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Baseline histopathological survey of a recently invading island population of 'killer shrimp', *Dikerogammarus villosus*

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ABSTRACT: *Dikerogammarus villosus*, an invasive amphipod, has recently been detected in UK freshwaters. To assess the potential for pathogen introduction with the invader, a year-long histopathology survey of the *D. villosus* population inhabiting the initial site of detection (Grafham Water, Cambridgeshire, UK) was conducted. Additional samples were collected from 2 other subsequently identified populations within the UK (Cardiff Bay and Norfolk Broads), and from established populations in France (River Rhine) and Poland (River Vistula). The data revealed a range of pathogens and commensals. Several pathogens occurring within continental populations were not present within the UK populations. Microsporidian parasites and a novel viral pathogen were amongst those not observed in the UK. The absence of these pathogens at UK sites may therefore impart significant survival advantages to *D. villosus* over native fauna, thereby increasing its success as an invader. The contrast in pathogen profile between UK and continental-invasive populations of *D. villosus* provides preliminary evidence for so-called 'enemy release' in UK populations of *D. villosus* and is suggestive of single-point introductions, rather than continual incursion events as previously observed throughout its continental invasive range. This baseline survey provides important data on the pathogen and commensal profile of a high-impact, invasive species early in its invasion history of the UK. It can be utilised to assess potential for temporal pathogen acquisition by non-native invasive aquatic species and to investigate competitive advantages placed upon this invader due to absence of important pathogens experienced within its native range.

KEY WORDS: Amphipod · Non-native invasive species · Biodiversity · Virus · Microsporidia · Parasite · Commensal

INTRODUCTION

Biological invasions often lead to altered host–parasite relationships that in turn influence their impact on the recipient community (Dunn 2009, Dunn & Perkins 2012). Invasive species may introduce novel parasites and pathogens to their new range (Stebbing et al. 2012, Strauss et al. 2012). Some acquire parasites previously unreported in the invasive species from the new environment, or benefit from a loss

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of natural enemies, including parasites, freeing resources that would otherwise be devoted to immune responses, allowing increased investment in growth and reproduction (Mitchell & Power 2003, Torchin et al. 2003, Roy & Handley 2012).

Dikerogammarus villosus (Sowinsky, 1894) is a freshwater amphipod native to the Ponto-Caspian region of Eastern Europe. Several life history characteristics of *D. villosus* are important for its success as an invader; these include a high salinity tolerance (Bruijs et al. 2001, Piscart et al. 2011), an ability to survive and grow in a wide range of temperatures (Maazouzi et al. 2011), rapid growth and early maturation, with short interbrood periods (Piscart et al. 2003, Devin et al. 2004), high fecundity (Kley & Maier 2003, Pöckl 2007), an omnivorous diet (Krisp & Maier 2005) and a capability to compete with, and prey upon, a range of cohabiting taxa, including those several trophic levels above itself (Dick et al. 2002, Krisp & Maier 2005, MacNeil et al. 2013). These attributes have resulted in *D. villosus* out-competing other gammarids within Europe, including *Gammarus pulex* (Boets et al. 2010), *G. tigrinus* and *G. duebeni* (Dick & Platvoet 2000). The invasion of continental Europe by *D. villosus* began with the construction of the Danube-Main-Rhine canal in 1992 which linked waterways of the Ponto-Caspian region to those of northwest mainland Europe, creating 'invasion corridors' along which *D. villosus* rapidly spread (Bij de Vaate et al. 2002). Ponto-Caspian populations of *D. villosus* are expected to have further colonised adjoining river systems and have invaded larger areas of continental Europe via shipping and natural upstream migration using the Danube-Main-Rhine canal (Bij de Vaate et al. 2002, Grigorovich et al. 2003, Casellato et al. 2006). In 2010, populations of *D. villosus* were detected for the first time in the UK, at Grafham Water, Cambridgeshire, and subsequently at Cardiff Bay and Eglwys Nunydd reservoir in South Wales. By 2012, the invasion reached The River Ant and Barton Broad in Norfolk.

Previous studies on pathogens of *Dikerogammarus villosus* are restricted to samples collected from continental Europe. Four species of gregarines (Apicomplexa) were described in populations from Poland: *Cephaloidophora similis, C. mucronata, Uradiophora longissima* and *U. ramosa* (Ovcharenko et al. 2009). The peritrich ciliate *Lagenophrys pontocaspica* was reported attached to the gills and oostegites of amphipods in the Ukraine, including *D. villosus*, at a prevalence of up to 80% (Boshko 1996). The trematode *Plagioporus skrjabini* was reported in *D. villosus* from the Volga River in central Russia (Chernogorenko et al. 1978). Several microsporidians have been reported to infect *D. villosus,* including *Cucumispora dikerogammari* (= *Nosema dikerogammari* Ovcharenko and Kurandina, 1987) (Ovcharenko et al. 2010), *Nosema granulosis*, *Dictyocoela roeselum, Dictyocoela muelleri* and *Dictyocoela berillonum* (Wattier et al. 2007). Despite these descriptions, to date, there have been no published reports which provide comprehensive data on the pathogen profile of *D. villosus* within its native range.

Wattier et al. (2007) surveyed 34 *Dikerogammarus villosus* populations in Europe and found no evidence for reduced genetic diversity or for loss of microsporidian parasites in the continental-invasive range of the host. This led to the conclusion that the invasion within continental Europe was either massive, recurrent or both and led to a concurrent establishment of a pathogen fauna, across the continental invasive range of *D. villosus*. In contrast, the establishment of the recently identified UK *D. villosus* populations is likely to be the result of isolated introductions. This different introduction pathway poses the question of whether the pathogen fauna in UK populations may be depleted when compared to that found within native or continental-invasive range populations of this species. This is hinted at by a survey which included a small number of samples of *D. villosus* obtained from Grafham Water in which no evidence was found for microsporidia within the genus *Dictyocoela* (Wilkinson et al. 2011).

MATERIALS AND METHODS

Field sampling

Dikerogammarus villosus were sampled monthly (n = ~150 mo^{-1}) from Grafham Water, Cambridgeshire, between September 2011 and August 2012. Since *D. villosus* inhabit gaps within rocky substrates, unbaited 'colonisation samplers' constructed from air bricks were deployed on the shoreline of the reservoir. During colder months, *D. villosus* were less frequently found within colonisation samplers. At these times, *D. villosus* were collected from within rope-growing populations of zebra mussels *Dreissena polymorpha*, a co-existing invasive species at this site. In addition to the seasonal survey of *D. villosus* from Grafham Water, animals were also collected from other UK populations at Cardiff Bay and the Norfolk Broads, and from sites within its continental-invasive range in France (Chalampé, Rhine River) and Poland (Vistula River) (Table 1).

Table 1. Site-specific sampling dates (dd/mm/yyyy), locations and parasite prevalence. DvBV: *Dikerogammarus villosus* bacilliform virus

Collection site	Grid reference/ coordinates	Date sampled	Sample size (n)	Micro-sporidia (n)	Nema-tode (n)	Digenea (n)	Ciliate (n)	Isopod (n)	Bryo-zoan (n)	Commensal worm (n)	Parasitic worm (n)	Gregarine (n)	Bacterial infection (n)	DvBV (n)
Grafham Water	TL150680	01/09/2011	144	0	0	2	23	10	0	0	0	65	0	0
Grafham Water	TL150680	14/09/2011	151	0	0	3	28	7	1	0	0	60	0	0
Grafham Water	TL150680	26/10/2011	147	0	1	1	116	0	9	0	0	79	0	0
Grafham Water	TL150680	10/11/2011	122	0	0	1	34	0	1	0	0	64	0	0
Rhine River, France	47.81° N, 7.54° E	19/11/2011	291	41	0	0	68	0	0	0	2	176	1	0
Grafham Water	TL150680	19/12/2011	131	0	0	0	66	0	2	3	0	74	0	0
Grafham Water	TL150680	19/01/2012	168	0	0	0	117	0	8	0	0	89	1	0
Grafham Water	TL150680	20/02/2012	167	1	0	1	97	1	9	0	0	108	0	0
Grafham Water	TL150680	19/03/2012	167	0	0	1	120	4	4	0	0	89	0	0
Grafham Water	TL150680	16/04/2012	184	0	0	0	106	1	0	0	0	96	0	0
Norfolk Broads	TG390183	02/05/2012	178	0	0	0	144	0	0	0	0	0	0	0
Vistula River, Poland	54.33° N, 18.93° E	20/05/2012	298	28	0	0	155	1	0	2	4	130	1	4
Grafham Water	TL150680	21/05/2012	169	0	0	2	16	8	0	0	0	89	0	0
Grafham Water	TL150680	18/06/2012	119	0	0	0	54	1	1	0	0	35	0	0
Grafham Water	TL150680	16/07/2012	109	0	0	1	14	2	3	0	0	49	0	0
Grafham Water	TL150680	06/08/2012	159	0	0	3	31	3	6	0	0	17	0	0
Cardiff Bay	ST182759	08/08/2012	195	0	0	0	169	1	0	0	0	46	0	0
		TOTAL =	2899											

Histopathology

Individual specimens (n = 2899) were euthanised by initially being placed on ice following fixation for histology by direct injection with and submersion in Davidson's freshwater fixative (Hopwood 1996) over the study period. Of these, 2310 animals were sampled from sites within the UK, 291 were sampled from France, and 298 were sampled from Poland (Table 1).

After 24 h, samples were transferred to 70% industrial methylated spirit (IMS). Whole-animal histological preparations of *Dikerogammarus villosus* were created by initial placement of fixed specimens in 'Rapid Decalcifier II' for 30 min with frequent agitation. Paraffin wax infiltration was completed using a vacuum infiltrating automated tissue processor (Poloris, Leica Microsystems) before specimens were embedded longitudinally into paraffin wax blocks. Blocks were sectioned using a rotary microtome (Finesse E/NE, Thermofisher) at 3 to 4 µm thickness. Sections were mounted onto glass slides via a water/ Sta-on mix prior to rehydration and staining with haematoxylin and alcoholic eosin (H&E) using an autostainer (Surgipath). Stained slides were cover-slipped (Consul, Thermofisher) prior to reading with bright field light microscopy. Longitudinal sections of individual *D. villosus* were screened for previously reported pathogens (Table 2) and for novel infections according to the ISO17025 accredited system for the screening of crustacean pathogens via histology, in place at the Cefas Weymouth Laboratory. Digital images were obtained using a Nikon Eclipse E800 light microscope with an integrated LEICA™ (Leica) camera and LuciaG software (Nikon).

In several cases, electron microscopy preparations were made from samples previously processed for wax histology. In such cases, reprocessing involved resectioning of the specific region of interest from wax blocks. The resection was subsequently divided into small cubes (ca. 1–2 mm^3) prior to submerging in Clearene. Clearene was changed regularly to systematically remove residual wax from the sample. The process was continued overnight to ensure wax removal prior to replacement of the Clearene with 70% IMS, which was

Table 2. Previously identified pathogens of *Dikerogammarus villosus*

Taxon	Source
Microsporidia	
Cucumispora dikerogammari	Ovcharenko et al. (2010)
Nosema granulosis	Wattier et al. (2007)
Dictyocoela muelleri	Wattier et al. (2007)
Dictyocoela roeselum	Wattier et al. (2007)
Dictyocoela berillonum	Wattier et al. (2007)
Apicomplexa	
Cephaloidophora similis	Ovcharenko et al. (2009)
Cephaloidophora mucronata	Ovcharenko et al. (2009)
Uradiophora longissima	Ovcharenko et al. (2009)
Uradiophora ramosa	Ovcharenko et al. (2009)
Ciliata	
Lagenophrys pontocaspica	Boshko (1996)
Trematoda	
Plagioporus skrjabini	Chernogorenko et al. (1978)

again changed regularly and left overnight to ensure optimum infiltration. IMS was then washed away using distilled water prior to placing the sample into 2.5% glutaraldehyde in 0.1% sodium cacodylate buffer for 1 h. Fixed specimens were sectioned transversely into several pieces using a razor blade. Specific samples were stained using 1% osmium tetroxide (OsO_4) before dehydration using an acetone dilution series. Each sample was embedded into Agar 100 resin after initial infiltration of the resin via a resin–acetone dilution series (1 h per dilution). Polymerisation was achieved via heating at 60°C for 16 h. Blocks were cut into semi-thin sections using a Reichert Ultracut Microtome equipped with glass blades and stained with Toluidine Blue for screening using an Olympus BH-2 light microscope. Ultra-thin sections were taken using a Diatome diamond blade and stained using uranylacetate and Reynolds Lead Citrate (Reynolds 1963) for reading on a Jeol JEM-1400 transmission electron microscope (TEM).

RESULTS

In total, we assessed 2899 individual *Dikerogammarus villosus* using histology for the presence of known and novel pathogens (Table 1). In addition, a histological atlas of normal (uninfected tissues and organs) was constructed and forms part of the Registry of Aquatic Pathology available at the Cefas Weymouth Laboratory. Longitudinal sectioning of whole specimens of *D. villosus* allowed for consistent assessment of all major organ and tissue types. Direct fixation via injection ensured that the integrity of these tissues and organs remained consistently high throughout the study. The anatomical placement of major organs and tissues within *D. villosus* was consistent with that of other gammarid amphipods.

Grafham Water and other UK sites

Dikerogammarus villosus obtained from sites within the UK were associated with several pathogens and commensals previously described from this host (Table 2) as well as a number of commensals not previously reported. Gregarines were the most common commensal observed within *D. villosus* from Grafham Water. Gregarines occurred within the midgut and occasionally within the hepatopancreas of up to 65% of the animals surveyed in each of the months. Whilst the taxonomic status of these gregarines was not confirmed, 2 main morphotypes were recorded. Morphotype 1 was elongate with no distinctive epimerite (at least via histology) and may be homologous to the previously described *Uradiophora* sp., associated with *D. villosus* in its continental-invasive range. In contrast, morphotype 2 was defined by a large rounded deutomerite and distinct epimerite (Fig. 1a). In this respect, it resembles *Cephaloidophora* sp., another gregarine found within *D. villosus* from its continental-invasive range. Whilst gregarines often reached significant numbers within the gut lumen (Fig. 1a–c), in the vast majority of cases, they were not associated with an obvious host pathology. In this respect, we have used the term commensal rather than parasite. However, in rare cases, sloughing of epithelial cells from the basement membrane, in association with breach of the basement membrane, led to pronounced host reactions within the surrounding regions. In these instances, host haemocytes aggregated at the wound site and elicited a granulomatous response leading to formation of melanin (Fig. 1c). Numerous life stages of these gregarines were observed in *D. villosus* from Grafham Water. These included intracellular life stages within gut epithelial cells (Fig. 1d) and gregarine cells undergoing syzygy (pairing) to form zygotes (Fig. 1e,f).

The metacercariae of digenean parasites were observed at low prevalence (<2%) throughout the sampling period (Fig. 2a). In the majority of cases, amphipods possessed a single digenean metacercaria in individual tissue sections, usually within the connective tissues surrounding the gut, hepatopancreas or gonad and in one instance within the gill. In rare cases, more than one metacercaria were observed in a single tissue section. Pathologies associ-

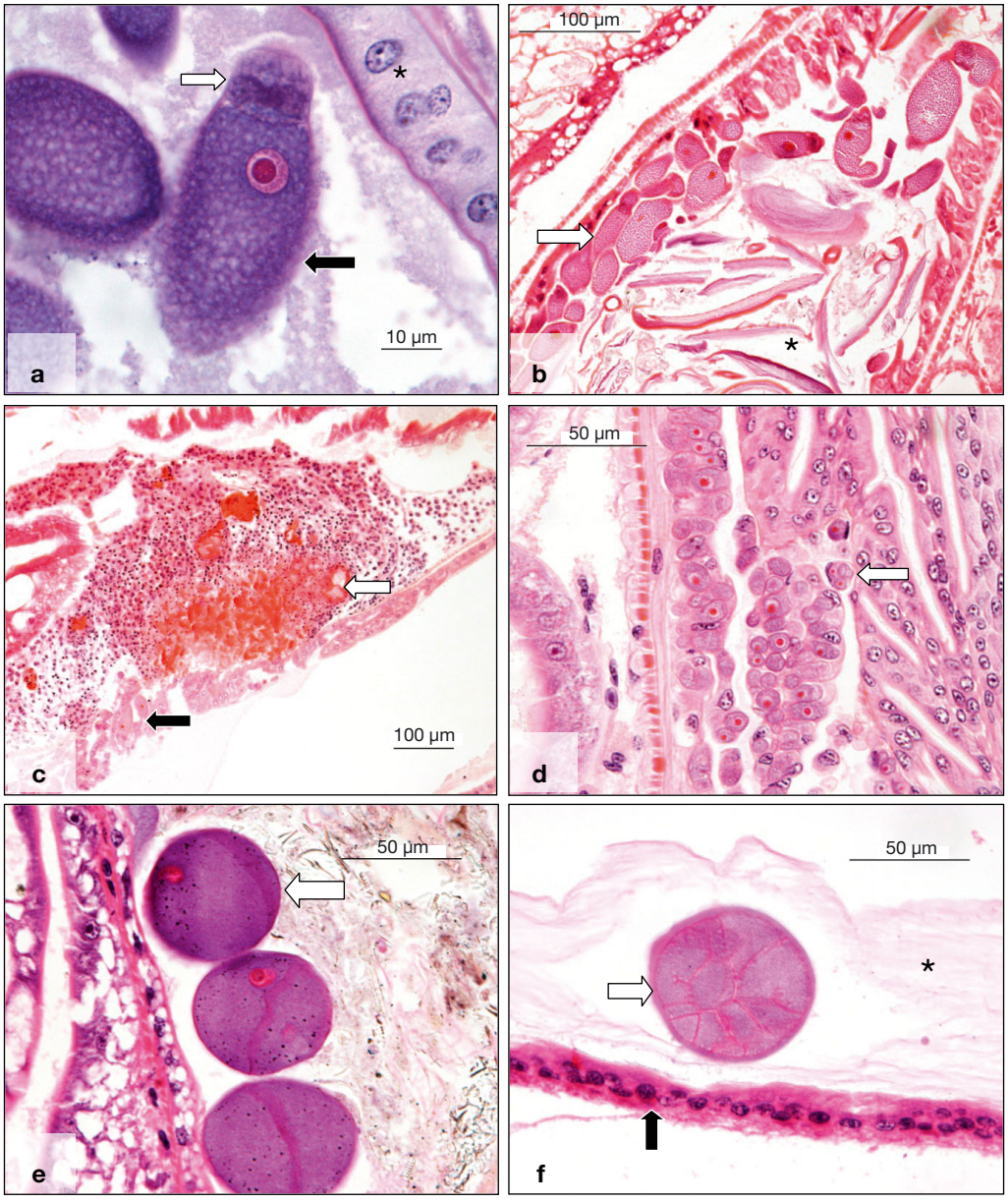

Fig. 1. Unidentified gregarine pathogens inhabiting the gut lumen of *Dikerogammarus villosus*. (a) Individual gregarine parasite in gut lumen. Protomerite (white arrow), deutomerite (black arrow), * = gut epithelium. (b) Congestion of gut lumen with masses of gregarine parasites (white arrow). The gut lumen (*) contains a food bolus comprised of insect or crustacean material. (c) Breach of gut epithelium caused by gregarine infection. Resulting haemocyte aggregation and melanisation at the site of the wound (white arrow) associated with the presence of gregarines (black arrow). (d) Early-stage infection showing developing gregarine parasites within mid-gut epithelial cells (white arrow). (e) Gregarines undergoing gamete formation (syzygy; white arrow). (f) Zygote formation leading to spore development. The gregarine zygote (white arrow) remains in direct contact with the gut lumen (*) and detached from the gut epithelium (black arrow)

ated with metacercaria included 'pearling', haemocyte aggregation and occasionally, a melanisation response. Whether the digenean is *Plagioporus skrjabini* (Chernogorenko et al. 1978), a digenean trematode reported to infect *Dikerogammarus villosus* in the Volga River, Russia, remains to be shown.

Epibiotic peritrichious and stalked ciliates were commonly observed attached to the carapace, gills, appendages and marsupium of *Dikerogammarus villosus* (Fig. 2b). The prevalence of these epibionts ranged from 9.5% (May 2012) to 78.9% (October 2011). The presence of these ciliates appeared to elicit

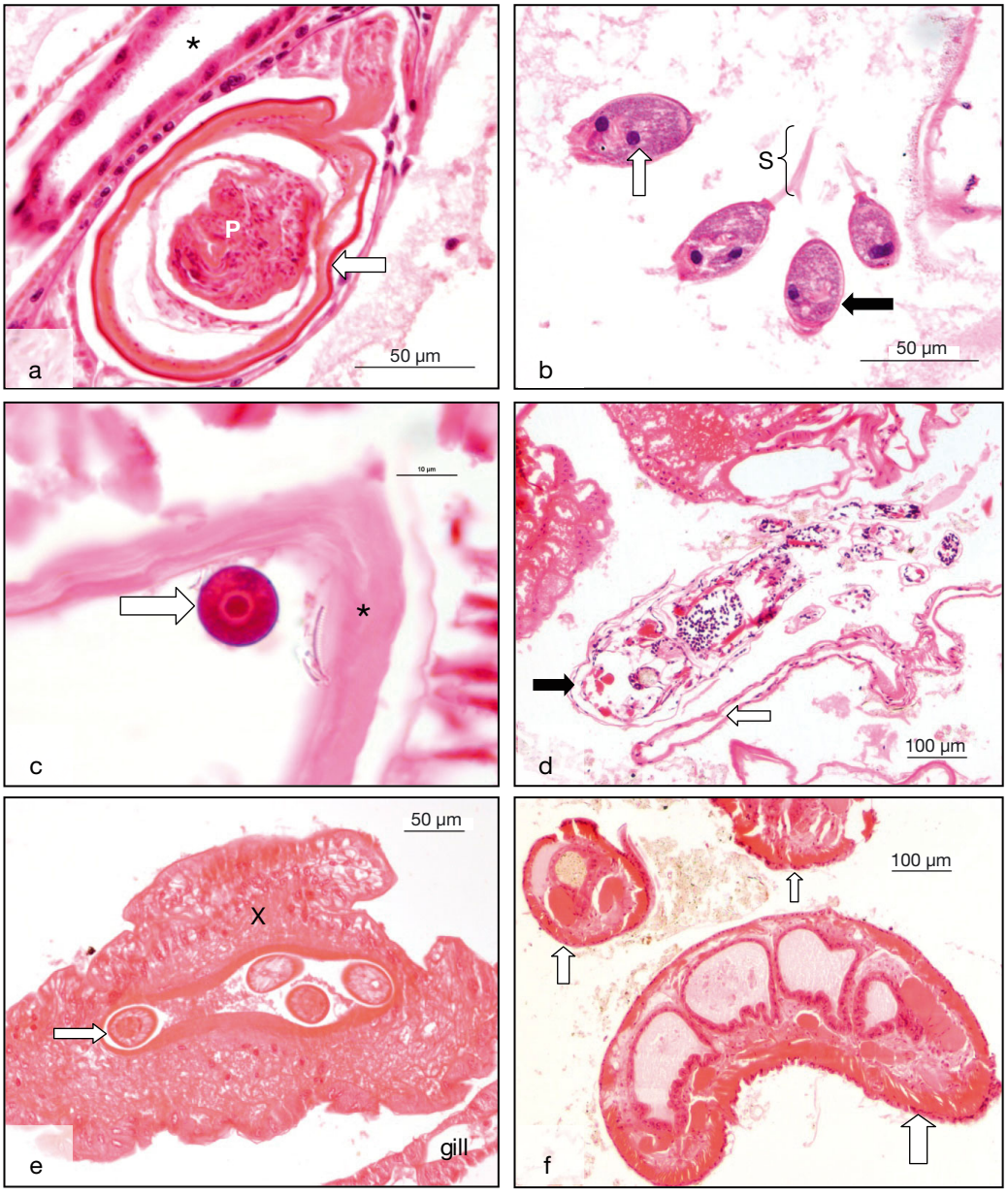

Fig. 2. Observations of novel parasites and symbionts associated with *Dikerogammarus villosus* sampled from Grafham Water, UK. (a) Digenean metacercaria within the gonad of a female (white arrow). Parasite pharynx (P), gut lumen (*). (b) Peritrichious ciliates attached to the carapace (black arrow). A section through the U-shaped nucleus of a ciliate (white arrow). Attachment stalks for anchorage to the carapace/gills/marsupium (S). (c) Putative bryozoan statoblast (white arrow) attached to the carapace (*). (d) Isopod (black arrow) residing within the gill cavity (white arrow) of a male. (e) Nematode infection (white arrow) within the gill cavities. Haemocytic aggregate in response to nematode infection (X) adjacent to normal gill (gill). (f) Commensal oligochaete worm (white arrows) residing within the gill cavity

no pathology. Commensal bryozoans, usually present as a single statoblast, were occasionally observed in most survey months, reaching their highest prevalence in October 2011 (6.1%). Bryozoa were observed at the joints of the appendages. On rare occasions, other life stages were also observed, but in all cases, no associated pathology accompanied colonisation.

Parasitic isopods were occasionally observed associated with *Dikerogammarus villosus* sampled from Grafham Water (Fig. 2d). Their prevalence was low in most months (reaching 4.7% in May 2012). Isopods were associated with the gill cavity, appendages and most commonly, the marsupium. Although relatively large symbionts, the association did not appear to

elicit significant negative effects on the host, although on occasion, haemocytic aggregates and melanisation were observed within the vicinity of isopods.

Rare cases (n = 3 over the study period) involving colonisation by oligochaetes were observed in the Grafham Water population of *Dikerogammarus villosus* sampled in December 2011 (Fig. 2f). Worms were found either associated with the gill cavity, or with the appendages of affected hosts. Once again, no pathology was associated with the presence of these worms. A nematode infection was observed within the gill lamellae of a single specimen of *D. villosus* sampled from Grafham Water in October 2011 (Fig. 2e). This is the first observation of a nematode infection in this species. Haemocyte aggregation was associated with the nematode infection.

A single bacterial infection was observed in the gonad of an animal sampled from Grafham Water in January 2012. This infection was confined to a single oocyte and did not elicit any immune response, suggesting that the infection was at an early stage. Other than this single event, no further bacterial infections were identified in UK populations of *Dikerogammarus villosus*.

A microsporidian infection was observed infecting the peripheral sarcolemma of skeletal muscle fibres in a single specimen of *Dikerogammarus villosus* sampled from Grafham Water (Fig. 3d–f). Reprocessing of this histological sample for TEM revealed that the microsporidian spores were likely contained within a sporophorous vesicle, were monokaryotic and possessed 10 coils of the polar filament (Fig. 3d–f). These features were inconsistent with the currently known microsporidian pathogens infecting this host within its native or continental-invasive range (Table 2). Due to post-fixation of the specimen for histology, detail in Fig. 3e,f may not be accurate when measured. However, distinct organelles are present and the number of coils of the polar filament can be distinguished, as well as the number of nuclei, leading us to conclude that this is a separate morphology when compared to the only other morphologically derived microsporidian specimen from a *D. villosus*, viz. *Cucumispora dikerogammari* (Ovcharenko et al. 2010).

The pathogen and commensal fauna observed in the single sample of 195 *Dikerogammarus villosus* collected from Cardiff Bay was similar to those observed within animals obtained from Grafham Water in that month (April 2012) of the seasonal survey. The prevalence of gregarines was lower (24%) whilst epibiotic ciliates were commonly observed (87%). A single case of isopod colonisation was observed. No microsporidian infections were observed in samples from Cardiff Bay.

Analysis of the single sample of 178 *Dikerogammarus villosus* collected from Barton Broads, Norfolk, revealed an apparent absence of all aforementioned pathogens and commensals with the exception of epibiotic ciliates inhabiting the carapace, appendages, gills and marsupium. The ciliates were present, usually in large numbers, in 81% of the specimens, but once again, were not associated with any obvious host pathology.

Continental Europe

Histopathology of *Dikerogammarus villosus* obtained from 2 individual samples from France and Poland revealed a range of symbionts and pathogens. As observed in the Grafham Water survey, gregarines were commonly observed in samples of *D. villosus* obtained from France (57% prevalence) and Poland (43% prevalence). Further, the observed life stages and morphotypes were consistent with those observed in *D. villosus* obtained from Grafham Water in the UK (Fig. 1). Unidentified species of peritrichious and stalked ciliates were observed in samples of *D. villosus* obtained from France and Poland. The ciliates were similar to those observed colonising the gills and appendages of *D. villosus* from Grafham Water, although at a generally lower prevalence (France: 23%, Poland: 52%).

The most significant pathogenic agent observed in *Dikerogammarus villosus* obtained from continental samples was a microsporidian infecting the skeletal musculature. The musculature of infected animals was progressively replaced by masses of parasite spores, causing degradation of muscle fibres and often eliciting a significant haemocytic response, presumably to ruptured muscle fibres (Fig. 3a–c). The location of infection (musculature) and associated pathology was consistent with *Cucumispora dikerogammari* (Ovcharenko et al. 2010), previously known to infect *D. villosus* within continental Europe. Infection prevalence was 14% in the sample obtained from France and 9% in the sample obtained from Poland.

A pathology consistent with infection by a DNA virus (hereby reported as *Dikerogammarus villosus* bacilliform virus = DvBV) was noted in 4 specimens (<1%) of *D. villosus* sampled from Poland. The pathology, recorded in the hepatopancreas of affected amphipods, included hypertrophy of the host cell

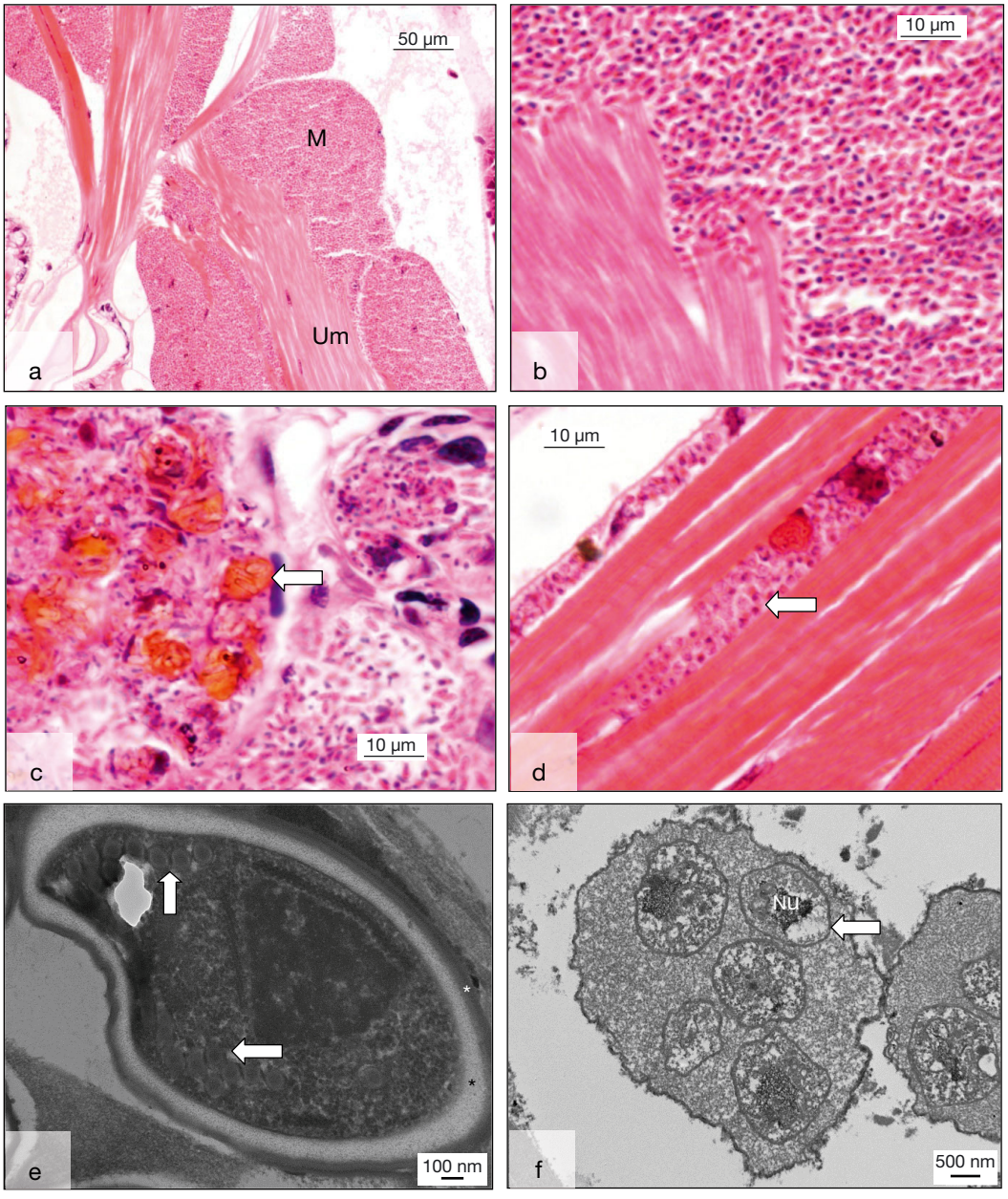

Fig. 3. Microsporidian parasite infection of the musculature of *Dikerogammarus villosus* sampled from France (a–c) and from Grafham Water, UK (d–f). (a) Skeletal muscle tissue infected with the microsporidian parasite *Cucumispora dikerogammari* (Ovcharenko et al. 2009); replacement of normal fibres (Um) with microsporidian life stages (M). (b) Higher magnification of (a): Microsporidian spores displace normal fibre structure and are closely associated with individual myofibrils. (c) Haemocytic aggregation and melanisation associated with a ruptured muscle fibre containing parasite life stages. Melanisation was associated with individual spores (white arrow). (d) Skeletal muscle tissue infected with an unidentified microsporidian parasite. Small parasitic cysts (white arrow) displaced otherwise normal myofibres. (e) TEM image from re-processed histological material showing a microsporidian spore found within muscle tissue. The spore was unikaryotic, contained a thick exospore, an electron-dense endospore and 9 to 10 turns of an isofilar polar filament (white arrows). (f) TEM image from other regions within infected musculature showing apparent meront plasmodia containing multiple unikaryotic nuclei (Nu, white arrow)

nucleus and margination of chromatin, consistent with displacement by a viroplasm. Infected cells became detached from neighbouring epithelial cells and from the basement membrane and were occasionally observed undergoing apoptosis (Fig. 4a,b). This pathology appears similar to that caused by other DNA viruses in crustaceans, such as the white clawed crayfish *Austropotamobius pallipes* (Long-

shaw et al. 2012). *Austropotamobius pallipes* bacilliform virus is a DNA virus found infecting the hepatopancreas of the white clawed crayfish and although not exactly the same, the relative pathologies indicate that DvBV is most likely bacilliform in nature. Re-processing of histological material for TEM from infected amphipods revealed enlarged nuclei containing masses of rod-shaped virus particles (Fig. 4c,d). Virions consisted of an electron-dense core surrounded by an envelope. Enveloped virions (n = 30) measured approximately 278 ± 25 nm in length, 68 ± 28 nm in diameter with the core measuring 246 ± 7 nm in length and 45 ± 4 nm in diameter.

Incidental observations within *Dikerogammarus villosus* sampled from the continental-invasive range included 2 cases of a large metazoan parasite occupying the haemocoel between the ventral nerve cord and the hepatopancreas of 2 specimens from France (Fig. 5) and 4 specimens from Poland. This metazoan may be similar to, or the same as, *Pomphorhynchus tereticollis*, a recently discovered parasite targeting *D. villosus* in continental Europe (Emde et al. 2012).

In the Polish sample, several cases of bacterial infection were observed, with the majority resulting in small melanised lesions within the haemocoel and connective tissues. A single, localised, bacterial infection was found in a specimen from France. The infection was targeted to an immature oocyte in the gonad. Melanisation reactions could be seen surrounding the infection.

DISCUSSION

This study provides a baseline dataset for the pathogens and commensals of an important invasive species, early within its invasion history of the UK. By comparing our findings with previously recorded pathogens from continental-invasive populations of *Dikerogammarus villosus* and from samples collected from continental Europe in this study, the data have demonstrated that the pathogens present at discrete sampling sites are different and in some cases, previously recorded pathogens are absent altogether. The study provides important baseline infor-

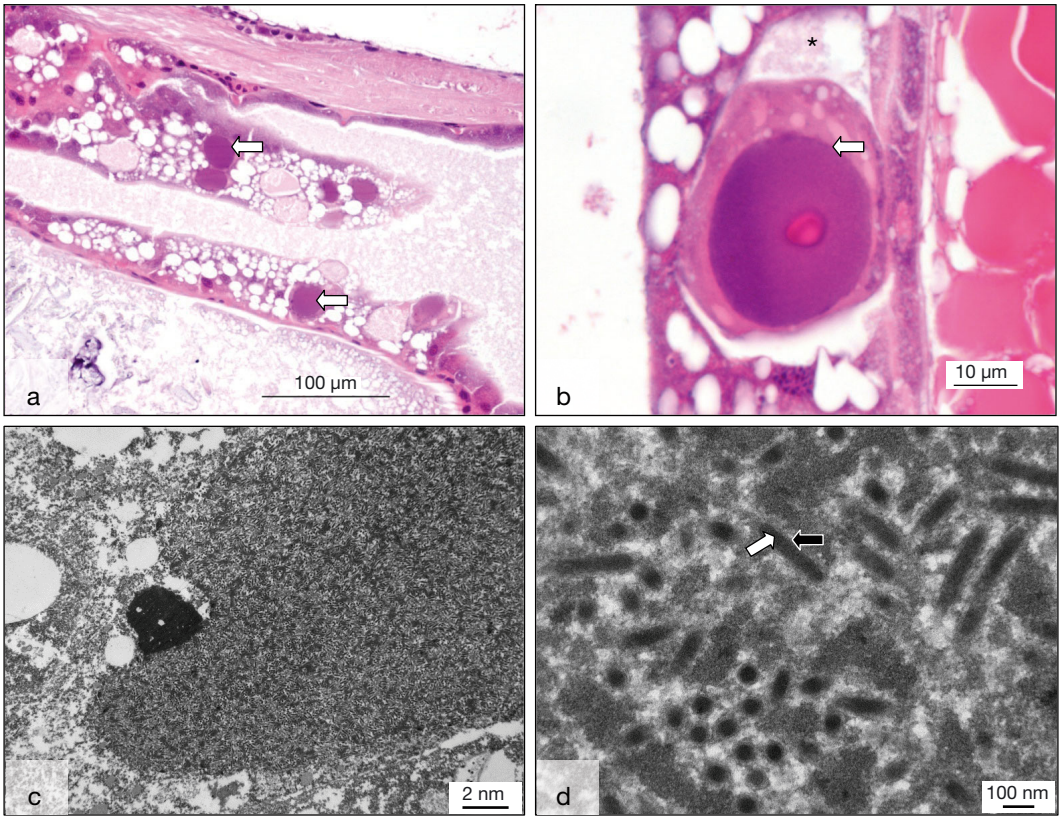

Fig. 4. Rod-shaped virus within the hepatopancreas of *Dikerogammarus villosus*. (a) Hypertrophied nuclei visible distributed throughout the hepatopancreas (white arrows). (b) Margination of the chromatin (white arrow) is evident and affected cells appear to become detached from their neighbours (*). (c) Hypertrophied nuclei found to contain multiple rod-shaped virions. (d) Virions consist of a rod-shaped electron-dense core (white arrow) surrounded by an envelope (black arrow)

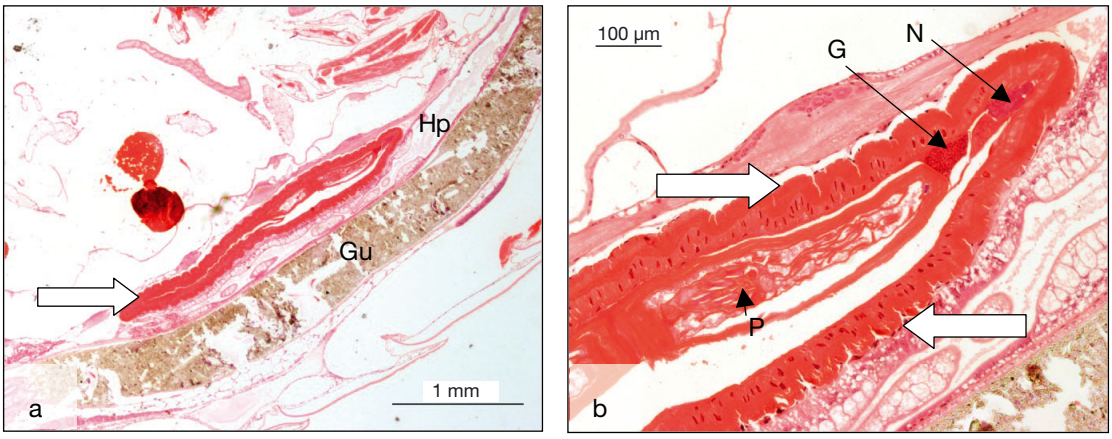

Fig. 5. Unidentified metazoan infecting *Dikerogammarus villosus* sampled from France. (a) Low-power image depicts presence of parasite (white arrow) adjacent to gut (Gu) and hepatopancreas (Hp) of the host. (b) Higher magnification image of the parasite shown in (a). White arrows highlight the close connection of the folds of the parasite integument with host nervous system and hepatopancreas. The pharynx (P) is visible, and towards the right of the image are the nervous tissue (N) and gonads (G)

mation for further assessment of so-called 'enemy release' in aquatic invasive arthropods and also, is an important resource for future studies of pathogen acquisition in an invading host. Specifically, *D. villosus* from Grafham Water hosted apicomplexans, trematodes, epibiotic and stalked ciliates, bryozoans, isopods, oligochaetes, nematodes and a novel microsporidian (in a single specimen). The Cardiff Bay population hosted a similar range of pathogens and commensals, whilst the population sampled from the Norfolk Broads displayed a distinct lack of associations, with only epibiotic and stalked ciliates observed. Populations sampled from within the continental-invasive range of *D. villosus* hosted a similar range of pathogens and commensals to those observed in Grafham Water, with the notable addition of pathogenic microsporidia, unidentified endoparasitic metazoans, and in the case of the Polish subsample, a novel DNA virus (DvBV) infecting the gut epithelia. To our knowledge, this is the first report of a bacilliform virus infecting an amphipod.

We have also demonstrated the utilisation of histopathology as a screening tool for the detection of known and novel pathogens in amphipods such as *Dikerogammarus villosus*, thereby supporting previous work from our laboratory on decapod crustaceans (Stentiford & Feist 2005, Stentiford 2008, Bateman et al. 2011). The tool is useful in that it provides a non-targeted approach to pathogen screening and hence allows for detection of novel pathogens that would not be easily detected via other means (e.g. the identification of DvBV in Polish populations). Despite its utility, the small size of the study animal does pose certain limitations on the co-collection of samples for molecular diagnostics and for TEM. Accordingly, confirmation of specific taxa (e.g. of gregarines) was somewhat limited in the current baseline study. Future efforts will attempt to optimise co-collection strategies for samples from individual amphipods. The use of laser dissection microscopy may assist with this. Small et al. (2008) described the use of this technique to further characterize 2 species of dinoflagellate (*Hematodinium* spp.) in *Cancer pagurus* and *Portunus tritubercalatus* tissues fixed using Davidson's fixative, which is destructive to DNA, but crucial for histological analysis. Small et al. (2008) were able to provide genetic data from the inappropriately fixed specimens. This technique would aid in obtaining DNA from pathogens infecting *D. villosus* fixed in Davidson's fixative and other fixatives that are inappropriate for DNA preservation, thus obtaining molecular data for relatively rare pathogens.

Judging by the lack of associated pathology, the majority of the newly observed associations in *Dikerogammarus villosus* collected from Grafham Water (bryozoans, isopods, oligochaetes, ciliates) appear to pose little threat to the general health status of *D. villosus* inhabiting UK invasion sites. Digenean trematodes have a complex life cycle involving 2 or more hosts (Chernogorenko et al. 1978); hence parasites located near to the native range of *D. villosus* which could be introduced with the invader may be less likely to find a suitable penultimate host in which to complete their life cycle. In this respect, it is probable that the trematode infections observed in *D. villosus*, in this study, came from Grafham Water, the newly invaded habitat. This could be an example of *D. villo-*

sus becoming an opportunistic host or even vector for trematodes acquired from Grafham Water. Similarly, the ultrastructure of the microsporidian observed in a single *D. villosus* sampled from Grafham Water differed from those previously recorded in *D. villosus* from Continental Europe by Wattier et al. (2007) and Ovcharenko et al. (2010). *Cucumispora dikerogammari* is the only morphologically studied microsporidian found in *D. villosus* (Ovcharenko et al. 2010), and so morphological comparison can only be made with this parasite. Other microsporidia found associated with *D. villosus* were identified by PCR (Wattier et al. 2007). The microsporidian found infecting *D. villosus* in Grafham Water has 10 turns of the polar filament and is unikaryotic, whereas *C. dikerogammari* has 6 to 8 turns of the polar filament and is diplokaryotic. As no molecular information could be derived from the histology-fixed specimen infected with the microsporidian from Grafham Water, no molecular comparison can be made at this stage.

It is likely that this microsporidian is a post-invasion acquisition from a co-habiting native species. This is further supported by observations of very low infection intensity (individual muscle fibres implicated) and prevalence in *Dikerogammarus villosus* collected from Grafham Water (0.05% over the year sample period). This low prevalence can be indirectly compared to the relatively high prevalence of microsporidian pathogens infecting *D. villosus* collected from the continental-invasive range (France: 14%, Poland: 9%). No other microsporidian infections have been observed in any of the UK populations studied. The isolated cases of microsporidian (and nematode) infections in individuals sampled from Grafham Water may therefore be opportunistic infections acquired from consumption of infected prey or via wounding, at the invasion site. Such explanations are consistent with high predatory capabilities of *D. villosus* (Dick et al. 2002). The relative lack of pathogens and symbionts in *D. villosus* obtained from the Norfolk Broads population may be used to hypothesize that the former site was seeded from another UK population (Grafham Water, Cardiff Bay, or possibly another yet to be identified population) and not directly from continental-invasive populations. The lack of gregarine associations in specimens from the Norfolk Broads site seems particularly surprising since these associations are observed in at least 30% of specimens from all other sites tested (including those in the UK). Further work may be required to understand how the *D. villosus* population at Norfolk Broads can be almost completely unaffected by parasites.

Have *Dikerogammarus villosus* undergone 'enemy release'?

In an extensive study of populations of *Dikerogammarus villosus* across their continental-invasive range, Wattier et al. (2007) found no evidence for freedom from microsporidian parasites. Our data for sites within the continental-invasive range (France and Poland) are in accord with these findings, showing microsporidian prevalence at up to 14% within these populations. Wattier et al. (2007) utilised these data to propose that *D. villosus* had likely undergone massive or recurrent introductions during the colonisation of Europe, and hence had moved into this new range replete with those pathogens occurring within its native range. In contrast, the absence of important pathogens (microsporidia) observed within UK populations of *D. villosus* sampled during our study may instead reflect focal, non-recurrent introductions, leading to a loss of pathogen fauna during the invasion process. Further data and comparison with *D. villosus* from the Ponto-Caspian native range would be needed to confirm this. Dependent on the nature of the introduction(s), pathogen loss from the invading founder populations may be facilitated by either low initial host (and pathogen) density at the invasion site, or death of heavily infected animals (and their pathogens) during the translocation process (Torchin et al. 2003, Torchin & Mitchell 2004). The fact that translocation to Grafham Water and other UK sites likely occurred via anthropogenic activities, as discussed in several papers, (e.g. via sailing craft) may have assisted the selection of the fittest/less parasitized individuals to survive in transit to the new location (Bij de Vaate et al. 2002, Grigorovich et al. 2003, Casellato et al. 2006, Yang et al. 2010). Similar scenarios have been discussed for UK invasions of the Chinese mitten crab *Eriocheir sinensis* and its pathogens (Stentiford et al. 2011).

Biosecurity and biocontrol considerations

Understanding introduction pathways and the subsequent control of invasive species is a high-priority topic for national and international governments. The priority is based upon the negative effects of invasive species on native biodiversity (illustrated for *Dikerogammarus villosus* by MacNeil et al. 2013). Given the observed absence of important pathogenic agents (e.g. *Cucumispora dikerogammari*), and the apparent population success, in UK populations of *D. villosus*, it is interesting to speculate how this rel-

ative 'disease freedom' may be manipulated in order to reduce the relative competitive advantage of this species. So-called 'biological control' refers to the mitigation of pest populations by a range of natural mechanisms from predation through to parasitism. Although the technique is widely used as part of integrated pest management strategies to control insect populations in agricultural settings, studies within aquatic habitats have mainly focussed on the control of aquatic plants via arthropod consumers (Reeves & Lorch 2012). Other aquatic pests, in particular invertebrates, are relatively unstudied, with very few investigations attempting to provide context to the potential use of aquatic pathogens to control invasive species (Davidson et al. 2010).

This year-long study revealed *Dikerogammarus villosus* from Grafham to be missing 3 pathogens observed in European populations: microsporidia, endoparasitic metazoa and DvBV. As such, the absence of pathogenic agents in these populations may provide an opportunity to develop aquatic pathogen-oriented biological control. The microsporidian parasite *Cucumispora dikerogammari* and other horizontally transmitted microsporidia occur at prevalences up to 20% in *D. villosus* continental-invasive ranges (Wattier et al. 2007, Ovcharenko et al. 2010, this study) but are absent from UK sites (Wilkinson et al. 2011, this study). Further, recent observations by Bacela-Spychalska et al. (2012) suggest that *C. dikerogammari* may have low capability to infect and become established in non-target hosts. The observed microparasite loss, coupled with an infrequent introduction regime and the focal nature of pioneer populations, may provide appropriate circumstances for testing potential biocontrol agents against *D. villosus*. Host-specific microsporidian and viral pathogens may be ideal candidates for such an approach through appropriate assessment of risk-based approaches to biocontrol coupled with vital research to understand suggested pathogens. Pest and invasive species control in real-world scenarios such as the *D. villosus* invasion are clearly required.

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