, symbionts are sometimes introduced along with their host in an invasion event, and can form part of a disease introduction event, resulting in the potential for host switching events and the emergence of wildlife pathogens (Roy et al. 2017), or could help regulate the impact of their invasive host in the invasive range (Dunn & Hatcher 2015).

Co-introduced pathogens can regulate an invasive host population, and its impact, by decreasing invasive behaviours and increasing mortality rates (Dunn & Hatcher 2015). Parallels can be drawn between this process and biological control, which aims to decrease the impacts and population size of a pest through the use of its natural enemies, such as microsporidians and viruses (Lacey et al. 2001, de Faria & Wraight 2007, Hajek et al. 2007). One group of invaders that require control are invasive amphipods in the UK. The identification of symbionts that regulate invasive amphipod behaviour and survival will help to provide information on how to control their populations at invasion sites.

The invasive 'demon shrimp' *Dikerogammarus haemobaphes* is an amphipod native to the Ponto-Caspian region, which invaded the UK in 2012 and carried with it the microsporidian parasite *Cucumispora ornata* (Bojko et al. 2015). *D. haemobaphes* is considered a low-impact, non-native relative of *Dikerogammarus villosus* (the 'killer shrimp'), which invaded the UK in 2010 without microsporidian, acanthocephalan, or viral symbionts (MacNeil et al. 2010, Bojko et al. 2013, Dodd et al. 2014, Bovy et al. 2015). Whether parasites are the determining factor amongst several possible factors (e.g. fecundity, niche occupation) that regulate *D. haemobaphes* populations, and lower their impact relative to *D. villosus* in the UK, is currently unclear.

In this study, we compare the activity, aggregation, and rate of survival between healthy and infected *D. haemobaphes*, collected directly from their invasive habitat. First, histological diagnostics were applied to animal carcasses after behavioural trials to determine their individual symbiont profiles, to compare parasitism and behaviour. Second, animals were collected from the invasion site and screened for the presence of Microsporidia, to assess the host range of *C. ornata* in the environment. Finally, survival trials were used to assess the transmissibility of *C. ornata* and the effects of selected parasites on host survival in 3 species: the type host *D. haemobaphes*, a close relative *D. villosus*, and the native keystone species *Gammarus pulex*.

2. MATERIALS AND METHODS

Due to the wide range of methods utilised in this study, we have provided a flow chart of how the experiment was conducted in Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d136p063_supp.pdf.

2.1. Field sampling

Dikerogammarus haemobaphes were collected from Carlton Brook (Leicestershire, UK) (British National Grid [BNG] ref: SK3870004400) for behavioural assessment prior to histological screening and to take part in a survival trial with the microsporidian parasite *Cucumispora ornata* (Table 1). In addition, freshwater mussels, beetle larvae, fly larvae, annelids, isopods, freshwater snails, and the freshwater amphipod *Crangonyx pseudogracillis* were also collected and fixed on site in ethanol for microsporidian PCR diagnostics (Table 1).

Dikerogammarus villosus were collected from Grafham Water (BNG ref: TL1442767283), and 2 collections were also made of *Gammarus pulex* — one from a population found co-occurring at the Carlton Brook site alongside *D. haemobaphes* and a second population from Meanwood Park, Leeds (BNG ref: SE2803737255), where *D. haemobaphes* have not been detected to date — for a transmission and survival trial to observe potential infection with *C. ornata* (Table 1).

2.2. Behavioural trials for *Dikerogammarus haemobaphes*

Individual *D. haemobaphes* (n = 282) underwent measurement of various morphological characteristics, including sex, presence and number of offspring, length, weight, and pair status. Animals were transported to the University of Leeds and acclimatised in canal water with vegetation at 14°C for a minimum of 24 h before use in behaviour trials. The activity trial included 120 animals, the aggregation trial included 63 animals, and the remaining 99 were measured and screened using histology but not included in a behavioural trial. Each animal was only used once, and on completion of the behavioural trial were fixed for histological screening.

2.2.1 Activity assessment

Activity assessment was conducted in a similar fashion to the study by Bacela-Spychalska et al.

Table 1. A breakdown of the animals sampled during the study to explore the behaviour, transmission trial, survival, and host range experimentation. Dates given as dd/mm/yyyy

Species/organism	Sample date	Sample location	n	Reason for collection
Dikerogammarus haemobaphes	18/05/2015	Carlton Brook	282	Activity and aggregation behaviour trials and to collect physiology data
	19/07/2015	Carlton Brook		
	27/07/2015	Carlton Brook		
	03/08/2015	Carlton Brook		
	14/08/2016	Carlton Brook	56	Survival trial (control n = 29, exposed = 27)
Dikerogammarus villosus	20/09/2016	Grafham Water	87	Survival trial and on-site fixed samples (control n = 29, exposed = 28)
Gammarus pulex	14/08/2016	Carlton Brook	36	Survival trial and on-site fixed samples (control n = 9, exposed = 10)
	01/11/2016	Meanwood Park	57	Survival trial and on-site fixed samples (control n = 13, exposed = 14)
Crangonyx pseudogracillis	18/05/2015	Carlton Brook	1	*Cucumispora ornata* screening
Freshwater mussels	18/05/2015	Carlton Brook	4	*Cucumispora ornata* screening
Beetle larvae	18/05/2015	Carlton Brook	3	*Cucumispora ornata* screening
Fly larvae	18/05/2015	Carlton Brook	7	*Cucumispora ornata* screening
Annelids	18/05/2015	Carlton Brook	4	*Cucumispora ornata* screening
Isopods	18/05/2015	Carlton Brook	2	*Cucumispora ornata* screening
Freshwater snails	18/05/2015	Carlton Brook	5	*Cucumispora ornata* screening

(2014). Individual *D. haemobaphes* (n = 120) were placed into uniform transparent pots bisected equally with a black line. Animals were placed on this line at 00:00 min and provided with 02:00 min to acclimatize to the new surroundings. After 02:00 min, activity (crosses of the black line) was recorded between 02:00 and 04:00 min, 06:00 and 08:00 min, and 10:00 and 12:00 min providing a total 6 min of activity data collection per individual. Animal activity was not recorded between 00:00 and 02:00 min (acclimatisation period), 04:00 and 06:00 min, and 08:00 and 10:00 min. After each experiment the size, weight, gravidity, egg clutch size, mating pair status, and visible infection status with microsporidia were assessed for each animal.

2.2.2. Aggregation assessment

The aggregative behaviour of *D. haemobaphes* (n = 63) was assessed using an experimental set-up (see Fig. S2 in the Supplement) that consisted of a white tray bisected by a black line, with a buffer zone (2 cm locus). This white tray contained 2 gauze cages of 8 cm^3 volume with 0.5 mm mesh size, 1 containing 4 male *D. haemobaphes* and the second empty at either end of the tray. Gauze cages were placed equidistant to the black line. The side of the tray containing the gauze cages present with animals was designated the 'social zone' and the side without animals the 'null zone'. Each experiment was conducted with fresh, de-chlorinated water and gauze cages in the water for 03:00 min prior to the test subject. The test subject was placed into a black tube on the buffer zone to acclimatize for a further 02:00 min. Once acclimatised, the test subject was released from the black tube and its time spent in either zone was measured over a 10:00 min period. Time data collected from this experiment were used to determine the percentage of time spent in each area. Time spent in the buffer zone was excluded to ensure that the preferences corresponded to a strong choice between the social and null zones.

2.3. Survival trial and data collection

An inoculum was produced by homogenising the carcasses of *D. haemobaphes* visibly infected with *C. ornata*, which was tested to be positive for *C. ornata* via nested PCR, but not for virus via PCR. This was fed to half the animals included in the transmission and survival trial. The composition of animals in each trial is outlined in Table 1. For *D. villosus* and *G. pulex*, 30 animals were collected on site and immediately fixed in ethanol to identify the background prevalence of *C. ornata* in the wild population.

Animals used in the survival trial were separated into individual Petri dishes to prevent them interacting whilst taking part in the trial. The Petri dishes were split into oxygenated tanks (3 control tanks and 3 exposure tanks). Six litres of de-chlorinated water

were used in each tank. The trials consisted of a 48 h starvation period before providing 15 mg of food pellets ('Crustacean Cuisine' food pellets — uninfected material) to each Petri dish in the control group and 15 mg of demon shrimp homogenate to the exposure group. Each group was cultured for 30 d after initial starvation and survival rate was measured at 12:00 pm on a daily basis. During (if mortality occurred) or after the trial, *D. haemobaphes* were fixed in 100% ethanol for molecular diagnostics to assess for *C. ornata* and *D. haemobaphes* bi-facies-like virus (DhbflV) presence. *D. villosus* and *G. pulex* were cut in half for dissection to allow for pathogen screening using both molecular diagnostics (head and I–III pereon segment) and histology (IV pereon segment to telson) to detect infection with *C. ornata*.

2.4. Histology and electron microscopy

Specimens were anaesthetised using carbonated water and dissected, removing the urosome for DNA extraction and molecular diagnostics with the rest of the animal being fixed for histological analysis. This same procedure took place after each behavioural experiment for each test subject. A single specimen displaying a rare viral infection was removed from the wax block that it was initially preserved in for histology, to be re-processed for transmission electron microscopy (TEM) analysis. A stock specimen collected by Bojko et al. (2015) was used to gather TEM evidence for the *D. haemobaphes* bacilliform virus (DhBV) infection of the hepatopancreas.

D. haemobaphes displaying *C. ornata* infection in the histology were assigned a burden intensity ranging from uninfected (score = 0) through to heavy infection (score = 3) (see Fig. 1). Animals displaying DhBV infection were assigned a percentage burden estimation based on the number of infected nuclei of the hepatopancreatic epithelial cells divided by the total number of epithelial cell nuclei in the hepatopancreas. Other infections were not assessed for burden but recorded in binary as infected or uninfected (0 or 1).

For full details of the histological and TEM procedures, including recovery of TEM samples from wax blocks, refer to Bojko et al. (2015).

2.5. Genetic sequencing and diagnostics

DNA was extracted from individuals used in the survival trial, from the inoculum used, and from animals collected on-site to check for background prevalence of Microsporidia infection (*D. villosus*, *G. pulex*, other macroinvertebrates). DNA extraction was conducted using the phenol–chloroform procedure detailed in Bojko (2017), after an overnight Proteinase K digestion. In cases where animals were too large to conduct a whole-animal extraction, the extraction was conducted on a biopsy or halved specimen.

All potential hosts, and the homogenate used as infected feed source in the transmission experiments, were assessed for microsporidian infection using the general MF1 (5′-CCG GAG AGG GAG CCT GAG A-3′) and MR1 (5′-GAC GGG CGG TGT GTA CAA A-3′) primer set developed by Tourtip et al. (2009) as used by Bojko et al. (2015). Infection by the microsporidian *C. ornata* was detected using a nested PCR approach; the Mic18/19F (5′-ATA GAG GCG GTA GTA ATG AGA CGT A-3′) and Mic18/19R (5′-TTT AAC CAT AAA ATC TCA CTC-3′) primers developed by Grabner et al. (2015) were used in a 50 µl PCR mix for the second round after initial amplifica-

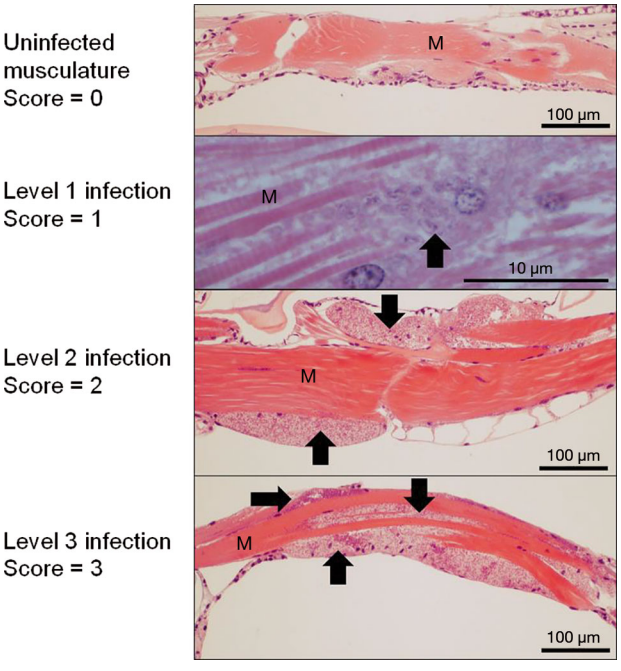


Fig. 1. Microsporidian intensity scale used to histologically quantify the burden of a microsporidian infection in the muscle tissue (M). The scale starts at 0 (uninfected) and moves through to level 3 (heavy burden infection) as shown to the left of the diagram. The black arrows indicate the infected areas in all images. Scale 1 identifies the presence of microsporidian development stages at the lowest burden, perhaps even without spore formation as shown. Scale 2 shows sarcolemma infection (can include connective tissue infection). Scale 3 shows the highest burden where myofibrils and sarcolemma are infected throughout the host

tion by the MF1/MR1 primer set. The 50 µl Go-Taq PCR reaction consisted of 1.25 U of *Taq* polymerase (Qiagen), 1 µM of each primer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, and 2.5 µl of genome template or PCR product for each sample. Thermocycling conditions (Bio-Rad thermocycler) were 94°C (5 min); followed by 35 cycles of 94°C (1 min), 58°C (1 min), and 72°C (1 min); and finally, 72°C (10 min).

The inoculum and *D. haemobaphes* used in the survival trial were tested for infection by DhbflV using a novel PCR method. Amplification of DhbflV helicase gene was accomplished using a standard PCR protocol in 50 µl quantities with the DHhelicaseF (5′-CGT GTG TTT AGG TAC AAG AAC-3′) and DHhelicaseR (5′-TAG AGA AGG TGG AAA TGA CTA-3′) primer set. These primers were developed from unpublished metagenomic data derived from infected shrimp (Bojko 2017). The 50 µl Go-Taq PCR reaction consisted of 1.25 U of *Taq* polymerase, 1 µM of each primer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, and 2.5 µl of genome template for each sample. Thermocycling conditions (Bio-Rad thermocycler) were 94°C (5 min); followed by 35 cycles of 94°C (1 min), 52°C (1 min), and 72°C (1 min); and finally, 72°C (10 min). Viral amplicons were produced at ~500 bp.

In all cases, PCR amplicons were visualised on a 2% agarose gel alongside a hyperladder (100 to 2000 bp), or 1 kb ladder (Promega), to diagnose infection by amplicon size. In ad-hoc cases, gel bands attained from the viral and microsporidian PCRs were excised and purified before being sent for forward and reverse sequencing via Eurofins sequencing barcode service (https://www.eurofinsgenomics.eu/en/custom-dna-sequencing.aspx). Sequence data for a novel microsporidian isolate (18S RNA), and for DhbflV (predicted D5 helicase), has been deposited under accession numbers MF428413 and MG661262, respectively.

2.6. Statistical analyses

Statistical analyses were conducted in R version 3.2.1 (R Core Team 2013) through the Rstudio interface. Prior to analysis, continuous and count data collected from individuals (physical measurements, viral burden data, activity, and aggregation) were tested for normality using the Shapiro-Wilk normality test, determining that all continuous data from the study was not normally distributed. Log transformation of the weight and length measurements allowed conformation to normality based on a search for linearity using quantile–quantile plots, and allowed the use of parametric statistics. The square root of the activity data allowed for the use of parametric tests. The rest of the data were not normally distributed and were analysed using non-parametric statistics such as Wilcoxon test (with continuity correction), Kruskal-Wallis test (KW), and Spearman's rank correlation. Weight and length data comparisons were conducted using parametric statistics (independent *t*-tests with equal variance) or generalised linear models, which account for non-normal data (quasi-Poisson error distribution). Generalised linear models were also used to compare count data (egg counts) (without female size as an interaction) between infected and uninfected animals, and fitted with a quasi-Poisson error distribution to account for over-dispersion. Factorial data comparisons were conducted using Pearson's chi-squared test with Yates' continuity correction.

Analysis of survival data employed the 'coxme' package developed by Therneau (2015a) and the 'survival' package developed by Therneau (2015b). First, a survival fit was created to describe survival variation in time to death between different groups. A Cox proportional hazards model was used to test the significance of different factors (microsporidian infection, DhbflV infection, tank number) in determining differences in the time to death. Survivorship models contained the infection status of each individual as a fixed effect along with the food treatment as a random blocking effect. Fisher's exact probability tests were applied to prevalence statistics for the animals involved in the survival trial to determine the likelihood of microsporidian acquisition from experimental analysis via a comparison between control and exposure groups.

3. RESULTS

3.1. Histopathology and ultrastructure of novel pathogens

During the study, several novel infections were observed alongside the previously described *Cucumispora ornata*. These included 2 novel viruses infecting the hepatopancreas and haemocytes, gregarines in the gut lumen, and digenean trematodes encysted within the connective tissues around the gut and gonad. Based on histology, *C. ornata* was noted at 85.5% prevalence in the 282 specimens of *Dikerogammarus haemobaphes* collected for physiological and behavioural observations.

3.1.1. *Dikerogammarus haemobaphes* bacilliform virus (DhBV)

This is the first report of a viral infection in *D. haemobaphes*. The viral pathology noted during histological analysis revealed hypertrophic nuclei in the hepatopancreas of *D. haemobaphes* (Fig. 2a,b). The host chromatin was condensed to the margins of the nucleus (Fig. 2a) and the cytoplasm of cells was additionally condensed due to the hypertrophic nucleus. In some cases, a deep purple staining occlusion body was present (Fig. 2b). No immune responses, such as melanisation of surrounding tissues or recruitment of granulocytes, were observed in response to this infection. Infected individuals varied in the intensity of infection, with some animals exhibiting only 1 or 2 infected nuclei and others with more extensive infections across the entire hepatopancreas. In all cases, the infection was limited only to the nuclei of hepatopancreatocytes. Infection prevalence across the 282 sampled individuals was 77.7%. Individuals showed no external clinical signs of infection based on the observations made during this study before histological preservation.

TEM of infected individuals revealed that infected nuclei were filled with a viroplasm that consisted of fully formed and partially formed bacilliform virions, which were not in any crystalline order (Fig. 2c). Individual virions consisted of a rod-shaped electron-dense core and an enveloping membrane that maintains a close association with the core genetic material (Fig. 2c, inset). The electron-dense core measured approximately (n = 30, mean ± SD) 302 ± 13 nm in length and 55 ± 4 nm at its diameter. The outer membrane measured approximately 410 ± 25 nm in length and 98 ± 6 nm in width. Based on viral morphology using electron microscopy, this study suggests it be referred to as '*Dikerogammarus haemobaphes* bacilliform virus' (DhBV) until genetic data is available for a full taxonomic description.

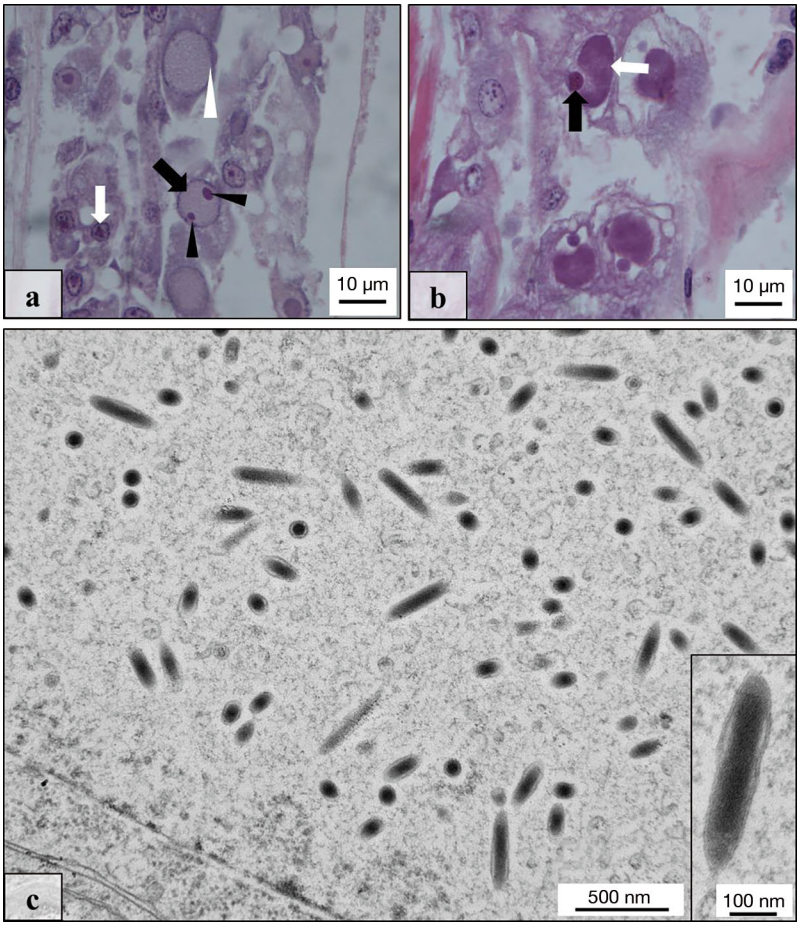


Fig. 2. Histopathology and ultrastructure of *Dikerogammarus haemobaphes* bacilliform virus. (a) Early infections reveal a growing viroplasm (black triangles) within the nucleus of the hepatopancreatocytes (black arrow) and the host chromatin is marginated (white triangle). An uninfected nucleus is highlighted by a white arrow. (b) Later stage infections are deep purple under haematoxylin and eosin (white arrow) and are present with occlusion bodies (black arrow). (c) Transmission electron microscopy identified rod-shaped viruses in the nuclei, one of which is highlighted in greater detail in the inset

3.1.2. *Dikerogammarus haemobaphes* bi-facies-like virus (DhbfIV)

Histology revealed the presence of a second viral pathology in the haemolymph (haemocytes and granulocytes), connective tissues, and haematopoietic tissues around the carapace. Infected cells contained hypertrophic nuclei filled with a pink-purple staining viroplasm (Fig. 3a). This infection was noted in 3 individuals in the population of invasive *D. haemobaphes* from Carlton Brook in the UK. No immune responses were observed in relation to this virus and on all occasions infection intensity was pronounced with most haemocytes infected. Via TEM, the nuclei of infected cells were shown to have a viroplasm consisting of a labyrinthine network of DNA and protein (Fig. 3b). In advanced infection, the viroplasm

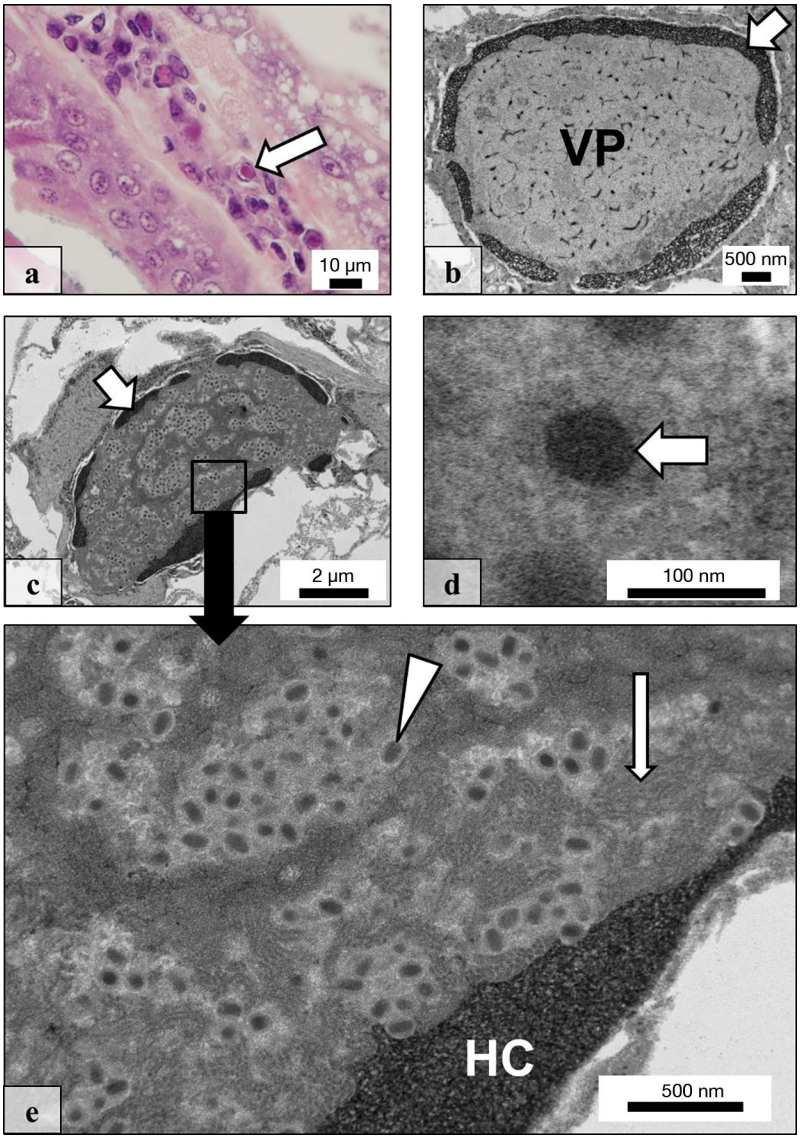


Fig. 3. Histopathology and transmission electron microscopy (TEM) of *Dikerogammarus haemobaphes* bi-facies-like virus. (a) Haemocyte nuclei (white arrow) infected with the virus. (b) TEM image of a growing viroplasm (VP) in a haemocyte nucleus (white arrow). (c) A late stage nucleus (white arrow) with several virions. (d) High magnification of a single virion core (white arrow) identifies it with a pentagonal cross-section. (e) Higher magnification image of (c) identifies a labyrinthine network for viral assembly (white arrow), several virions (white triangle), and host chromatin (HC)

had arranged to form discrete virions (Fig. 3c), each with a pentagonal cross-section (Fig. 3d). Virions could be seen amongst complex networks of membranes, proteins, and nucleic acids (Fig. 3e). Each virion had a central, electron-dense core measuring 52 ± 6 nm in width and 105 ± 19 nm in length, and was surrounded by a membrane measuring 111 ± 9 nm in width and 149 ± 14 nm in length. No genetic information is currently available for this virus. This virus has been termed '*Dikerogammarus haemobaphes* bi-facies-like virus' (DhbflV) given its similarity in form to the virus described in Johnson (1988). However, further genomic information is required for accurate taxonomy.

3.1.3. Apicomplexa and Digenea

Gregarine parasites (Apicomplexa) were noted in 51.8% of the 282 *D. haemobaphes* collected for assessment. The gregarines were often present in 1 of 3 life-stages: (1) intracellular stage, within the gut epithelium of the host (Fig. 4a,b); (2) in the mid-gut lumen of the host (Fig. 4c); or (3) undergoing syzygy in the hind-gut. In all cases of infection, no observable immune response was elicited by the presence of gregarines.

Digenean trematodes were present in a single individual (1/282). Digenea were observed to encyst within the connective tissues of their host, presenting with an eosinophilic layer surrounding an encysted metacercariae (Fig. 4d). Digeneans were not seen to elicit any visible host immune response.

3.2. Pathogen effects on host fitness

Sex, size, pair status, and the presence and number of offspring were recorded for each *D. haemobaphes* (n = 282) undergoing behavioural and physiological assessment. These characteristics were assessed relative to the infection status of individuals by the parasites and pathogens detected by histology.

Sex was recorded as male, female, or intersex, with the latter being rare in the Carlton Brook population (<1%); this category was subsequently removed from the sex analysis. *C. ornata* prevalence was 85.5%, and did not differ significantly between males and females (chi-squared test, $\rho^2_{df=1}$ = 1.559, p = 0.212). In addition, infection by *C. ornata* was not associated with either length (*t*-test, *t* = 1.021, df = 280, p = 0.308) or weight (*t*-test, *t* = 1.129, df = 280, p =

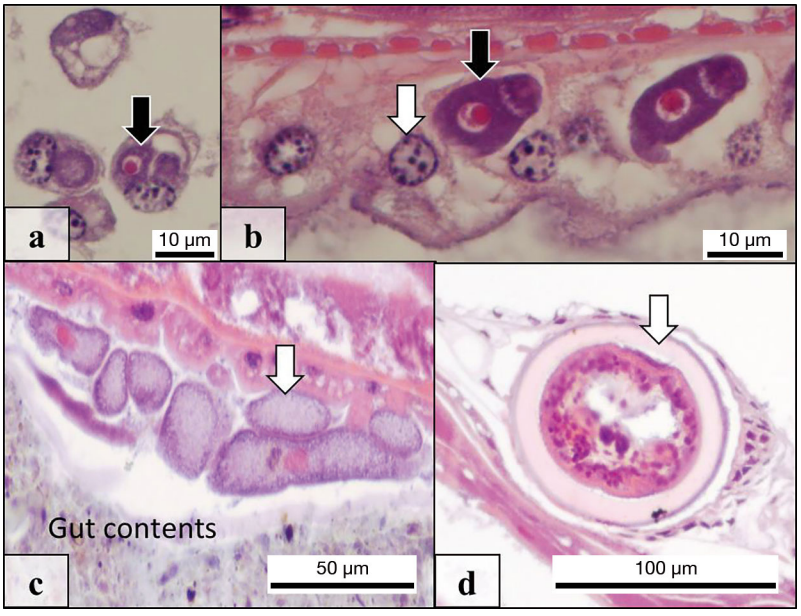


Fig. 4. Gregarines and digeneans infecting *Dikerogammarus haemobaphes* from Carlton Brook. (a) Intracellular life stage of gregarine development (black arrow). (b) Gregarines (black arrow) enlarge and mature before emerging from the cells into the gut lumen. A host nucleus is identified by the white arrow. (c) Gregarines (white arrow) align along the gut wall. (d) A digenean cyst (white arrow) within the connective tissues of the host

0.260) of individual amphipods. *C. ornata* prevalence did not differ between individuals found in pairs versus singletons (chi-squared test, $\rho^2_{df=1}$ = 0.233, p = 0.630). For females, gravidity was not associated with the presence of *C. ornata* (chi-squared test, $\rho^2_{df=1}$ = 3.315, p = 0.069), nor the size of the egg clutch (egg number) (quasi-Poisson generalised linear model [GLM], dispersion parameter = 44.436, *t* = 0.748, df = 109, p = 0.456), nor was clutch size associated with *C. ornata* burden (quasi-Poisson GLM, chi-squared test on model, $\rho^2_{df=3}$, deviance = 4141.1, p = 0.063).

The prevalence of DhBV was 77.7% and did not differ significantly between males and females (chi-squared test, $\rho^2_{df=1}$ = 0.000, p = 1.000), length (*t*-test, *t* = −1.238, df = 280, p = 0.217), or weight (*t*-test, *t* = −0.687, df = 280, p = 0.492). There was no difference in the pairing status (paired vs. single) of infected or uninfected individuals (chi-squared test, $\rho^2_{df=1}$ = <0.001, p = 0.996). The virus was not more prevalent in gravid females (chi-squared test, $\rho^2_{df=1}$ = 0.037, p = 0.847). Female clutch size was not affected by DhBV presence (quasi-Poisson GLM, dispersion parameter = 45.719, *t* = 0.263, df = 109, p = 0.793), although there was a slight negative correlation of clutch size with DhBV burden (quasi-Poisson GLM, dispersion parameter = 43.946, *t* = −1.236, df = 109, p = 0.219).

Gregarines were more commonly associated with males (prevalence = 97/171) than females (prevalence = 48/110) (chi-squared test, $\rho^2_{df=1}$ = 4.297, p = 0.038). The length (*t*-test, *t* = −0.555, df = 280, p = 0.579) and weight (*t*-test, *t* = −0.896, df = 280, p = 0.371) of the host was not associated with the presence of gregarines. There was no difference in the pairing status of infected versus uninfected individuals (chi-squared test, $\rho^2_{df=1}$ = 0.083, p = 0.773). Gravid females were not associated significantly with gregarine infection (chi-squared test, $\rho^2_{df=1}$ = 0.668, p = 0.414) and the clutch size of gravid females appeared not to be affected by the presence of gregarines (quasi-Poisson GLM, dispersion parameter = 43.708, *t* = −1.345, df = 109, p = 0.181).

The prevalence of Digenea and DhbfIV was too low to conduct statistical assessment of the effects on host physiology.

3.3. Activity assessment

The activity of *D. haemobaphes* was affected by physiology and morphology. Sex, gravidity, clutch size, and pair status were all significant factors affecting the activity of *D. haemobaphes*; where males were more active than females (quasi-Poisson GLM, dispersion parameter = 16.427, *t* = 3.663, df = 128, p < 0.001), gravid females were not more active than females without young (quasi-Poisson GLM, dispersion parameter = 13.037, *t* = 2.241, df = 61, p = 0.029), activity was correlated with the size of the egg clutch (Spearman rank, ρ = 0.327, *S* = 26 725, p = 0.009), and single animals were more active than those that were paired (quasi-Poisson GLM, dispersion parameter = 17.030, *t* = −2.787, df = 130, p = 0.006). Weight (quasi-Poisson GLM, dispersion parameter = 18.696, *t* = 1.604, df = 130, p = 0.111) and length (quasi-Poisson GLM, dispersion parameter = 18.579, *t* = 1.809, df = 130, p = 0.073) did not significantly affect activity.

Histological screening revealed 112 *C. ornata* infected individuals out of 131 tested after the activity assay. Individuals were split into 1 of 4 groups: uninfected (score = 0) (n = 19); low-level infection (score = 1) (n = 79); medium-level infection (score = 2) (n = 13); and high-level infection (score = 3) (n = 20), according to Fig. 1. Analysis revealed that the status of 'infected'

or 'uninfected' could not be correlated with variation in the activity of the host (quasi-Poisson GLM, dispersion parameter = 18.666, t = –0.240, df = 130, p = 0.810) (Fig. 5). However, heavier burden (score = 3) microsporidian infections were significantly correlated with reduced host activity (ANOVA, $F_{3,127}$ = 8.087, p < 0.001) (Fig. 5).

Histological screening revealed 97 DhBV infected individuals out of 131 tested after the activity assay. The presence or absence of DhBV was not associated with altered activity (quasi-Poisson GLM, dispersion parameter = 18.504, t = 1.278, df = 130, p = 0.203) (Fig. 6). However, when burden (defined by the number of infected nuclei relative to the number of unin-

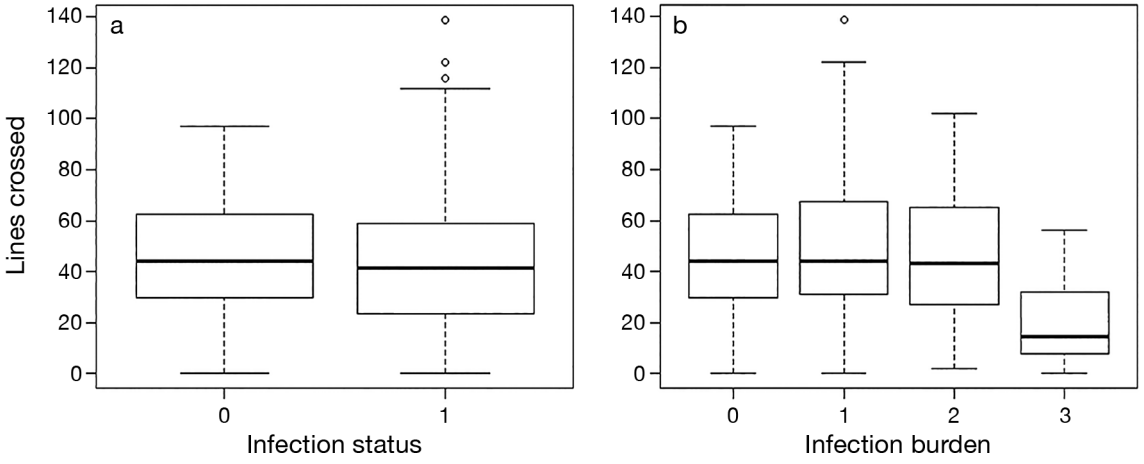
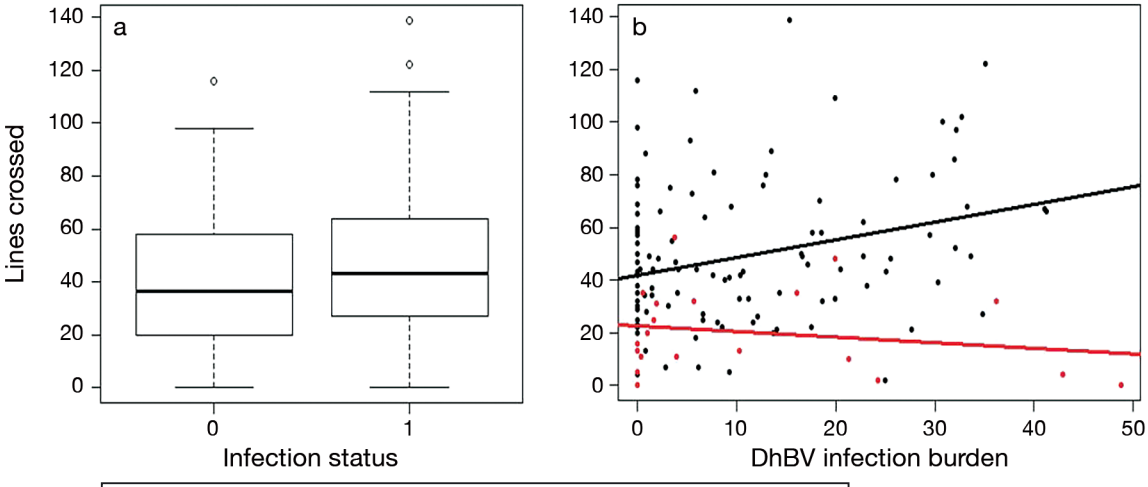


Fig. 5. *Dikerogammarus haemobaphes* activity (a) affected by *Cucumispora ornata* presence (1) or absence (0) of infection and (b) microsporidian burden according to the infection intensity scale in Fig. 1. The activity proxy accounts for the sum of the number of lines crossed by the host during the allotted times. Bar: median; box: 25th–75th percentiles; whiskers: min.–max. within 1.5× interquartile range (IQR) of the data; dots: outliers at >1.5× IQR



c

Measurement	Estimate	Error	*t*-value	p-value
DhBV burden	0.013	0.004	2.997	0.003
Microsporidian (level 3)	–0.628	0.250	–2.507	0.013
DhBV:Microsporidian (level 3)	–0.024	0.015	–1.618	0.108

Fig. 6. *Dikerogammarus haemobaphes* activity (a) affected by DhBV presence (1) or absence (0), and (b) against viral burden (boxplot features as in Fig. 5). The scatter plot in (b) identifies all data points; however, those in red also have a high microsporidian burden (level 3). The black line identifies the increased activity observed by *D. haemobaphes* bacilliform virus (DhBV)-infected animals at various burdens of infection. The red line identifies the activity trend observed for those animals with not only DhBV infection, but also level 3 microsporidian infection. (c) Interaction between DhBV burden and microsporidian level 3 infection

fected nuclei) was considered, a significant correlation between increased activity and higher viral burden was observed (quasi-Poisson GLM, dispersion parameter = 17.802, t = 2.147, df = 130, p = 0.034) (Fig. 6). High-level (level 3) microsporidian infections (noted in red on Fig. 6) also strongly correlated with lower host activity; an interaction analysis identified a non-significant interaction, which shows that the relationship between activity and DhBV infection intensity does increase host activity, and is not altered by microsporidian infection level (quasi-Poisson GLM, dispersion parameter = 15.143, t = −1.618, df = 130, p = 0.108) (Fig. 6c).

Histological detection of gregarine infections was from 73 individuals out of 131 tested after the activity assay. The presence or absence of gregarines was also analysed against the activity data, revealing that the presence of gregarines did not affect the activity of their host (quasi-Poisson GLM, dispersion parameter = 18.539, t = 0.567, df = 130, p = 0.572). Due to the histology-oriented data collection method, accurate assessment of parasite burden could not be determined for gregarine infections as sections of the gut could not be standardised accurately.

3.4. Aggregation assessment

Only male animals were used to measure behaviour in the aggregation assessment, in which individuals had a choice between an empty shelter (null zone) and a shelter containing 4 males (defined as the social zone).

The length (Spearman rank, ρ = −0.147, S = 47774, p = 0.251), weight (Spearman rank, ρ = −0.172, S = 48850, p = 0.177), or pair status (Wilcoxon test, W = 154.5, p = 0.818) of male individuals was found not to be significantly associated with amount of time in the social zone, where individuals had a choice between an empty shelter and a shelter containing 4 males. There was no association between infection with *C. ornata* and time spent in the social zone (Wilcoxon test, W = 283.5, p = 0.733), nor was aggregation behaviour affected by parasite burden (KW test, $\rho^2_{df=3}$ = 0.373, p = 0.946).

Similarly, the presence or absence of DhBV did not significantly affect the amount of time spent in the social zone (Wilcoxon test, W = 456.5, p = 0.119); nor did DhBV burden (Spearman rank, ρ = −0.114, S = 46402, p = 0.375), or the presence of gregarines (Wilcoxon test, W = 509, p = 0.321).

3.5. *Cucumispora ornata* in aquatic invertebrates

From the samples of aquatic invertebrates from Carlton Brook, the general primers (MF1/MR1) provided 4 amplicons: 2 that were too weak to sequence, 1 non-microsporidian eukaryote SSU DNA sequence (220 bp) (freshwater mussel *Sphaerium nucleus* [KC-429383.1]; 87% coverage; 96% identity; E-value = 1×10^{-82}), and 1 amplicon (884 bp) from a likely novel microsporidian taxon with closest sequence similarity to *Encephalitozoon cuniculi* isolated from the kidney of a blue fox from China (KF169729) (99% coverage; 87% identity; E-value = 0.0) (Table 2). The specific primer set (Mic18/19) yielded 5 amplicons: 2 from freshwater mussels, 1 from a mosquito larva, 1 from a beetle larva, and 1 from a freshwater snail (Table 2). All of these amplicons shared 99–100% sequence identity, and 99–100% coverage, with *C. ornata*. The final 2 amplicons from the mosquito

Table 2. The macroinvertebrates collected alongside *Dikerogammarus haemobaphes* and *Gammarus pulex* at the Carlton Brook site. Each specimen underwent DNA extraction and was tested for the presence of *Cucumispora* via nested PCR

Taxonomy of the host	n	Infected	Nested first round MF1, MR1 (Tourtip et al. 2009)	Nested second round Mic18/19F, Mic18/19R (Grabner et al. 2015)
Sphaeriidae	4	3	Host amplicon (~800 bp)	*Cucumispora ornata* +ve (×2)
Coleopteran larvae 1	2	0	No amplification	No amplification
Coleopteran larvae 2	1	1	No amplification	*Cucumispora ornata* +ve
Trichoptera	1	0	No amplification	No amplification
Clitellata	4	0	No amplification	No amplification
Asellus aquaticus	2	1	Unconfirmed sequence	No amplification
Ephemeroptera	3	0	No amplification	No amplification
Tipulidae	2	0	No amplification	No amplification
Planorbis sp.	1	0	No amplification	No amplification
Lymnaea	4	1	No amplification	*Cucumispora ornata* +ve
Culicidae	1	1	No amplification	Unconfirmed positive
Crangonyx pseudogracillis	1	1	*Encephalitozoon microsporidian*	No amplification

larvae and second freshwater mussel were not sequenced due to low concentration of product.

The same screening protocol was applied to *Dikerogammarus villosus* tissue from 30 individuals collected from Grafham Water, and did not reveal any amplicons. One out of 17 *G. pulex* (5.9%) fixed on-site at Carlton Brook was PCR positive for *C. ornata*, confirming the presence of this microsporidian in wild native amphipod populations. *C. ornata* was not detected in the 30 *G. pulex* that were fixed on-site at Meanwood Park, Leeds, via nested PCR.

3.6. Host range and mortality assessment of demon shrimp pathogens

3.6.1. *Dikerogammarus haemobaphes* mortality in response to infection

Individuals that were positively diagnosed with *C. ornata* after the survival trial showed higher mortality rates than uninfected individuals (Score [logrank] test, $p < 0.001$) (Fig. 7a). A Fisher's exact probability test identified the likelihood of microsporidian acquisition from the inoculum as not significant, likely meaning that some animals were present with the infection on arrival into the laboratory ($p = 0.22$).

Due to the availability of a PCR diagnostic for the haemocyte virus, DhbflV, it was possible to diagnose infection from *D. haemobaphes* used in the survival trial. Individuals that were PCR positive for DhbflV (9/56) showed a higher mortality rate (Score [logrank] test, $p < 0.001$) (Fig. 7b). The inoculum was PCR negative for this virus, so it is assumed that those *D. haemobaphes* positive for infection carried it into the laboratory and may have been infected at different burdens at the start of the trial.

D. haemobaphes that were fed with inoculum showed greater mortality than those in the control group, which were fed on food pellets (Score [logrank] test, $p < 0.001$). The relative difference in mortality between all individual tanks was also significant (Score [logrank] test, $p = 0.001$).

3.6.2. Mortality in *Dikerogammarus villosus* when fed on demon shrimp carcasses

Based on the nested PCR diagnostic, no *D. villosus* that were used in the experiment became infected with *C. ornata* (0/57). The animals fixed on-site at Grafham Water were also confirmed to have a 0% prevalence of *C. ornata* infection. Histological screening revealed 1 individual from the exposure group with a low-grade microsporidian infection; however, this did not provide a positive PCR result in either the first or second round of the PCR diagnostic.

Assessment of whether the exposure group differed in mortality from the control group was not significant (Score [logrank] test, $p = 0.071$), nor was the mortality difference between individual tanks (Score [logrank] test, $p = 0.082$).

3.6.3. *Cucumispora ornata* in *Gammarus pulex* co-occurring at Carlton Brook

G. pulex in the laboratory trials showed a significant increase in mortality if positively diagnosed with *C. ornata* via nested PCR, relative to uninfected individuals (Score [logrank] test, $p = 0.042$) (Fig. 8). The prevalence of infection at the end of the trial was

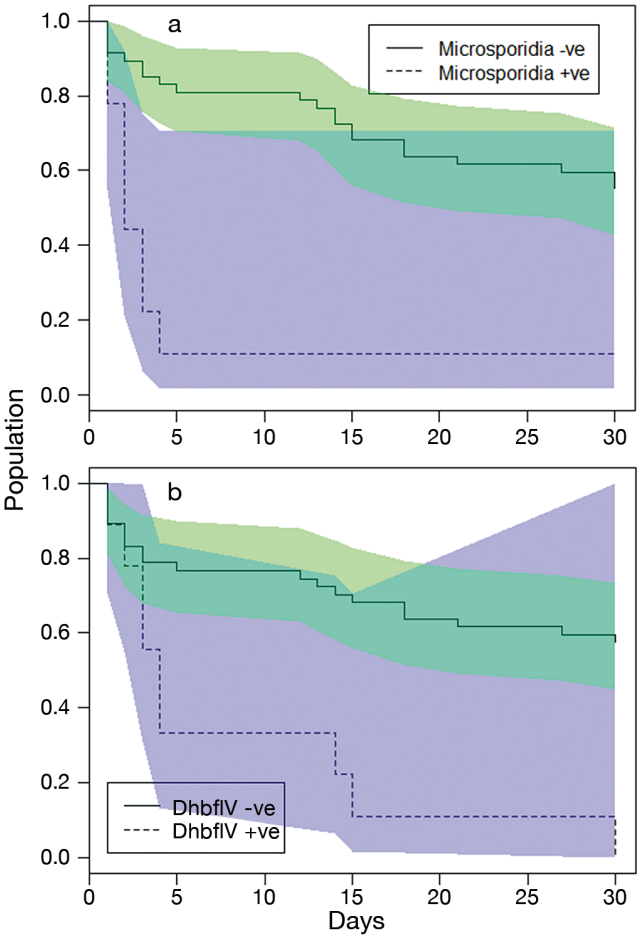


Fig. 7. *Dikerogammarus haemobaphes* survival rate with (a) *Cucumispora ornata* and (b) DhbflV infections. The *x*-axis (days) is the number of days the specimen survived and the *y*-axis (population) identifies the population size decrease over time. Shaded areas = 95% CI

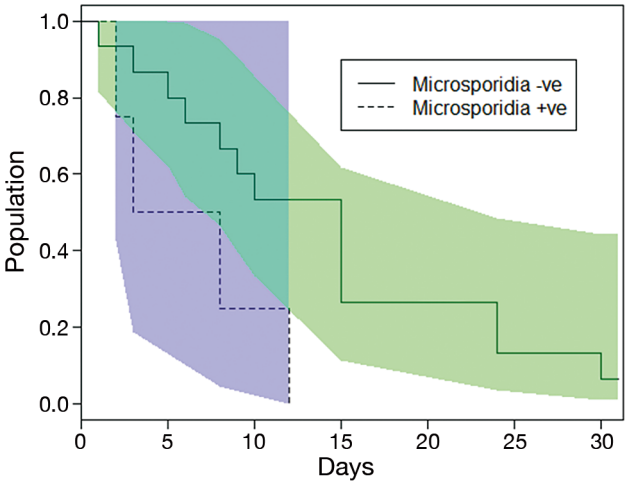


Fig. 8. *Gammarus pulex* (from Carlton Brook) survival rate comparison between those animals with *Cucumispora ornata* infection (Microsporidia +ve) and those without (Microsporidia –ve). Graph descriptions as in Fig. 7

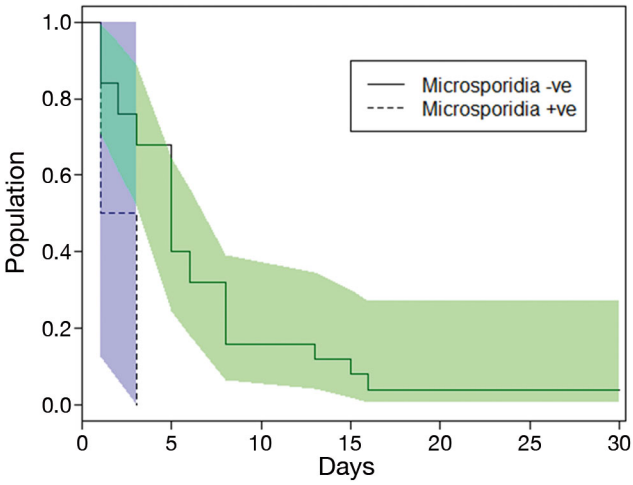


Fig. 9. *Gammarus pulex* (from Meanwood Park) survival rate comparison between those animals with *Cucumispora ornata* infection (Microsporidia +ve) and those without (Microsporidia –ve). Graph descriptions as in Fig. 7

4/19. Of the *G. pulex* collected and fixed in ethanol on-site, 1/17 animals was positive for *C. ornata*, confirming infection is present in the environment.

The effect of being present in either the control (uninfected feed) or exposure group (infected feed) was not significantly associated with mortality (Score [logrank] test, p = 0.537). Histological screening of the remaining carcass identified 1 of the PCR positive animals with a visible microsporidian infection in the musculature. Fisher's exact probability test indicated a higher prevalence in the exposed group than the control group (p = 0.054), suggesting transmission from the infected feed.

3.6.4. *Cucumispora ornata* in *Gammarus pulex* from a naïve population

No individuals were detected to be infected with *C. ornata* from the control group; however, 2 were positive for unknown microsporidian species in the first round of the PCR. Two individuals were PCR positive for *C. ornata* after mortality in the laboratory trial, both present in the 'exposure' group and fed on infected material. Those animals positive for *C. ornata* infection were associated with increased mortality relative to uninfected individuals (Score [logrank] test, p = 0.033) (Fig. 9). The on-site collection at Meanwood Park gave an overall prevalence of *C. ornata* infection in the environment as 0/30.

Whether the animals were present in either laboratory trial (control or exposure) did not associate with mortality (Score [logrank] test, p = 0.511). Histological screening revealed 1 of the second-round PCR positive animals to have a microsporidian infection in the musculature. Fisher's exact probability test did not provide a likelihood of horizontal transmission from the inoculum (p = 0.23).

4. DISCUSSION

This study aimed to explore the diversity and impacts of pathogens (including viruses, gregarines, digeneans, and microsporidians) in non-native *Dikerogammarus haemobaphes* in the UK and to test the potential for pathogen transmission to other species. We show that *D. haemobaphes* are less active when infected with high burdens of the co-introduced microsporidian pathogen *Cucumispora ornata*, but are potentially more active when infected with high burdens of DhBV infection. None of the parasites affect aggregation behaviours in their host.

C. ornata has been detected in invasive populations of *D. haemobaphes* in Germany (Grabner et al. 2015) and Poland (NCBI accession: KP699690), in addition to the Carlton Brook site in the UK where it was initially described (Bojko et al. 2015). This microsporidian was detected via nested PCR in 5 novel hosts from Carlton Brook: a freshwater mussel, a beetle larva, a freshwater snail, a native amphipod (*Gammarus pulex*), and a mosquito larvae. *C. ornata* was also detected in the *G. pulex* population collected at the same site (1/17; 5.9%) and during experimental transmission to the exposed group in the laboratory (4/10; 40%). This identifies that the

microsporidian is already present (possibly as a contaminant) in several native species and constitutes a threat to native susceptible taxa. Transmission of *C. ornata* to naïve *G. pulex* occurred (14.3%) while transmission to invasive killer shrimp *Dikerogammarus villosus* did not. Mortality correlated with the presence of *C. ornata* infection in all cases, and these non-target effects (specifically the increased mortality of the keystone shredder *G. pulex*) likely mean that this parasite cannot be adapted as a control agent and is potentially a threat to native susceptible taxa.

4.1. *Cucumispora ornata*: 'wildlife threat' or 'control agent'?

Due to the increased research effort on the symbionts of the demon shrimp, it seems prudent to review those now known and provide a pathogen profile for this species in both its native and invasive range(s) (Table 3). An understanding of microbial diversity in this species provides insights into possible biocontrol development and further risk assessment for pathogens that have potential for transfer to native susceptible hosts.

The microsporidian parasite *C. ornata* was identified to infect native *G. pulex* from 2 UK sites and has been detected in 1 *G. pulex* collected onsite from Carlton Brook. This is also the case for some insects and molluscs sampled from Carlton Brook. It is yet to be determined whether the molluscs and insects are truly infected by *C. ornata* or whether liberated spore stages are being detected as environmentally derived contaminants. For example, mussels are filter-feeding species and microsporidian spores may concentrate within the animal through bioaccumulation (Willis et al. 2014).

Survival analysis has shown that the detection of *C. ornata* in *G. pulex* is significantly associated with decreased survival rate. The analyses for this species included a low sample size due to difficulties in housing the population in the laboratory, resulting in a higher than expected control mortality. Despite the low sample sizes used in this study, is seems that *C. ornata* has

Table 3. The parasites and pathogens that have been detected from *Dikerogammarus haemobaphes* from available literature

Parasite	Species	Location	Reference
Viruses	*Dikerogammarus haemobaphes* bacilliform virus	Carlton Brook, UK	Bojko (2017), this study
	Dikerogammarus haemobaphes bi-facies-like virus	Carlton Brook, UK	Bojko (2017), this study
	Unidentified circovirus	Carlton Brook, UK	Bojko (2017)
Bacteria	*Krokinobacter* sp.	Carlton Brook, UK	Bojko (2017)
	Thiothrix sp.	Carlton Brook, UK	Bojko (2017)
	Trachelomonas sp.	Carlton Brook, UK	Bojko (2017)
	Deefgea rivuli	Carlton Brook, UK	Bojko (2017)
Apicomplexa	*Cephaloidophora mucronata*	Danube Delta	Codreanu-Balcescu (1995)
	Cephaloidophora similis	Danube Delta	Codreanu-Balcescu (1995)
Oomycete	*Saprolegnia* sp.	Carlton Brook, UK	Bojko (2017)
Microsporidia	*Cucumispora* (=*Nosema*) *dikerogammari*	Goslawski Lake and Bug in Wyszków	Ovcharenko et al. (2009)
	Thelohania brevilovum	Goslawski, Poland	Ovcharenko et al. (2009)
	Dictyocoela mulleri	Goslawski, Poland	Ovcharenko et al. (2009)
	Dictyocoela spp. ('Haplotype: 30-33')	Goslawski, Poland	Wilkinson et al. (2011)
	Dictyocoela berillonum	Unknown/Wallingford Bridge and Bell Weir, UK	Green Etxabe et al. (2015), Bojko (2017), accession no. KF830272
	Cucumispora ornata	River Trent, UK	Bojko et al. (2015)
Acanthocephala	*Acanthocephalus* (=*Pseudoechinirhynchus*) *clavula*	Danube Delta	Komarova (1969)
	Pomphorhynchus laevis	Volga River	Djikanovic et al. (2010)
Cestoda	*Amphilina foliacea*	Caspian Sea	Bauer et al. (2002)
	Bothriomonas fallax	Caspian Sea	Bauer et al. (2002)
Nematoda	*Cystoopsis acipenseris*	Volga River, Russia	Bauer et al. (2002)
Trematoda	*Nicolla skrjabini*	Danube Delta	Kirin et al. (2013)
	Undetermined Digenean	Carlton Brook, UK	This study

the potential to drive mortality in this native species. The question of nutritional value must also be noted between the artificial food pellets and the homogenate demon shrimp tissues, which could affect host survival; however, this is unlikely to have caused significant alterations to host mortality because the factor of food presence and tank was considered in the survival analysis. Cumulatively, this suggests that *C. ornata* can transmit to *G. pulex* in the UK, and this could be because it has an evolutionary history of infecting this host, or the parasite may be capable of infecting this host due to a shared lack of resistance between *D. haemobaphes* and *G. pulex*. Grabner et al. (2015) have identified this microsporidian from *D. haemobaphes* populations in Germany, and also from *G. roeselii* populations in the same area, but not from *G. pulex*, providing diagnostic data for the potential of *C. ornata* to infect some *Gammarus* sp. A parallel study by Bacela-Spychalska et al. (2012) looked at *Cucumispora dikerogammari* in invaders in France. They identify that this parasite is present in several other hosts (*Chelicorophium curvispinum*, *Chaetogammarus ischnus*, and *D. haemobaphes*) but is more common in environmental samples when *D. villosus* show high levels of infection; the high prevalence of *C. ornata* in *D. haemobaphes* in this study may increase risk to native fauna. In addition, Bacela-Spychalska et al. (2012) report a lack of *C. dikerogammari* infection in *Gammaus roeselii*, despite a similar microsporidian (*Cucumispora roeselii*) being described (Bojko et al. 2017).

C. ornata has been shown to lower the activity of its type host at high burden, and has been significantly associated with decreased survival rate, suggesting that this parasite limits its host's invasive capability, despite itself being a potential threat to UK wildlife. Increased activity and survival have been associated with invasiveness, as has also been determined for several invaders, including invasive grey squirrels across Europe (Wauters et al. 2005) and invasive *Carcinus maenas* (Torchin et al. 2001), which have parallels with amphipod populations. A decrease in activity and survival caused by the presence of pathogens may explain why *D. haemobaphes* is considered a low-impact species in the UK relative to uninfected *D. villosus* (Bovy et al. 2015). In this study *D. haemobaphes* were defined by morphology, but assessment of haplotype and infection susceptibility could further our knowledge of resistance to infection. The lack of detectable experimental transmission of *C. ornata* to invasive *D. villosus* from Grafham Water (again, haplotype not assessed) suggests it is resistant and a greater understanding of how this resistance works would be highly informative.

4.2. Effect of viruses on the activity and survival of the demon shrimp

This study has identified 2 newly discovered viruses, DhBV and DhbflV. DhBV has been observed to infect the hepatopancreas of its host and is now the third virus isolated from the hepatopancreas of an amphipod and potentially a member of the Nudiviridae (Bojko et al. 2013, 2017). This virus does not yet have a means of being detected via PCR, restricting detection to either histology or TEM.

DhBV was found at high prevalence in the UK population of *D. haemobaphes* and was significantly associated with increased activity, relative to increased viral burden. This relationship suggests that DhBV may be increasing the invasive capabilities of its host by making it more active; likely a side-effect of increasing viral transmission. However, an understanding of host survival in reference to DhBV still requires assessment. For invasive species, the presence of 'beneficial' microorganisms could provide a symbiotic relationship that increases invasiveness; a process that has been observed between invasive amphipods and their sex-distorting microsporidian pathogens, where sex change may benefit invasion success (Slothouber Galbreath et al. 2004). Studies using homopterans have found that viral infection can alter certain activities to increase viral transmission (Fereres & Moreno 2009) and this study system may have parallels for crustacean viruses and their hosts. No behavioural assays involving hosts specifically infected with nudiviruses are available to corroborate these findings, but future studies could determine whether this group of viruses are accessory to invasion capability. Roossinck (2011) explores a variety of beneficial viruses in a review, such as parvoviruses that stimulate the development of wings in aphids (conditional mutualism); polydnaviruses, which increase egg survival of parasitic wasps in their host (symbiogenic relationship); and pararetroviruses that protect plants against pathogenic viruses (symbiogenic relationship). Baculoviruses (relatives of nudiviruses) have been shown to cause behavioural change in their host, causing them to move upward (phototactic response) so that on decomposition the virions would increase their dispersal and increase their chance to infect further susceptible hosts (van Houte et al. 2014). Entomopathogenic fungi also have behavioural effects on their hosts, primarily by causing them to move higher within the canopy to spread fungal spores further — an activity-increasing behavioural response (Gryganskyi et al. 2017). Whether DhBV infection in *D. haemobaphes* also reflects a

phototactic response is unknown, but should be tested in future assays to better understand the transmission, host survival and host-effect of DhBV infection.

DhbflV is much rarer than DhBV, only being detected in hosts that underwent experimental manipulation in the laboratory. This virus infects the haemocytes of the host, causing hypertrophy of the nucleus and likely reducing its host's immunological capabilities. Similar pathognomonic signs have been reported for Caribbean spiny lobsters infected by *Panulirus argus* virus 1 (Butler et al. 2008). DhbflV was significantly associated with a decrease in survival rate; however, the histological detection of the virus revealed too few individuals to conduct adequate behavioural statistical analyses to correlate with activity or aggregation. The inoculum was PCR negative for this virus so assessment of experimental host range could not be conducted at this time. Manifestation of infection associated with this virus indicates that infected *D. haemobaphes* were likely carrying the virus prior to collection and experimental trial, suggesting that stress may trigger progression of infection to disease. These data suggest that DhbflV, alongside *C. ornata*, is a mortality driver in this invasive population and could be helping to regulate invasion impact on the UK freshwater environment.

4.3. Concluding remarks

D. haemobaphes has been considered a low-impact invader that has carried pathogens and parasites into its invasive range (Bojko et al. 2015, Green Etxabe et al. 2015, this study), a process that has also been noted for other non-native amphipod species (Bojko et al. 2017). The effects of pathogens and parasites on the *D. haemobaphes* population at Carlton Brook might explain the low direct impact of this host; however, some of these invasive pathogens are capable of infecting alternative hosts, such as the keystone shredder and native species, *G. pulex*, resulting in significant fitness costs. Hence, we need a nuanced approach to monitoring invasion risk through indirect trophic links that takes into account the entourage of invasive pathogens that impact, and are carried by, both invaders and native species.

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