

Disease risks associated with the importation and release of non-native crayfish species into mainland Britain

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ABSTRACT: A full histological survey of 782 non-indigenous crayfish established in riverine habitats or imported into mainland Britain through the aquarium trade was conducted. The selected populations were subjected to further bacteriological, molecular and ultrastructural studies to characterise disease conditions. *Pacifastacus leniusculus*, *Orconectes virilis* and *Astacus leptodactylus* were obtained from 16 rivers in mainland Britain. Additionally, *Cambarellus patzcuarensis*, *Cherax quadricarinatus*, *Procambarus clarkii* and *P. fallax* were obtained from 8 pet shops, whilst *C. patzcuarensis*, *Cherax peknyi*, *C. quadricarinatus* and *P. clarkii* were seized at a point of entry into Britain. Tests for infections were negative in the majority of *P. leniusculus* (66.4 %); the rest were infected with at least one pathogen or commensal, including an intranuclear bacilliform virus and a novel *Spiroplasma* sp. of male Sertoli cells. Low level bacterial and ciliate infections and commensal mites and ostracods also occurred on or in established signal crayfish. The established population of *O. virilis* was found to be negative for any visible infections; one shipment of *P. clarkii* and one aquarium-held population of *C. quadricarinatus* were also found to contain no visible infections. One shipment of *P. clarkii* from Singapore was infected with white spot syndrome virus (WSSV). The bacterial species isolated from crayfish included *Aeromonas hydrophila*, *A. sobria*, *Citrobacter freundii*, *Grimontia hollisae*, *Hafnia alvei*, *Pasteurella multocida* and *Weeksellia virosa*. The results are discussed in relation to the enemy release hypothesis, and the risk associated with the transboundary trade in non-indigenous crayfish is considered as a potential source of disease to native crayfish species.

KEY WORDS: Crustacean hosts · Virus · Parasite · Transboundary disease · Live animal trade · Enemy release hypothesis

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INTRODUCTION

The deliberate or accidental release of an alien species into a new area brings with it the risk of altering the community structure of native populations through competition, predation, habitat alteration or disease transfer (Calvo-Ugarteburu & McQuaid 1998). Introduced species may be successful in establishing in a new area and becoming invasive if they are released from control by their natural enemies,

including disease agents (Clay 2003). If an alien species successfully establishes in a new area, it can either lose or leave behind some or all of its parasites and diseases (Torchin et al. 2003) or acquire indigenous parasites after introduction, with either a limited impact or an enhancement of their invasiveness (Dick et al. 2010). The enemy release hypothesis, whereby an alien host advantageously loses its parasites, has been used to explain the establishment success of a number of invasive species in terrestrial and

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aquatic ecosystems (Torchin et al. 2001). Torchin et al. (2003), in reviewing the literature on species introductions, suggested that whilst on average 16 parasite species were recorded from native populations, only an average of 3 parasite species successfully accompanied an invader to its introduced range. Alien species subsequently only acquire an average of 4 'native' pathogens. The invasive European shore crab *Carcinus maenas* is host to a number of parasites that are known to affect its growth, mortality and reproduction in its native range; in its invasive range, where it is a major pest species, the crabs were less parasitized and larger, suggesting that survival or growth was improved as a direct result of the loss of its parasite fauna (Torchin et al. 2001).

Holdich et al. (2009) listed 10 non-indigenous crayfish species (NICS) that are established in European waterways, compared with only 5 indigenous crayfish species (ICS). The 3 most widespread NICS in Europe, *Pacifastacus leniusculus*, *Orconectes limosus* and *Procambarus clarkii*, are of North American origin and were introduced prior to 1975 (Holdich et al. 2009). *Astacus leptodactylus* from eastern Europe and *Cherax quadricarinatus* from Australia have a narrower geographical range but are still considered detrimental to ecosystems (Holdich et al. 2009, Savini et al. 2010). The concern regarding invasive crayfish species has been in part due to competition between NICS and ICS but also because some invasive species are potential carriers of *Aphanomyces astaci*, the causative agent of so-called crayfish plague (Alderman et al. 1990). Non-indigenous crayfish have been moved into Europe via the aquaculture and aquarium trades. Subsequently, the deliberate or accidental release of NICS into the environment has allowed some of them to become established in Europe and other parts of the world (Belle & Yeo 2010).

Numerous disease conditions and parasites of crayfish have been reported in the literature (for reviews, see Edgerton et al. 2002 and Longshaw 2011). However, as noted by Longshaw (2011), the profile of parasites and pathogens described in each report often reflect the particular interest of specific researchers rather than a true representation of the full range of pathogens present. For example, whilst *Cambarellus patzcuarensis* is listed as a host for 24 ciliate species, no other infections have been reported (Mayén-Estrada & Aladro-Lubel 2001). Likewise, in describing the reproductive elements of the marble crayfish *Procambarus fallax*, Vogt et al. (2004) described a coccidian and a *Rickettsia*-like organism. Although no further disease surveys of marbled crayfish have been conducted, Martin et al. (2010) suggested that the

species may be capable of transmitting *Aphanomyces astaci* and should therefore be considered a threat to native crayfish species, particularly because modelling studies suggest that it will be able to successfully establish in a number of countries, including the USA, Madagascar and parts of Europe (Feria & Faulkes 2011). *P. clarkii* in the USA has been recorded as a host for *Psorospermium* spp., ~8 species of Digenea, at least 3 ciliate species, several branchiobdellids and the acanthocephalan *Southwellina dimorpha* (Edgerton et al. 2002, Longshaw 2011). *P. clarkii* transferred to Italy were recorded as hosts for a small number of 'native' pathogens, including ostracods, nematodes and branchiobdellids as well as *Cambarincola mesochoreus* from the USA (Gelder et al. 1999, Quaglio et al. 2006), whilst *P. clarkii* in China were noted as being infected with a systemic *Spiroplasma* sp. (Bi et al. 2008) and white spot syndrome virus (WSSV) (Du et al. 2008). Baumgartner et al. (2009) noted the presence of WSSV in wild populations of *P. clarkii* in the USA.

The present study describes the results of a disease survey of non-native crayfish species that are either established in freshwater ecosystems in mainland Britain, seized from commercial pet shops or seized at border inspection posts (BIPs) at a point of entry into the country. The data is put into the context of the enemy release hypothesis, and the wider risks associated with the transboundary trading of non-native crayfish for indigenous species as well as disease risks to non-indigenous species from native pathogens are considered.

MATERIALS AND METHODS

Crayfish collection and measurements

Sixteen *Pacifastacus leniusculus* populations in 15 rivers, one *Orconectes virilis* population and one *Astacus leptodactylus* population within Britain were sampled using traps between July 2007 and November 2010 (see Table 1). In addition, *Cambarellus patzcuarensis*, *Cherax quadricarinatus*, *Procambarus clarkii* and *P. fallax* were seized from 8 pet shops in England, whilst illegally imported *C. quadricarinatus* and *P. clarkii* from Singapore and *Cherax peknyi* from Indonesia and Singapore were seized at the BIP at Manchester Airport (see Table 2).

Live crayfish were examined for external conditions, including fouling and damage to carapace and legs, then placed on ice for ~20 min to euthanize them prior to sampling for histology, electron microscopy, bacteriology and molecular studies.

Screening for bacteriology

Approximately 2 ml of haemolymph was collected from selected populations of crayfish and mixed with an equal volume of neutral buffered formalin, smeared onto a slide and left to air dry. These slides were subsequently stained using May-Grünwald Giemsa stain. A further sample of haemolymph was swabbed onto tryptic soy agar (TSA) plates and incubated at 22°C for up to 24 h. Any bacterial colonies developing after 24 h were re-plated onto TSA plates. Pure bacterial isolates were identified using a series of biochemical and morphological tests, including catalase, oxidase, morphology and motility. In addition, the isolates were further identified using the API (Biomerieux) system. Oxidase-positive isolates were identified with the 20NE system, and oxidase-negative isolates were identified with the 20E system.

Histological screening

Crayfish with a carapace length <5 cm were fixed whole in Davidson's freshwater fixative. Larger animals were dissected, and samples of the carapace, abdominal and cheliped muscle, gill, gonad and hepatopancreas were preserved in Davidson's freshwater fixative for 24 h then transferred to 70% industrial methylated spirits (IMS). If required, the tissue samples were decalcified in a rapid decalcification solution. The tissues were processed to wax blocks using an automatic vacuum infiltration tissue processor (Vision Biosystems Peloris). Sections were cut at 3 to 5 µm and routinely stained with haematoxylin and eosin (H&E) in an automatic tissue stainer. The tissues were examined on a light microscope using brightfield illumination. A record was made of any pathologies or pathogens in tissues and, where appropriate, an indication of the level of infection severity. Images were captured using a LUCIA™ (Nikon) screen measurement system.

Electron microscopy

Small cubes measuring 1 to 2 mm³ of hepatopancreas were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. In addition, the tissue samples were processed for transmission electron microscopy (TEM) from wax blocks, and the tissue of interest was identified and cut from the wax block. The tissue was de-waxed, rehydrated and rinsed thoroughly in 0.1 M

sodium cacodylate buffer (pH 7.4) before being fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer as above. The fixed tissue samples were rinsed in 0.1 M sodium cacodylate buffer and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The specimens were washed in 3 changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. The specimens were embedded in Agar 100 epoxy resin (Agar Scientific, Agar 100 pre-mix kit, medium) and polymerised overnight at 60°C in an oven. Semi-thin (1 to 2 µm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70 to 90 nm) of these areas were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope, and digital images were captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software. In a small number of cases, the wax-embedded tissue blocks were reprocessed for electron microscope studies according to the methods of Watson et al. (1972). Sections were stained with lead citrate and uranyl acetate and examined on a JEOL JEM 1210 as above.

Molecular screening

Samples of gonad, muscle, gill and hepatopancreas tissue were preserved in ethanol. In addition, bacterial colonies for molecular identification were emulsified in molecular-grade water containing 1 ml DNazol® and centrifuged at 4472 × *g* for 10 min. The supernatant was resuspended in 99.9% ethanol and centrifuged for a further 30 min, after which the supernatant was discarded, and the pellet was resuspended in molecular-grade water and heated to 65°C. Samples were lysed in 44 µl of 1% sodium dodecyl sulphate (SDS), 5 µl of Proteinase K (20 µl ml⁻¹) and 400 µl of Tris-ethylenediaminetetraacetic (TE) buffer for 1 h at 60°C. The samples were then extracted twice with 400 µl of phenol/chloroform/isoamyl alcohol (50:50:1) and 40 µl of 3 M sodium acetate. Genomic DNA was precipitated in absolute ethanol and centrifuged at 13 000 × *g* for 15 min, and the ethanol was removed. The resultant pellet was air-dried, resuspended in 20 µl of RNase/DNase-free water and stored at -20°C. The bacterial 16S rDNA was amplified by a single-round PCR using the primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 536R (5'-GWA TTA CCG CGG CKG CTG-3'). The PCR was

Table 1. Prevalence of infection (%) of all disease conditions and parasites of *Pacifastacus leniusculus* (P. L.), *Astacus leptodactylus* (A. L.) and *Orconectes virilis* (O. v.) collected between July 2007 and January 2011 from established populations in rivers of Britain. Prevalence data for *Spiroplasma* sp. refers to male crayfish only in that sample. PIBV: *P. leniusculus* bacilliform virus

River	Date	Number sampled	Crayfish species	Negative	Gill ciliates	Carapace ciliates	PIBV	Granuloma	Psorospermium sp.	Ostracod	<i>Spiroplasma</i> sp.	Mite
Avon	Sep 2009	26	P. L.	76.9	23.1	0	0	0	0	0	0	0
Bach Howey	May 2008	19	P. L.	10.5	84.2	0	0	0	0	26.3	5.3	10.5
Bourne	Nov 2007	14	P. L.	100	0	0	0	0	0	0	0	0
Calder	Sep 2009	20	P. L.	20	35	0	65	0	0	0	0	0
Frome	Jun 2008	30	P. L.	73.3	36.4	0	0	0	0	0	0	0
Great Ouse	Sep 2009	25	P. L.	96	4	0	0	0	0	0	0	0
Lee	Oct 2007	40	A. L.	37.5	0	0	0	0	62.5	0	0	0
Lee	May 2008	16	A. L.	18.8	68.8	0	0	0	56.3	0	0	0
Lee Navigation	May 2008	30	P. L.	53.3	33.3	0	0	6.7	0	6.7	0	0
Lee Navigation	Oct 2007	15	O. v.	100	0	0	0	0	0	0	0	0
Medway	Nov 2007	6	P. L.	0	83.3	0	0	0	0	50	16.7	0
Ock	Jul 2007	11	P. L.	100	0	0	0	0	0	0	0	0
Ock	Sep 2007	20	P. L.	75	10	0	0	5	0	10	0	0
Ock	Mar 2008	4	P. L.	75	25	0	0	0	0	0	0	0
Ock	May 2008	32	P. L.	59.4	21.9	0	0	0	0	12.5	0	0
Ray	Sep 2007	28	P. L.	64.3	25	7.1	0	0	0	7.1	0	0
Ray	Nov 2007	34	P. L.	100	0	0	0	0	0	0	0	0
Ray	Mar 2008	28	P. L.	71.4	28.6	0	0	0	0	7.1	0	0
Ray	May 2008	32	P. L.	75	21.9	0	0	3.1	0	0	0	0
Ribble	Sep 2009	25	P. L.	84	4	0	0	4	0	4	5	0
Stour	Aug 2007	27	P. L.	25.9	33.3	29.6	0	0	0	0	18.5	0
Trent	Sep 2009	25	P. L.	96	0	0	50	4	0	0	25	25
Trent	Sep 2010	4	P. L.	50	0	0	100	20	0	0	0	20
Trent	Oct 2010	5	P. L.	0	0	0	27.3	0	0	45.5	0	9.1
Trent	Nov 2010	11	P. L.	27.3	45.5	0	33.3	22.2	0	11.1	0	11.1
Trent	Jan 2011	9	P. L.	33.3	0	11.1	0	0	0	12.5	0	0
Tweed	Jul 2007	8	P. L.	62.5	25	0	0	4	0	0	50	0
Wharfe	Sep 2009	25	P. L.	68	16	0	0	0	0	0	0	0

performed in standard 100 µl reactions (containing tris-HCl buffer, 2.5 U of *Taq* DNA polymerase, 200 µM of each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 0.1 µM of both primers and distilled water), and the temperatures were cycled in a Stratagene Robocycler 40. A total of 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C were used in the amplification. The process was ended by a 10 min extension at 72°C. The PCR products were resolved for 20 min at 140 V on a 2% agarose gel containing ethidium bromide. The DNA was visualised with UV light, and the DNA fragment of interest was excised from the agarose gel and separated from the agarose using the Wizard® SV Gel and PCR Clean-up system following the manufacturer's protocol. The template DNA was cycle sequenced at 94°C for 30 s, 96°C for 10 s, 50°C for 10 s, and 60°C for 4 min for 30 cycles and then held at 4°C. The samples were then run through a sequencer, and sequences were analysed with WU-BLAST2 to identify bacterial species. The molecular identification of WSSV followed standard protocols as defined by the Office International des Epizooties Manual of Diagnostic Tests for Aquatic Animals (OIE 2009).

RESULTS

Health status of established non-native crayfish

A total of 498 signal crayfish *Pacifastacus leniusculus* were collected from 16 sites on 15 rivers in Britain; of these, 331 were negative for any infections (prevalence of 66.4 %). All of the signal crayfish collected from the River Ray in November 2007, the River Ock in July 2007 and the River Bourne in November 2007 were negative for infections. The crayfish populations from the remaining sites contained at least one animal infected with a pathogen. Prevalence data for signal crayfish infections are shown in Table 1.

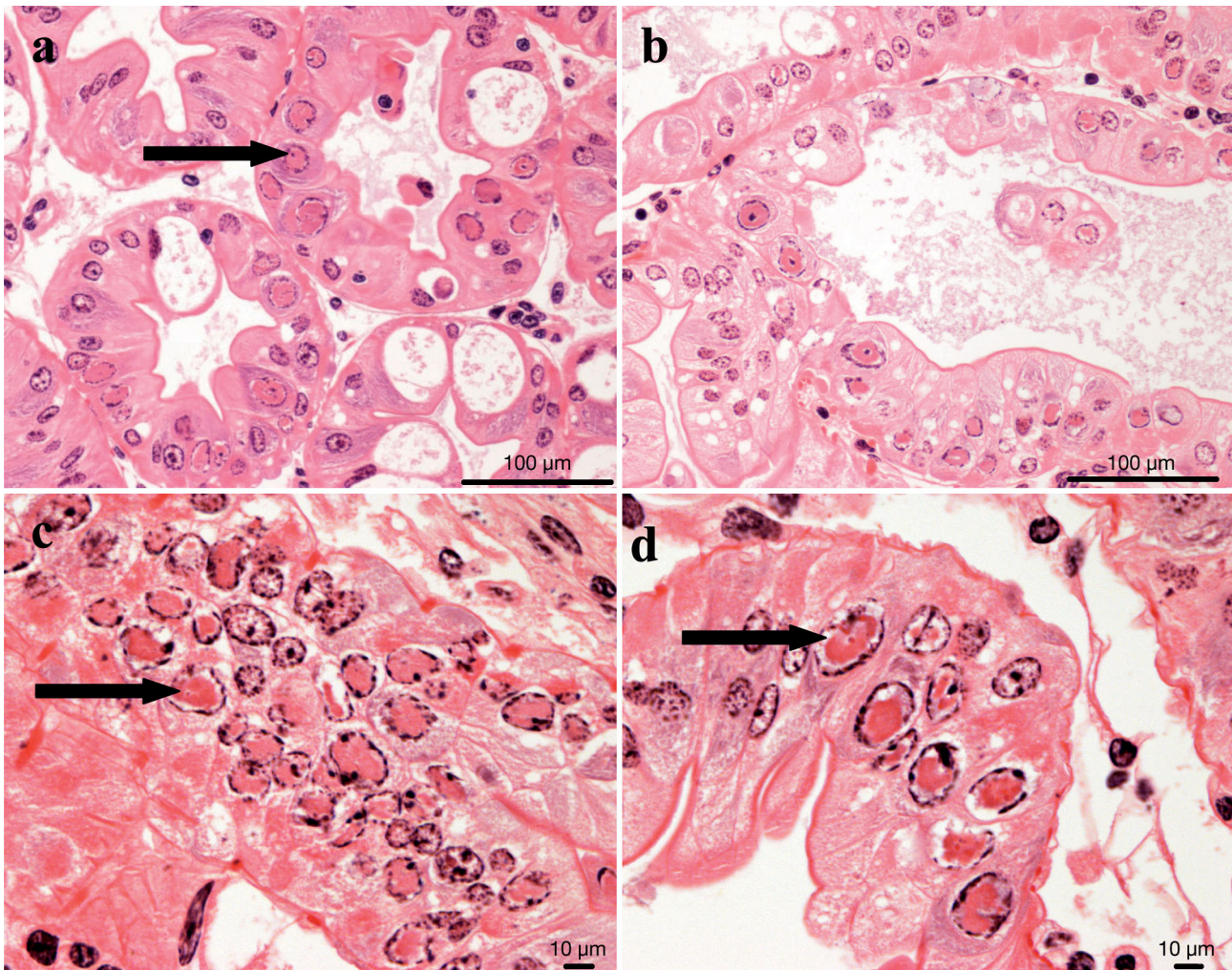


Fig. 1. *Pacifastacus leniusculus* bacilliform virus (PIBV) within epithelial cells of crayfish hepatopancreas tissues, based on H&E staining. (a) Low power view of several hepatopancreatic tubule epithelial cell nuclei showing typical enlarged appearance with emarginated chromatin and large eosinophilic inclusion (arrow). (b) Hepatopancreatic tubule infected with PIBV. Note the presence of a sloughed area of tubule cells in tubule lumen. (c) High power view of PIBV-infected hepatopancreatic tubule. Infected nucleus marked with arrow. (d) High power view of PIBV-infected hepatopancreatic tubule. Note emarginated chromatin and inclusion body typical of infection in signal crayfish (arrow)

An intranuclear bacilliform viral infection of the midgut epithelium and the hepatopancreas was noted in 4 out of 5 samples of *Pacifastacus leniusculus* from the River Trent at prevalences up to 100% and from 65% of signal crayfish collected from the River Calder in September 2009. The nuclei of the affected hepatopancreatocytes were enlarged with emarginated chromatin (Fig. 1). In a small number of individuals, some of the infected cells were sloughed into the tubule lumina (Fig. 1c). Electron microscopy revealed multiple infected nuclei; the host chromatin was marginalised, and the nuclear membrane appeared swollen (Fig. 2a). Fully formed virions appeared to accumulate at the nuclear membrane in some nuclei, forming paracrystalline arrays (Fig. 2b),

and were rod shaped, consisting of an electron-dense nucleocapsid surrounded by a closely fitting trilaminar membrane (Fig. 2c,d). The virions measured on average 228.8 nm in length and 71.9 nm in width and contained a nucleocapsid measuring on average 195.4 nm in length and 46.9 nm in width ($n = 40$). Virion development appeared to be associated with double membrane-bound vesicles within the nuclei and rod-shaped filaments (Fig. 2e,f).

An intracytoplasmic inclusion in the Sertoli (nurse) cells of male gonads was recorded in *Pacifastacus leniusculus* from the Rivers Trent, Stour, Medway, Wharfe, Ribble and Bach Howey Stream. Infected tubules occurred in clusters within the testicular lobes, and in low level infections, the inclusions were

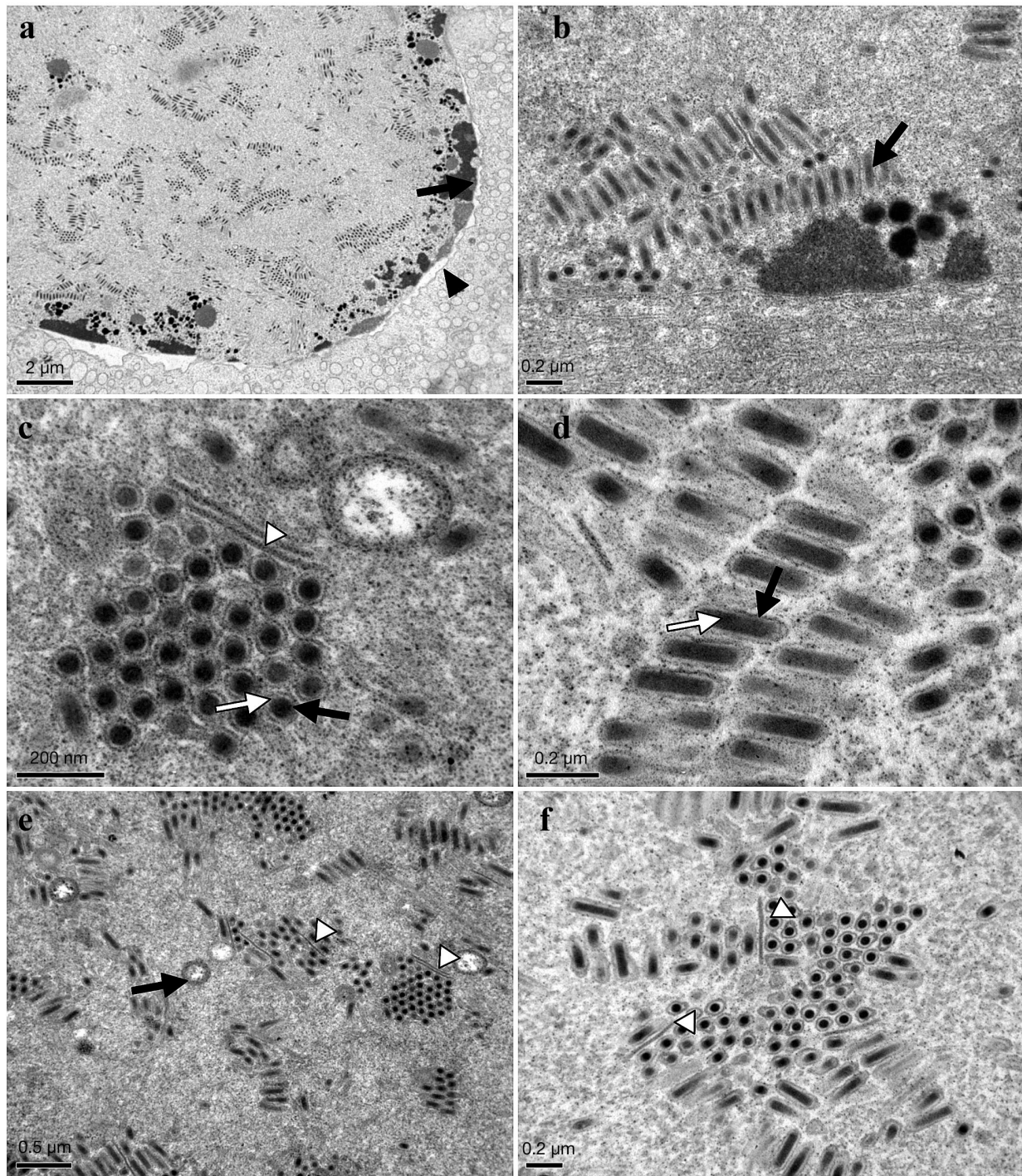


Fig. 2. *Pacifastacus leniusculus* bacilliform virus (PIBV) within epithelial cells of crayfish hepatopancreas tissues. Transmission electron microscopy (TEM). (a) Nucleus from a PIBV-infected cell containing rod-shaped bacilliform virions. Host chromatin is marginalised (arrow), and the nuclear membrane appears swollen (arrowhead). (b) Fully formed virions appeared to accumulate at the nuclear membrane in some nuclei, forming paracrystalline arrays (arrow). (c) Transverse section of virions, electron-dense nucleocapsid (black arrow) can be seen within trilaminar membrane (white arrow). Note presence of rod-shaped filaments closely associated with developing virions (arrowhead). (d) Longitudinal section of rod-shaped virion consisting of an electron-dense nucleocapsid (black arrow) surrounded by a closely fitted trilaminar membrane (white arrow). (e) Virion development appeared to be associated with double membrane bound vesicles within the nuclei (arrow) and rod shaped filaments (white arrowheads). (f) Virion development appeared as rod-shaped filaments (white arrowheads) with fully formed virions accumulating around these structures within the nuclei

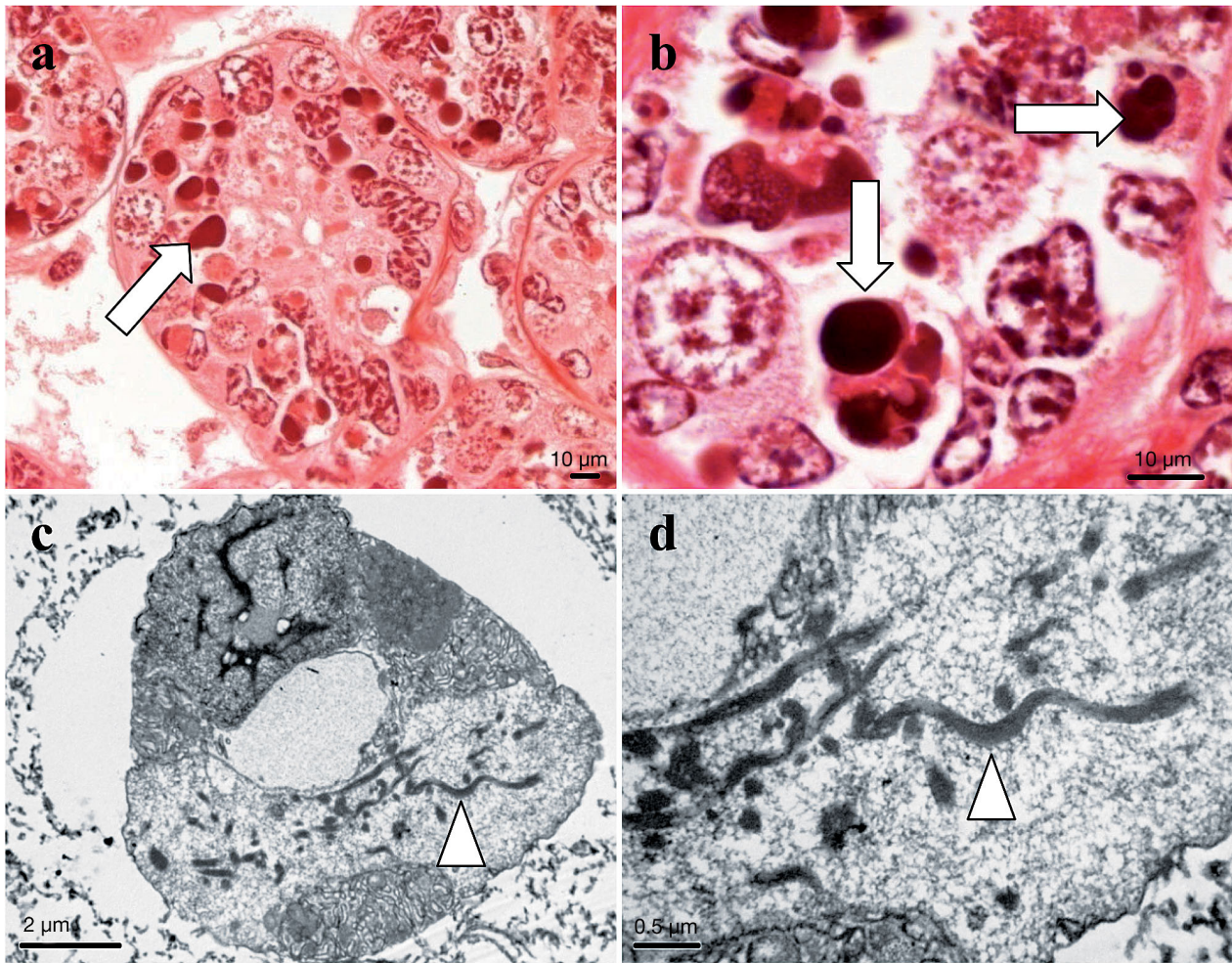


Fig. 3. *Spiroplasma* sp. infection of *Pacifastacus leniusculus*. (a) Low power view of gonadal tubule showing inclusion bodies (arrow) in the cytoplasm of a Sertoli cell. Note the lack of sperm within the lumen and the general degeneration of cells immediately surrounding infected cells. H&E staining. (b) High power view of male gonad with infected Sertoli cells containing inclusion body (arrows). H&E. (c) Single Sertoli cell with inclusion body containing helical structures in cytoplasm (arrow head). TEM. (d) High power view of *Spiroplasma* sp. in cytoplasm of inclusion body. Note obvious helical arrangement of *Spiroplasma* sp. (arrowhead). TEM

restricted to single lobes (Fig. 3a). The infection was absent from cells of the collecting ducts and the vas deferens. Mature sperm was generally absent from the lumens of affected tubules. Degeneration of the epithelial and Sertoli cells of the tubules occurred in latter stages of the infection (Fig. 3b). Ultrastructurally, a single, membrane-bound inclusion was noted in the cytoplasm of the Sertoli cells (Fig. 3c). Despite the use of material from wax blocks, the preservation was of sufficient quality to discern spiral-shaped organisms resembling *Spiroplasma* spp. within the inclusion that lacked a cell wall and measured ~20 nm in diameter and 5 to 10 µm in length (Fig. 3d). No dividing stages were noted.

Peritrichous ciliates were recorded in the gills of *Pacifastacus leniusculus* from all sites, with the

exception of the River Bourne. No pathology was noted with these infections, although levels were considered low, with a maximum of 20 individual ciliates being visible in a histological section. A stalked ciliate on the legs and carapace was noted in *P. leniusculus* from 2 sites at a maximum prevalence of 29.6%. Ectocommensal ostracods and mites were associated with the gills and legs of *P. leniusculus* from 8 and 2 sites, respectively. No pathology was noted with either of these ectocommensals.

Enteric bacteraemia typified by the presence of granulomas in the hepatopancreas tubules, and mid-gut epithelium was apparent in 7 samples of *Pacifastacus leniusculus*. In 2 animals, these granulomas had progressed to form large necrotic areas encapsulated by the deposition of melanin. Additionally, bac-

teria were isolated from the haemolymph of *P. leniusculus*, including *Aeromonas hydrophila*, *Hafnia alvei* and *Vibrio alginolyticus* (Table 2); molecular sequencing of these isolates provided >99% homology with these species and those recorded in the WU-Blast2 database.

A total of 15 *Orconectes virilis* were caught in the Lee Navigation in October 2007. No infections were detected in these animals (Table 1). A total of 40 *Astacus leptodactylus* were collected from the River Lee in October 2007, and a further 16 were caught in the same river in May 2008 (see Table 1). A multiplate *Psorospermium* sp. was noted in the hepatopancreas, cuticular epithelium, gills and gonads of 25 *A. leptodactylus* in October 2007 and in the cuticular epithelium, muscle and gonads of 9 animals in May 2008 (Fig. 4a). Peritrichous ciliates were noted on the gills of 11 *A. leptodactylus* caught in May 2008. Pathology associated with the infection was negligible.

Health status of captive non-native crayfish

Summary data for infections detected in captive non-native crayfish are presented in Table 3. No histological evidence of infections was detected in 3 *Cambarellus patzcuarensis* obtained from a fish

dealer in Northampton. However, the bacteria *Aeromonas sobria*, *Citrobacter freundii* and *Weeksella virosa* were isolated from 3 different individuals and identified using primary tests, API20E and API20NE (see Table 2). A low level infection with an *Epistylis*-like organism was detected on the legs of the crayfish obtained from a dealer in Guildford and on 1 of 5 individuals from a dealer in Colchester. In addition, a single granuloma was noted in the hepatopancreas of a single crayfish from Guildford.

Cherax peknyi imported from Indonesia in August 2007 and from Singapore in September 2007 and April 2008 were seized at a port of entry. A multiplate *Psorospermium* sp. occurred in the gills, hepatopancreas and cuticular epithelium of 2 animals from Indonesia (Fig. 4b) and in the muscle of 1 *C. peknyi* from Singapore in September 2007 (Fig. 4c). A biplate *Psorospermium* sp. occurred in the gills, gonadal tissues, stomach, hepatopancreas, muscle and cuticular epithelium of 75% of the studied crayfish from Indonesia (Fig. 4d), in 10 individuals from Singapore collected in September 2007 (Fig. 4e) and in the gills, hepatopancreas and gonads of 4 individuals seized in April 2008. The biplate form did not co-occur with the multiplate form in any host. Unidentified temnocephalids were noted in the gills of 10% of the *C. peknyi* seized from Indonesia; no pathology was n.

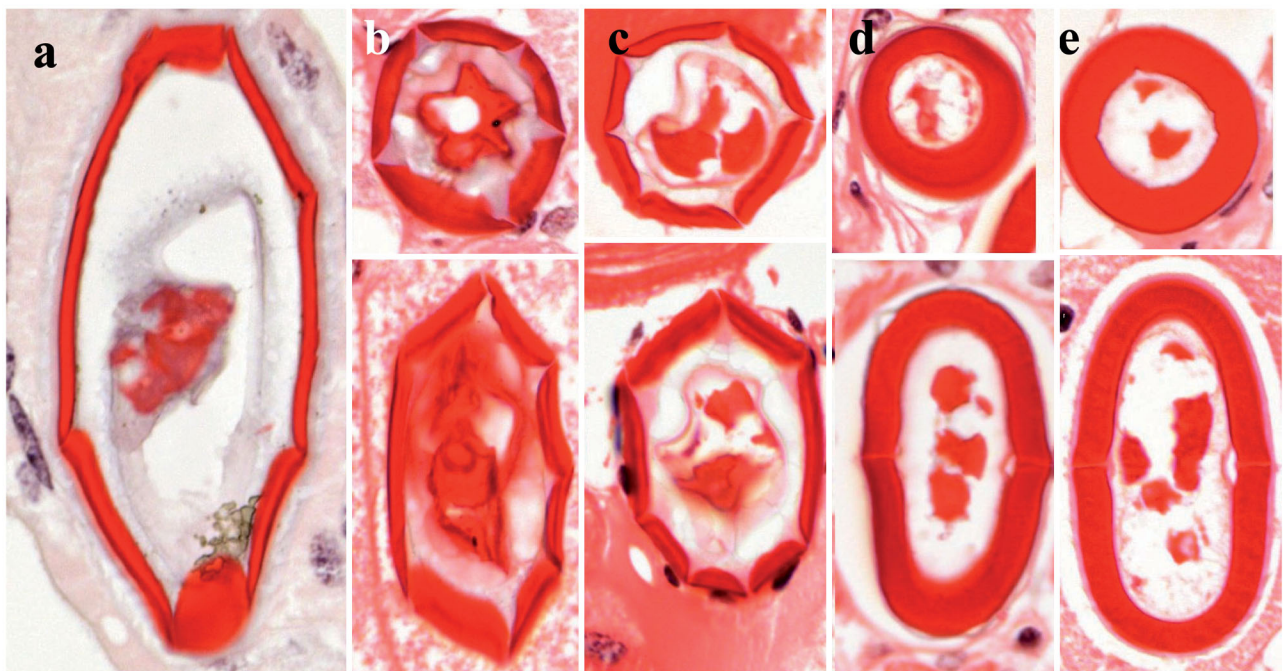


Fig. 4. Histological sections (H&E stained) through *Psorospermium* spp. from multiple crayfish hosts. Multiplate form from (a) Turkish crayfish collected in the UK, (b) zebra crayfish imported from Indonesia, and (c) zebra crayfish imported from Singapore. Biplate form isolated from (d) zebra crayfish imported from Indonesia, and (e) zebra crayfish imported from Singapore. Transverse (top) and longitudinal (bottom) sections shown in (b) to (e); longitudinal section only shown in (a)

Table 2. Results of primary tests for bacteria isolated from the haemolymph of crayfish. *P.l.*: *Pacifastacus leniusculus*; *P.f.*: *Procamburus fallax*; *C.pa.*: *Cambarellus patzcuarensis*

Bacterial species	Morpho- logy	Gram	Oxidase	Catalase	Motility	Fermen- tive	API20E (%)	API20NE (%)	Crayfish hosts	Collection site	Collection date	No. of ind. infected
<i>Aeromonas hydrophila</i>	Rod	-	+	+	+	+	61.3	-	<i>P.l.</i>	River Stour, UK	Aug 2007	1/27
<i>Aeromonas hydrophila</i>	Rod	-	+	+	+	+	-	99.6	<i>P.l.</i>	River Bourne, UK	Nov 2007	2/14
<i>Aeromonas hydrophila</i>	Rod	-	+	+	+	+	-	99.7	<i>P.l.</i>	Bach Howey Stream, UK	May 2008	2/19
<i>Aeromonas sobria</i>	Rod	-	+	+	+	+	-	95.6-98.4	<i>P.f.</i>	Southampton, UK	Nov 2007	4/8
<i>Aeromonas sobria</i>	Rod	-	+	+	+	+	-	99.1	<i>C.pa.</i>	Northampton, UK	Jan 2008	1/3
<i>Citrobacter freundii</i>	Rod	-	-	+	+	+	99.9	-	<i>P.f.</i>	Southampton, UK	Nov 2007	2/8
<i>Citrobacter freundii</i>	Rod	-	-	+	+	+	83.9	-	<i>C.pa.</i>	Northampton, UK	Jan 2008	1/3
<i>Grimontia hollisae</i>	Rod	-	+	+	+	?	-	97.1	<i>P.f.</i>	Southampton, UK	Nov 2007	2/8
<i>Hafnia alvei</i>	Rod	-	-	+	-	+	99.7	-	<i>P.l.</i>	River Stour, UK	Aug 2007	2/27
<i>Hafnia alvei</i>	Rod	-	-	+	-	+	46.2	-	<i>P.l.</i>	River Bourne, UK	Nov 2007	2/14
<i>Hafnia alvei</i>	Rod	-	-	+	-	+	93.6	-	<i>P.l.</i>	River Lee, UK	May 2008	1/30
<i>Pasteurella multocida</i>	Rod	-	+	+	+	?	-	83.1	<i>P.f.</i>	Southampton, UK	Nov 2007	1/8
<i>Vibrio alginolyticus</i>	Rod	-	+	-	-	+	-	78.9	<i>P.l.</i>	River Lee, UK	May 2008	1/30
<i>Weeksella virosa</i>	Rod	-	+	+	-	?	-	84.3	<i>C.pa.</i>	Northampton, UK	Jan 2008	1/3

Table 3. Number and source of crayfish seized from pet shops or as illegal imports at Manchester Airport, UK. Numbers of infected crayfish are shown for each condition. Bacteria isolated from the haemolymph not shown here; data shown in Table 2. N: number sampled; WSSV: white spot virus syndrome virus; Bi-P. sp.: biplate *Psorospermium* sp.; multi-P. sp.: multiplate *Psorospermium* sp.; temno.: temnocephalid; *C.pa.*: *Cambarellus patzcuarensis*; *C.pe.*: *Cherax peknyi*; *C.q.*: *Cherax quadricarinatus*; *P.c.*: *Procamburus clarkii*; *P.f.*: *Procamburus fallax*

Source	Date	Crayfish species	N	Negative for visible infections	WSSV	Granuloma	Ciliates	Cothurnia	Bi-P. sp.	Multi-P. sp.	Ostracod	Mites	Digenaea	Temno.
Northampton, UK	Jan 2008	<i>C.pa.</i>	3	3	0	0	0	0	0	0	0	0	0	0
Guildford, UK	Mar 2008	<i>C.pa.</i>	1	0	0	1	1	0	0	0	0	0	0	0
Colchester, UK	Feb 2008	<i>C.pa.</i>	5	4	0	0	1	0	0	0	0	0	0	0
Indonesia	Aug 2007	<i>C.pe.</i>	20	3	0	0	0	0	15	2	0	0	0	1
Singapore	Sept 2007	<i>C.pe.</i>	12	1	0	0	1	0	10	1	0	0	0	0
Singapore	Apr 2008	<i>C.pe.</i>	4	0	0	0	0	0	4	0	0	0	0	0
Cambridgeshire, UK	Mar 2008	<i>C.q.</i>	3	3	0	0	0	0	0	0	0	0	0	0
Singapore	Feb 2008	<i>C.q.</i>	16	7	0	0	0	1	0	0	0	0	0	9
Dorset, UK	May 2008	<i>P.c.</i>	7	6	0	0	1	0	0	0	0	0	0	0
Hertfordshire, UK	May 2008	<i>P.c.</i>	26	22	0	0	4	0	0	0	0	0	0	0
Singapore	May 2008	<i>P.c.</i>	4	4	0	0	0	0	0	0	0	0	0	0
Singapore	July 2008	<i>P.c.</i>	1	0	0	0	0	0	0	0	0	0	1	0
Singapore	Feb 2010	<i>P.c.</i>	8	0	8	0	1	0	0	0	0	0	0	0
Southampton, UK	May 2007	<i>P.f.</i>	16	13	0	0	3	0	0	0	0	0	0	0
Basildon, UK	Sept 2007	<i>P.f.</i>	19	0	0	1	5	0	0	0	1	6	0	0
Southampton, UK	Nov 2007	<i>P.f.</i>	68	65	0	3	0	0	0	0	0	0	0	0

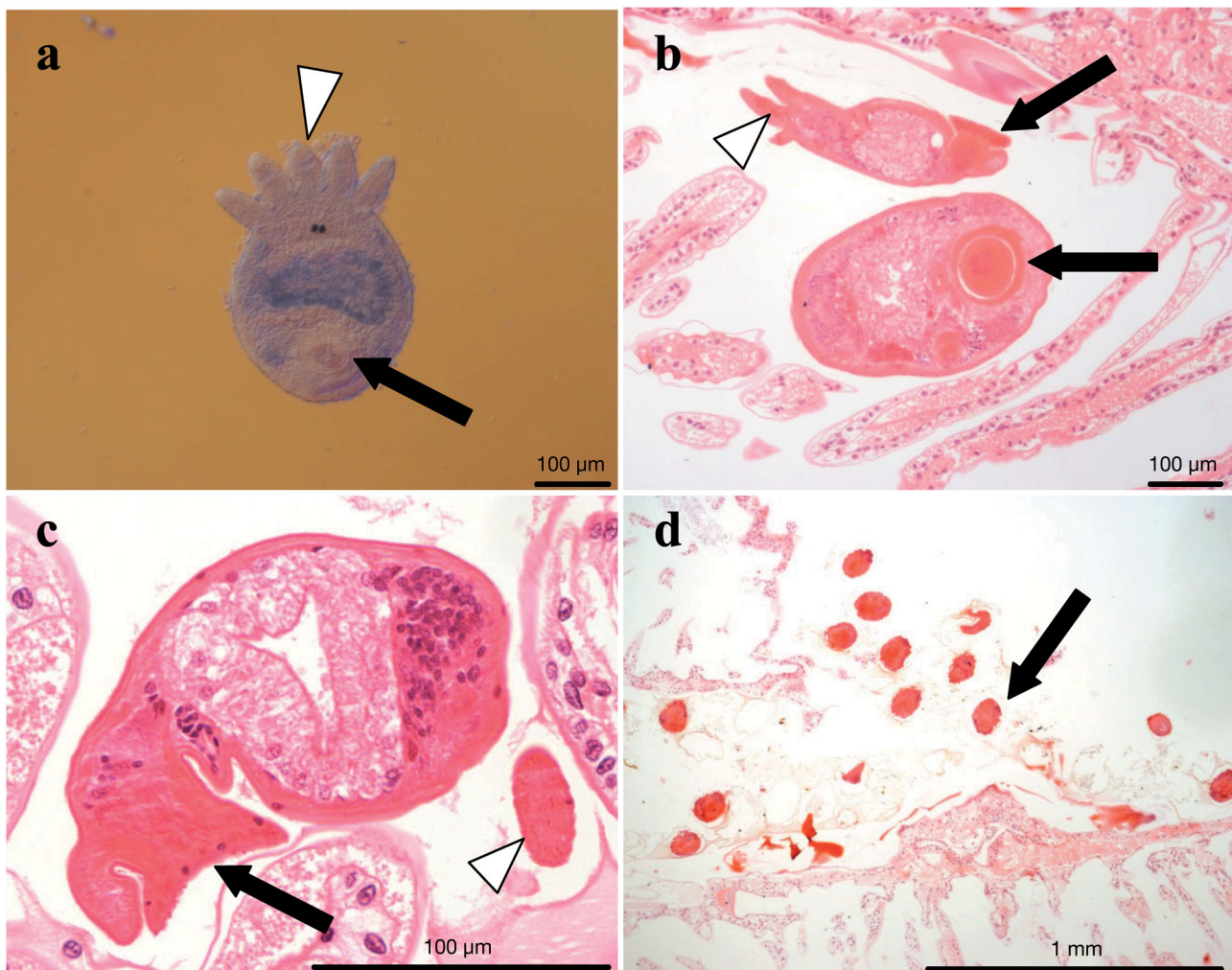


Fig. 5. Temnocephalid infection of *Cherax quadricarinatus*. (a) Entire temnocephalid removed from crayfish following fixation in Davidson's freshwater fixative. Five digitate tentacles are visible at anterior end of the animal (arrowhead), with the subterminal posterior adhesive disk marked with a black arrow. Two eyespots are also visible at the anterior end. Differential interference contrast (DIC) microscopy. (b) Histological section through 2 temnocephalids associated with the gills. Three of the 5 tentacles are seen in section (arrowhead) as well as posterior adhesive disk (arrows). H&E. (c) High-power view through a single temnocephalid. Digitate tentacle marked with arrowhead and posterior adhesive disk (arrow) associated with gill. H&E. (d) Eggs of unidentified temnocephalid (arrow) associated with gills. H&E

oted, whilst a low level peritrichous ciliate infection was recorded on the gills of a single *C. peknyi* from Singapore seized in September 2007.

No infections were detected in 3 *Cherax quadricarinatus* seized from a fish dealer in Cambridgeshire. The 9 *C. quadricarinatus* seized from a shipment from Singapore were infected with temnocephalids in the gills, and pathology was limited (Fig. 5). A concomitant low level *Cothurnia*-like infection was detected on the gills of 1 individual.

Peritrichous ciliates were noted on the gills of 4 out of 26 *Procambarus clarkii* from an aquarium in Hertfordshire and on 1 out of 7 from Dorset. Infection levels were low, with the exception of 1 individual

from Hertfordshire, which was found to have a particularly high number of peritrichous ciliates associated with the carapace. No other infections were detected in these samples. Four individuals illegally imported from Singapore in May 2008 were negative for infection, whilst 1 digenean was detected in the muscle of the 1 *P. clarkii* imported from Singapore in July 2008. All 8 *P. clarkii* imported from Singapore in February 2010 were positive for infection with WSSV. The virus was noted histologically in all epithelial tissues. In particular, hypertrophied nuclei with obvious inclusions were found in the cuticular epithelium, gills and midgut epithelium (Fig. 6). Subsequent PCR and sequencing of the amplicon con-

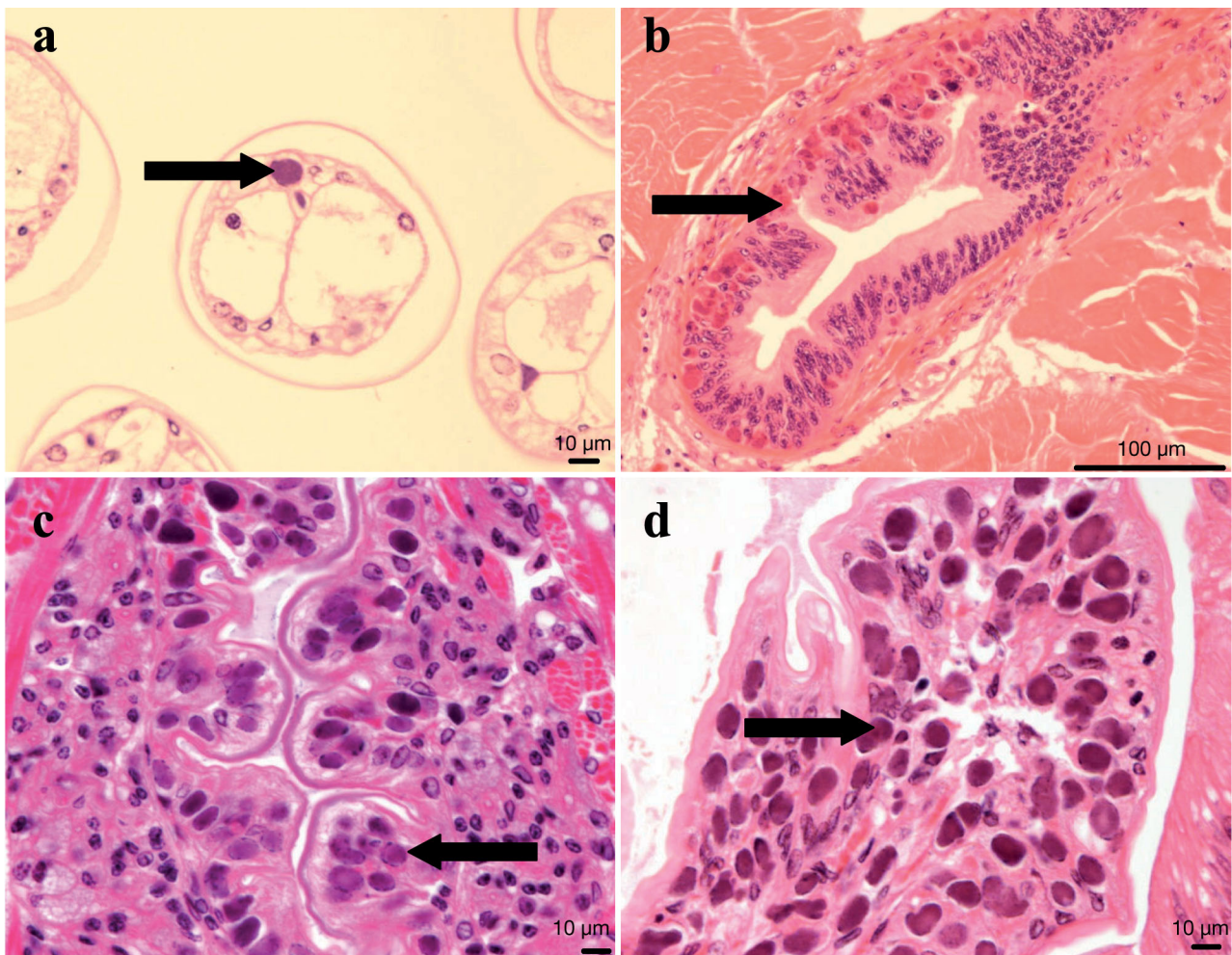


Fig. 6. Histology of white spot syndrome virus (WSSV) in *Procambarus clarkii* based on H&E staining. (a) WSSV-infected nucleus in gill (arrow). (b) Low-power view of hindgut showing numerous infected nuclei (arrow). (c,d) Midgut epithelium nuclei with inclusions and emarginated chromatin clearly visible (arrow)

firmed the identity as WSSV in these individuals; peritrichous ciliates were noted in the gills of 1 crayfish at a low level.

Peritrichous ciliates were recorded on the gills of 3 *Procambarus fallax* from a fish dealer from Southampton in May 2007 and from 5 *P. fallax* from Basildon in September 2007. In addition, mites were detected on the gills of 6 *P. fallax*, and a single ostracod was noted in 1 individual seized from an aquarium in Basildon. Granulomas were recorded in the heart, hepatopancreas, gills and connective tissue of the gonads of 3 *P. fallax* seized from Southampton in November 2007. Haemolymph was only extracted from 8 marbled crayfish collected in Southampton. The bacteria identified using primary tests and API 20E or API 20NE included *Citrobacter freundii*, *Aeromonas sobria*, *Grimontia hollisae* and *Pasteurella multocida*. No other infections were recorded.

DISCUSSION

The present study is the first wide-scale survey of non-native crayfish species established in rivers or imported into mainland Britain and provides evidence that these animals are host to a small number of parasites, commensals and potential pathogens. The present study also demonstrated the presence of previously unrecorded novel infections in *Pacifastacus leniusculus* and *Cherax peknyi*. In the original description of *C. peknyi* by Lukhaup & Herbert (2008), no pathogens or commensals were reported; the current study has identified the presence of 2 *Psorospermium* forms and an unidentified temnocephalid. However, this species was originally described from Papua New Guinea, while the samples examined in the current study were obtained from a shipment imported from Indonesia, and thus,

it is not possible to determine where these animals became infected. Crayfish seized at a port of entry or in pet shops were found in almost all cases to carry innocuous or low level infections. However, their potential role in further mortalities of indigenous crayfish species is unknown. Importantly, the apparent absence of infections in these species provides further evidence of the potential success of these hosts if released into our waterways. In the event that the NICS were to be released into a watercourse in Britain, deliberately or otherwise, and assuming that the abiotic factors present are conducive to survival, the limited number of pathogens present would be advantageous to the NICS. Transmission of pathogens from NICS to ICS has been shown to occur, sometimes with dire consequences (Alderman 1993, Chinain & Vey 1988, Ohtaka et al. 2005, Füreder et al. 2009, Volonterio 2009).

The most widespread and invasive crayfish species in Britain is *Pacifastacus leniusculus*, originally introduced from California, USA and Sweden for aquaculture purposes in 1976 (Holdich et al. 2009). Alderman (1993) suggested that although several of the original imports of signal crayfish from the USA were destined directly for quarantine facilities with limited possibility of disease transfer, others may have been imported directly from North America with no disease screening occurring and potentially without entering such facilities. Thus, at least some British populations are potentially derived directly from wild North American stocks. In its native range, however, signal crayfish have only been noted as hosts for a previously undescribed intranuclear bacilliform virus (Longshaw 2011) and for 2 branchiobdellids *Sathodrilus attenuatus* and *Xironogiton victoriensis* (Gelder & Siddall 2001, Williams et al. 2009). *X. victoriensis* has subsequently been recorded on signal crayfish in Italy (Oberkofler et al. 2002), and both branchiobdellids were recorded in Japan following the introduction of signal crayfish into Japan (Ohtaka et al. 2005). In introduced areas, signal crayfish have been shown to carry or be susceptible to WSSV, *Aeromonas hydrophila*, *Fusarium* sp., *Aphanomyces astaci*, *Psorospermium haeckeli*, *Thelohania contejeani* and 3 undescribed microsporidia (Alderman et al. 1990, Diéguez-Uribeondo et al. 1993, Dunn et al. 2009). Signal crayfish are considered to be responsible for the spread of *A. astaci* across Europe (Lilley et al. 1997, Longshaw 2011).

In the current study, no visible infections were noted in two-thirds of the signal crayfish examined, and in the rest, few parasites, disease agents or commensals were noted. Thus, the surveyed signal cray-

fish populations fulfil the criteria of being successful invaders with few detrimental pathogens limiting their survival. Furthermore, 5 of the 7 infections noted in this species during the current study are considered non-specific, including ciliates, ostracods and mites. Although these commensals were not identified to species, they are unlikely to be a major mortality driver for signal crayfish because the numbers were generally low and no pathology was noted. Torchin et al. (2003) suggested that on average successful invaders transfer 3 infections from their native range and acquire an average of 4 infections, a pattern that is mirrored in the current study.

The viral infection in signal crayfish appears to be similar to the B-virus in the shore crab *Carcinus maenas*, B2 virus in *Carcinus mediterraneus* and Baculo-B in the blue crab *Callinectes sapidus*, all of which are viral infections of the haemocytes that show an association of the developing viral particles to vesicles within the nucleus (Johnson 1988). A presumptively identical virus, the *Pacifastacus leniusculus* bacilliform virus, was reported from signal crayfish in California by Hauck et al. (2001); because there has been no reciprocal transfer of signal crayfish from Europe to the USA, it seems probable that the virus infection was introduced into Britain with imports of signal crayfish directly from North America. The wider distribution of the virus in signal crayfish in Britain and across Europe is unknown and deserves further investigation. Whilst similar bacilliform viruses have been noted in other crayfish species, they are all likely to represent distinct virus species with strict host specificity (Longshaw 2011). With the exception of the *Cherax quadricarinatus*-bacilliform virus (CqBV), apparently causing mortality (Romero & Jiménez 2002), intranuclear bacilliform viruses in crayfish do not appear to be particularly detrimental to their hosts (Edgerton et al. 1996, Edgerton 2003).

A novel *Spiroplasma*-like organism causing marked pathology in the gonadal tissue of male crayfish in several populations was noted. Sperm production in the affected animals was compromised, and the pathology of the infected tubule was marked. Whilst it has not been possible in the current study to fully assess the effect of the infection on fecundity or survival, *Spiroplasma* spp. have been reported in a number of plant and animal hosts, and some are considered to act as male killing agents, distorting sex ratios (Nienhaus & Sikora 1979, Regassa & Gasparich 2006). In freshwater Crustacea, including crayfish, *Spiroplasma* spp. have been associated with mortality (Nunan et al. 2005, Wang et al. 2010). The relationship between the *Spiroplasma* sp. detected in the

current study and the systemic form reported in *Procambarus clarkii* by Wang et al. (2005) needs to be elucidated, and thus, further work, including experimental transmission trials, 16SrRNA sequencing, serology and ultrastructural studies on material collected specifically for TEM, will be required to fully characterise this infection. The wider distribution and origin of the *Spiroplasma* of signal crayfish is unknown, and in particular, the role of the bacteria as a biological control agent or as a barrier for the invasion success of signal crayfish throughout its range needs consideration.

Virile crayfish *Orconectes virilis* were found to be negative for any visible infections in the current study. Virile crayfish were first recorded in Britain in a concrete-lined pond on the River Lee system in 2004 and thought to be due to the illegal disposal of the animals by a local householder (Ahern et al. 2008). The crayfish have subsequently successfully migrated into adjacent watercourses some 7 km away from the original point of release in that system; in the Netherlands, the species has become established in several hundreds of kilometres of waterway (Holidich et al. 2009). It is interesting to speculate that the successful establishment and movement of the virile crayfish in the Lee catchment is due in part to a lack of disease agents. However, with the exception of a report of a *Thelohania* sp. in Canada (France & Graham 1985, Graham & France 1986), of 2 branchiobdellids in Canada (Williams et al. 2009) and evidence of susceptibility to WSSV (Davidson et al. 2010), no data exist on the pathogens of virile crayfish in its native range, making it difficult to unequivocally attribute its success to a lack of pathogens compared with its native range.

In contrast, *Astacus leptodactylus* has been shown to be susceptible to at least 6 fungal agents, approximately different 12 protists, a *Psorospermium* sp. and branchiobdellids in its native range (Edgerton et al. 2002, Fard et al. 2011, Longshaw 2011). Although *A. leptodactylus* has been recorded in a number of sites in mainland Britain, to date, no concerted disease survey of the species has been carried out. In the current study, they were only visibly infected with *Psorospermium* sp. in the sample taken in October 2007 and with *Psorospermium* sp. and peritrichous ciliates in the sample taken in May 2008; both of these parasites or commensals are relatively non-specific. Thus, the almost complete absence of visible infections in this population compared with the wide range of infections noted in its native range strongly suggest that its successful invasion and establishment is in part due to the absence of pathogens.

The relative absence of pathogens in *Cherax quadricarinatus* in the current study is of concern because in its native range, it has been recorded as a host for at least 8 different viral infections, 6 ciliate species, at least 5 species of temnocephalids, bacteria, fungi and *Psorospermium* (Herbert 1988, Eaves & Ketterer 1994, Edgerton & Owens 1999, Edgerton et al. 2000, La Fauce & Owens 2007, Owens & McElnea 2000). However, in areas outside its native ranges where it is considered a pest species (Belle et al. 2011), limited pathogens have been recorded (Hauck et al. 2001, Romero & Jiménez 2002, Volonterio 2009, Longshaw 2011), which supports the enemy release hypothesis. In the current study, the animals obtained from a pet shop were negative for infections, whilst the shipment from Singapore only contained temnocephalids and a single example of a ciliate infection of the gills. This species has become established in a number of water catchments in Singapore (Ahyong & Yeo 2007), and whilst not expressly commented on by those authors, it is interesting to speculate on its success in Singapore being due to an absence of pathogens.

The potential introduction of the EC Directive 2006/88 listed pathogen WSSV (Stentiford et al. 2010) through the importation of *Procambarus clarkii* is a real risk, with clear histological and molecular evidence of the virus in crayfish from Singapore. In its native and introduced range, *P. clarkii* is susceptible to the virus (Yan et al. 2007, Baumgartner et al. 2009). Even at low water temperatures, the virus is able to replicate, although at a lower rate than at higher temperatures (Du et al. 2008), and thus, it could establish with devastating consequences if introduced into the British Isles. However, current legislation does not limit the numbers of crayfish destined for the aquarium trade that are imported into Britain. Furthermore, there is no requirement for exporting nations to demonstrate freedom from notifiable diseases, such as WSSV, if animals are destined for the aquarium trade or for human consumption (see Stentiford et al. 2010 for context). Thus, the risk of importation of exotic pathogens via this route requires further analysis.

The current study has shown that established and imported non-native crayfish in mainland Britain tend to have a limited number of pathogens present, and with the possible exception of a viral and *Spiroplasma* infection, most of the pathogens are innocuous and are non-specific. The success of signal crayfish, introduced into mainland Britain in the 1970s for aquaculture purposes, is in part explained by the lack of pathogens present, in direct support of the

enemy release hypothesis. However, the pathogens present in its native range must be examined to determine if the successful invasion of the host throughout Europe is due to the lack of pathogens or some other factor. Further studies are required for all species imported into Europe to determine baseline data on the pathogens across their range and to confirm that the diseases of non-indigenous crayfish species in their new range are limited compared with their indigenous range. Successful invaders appear to have fewer pathogens.

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