

1 **Competition and parasitism in the native White Clawed Crayfish *Austropotamobius***
2 ***pallipes* and the invasive Signal Crayfish *Pacifastacus leniusculus* in the UK**

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1 **Abstract**

2 Many crayfish species have been introduced to novel habitats worldwide, often threatening
3 extinction of native species. Here we investigate competitive interactions and parasite infections
4 in the native *Austropotamobius pallipes* and the invasive *Pacifastacus leniusculus* from single
5 and mixed species populations in the UK. We found *A. pallipes* individuals to be significantly
6 smaller in mixed compared to single species populations; conversely *P. leniusculus* individuals
7 were larger in mixed than in single species populations. Our data provide no support for
8 reproductive interference as a mechanism of competitive displacement and instead suggest
9 competitive exclusion of *A. pallipes* from refuges by *P. leniusculus* leading to differential
10 predation. We screened fifty-two *P. leniusculus* and twelve *A. pallipes* for microsporidian
11 infection using PCR. We present the first molecular confirmation of *Thelohania contejeani* in the
12 native *A. pallipes*; in addition, we provide the first evidence for *T. contejeani* in the invasive *P.*
13 *leniusculus*. Three novel parasite sequences were also isolated from *P. leniusculus* with an
14 overall prevalence of microsporidian infection of 38 % within this species; we discuss the identity
15 of and the similarity between these three novel sequences. We also screened a subset of fifteen
16 *P. leniusculus* and three *A. pallipes* for *Aphanomyces astaci*, the causative agent of crayfish
17 plague and for the protistan crayfish parasite *Psorospermium haeckeli*. We found no evidence for
18 infection by either agent in any of the crayfish screened. The high prevalence of microsporidian
19 parasites and occurrence of shared *T. contejeani* infection lead us to propose that future studies
20 should consider the impact of these parasites on native and invasive host fitness and their
21 potential effects upon the dynamics of native-invader systems.

22

23 *Keywords:* *Austropotamobius pallipes*; competitive exclusion; differential predation;
24 invasion; microsporidia; *Pacifastacus leniusculus*; parasites

1 **Introduction**

2 Parasites can play important roles in biological invasions: invading species may bring with
3 them parasites or diseases which may detrimentally affect native species (Ohtaka et al. 2005;
4 Rushton et al. 2000), or may themselves acquire parasites from their new environment (Bauer
5 et al. 2000; Krakau et al. 2006). Alternatively invading species may lose their parasites,
6 potentially giving them an advantage over native species (Torchin et al. 2003; Torchin et al.
7 2001). Parasites have been shown to be important mediators of interspecific interactions
8 (Hatcher et al. 2006): they may confer a competitive advantage to the host species (Yan et al.
9 1998), alter dominance relationships and predation hierarchies (MacNeil et al. 2003a), and
10 may promote species exclusion or coexistence (MacNeil et al. 2003b; Prenter et al. 2004).
11 By mediating native-invader interactions, parasites can play a key role in the outcome of a
12 biological invasion (MacNeil et al. 2003a; MacNeil et al. 2003b; Prenter et al. 2004). For
13 example, in Northern Ireland, the acanthocephalan parasite *Echinorynchus truttae* reduces the
14 predatory impact of the invasive amphipod *Gammarus pulex* on the native *G. duebeni celticus*
15 (MacNeil et al. 2003b).

16

17 The North American Signal Crayfish, *Pacifastacus leniusculus* (Dana), has become
18 established throughout Britain as a result of escapes from farms (Holdich et al. 2004). The
19 species is highly invasive and commonly leads to the displacement of Britain's only native
20 crayfish *Austropotamobius pallipes* (Lereboullet) (Bubb et al. 2006; Kemp et al. 2003) As a
21 result, populations of *A. pallipes* are now concentrated in central and northern England
22 (Souty-Grosset et al. 2006) where they are of global importance, representing the densest
23 concentrations of the species within Europe (Holdich 2003). The mechanism by which *A.*
24 *pallipes* is displaced varies between populations. In some cases, the native species is
25 displaced through competitive interactions, (Bubb et al. 2006); however the exact mechanism

1 by which this occurs is unclear. In many water courses in the south of England, extinction of
2 *A. pallipes* has resulted from crayfish plague (Kemp et al. 2003). The invasive crayfish, *P.*
3 *leniusculus*, commonly acts as a reservoir for *Aphanomyces astaci* (the causative agent of
4 crayfish plague), which is fatal to the native species (Holdich 2003).

5
6 Also of interest are two further parasites. The microsporidian parasite *Thelohania*
7 *contejeani* (Henneguy), infects *Austropotamobius pallipes* causing porcelain disease and is
8 the most widely recorded parasitic infection of this species (Alderman and Polglase 1988).
9 Whilst the pathology of *T. contejeani* is not as severe as that of crayfish plague it can be a
10 serious threat within crayfish aquaculture (Edgerton et al. 2002) and may cause changes in the
11 ecology of its host through changes in diet (Chartier and Chaisemartin 1983); however the
12 consequences of infection by many pathogen groups in European freshwater crayfish are
13 largely poorly understood (Edgerton et al. 2004). Microsporidia are widespread in crustacean
14 hosts (Edgerton et al. 2002; Terry et al. 2004) and can cause significant mortality (Alderman
15 and Polglase 1988). A second parasite, the protist *Psorospermium haeckeli* (Haeckel) infects
16 crayfish and has recently been isolated from *A. pallipes* (Rogers et al. 2003) and *Pacifastacus*
17 *leniusculus* (Dieguez-Urbeondo et al. 1993). The influence of these parasites upon
18 native/invasive interactions in crayfish is unknown.

19
20 In the UK, Yorkshire is a stronghold for *A. pallipes*: although *P. leniusculus* is present
21 within the county in substantial numbers, it has not yet displaced many native populations and
22 mixed populations do exist (Peay and Rogers 1999). Here we investigate possible
23 competitive interactions by comparing the sizes of native and invading individuals in single
24 species versus mixed species populations. Secondly we use PCR screening and sequence
25 analysis to compare parasite diversity in the native and invasive crayfish, focusing on

1 microsporidian parasites.

2

3 **Materials and Methods**

4 *Animal collection and measurement*

5 A total of seven *A. pallipes* populations, four *P. leniusculus* populations and three mixed
6 species sites were surveyed between June and August 2005 (Table 1). Sites in the Wharfe
7 catchment were similar to each other and were typified by boulders and smaller stones
8 overlying gravel. Sites in the Dearne catchment (including Cawthorne Dike) were also
9 similar to each other and were typified by boulders and small stones overlying deep silt. Sites
10 were surveyed for crayfish using a standardised manual survey of selected refuges within a
11 site (Peay 2003). Selection of similar sized refuges at each site ensures no size bias during
12 collection (Peay 2003). For each crayfish individual captured we recorded the species, size
13 (carapace length) and sex. In addition any signs of disease, breeding or moult were recorded:
14 microsporidian infections when at high burden typically cause opacity of muscle tissues as a
15 result of spore replication and muscle pathology (Alderman and Polglase 1988);
16 *Aphanomyces astaci* can be identified by the appearance of brown melanisations on the
17 exoskeleton of the infected animal (Alderman and Polglase 1988). Following assessment,
18 crayfish were set aside to prevent duplication of records, until the population assessment of
19 the site had been completed. All *Austropotamobius pallipes* were then released; *P. leniusculus*
20 were stored at -20 °C.

21

22 *Statistical analysis*

23 Statistical analyses were conducted using R version 4.2.1. (www.R-project.org). Linear mixed
24 effects models (LMM) were fitted to the size distribution data for each species separately
25 using Maximum Likelihood fits. Size was used as the dependent variable with population

1 (single vs. mixed species) and sex as fixed factors; site identity was included in the model as a
 2 random factor to control for any inter-site differences in size composition.

3

4 **Table 1.** Field sites sampled during the study. All populations were surveyed for size
 5 distribution; ^b denotes populations from which *P. leniusculus* or dead *A. pallipes* were
 6 obtained for parasite screening

Site Name	Watercourse	Site Grid Reference	Population composition
Cawthorne South	Cawthorne Dike	SE299087	<i>P. leniusculus</i>
Road Bridge ^b	Cawthorne Dike	SE295088	Mixed
Haigh ^b	River Dearne	SE300116	<i>P. leniusculus</i>
Burnsall ^b	River Wharfe	SE025622	<i>P. leniusculus</i>
Lobwood	River Wharfe	SE077518	Mixed
Addingham	River Wharfe	SE082500	Mixed
Footbridge	River Wharfe	SE122484	<i>A. pallipes</i>
Riverside Gardens	River Wharfe	SE113480	<i>A. pallipes</i>
Denton Stones ^b	River Wharfe	SE132482	<i>A. pallipes</i>
Fenay ^b	Fenay Beck	SE179160	<i>P. leniusculus</i>
Adel Dam	Adel Beck	SE275407	<i>A. pallipes</i>
Meanwood ^b	Meanwood Beck	SE281385	<i>A. pallipes</i>
Grange Park	Wyke Beck	SE341363	<i>A. pallipes</i>
Gipton	Wyke Beck	SE342353	<i>A. pallipes</i>

7

8 In order to determine whether parasite prevalence differed between sexes or sizes of *P.*
 9 *leniusculus*, a Generalized Linear Model (GLM) with binomial error distributions was fitted

1 to the data. Microsporidian presence or absence was used as the dependent variable with size
2 and sex as fixed factors.

3

4 Non-significant fixed factors were removed from the maximal models in a stepwise
5 fashion until only factors significant at the 5 % level remained.

6

7 *Screening for microsporidian parasites*

8 Fifty-two *P. leniusculus* from the field collection (Table 1) were screened for microsporidia
9 (Table 2). As *A. pallipes* is classified as vulnerable (IUCN 2004) and protected under
10 Schedule 5 of the Wildlife and Countryside Act (1981), we did not screen live animals
11 collected from the field; however twelve dead *A. pallipes* obtained from sites detailed in
12 Table 1 were screened for microsporidia. Sampling was carried out towards the end of the
13 breeding season when most young have hatched and dispersed (Holdich 2003). However, one
14 female *P. leniusculus* still had two eggs attached; as many microsporidia are vertically
15 transmitted (Dunn and Smith 2001) we also screened these to test for the presence of
16 vertically transmitted parasites.

17

18 Crayfish tissue (approximately 0.25g) was dissected from tail muscle between the 3rd
19 and 4th pleonites, being careful to avoid sampling gut tissue. Eggs from the single gravid
20 female sampled were collected and homogenised. DNA was extracted using a chloroform
21 extraction described by Doyle and Doyle (1987) with modifications described in McClymont
22 et al. (2005).

23

24

25

1 Table 2. Results of PCR screen for microsporidian infection in *P. leniusculus*. Summary of
 2 PCR results for *P. leniusculus*; for site grid references refer to Table 1.

Site	Number of individuals screened	Number of infected individuals	Observed Prevalence
Cawthorne Road Bridge	16	7	0.44
Burnsall	13	5	0.38
Haigh	4	3	0.75
Fenay	19	5	0.26
Total	52	20	0.38

3
 4 PCR of the host cytochrome C oxidase 1 (CO1) gene was used to confirm the quality of
 5 the DNA extraction before PCR for microsporidian SSU rDNA was carried out. Primers used
 6 for detection of host DNA were LCO1490 and HCO2198, which amplify a fragment of the
 7 CO1 gene (Folmer et al. 1994). The CO1 PCR protocol was as described in McClymont et al.
 8 (2005). Positive controls containing DNA extracted from microsporidium infected crayfish
 9 muscle stored in ethanol and negative controls containing deionised water in place of DNA
 10 were included for each reaction; the total reaction volume was 25 µl.

11
 12 Three primer sets were used for detection of microsporidian SSU rDNA. V1f
 13 (Vossbrinck and Woese 1986) and 1492r (Weiss et al. 1994) are specific for *T. contejeani*
 14 (Lom et al. 2001), whilst both V1f and 530r (Baker et al. 1995), and 18sf (Baker et al. 1995)
 15 and 964r (McClymont et al. 2005) are general microsporidian primers. The PCR reaction
 16 mixture and protocols are as described by McClymont et al. (2005); annealing temperatures
 17 and PCR product lengths are shown in Table 3.

1 Positive controls containing DNA extracted from microsporidium infected crayfish muscle
 2 stored in ethanol and negative controls containing deionised water in place of DNA were
 3 included for each reaction; the total reaction volume was 25 µl for initial parasite detection.
 4 PCR protocols were all carried out on a Hybaid Omn-E Thermal Cycler (Hybaid Ltd,
 5 Waltham, Massachusetts, USA).

6
 7 Table 3. PCR annealing temperatures and approximate expected product length for primers
 8 used in parasite detection and for sequencing

Primers	Annealing temperature/°C	Product length/bp
V1f-1492r	50	1500
18sf-964r	50	900
V1f-530r	60	600
350f-964r	60	800
18sf-350r	50	600
18sf-530r	50	700
HA3bf-HG4r	60	1500
HG4f-HG4r	50	1200
HG4f-1492r	50	600
Thelof-580r	50	1400
BACF-1492r	50	800

9
 10 *Sequencing and phylogenetic analysis of microsporidia*
 11 Different primer sets gave positive bands in different individuals suggesting the presence of
 12 more than one microsporidian parasite within *P. leniusculus*. Therefore additional primers
 13 were used in order to obtain longer sequences: these were 580r (Vossbrinck et al. 1993),

1 Ha3Bf (Gatehouse and Malone 1998), HG4r (Gatehouse and Malone 1998), 350f (Weiss and
2 Vossbrinck 1998), HG4f (Gatehouse and Malone 1998), 1342r (McClymont et al. 2005) and
3 350r (5'-CCAAGGACGGC-AGCAGGCGCGAAA-3'), together with new primers Thelof
4 (5'-TCGTAGTTCCG-CGCAGTAAACTA-3') and BACF (5'-
5 ATATAGGAACAGATGATGGC-3'). Annealing temperatures for all primer combinations
6 are given in Table 3. Where PCR products were to be sequenced the amounts of reagents in
7 the reaction mixture were doubled to give a total reaction volume of 50 µl.

8

9 50 µl of each PCR product were electrophoresed through a 2 % agarose TAE gel in
10 standard TAE buffer, stained with ethidium bromide and visualised by UV light to ensure
11 successful amplification of the PCR product. PCR products were excised from the gel and
12 purified using a QIAQuick Gel Purification Kit (Qiagen, Crawley, UK) and were sequenced
13 on an ABI 3130xl capillary sequencer at the University of Leeds.

14

15 The closest matching sequence to each sequence generated within this study was
16 determined using the NCBI-BLAST database (Altschul et al. 1997) and a percentage
17 sequence similarity calculated using the pairwise alignment function in BioEdit (Hall 2005).

18

19 *Screening for Aphanomyces astaci and Psorospermium haeckeli*

20 In addition, a subset of fifteen *Pacifastacus leniusculus* and three *Austropotamobius pallipes*
21 from the field collection were screened for the presence of *Aphanomyces astaci* and of
22 *Psorospermium haeckeli*

23

24 Tissue was dissected from the eye to screen for the presence of *A. astaci* as in the early
25 stages of the infection mycelium are known to be present within the cornea (Vogt 1999).

1 DNA extraction was performed and confirmed as described previously. Primers 525 and 640
2 were used to screen for *A. astaci*, with an expected product length of 115 bp (Oidtmann et al.
3 2004). The reaction mixture comprised 0.625 U of GoTaq Taq polymerase and 5 µl 5 x
4 GoTaq buffer (giving a final concentration of 1.5 mM MgCl₂ per reaction) (Promega,
5 Southampton, UK), 0.04 mM dNTPs, 10 pmol of each primer, 1 µl DNA and deionised water
6 in a total reaction volume of 25 µl. No positive control material was available; a negative
7 control containing deionised water in place of DNA was included for each PCR reaction. The
8 PCR protocol is as described in Oidtmann et al. (2004).

9

10 To screen for *Psorospermium haeckeli*, tissue was dissected from the subepidermal
11 connective tissue as high parasite burdens have been reported from this tissue type (Henttonen
12 1996). DNA extraction was performed and confirmed as described previously. Primers Pso-
13 1 (Bangyeekhun et al. 2001) and ITS-4 (White et al. 1990) were used to screen for *P. haeckeli*
14 with expected product lengths of 1300 or 1500 bp (Bangyeekhun et al. 2001). The reaction
15 mixture comprised 1.25 U of GoTaq Taq polymerase, 5 µl 5 x GoTaq buffer (Promega,
16 Southampton, UK), 2 mM MgCl₂, 0.08 mM dNTPs, 20 pmol of each primer, 1 µl of DNA
17 and deionised water in a total reaction volume of 25 µl. No positive control was available; a
18 negative control containing deionised water in place of DNA was included for each PCR.
19 The PCR protocol is as described in Bangyeekhun et al. (2001).

20

21 **Results**

22 *Sizes of animals in single and mixed populations*

23 *Austropotamobius pallipes*

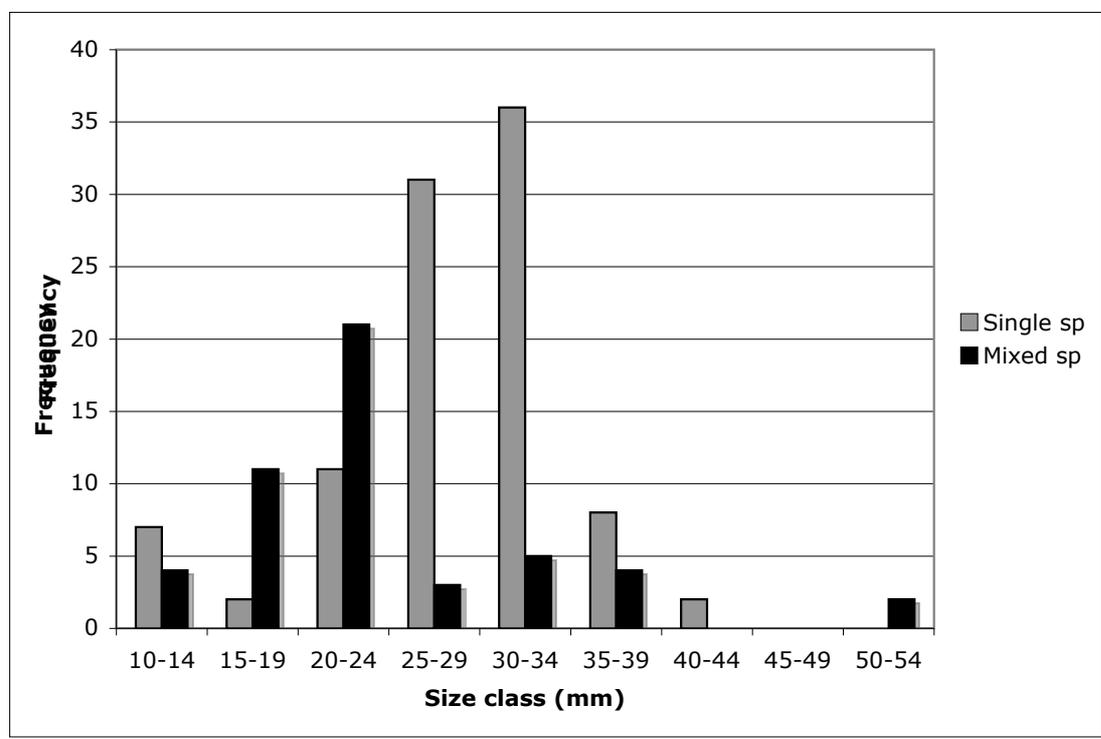
24 Following stepwise deletion of non-significant fixed effects from the Maximal model,
25 population composition (single vs. mixed species) was the only significant term remaining in

1 the Minimum Adequate Model (LMM, $F_{1,73}$, $p=0.025$) indicating a significant difference in
2 size composition of single and mixed species populations. The mean size of *A. pallipes* was
3 28.5 mm in single species populations and 22.5 mm in mixed populations (Fig. 1).

4

5 Fig 1. Size distributions of *Austropotamobius pallipes* in single species and mixed species
6 populations. *A. pallipes* individuals in single species populations were significantly larger
7 than those in mixed species populations (LMM, $F_{1,73}$, $p=0.025$)

8



9

10

11 *Pacifastacus leniusculus*

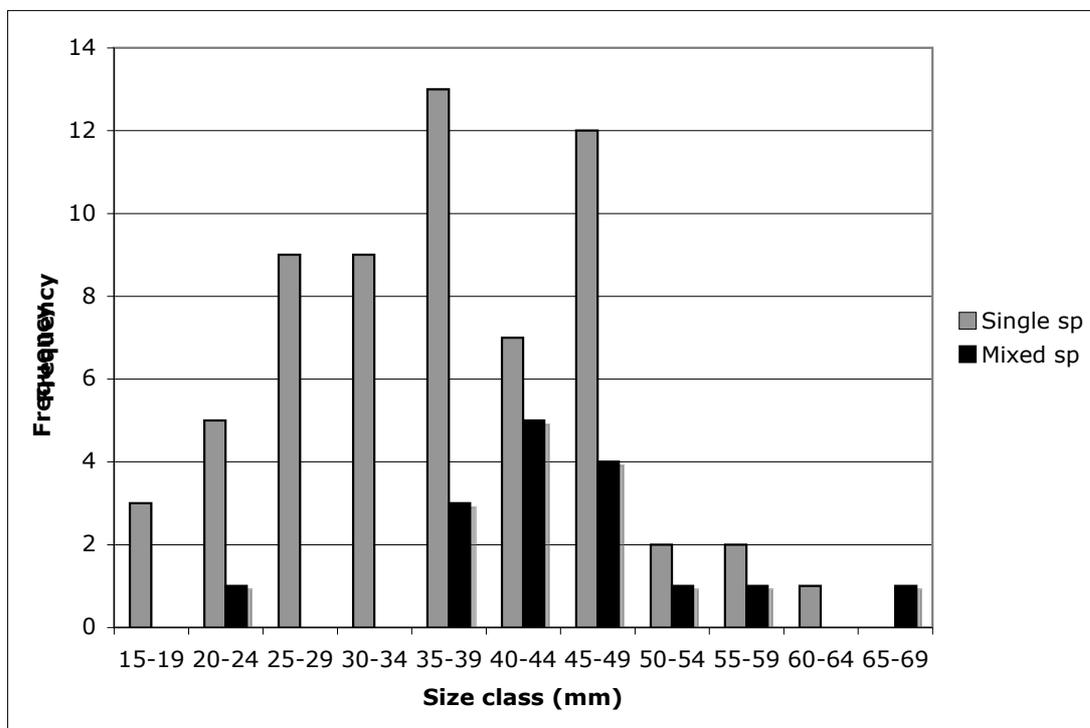
12 Following stepwise deletion of non-significant fixed effects, population composition (single
13 vs. mixed species) was the only significant term remaining in the Minimum Adequate Model
14 (LMM, $F_{1,72}$, $p=0.028$). *P. leniusculus* individuals in mixed populations were significantly
15 larger than their counterparts in single species populations with a mean size of 36.3 mm in

1 single species populations and 46.0 mm in mixed species populations (Fig. 2). Very few
2 juveniles were observed in the mixed species sites.

3

4 Fig 2. Size distributions of *Pacifastacus leniusculus* in single species and mixed species
5 populations. *P. leniusculus* individuals in single species populations were significantly
6 smaller than individuals in mixed species populations (LMM, $F_{1,72}$, $p=0.028$)

7



8

9 *Microsporidian parasites*

10 All twelve *A. pallipes* individuals tested showed clinical signs of microsporidian infection
11 through an opacity of the abdominal musculature; these all tested positive for microsporidian
12 infection through PCR screening. As we were only able to screen dead individuals from the
13 field, we were unable to estimate the prevalence of microsporidian infection for this species.

14

15 The prevalence of microsporidian infection in *P. leniusculus* ranged from 0.26 to 0.75,

1 with an overall prevalence across all populations of 0.38 (Table 2). Six of the twenty infected
2 individuals showed clinical signs of infection through an opacity of the abdominal
3 musculature; one of these was dead when collected. There was no significant difference
4 between the frequency of infection of males versus females (GLM, $p_{47}=0.181$) and there was
5 no significant difference in sizes of infected versus uninfected individuals (GLM, $p_{48}=0.831$).

6

7 *Parasite sequences*

8 We obtained multiple sequences from 4 distinct microsporidian parasite species (Table 4).
9 Three of these parasites, *Bacillidium* sp. PLFB32, *Microsporidium* sp. PLWB7A and
10 *Vittaforma* sp. PLDH3, had not previously been reported from crayfish hosts and represent
11 novel microsporidian sequences; the fourth, *Thelohania contejeani*, despite having been
12 previously recorded in crayfish, had not been sequenced from either of the two study species.

13

14 Forty-four sequences from 29 individuals were 98 % -100 % identical to *T. contejeani*
15 isolated from the crayfish *Astacus fluviatilis* in France (Lom et al. 2001). These sequences
16 were obtained from 17 *P. leniusculus* and 12 *Austropotamobius pallipes*. We detected two
17 strains of *T. contejeani* within each crayfish species, corresponding to strains TcC2 and TcC3
18 described by Lom et al. (2001). We found strain TcC2 in 7 individuals: 3 *A. pallipes* and 4 *P.*
19 *leniusculus*. We sequenced strain TcC3 from 18 individuals: 8 *A. pallipes* and 10 *P.*
20 *leniusculus*. Four samples were not sequenced across the variable region and so could belong
21 to either strain. In three cases we sequenced both strains from the same host, twice in *A.*
22 *pallipes* and once in *P. leniusculus*.

- 1 Table 4. Summary of microsporidian parasite diversity in *A. pallipes* and microsporidian diversity and prevalence *P. leniusculus*. It should be
 2 noted that only dead individuals of *A. pallipes* were screened for parasites, and so prevalence cannot be estimated for this species.

Parasite	% similarity	<i>A. pallipes</i>	<i>P. leniusculus</i>	Genbank Accession numbers
<i>Thelohania contejeani</i> isolates Tcc2PL, Tcc3PL, Tcc2AP and Tcc3AP	98-100% similarity to <i>Thelohania</i> <i>contejeani</i> (AF492593 and AF492594)	12/12	17/52	AM261747, AM261750, AM261751, AM261752, AM261753
<i>Vittaforma</i> sp. isolate PLDH3	95% similarity to <i>Microsporidium</i> sp. CRANFA (AJ966723) 93% similarity to <i>Vittaforma</i> -like parasite (AY375044)	Absent	1/52	AM261754
<i>Bacillidium</i> sp. isolate PLFB32	97% similarity to <i>Bacillidium</i> <i>vesiculoformis</i> (AJ581995)	Absent	1/52	AM261748
<i>Microsporidium</i> sp. isolate PLWB7A	75% similarity to <i>Bacillidium</i> <i>vesiculoformis</i> (AJ581995)	Absent	1/52	AM261749

1 Two sequences isolated from one *P. leniusculus* had 97 % sequence similarity
2 to *Bacillidium vesiculoformis*, a species that has to date only been described from the
3 oligochaete worm *Nais simplex* in Scotland. One sequence isolated from a *P.*
4 *leniusculus* egg had 75 % sequence similarity to *B. vesiculoformis*; the parent crayfish
5 tested negative for microsporidian infection.

6
7 Two sequences isolated from a single *P. leniusculus* host had 95 % sequence
8 similarity to *Microsporidium sp.* CRANFA isolated from the amphipod crustacean
9 *Crangonyx floridanus* in Florida (Galbreath 2005), and 93 % sequence similarity to a
10 *Vittaforma*-like parasite isolated from a human host (Sulaiman et al. 2003).

11
12 We found no clinical/visible signs of *Aphanomyces astaci* infection in any of
13 the individuals sampled. No evidence was found for infection by either *A. astaci* or
14 *Psorospermium haeckeli* in any of subset the individuals screened for these parasites
15 by PCR.

16
17 **Discussion**

18 *Competitive interactions*

19 In mixed populations the size distributions of both species differ from those in single
20 species populations. *Austropotamobius pallipes* tend to be smaller in mixed
21 populations (Fig. 1) whereas *Pacifastacus leniusculus* tend to be larger (Fig. 2).
22 Displacement mechanisms proposed in other native-invader crayfish systems include
23 reproductive interference (Westman et al. 2002); competitive exclusion from refuges
24 resulting in differential predation (Vorburger and Ribic 1999); and differential
25 susceptibility to diseases (Alderman and Polglase 1988).

1

2 In Finland, where *P. leniusculus* displaces the native *Astacus astacus*, it is
3 thought that reproductive interference by dominant *P. leniusculus* males results in the
4 majority of *A. astacus* females producing only sterile eggs (Westman et al. 2002).
5 Our data provide no support for this mechanism of displacement in our study system
6 as smaller *Austropotamobius pallipes* were more common in mixed populations (Fig.
7 1); this is in direct contrast to the pattern of fewer small *A. pallipes* in mixed
8 populations that would be predicted by reproductive interference (Westman et al.
9 2002).

10

11 Our data show large *A. pallipes* to be under-represented in mixed populations
12 (Fig. 1), which may reflect competitive exclusion by the larger (Lowery 1988) and
13 more dominant (Vorburger and Ribi 1999) invader from limited refuges (Bubb et al.
14 2006), since small *P. leniusculus* and large *A. pallipes* overlap in size (Fig. 1, 2). *P.*
15 *leniusculus* has been shown to oust other crayfish species from refuges (Söderbäck
16 1995) which would leave larger *A. pallipes* more vulnerable to predation (Söderbäck
17 1994, after Söderbäck 1992) and result in the reduction of large *A. pallipes* in the
18 mixed populations seen within our study.

19

20 The absence of juvenile *P. leniusculus* from mixed populations (Fig. 2) is
21 interesting, and implies that *A. pallipes* may in fact be influencing the population
22 structure of the invading species. The moulting of juvenile *P. leniusculus* is
23 synchronized, resulting in reduced intraspecific cannibalism (referenced in
24 Ahvenharju et al. 2005). However, interspecific predation by the native *A. pallipes*
25 (Gil-Sánchez and Alba-Tercedor 2006) as well as other predators such as fish

1 (Söderbäck 1992) may underpin the observed reduction in juvenile *P. leniusculus* in
2 mixed populations.

3 *Parasitism in native and invasive crayfish*

4 Four species of microsporidia were detected in the invasive crayfish *P. leniusculus*.
5 In contrast, only one microsporidian parasite was detected from *A. pallipes* although
6 the sample size was small. The overall prevalence of microsporidian infection in *P.*
7 *leniusculus* was 38 % (Table 2). This prevalence is higher than previous reports of
8 visible microsporidiosis in *A. pallipes* in Britain (9 %, (Brown and Bowler 1977); 26
9 % (Rogers et al. 2003); 30 % (Evans and Edgerton 2002)), France (0-8%, Chartier
10 and Chaisemartin 1983) and Spain (1%, Dieguez-Uribeondo et al. 1993), probably
11 reflecting a higher detection efficiency by PCR.

12
13 The *T. contejeani* sequences we obtained were identical to those previously
14 isolated from *Astacus fluviatilis* (Genbank accession numbers AF492593 and
15 AF492594, Lom et al. 2001). This is, to our knowledge, the first molecular
16 confirmation of *T. contejeani* infecting *P. leniusculus*, as well as the first report of the
17 parasite in an invasive species in Europe. Whilst *T. contejeani* has previously been
18 reported from *Austropotamobius pallipes* in the UK (Brown and Bowler 1977;
19 Edgerton et al. 2002; Rogers et al. 2003), these reports were based on light
20 microscopy and lack the ultrastructural or molecular information to confirm species
21 identity (Dunn and Smith 2001). This is the first molecular confirmation of the
22 presence of *T. contejeani* infecting *A. pallipes*.

23
24 The presence of *T. contejeani* in the invasive *P. leniusculus* leads to the question
25 of how the parasite has come to infect this species. Firstly, *P. leniusculus* may have

1 brought the parasite with it from its native range. *T. contejeani* has been reported
2 from a number of crayfish hosts (Graham and France 1986; Quilter 1976), and there is
3 a single report of *T. contejeani* from *P. leniusculus* in its native range in California
4 (McGriff and Modin 1983); but identification is based on spore size, and molecular or
5 ultrastructural confirmation is lacking. The pattern of infection in the current study
6 leads us to suggest that it is more likely that *P. leniusculus* in the UK has acquired *T.*
7 *contejeani* from the native host. *T. contejeani* was detected in *A. pallipes* only sites,
8 in mixed sites and in sites where only *P. leniusculus* occurred. Furthermore, identical
9 sequences were found in the native and invading species (Table 4). These data fit a
10 pattern of transmission from the native *A. pallipes* to the invading species in mixed
11 sites. Detailed studies of the fitness effects of *T. contejeani* and its mode of
12 transmission within and between crayfish species are required.

13
14 In addition, our *T. contejeani* sequences had 98-100 % sequence similarity to
15 the unclassified microsporidium, *Microsporidium* sp. JES2002H, which was detected
16 in three species of amphipod in France (Terry et al. 2004). We suggest that
17 *Microsporidium* sp. JES2002H and *T. contejeani* may be the same species, although
18 confirmation awaits ultrastructural analysis of *Microsporidium* sp. JES2002H.

19
20 In one *P. leniusculus* we found a microsporidium sequence with 97 % sequence
21 similarity to *Bacillidium vesiculoformis*, a parasite previously described from the
22 oligochaete worm *Nais simplex* in Scotland, UK (Morris et al. 2005). The sequence
23 similarity indicates that the parasite is likely to be in the same genus as *B.*
24 *vesiculoformis*; however further molecular and morphological analysis would be
25 required to confirm this. This is the first record of a *Bacillidium* spp. in crayfish and

1 supports Morris et al's (2005) suggestion that *B. vesiculoformis* is a generalist
2 parasite.

3

4 The *Vittaforma*-like parasite sequenced from *P. leniusculus* had closest
5 sequence similarity (95 %) to an unidentified *Microsporidium* sp. CRANFA
6 sequenced from *Crangonyx floridanus* in Florida (Galbreath 2005), which suggests
7 that this may be a parasite originating in the native range of *P. leniusculus*.

8

9 We also sequenced a novel parasite from a *P. leniusculus* egg. This parasite was
10 unlike other microsporidia and had the closest sequence similarity (75 %) to *B.*
11 *vesiculoformis*. The presence of the parasite in the egg suggests vertical transmission
12 (Terry et al. 2004), widespread amongst microsporidian parasites (Dunn and Smith
13 2001). Muscle tissue from the mother tested negative for microsporidian infection,
14 but ovarian tissue was not screened in this study.

15

16 We found no evidence of *Aphanomyces astaci* or *Psorospermium haeckeli*
17 within our study populations (although our results should be treated with caution
18 owing to the absence of positive control material). The absence of crayfish plague
19 may explain the persistence of mixed species populations in Yorkshire and highlights
20 the need for vigilance in preventing plague from spreading into these rivers.

21

22 In summary, our size distribution data are in accord with a pattern of
23 competitive exclusion of *Austropotamobius pallipes* from refuges leading to
24 differential predation. We provide the first molecular evidence for the presence of the
25 microsporidian parasite *T. contejeani* in both *A. pallipes* and *Pacifastacus leniusculus*.

1 We also detected three novel microsporidian sequences in *P. leniusculus*. This raises
2 the question of the effects of these parasites on host fitness as well as their potential
3 influence on native - invader interactions.

4

5

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1 **References**

- 2 Ahvenharju T, Savolainen R, Tulonen J et al. (2005) Effects of size grading on
3 growth, survival and cheliped injuries of signal crayfish (*Pacifastacus leniusculus*
4 Dana) summerlings (age 0+). *Aquac Res* 36: 857-867
- 5 Alderman DJ and Polglase JL (1988) Pathogens, Parasites and Commensals. In:
6 Holdich DM and Lowery RS (eds) *Freshwater Crayfish: Biology, Management and*
7 *Exploitation*. Croom Helm, London
- 8 Altschul SF, Madden TL, Schäffer AA et al. (1997) Gapped BLAST and PSI-
9 BLAST: a new generation of protein database search programs. *Nucleic Acids Res*
10 25: 3389 - 3402
- 11 Baker MD, Vossbrinck CR, Didier ES et al. (1995) Small Subunit Ribosomal DNA
12 Phylogeny of Various Microsporidia with Emphasis on AIDS Related Forms. *J*
13 *Eukaryot Microbiol* 42: 564 - 570
- 14 Bangyeekhun E, Ryyänen HJ, Henttonen P et al. (2001) Sequence analysis of the
15 ribosomal internal transcribed spacer DNA of the crayfish parasite *Psorospermium*
16 *haeckeli*. *Dis Aquat Organ* 46: 217 - 222
- 17 Bauer A, Trouve S, Gregoire A et al. (2000) Differential influence of
18 *Pomphorhynchus laevis* (Acanthocephala) on the behaviour of native and invader
19 gammarid species. *Int J Parasitol* 30: 1453 - 1457
- 20 Brown DJ and Bowler K (1977) A population study of the British freshwater crayfish
21 *Austropotamobius pallipes* (Lereboullet). *Freshwater Crayfish* 3: 33 - 49
- 22 Bubb DH, Thom TJ and Lucas MC (2006) Movement, dispersal and refuge use of co-
23 occurring introduced and native crayfish. *Freshwater Biol* 51: 1359 - 1368

1 Chartier L and Chaisemartin C (1983) Effect of *Thelohania* infection on populations
2 of *Austropotamobius pallipes* in granitic and calcareous habitats. Comptes Rendus des
3 Seances de L'Academie des Sciences, Paris 297: 441 - 443

4 Dieguez-Uribeondo J, Pinedo-Ruiz J, Cerenius L et al. (1993) Presence of
5 *Psorospermium haeckeli* (Hilgendorf) in a *Pacifastacus leniusculus* (Dana)
6 population of Spain. Freshwater Crayfish 9: 286 - 288

7 Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities
8 of fresh leaf tissue. Phytochem Bull 19: 11 - 15

9 Dunn AM and Smith JE (2001) Microsporidian life cycles and diversity: the
10 relationship between virulence and transmission. Microbes Infect 3: 381 - 388

11 Edgerton BF, Evans LH, Stephens FJ et al. (2002) Synopsis of freshwater crayfish
12 diseases and commensal organisms. Aquaculture 206: 57 - 135

13 Edgerton BF, Henttonen P, Jussila J et al. (2004) Understanding the Causes of
14 Disease in European Freshwater Crayfish. Conserv Biol 18: 1466 - 1474

15 Evans LH and Edgerton BF (2002) Pathogens, parasites and commensals. In: Holdich
16 DM (ed) Biology of freshwater crayfish. Blackwell Science, Oxford, United Kingdom

17 Folmer O, Black M, Hoeh W et al. (1994) DNA primers for amplification of
18 mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates.
19 Mol Mar Biol Biotechnol 3: 294 - 299

20 Galbreath JGS (2005) The impact of intercontinental invasion on host genetic and
21 microsporidian parasite diversity in the freshwater amphipod *Crangonyx*
22 *pseudogracilis*. Dissertation, University of Leeds

23 Gatehouse HS and Malone LA (1998) The ribosomal RNA gene of *Nosema apis*
24 (Microspora): DNA sequence for small and large subunit rRNA genes and evidence
25 of a large tandem repeat size. J Invertebr Pathol 71: 97 - 105

1 Gil-Sánchez JM and Alba-Tercedor J (2006) The Decline of the Endangered
2 Populations of the Native Freshwater Crayfish (*Austropotamobius pallipes*) in
3 Southern Spain: It is Possible to Avoid Extinction? *Hydrobiologia* 559: 113 - 122

4 Graham I and France R (1986) Attempts to transmit experimentally the
5 microsporidian *Thelohania contejeani* in freshwater crayfish (*Orconectes virilis*).
6 *Crustaceana* 51: 208 - 211

7 Hall T (2005) BioEdit: Biological sequence alignment editor for
8 Win95/98/NT/2K/XP. Accessed 07/06/2005.
9 <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>

10 Hatcher MJ, Dick JTA and Dunn AM (2006) How parasites affect interactions
11 between competitors and predators. *Ecol Lett* 9: 1253 - 1271

12 Henttonen P (1996) The Parasite *Psorospermium* in Freshwater Crayfish.
13 Dissertation, University of Kuopio

14 Holdich DM (2003) Ecology of the White-Clawed Crayfish. *Conserving Natura 2000*
15 *Rivers Ecology Series No. 1*. English Nature, Peterborough

16 Holdich DM, Sibley P and Peay S (2004) The White-Clawed Crayfish - a decade on.
17 *British Wildlife* 15: 153 - 164

18 IUCN (2004) IUCN Red List of Threatened Species. Accessed 07/04/2005.
19 www.redlist.org

20 Kemp E, Birkinshaw N, Peay S et al. (2003) Reintroducing the White-Clawed
21 Crayfish *Austropotamobius pallipes*. *Conserving Natura 2000 Rivers Conservation*
22 *Techniques Series No. 1*. English Nature, Peterborough

23 Krakau M, Thielges DW and Reise K (2006) Native Parasites Adopt Introduced
24 Bivalves of the North Sea. *Biol Invasions* 8: 919 - 925

1 Lom J, Nilsen F and Dykova (2001) *Thelohania contejeani* Henneguy, 1892:
2 dimorphic life cycle and taxonomic affinities, as indicated by ultrastructural and
3 molecular study. *Parasitol Res* 87: 860 - 872

4 Lowery RS (1988) Growth, moulting and reproduction. In: Holdich DM and Lowery
5 RS (eds) *Freshwater Crayfish: Biology, Management and Exploitation*. Croom Helm,
6 London

7 MacNeil C, Dick JTA, Hatcher MJ et al. (2003a) Parasite-mediated predation
8 between native and invasive amphipods. *P Roy Soc B-Biol Sci* 270: 1309 - 1314

9 MacNeil C, Fielding NJ, Dick JTA et al. (2003b) An acanthocephalan parasite
10 mediates intraguild predation between invasive and native freshwater amphipods
11 (Crustacea). *Freshwater Biol* 48: 2085 - 2093

12 McClymont HE, Dunn AM, Terry RS et al. (2005) Molecular data suggest that
13 microsporidian parasites in freshwater snails are diverse. *Int J Parasitol* 35: 1071 -
14 1078

15 McGriff D and Modin J (1983) *Thelohania contejeani* parasitism of the crayfish,
16 *Pacifastacus leniusculus*, in California. *Calif Fish Game* 69: 178 - 183

17 Morris DJ, Terry RS, Ferguson KB et al. (2005) Ultrastructural and molecular
18 characterization of *Bacillidium vesiculoformis* n. sp. (Microspora: Mrazekiidae) in the
19 freshwater oligochaete *Nais simplex* (Oligochaeta: Naididae). *Parasitology* 130: 31 -
20 40

21 Ohtaka A, Gelder SR, Kawai T et al. (2005) New records and distributions of two
22 North American branchiobdellidan species (Annelida: Clitellata) from introduced
23 signal crayfish, *Pacifastacus leniusculus*, in Japan. *Biol Invasions* 7: 149 - 156

1 Oidtmann B, Schaefer N, Cerenius L et al. (2004) Detection of genomic DNA of the
2 crayfish plague fungus *Aphanomyces astaci* (oomycete) in clinical samples by PCR.
3 Vet Microbiol 100: 269 - 282

4 Peay S (2003) Monitoring the White-clawed Crayfish *Austropotamobius p. pallipes*.
5 Conserving Natura 2000 Rivers Monitoring Series No. 1. English Nature,
6 Peterborough

7 Peay S and Rogers D (1999) The peristaltic spread of signal crayfish in the River
8 Wharfe, Yorkshire, England. Freshwater Crayfish 12: 665 - 677

9 Prenter J, MacNeil C, Dick JTA et al. (2004) Roles of parasites in animal invasions.
10 Trends Ecol Evol 19: 385

11 Quilter CG (1976) Microsporidian parasite *Thelohania contejeani* Henneguy from
12 New Zealand freshwater crayfish. New Zeal J Mar Fresh 10: 225 - 231

13 Rogers D, Hoffman R and Oidtmann B (2003) Diseases in selected *Austropotamobius*
14 *pallipes* populations in England. In: Management and Conservation of Crayfish.
15 Proceedings of a conference held on 7th November 2002. Environment Agency,
16 Bristol

17 Rushton SP, Lurz PWW, Gurnell J et al. (2000) Modelling the spatial dynamics of
18 parapoxvirus disease in red and grey squirrels: a possible cause of decline in the red
19 squirrel in the UK? J Appl Ecol 37: 997 - 1012

20 Söderbäck B (1992) Predator avoidance and vulnerability of two co-occurring
21 crayfish species, *Astacus astacus* (L.) and *Pacifastacus leniusculus* (Dana). Ann Zool
22 Fenn 29: 253 - 259

23 Söderbäck B (1994) Interactions among juveniles of two freshwater crayfish species
24 and a predatory fish. Oecologia 100: 229 - 235

1 Söderbäck B (1995) Replacement of the native crayfish *Astacus astacus* by the
2 introduced species *Pacifastacus leniusculus* in a Swedish lake: possible causes and
3 mechanisms. *Freshwater Biol* 33: 291 - 304

4 Souty-Grosset C, Holdich D, Noël P et al. (eds) (2006) Atlas of crayfish in Europe.
5 Muséum National d'Histoire Naturelle, Paris

6 Sulaiman IM, Matos O, Lobo ML et al. (2003) Identification of a New
7 Microsporidian Parasite Related to *Vittaforma corneae* in HIV-Positive and HIV-
8 Negative Patients from Portugal. *J Eukaryot Microbiol* 50: 586 - 590

9 Terry RS, Smith JE, Sharpe RG et al. (2004) Widespread vertical transmission and
10 associated host sex-ratio distortion within the eukaryotic phylum Microspora. *P Roy*
11 *Soc B-Biol Sci* 271: 1783 - 1789

12 Torchin ME, Lafferty KD, Dobson AP et al. (2003) Introduced species and their
13 missing parasites. *Nature* 421: 628 - 629

14 Torchin ME, Lafferty KD and Kuris AM (2001) Release from parasites as natural
15 enemies: increased performance of a globally introduced marine crab. *Biol Invasions*
16 3: 333 - 345

17 Vogt G (1999) Diseases of European freshwater crayfish, with particular emphasis on
18 interspecific transmission of pathogens. In: Gherardi F and Holdich DM (eds)
19 *Crayfish in Europe as Alien Species: How to make the best of a bad situation?* pp 87-
20 103. AA Balkema, Rotterdam, The Netherlands

21 Vorburger C and Ribi G (1999) Aggression and competition for shelter between a
22 native and an introduced crayfish in Europe. *Freshwater Biol* 42: 111 - 119

23 Vossbrinck CR, Baker MD, Didier ES et al. (1993) Ribosomal DNA Sequences of
24 *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: Species Identification and
25 Phylogenetic Construction. *J Eukaryot Microbiol* 40: 354 - 362

1 Vossbrinck CR and Woese CR (1986) Eukaryotic ribosomes that lack a 5.8S RNA.
2 Nature 320: 287 - 288

3 Weiss LM and Vossbrinck CR (1998) Microsporidiosis: molecular and diagnostic
4 aspects. In: Tzipori S (ed) Advances in Parasitology. Academic Press, San Diego

5 Weiss LM, Zhu X, Cali A et al. (1994) Utility of microsporidian rRNA in diagnosis
6 and phylogeny: a review. Folia Parasit 41: 81 - 90

7 Westman K, Savolainen R and Julkunen M (2002) Replacement of the native crayfish
8 *Astacus astacus* by the introduced species *Pacifastacus leniusculus* in a small,
9 enclosed Finnish lake: a 30-year study. Ecography 25: 53 - 73

10 White TJ, Bruns T, Lee S et al. (1990) Amplification and direct sequencing of fungal
11 ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ et al
12 (eds) PCR protocols: a guide to methods and applications. Academic Press, San
13 Diego

14 Yan G, Stevens L, Goodnight CJ et al. (1998) Effects of a tapeworm parasite on the
15 competition of *Tribolium* beetles. Ecology 79: 1093 - 1103

16

17