


# Crayfish plague affects juvenile survival and adult behaviour of invasive signal crayfish

John Rhidian Thomas<sup>1</sup> , Chloe V. Robinson<sup>2</sup>, Agata Mrugała<sup>3</sup>, Amy R. Ellison<sup>1,\*</sup>, Emily Matthews<sup>1</sup>, Siân W. Griffiths<sup>1</sup>, Sofia Consuegra<sup>2</sup> and Jo Cable<sup>1</sup>

## Research Article

\*Present address: Bangor University, School of Natural Sciences, Environment Centre Wales, Bangor LL57 2UW, UK.

**Cite this article:** Thomas JR, Robinson CV, Mrugała A, Ellison AR, Matthews E, Griffiths SW, Consuegra S, Cable J (2020). Crayfish plague affects juvenile survival and adult behaviour of invasive signal crayfish. *Parasitology* **147**, 706–714. <https://doi.org/10.1017/S0031182020000165>

Received: 22 August 2019  
Revised: 17 January 2020  
Accepted: 21 January 2020  
First published online: 12 February 2020

### Key words:

*Aphanomyces astaci*; behaviour; crayfish plague; invasive species; reservoir hosts; signal crayfish

### Author for correspondence:

John Rhidian Thomas,  
E-mail: [Thomasjr6@cardiff.ac.uk](mailto:Thomasjr6@cardiff.ac.uk)

## Abstract

The spread of invasive, non-native species is a key threat to biodiversity. Parasites can play a significant role by influencing their invasive host's survival or behaviour, which can subsequently alter invasion dynamics. The North American signal crayfish (*Pacifastacus leniusculus*) is a known carrier of *Aphanomyces astaci*, an oomycete pathogen that is the causative agent of crayfish plague and fatal to European crayfish species, whereas North American species are considered to be largely resistant. There is some evidence, however, that North American species, can also succumb to crayfish plague, though how *A. astaci* affects such 'reservoir hosts' is rarely considered. Here, we tested the impact of *A. astaci* infection on signal crayfish, by assessing juvenile survival and adult behaviour following exposure to *A. astaci* zoospores. Juvenile signal crayfish suffered high mortality 4-weeks post-hatching, but not as older juveniles. Furthermore, adult signal crayfish with high-infection levels displayed altered behaviours, being less likely to leave the water, explore terrestrial areas and exhibit escape responses. Overall, we reveal that *A. astaci* infection affects signal crayfish to a much greater extent than previously considered, which may not only have direct consequences for invasions, but could substantially affect commercially harvested signal crayfish stocks worldwide.

## Introduction

Parasites have a significant impact on communities and ecosystems by directly affecting host fitness, with subsequent impacts on population dynamics and overall biodiversity (Hudson *et al.*, 2006; Tompkins *et al.*, 2011; Cable *et al.*, 2017). Despite this, parasites are a fundamental component of healthy ecosystems with wide reaching impacts, from influencing the cycle of biogeochemical nutrients to regulating host density and functional traits (Hatcher *et al.*, 2014; Preston *et al.*, 2016). Parasites can also influence their host's behaviour, which can in turn alter the outcome of competitive interactions, reproductive behaviour and dispersal ability (Bakker *et al.*, 1997; Macnab and Barber, 2012; Barber *et al.*, 2017). During invasions by non-native species to new areas, parasites can play a key role facilitating or hindering the successful spread of invaders, while potentially having catastrophic effects on other related native species (Vilcinskas, 2015).

Crayfish are freshwater crustaceans that are commercially harvested in many countries, but can also reach high densities and exert a significant impact on ecosystems, with several species having become widespread, damaging invaders (Holdich *et al.*, 2014; James *et al.*, 2014; Ercoli *et al.*, 2015). For example, in Great Britain, the North American signal crayfish (*Pacifastacus leniusculus*) has become the most common crayfish species, having largely replaced the native white clawed crayfish (*Austropotamobius pallipes*, see Holdich *et al.*, 2014; James *et al.*, 2014). Crayfish are hosts to many parasites and symbionts, including viruses, bacteria, fungi and helminths that can cause chronic, long-term infections (Longshaw *et al.*, 2012; Kozubíková-Balcarová *et al.*, 2013). One such parasite, the oomycete *Aphanomyces astaci*, the causative agent of crayfish plague, is a key threat to crayfish biodiversity worldwide (Svoboda *et al.*, 2017), having eradicated many populations of native European crayfish (Filipová *et al.*, 2013; Kozubíková-Balcarová *et al.*, 2015) and recent evidence suggests it may have also caused a decline in commercially harvested North American crayfish stocks (Edsman *et al.*, 2015; Jussila *et al.*, 2015). This obligate parasitic oomycete penetrates host tissues (Söderhäll *et al.*, 1978) and produces motile reproductive zoospores (Cerenius and Söderhäll, 1984), which can reach high densities (up to several hundred zoospores per litre) during a crayfish plague outbreak (Strand *et al.*, 2014). An infected individual can release about 2700 zoospores per week (Strand *et al.*, 2012), and this number can be much higher when the crayfish is dying or moulting (Makkonen *et al.*, 2013; Svoboda *et al.*, 2013).

Generally, North American crayfish species which have co-evolved with *A. astaci* are considered to be chronic but largely asymptomatic carriers. They combat *A. astaci* through consistent production of prophenoloxidase, which activates a melanization cascade resulting in melanization of hyphae that prevents their invasion into host soft tissues (Cerenius *et al.*, 1987). Most native European crayfish, on the other hand, apparently only produce

prophenoloxidase only in response to infection, which is too slow to effectively melanize the hyphae that then spread into host tissues leading to paralysis and death (Cerenius *et al.*, 2003). The Australian yabby (*Cherax destructor*) also suffers high mortality as a result of crayfish plague, though this species shows some resistance to less virulent strains and survives longer when exposed to highly virulent strains compared to highly susceptible species (Mrugała *et al.*, 2016). In infected European crayfish, severe behavioural changes before death include a lack of coordination and paralysis (Gruber *et al.*, 2014), though to what extent carrier crayfish exhibit behavioural changes is largely unknown and this could play a vital role during new invasions and in commercial crayfish farms. Highly infected crayfish, for example, might be less likely to disperse, which would alter invasion success and introduction to new habitats.

Few studies have directly assessed the effect of the *A. astaci* on North American species, although there is some evidence that they can succumb to the disease and display altered behaviour if also stressed by other factors (Cerenius *et al.*, 1987; Aydin *et al.*, 2014; Edsman *et al.*, 2015). Co-infection of *A. astaci* and *Fusarium* spp., for example, results in eroded swimmeret syndrome (ESS) in signal crayfish, which causes females to carry fewer eggs (Edsman *et al.*, 2015). Mortality of adult signal crayfish has also been observed in experimental settings, though only when crayfish were exposed to very high zoospore numbers (Aydin *et al.*, 2014). Furthermore, vertical transmission of *A. astaci* (from adults to eggs) has been reported (Makkonen *et al.*, 2010), and little is known on how *A. astaci* might affect juvenile North American crayfish.

Here, we addressed two key issues regarding the effects of *A. astaci* on signal crayfish. First, we tested the hypothesis that juvenile signal crayfish would suffer high mortality upon infection by *A. astaci* zoospores, as it has previously been suggested that juvenile crayfish may be more susceptible to infection compared to adults (Mrugała *et al.*, 2016). Additionally, we assessed the effect of *A. astaci* on adult signal crayfish, hypothesizing that even if adults may not suffer significant mortality, behavioural changes would be apparent.

## Materials and methods

### Signal crayfish trapping

All adult signal crayfish were collected in February and March 2017 using cylindrical traps ('Trappy Traps', Collins Nets Ltd., Dorset, UK) baited with cat food and checked daily (trapping licence: NT/CW081-B-797/3888/02). The crayfish were collected from a population displaying negligible levels of infection (maximum agent level A1) when assessed in 2014 (Derw Farm pond, Powys, Wales, SO 13891 37557; James *et al.*, 2017). A small subset of individuals ( $n = 3$ ) re-tested by quantitative polymerase chain reaction (qPCR) (see 'Aphanomyces astaci culture and quantification' section) before the experiments began in May 2017 all revealed low levels of infection by *A. astaci*, although elevated compared to 2014 (agent level A2/A3). After removal from traps, crayfish were transferred to individual containers with 500 mL of pond water and transported to the Cardiff University Aquarium (holding licence: W C ILFA 002), where they were maintained individually in 20 L aquaria containing a plant pot refuge, gravel and air supply delivered *via* an airstone. The crayfish were held at  $13 \pm 1^\circ\text{C}$  under a 12 h light:12 h dark lighting regime and fed a mixture of frozen peas and *Tubifex* bloodworm (Shirley Aquatics, Solihull, West Midlands, UK) once every 2 days. A 50% water change was conducted 1 h after feeding to maintain water quality and remove excess food. Crayfish were acclimatized to the laboratory for at least 4 weeks

before the experiments began. Four females were carrying eggs, and upon hatching, the offspring were mixed, moved to 120 L communal aquaria and used in the juvenile infection experiment. Only male crayfish were used in the adult behavioural tests; since a relatively low number of females ( $n = 6$ ) were caught and therefore it was not possible to test an equal number of males and females in this experiment.

### Aphanomyces astaci culture and quantification

Crayfish in the current study were exposed to a group B strain (Pec14) of *A. astaci* provided by Charles University in Prague. This strain was isolated from dead *Astacus astacus* from a crayfish plague outbreak in the Černý Brook, Czech Republic (Kozubíková-Balcarová *et al.*, 2015) and demonstrated similarly high virulence towards European *A. astacus* (see Becking *et al.*, 2015) as the strains from group B (PsI) used in other experimental studies (Makkonen *et al.*, 2012; Jussila *et al.*, 2013). The culture was maintained in Petri dishes of RGY agar (Alderman, 1982; Becking *et al.*, 2015; Mrugała *et al.*, 2016) and zoospores were produced according to the methodology of Cerenius *et al.* (1987). Briefly, two to four agar culture plugs ( $\sim 2\text{ mm}^2$ ) were cut from an RGY culture and placed in flasks containing 200 mL of liquid RGY-medium. Multiple replicates were done each time in order to produce a sufficient number of zoospores. These cultures were allowed to grow at  $16^\circ\text{C}$  for 2–4 days on a shaker. Once sufficient mycelial growth had occurred, the cultures were washed to induce sporulation and transferred to separate flasks (containing 500 mL of distilled water). The washing was repeated in distilled water three to four times over  $\sim 8$  h. Then, the cultures were incubated at  $13 \pm 1^\circ\text{C}$  for 24–36 h until motile zoospores were produced. The number of zoospores was quantified using a haemocytometer.

Following both experiments, crayfish were euthanized by freezing at  $-20^\circ\text{C}$  for 1 h. For juveniles, the whole crayfish was lysed (TissueLyser, Qiagen) and DNA extracted using a Qiagen DNeasy extraction kit (Qiagen). For adult crayfish, a section of tail-fan and soft-abdominal tissue was removed by dissection, lysed (TissueLyser, Qiagen), and both tissues were pooled ( $\sim 20$  mg total), and the DNA extracted using the same kits. Infection intensity was estimated based on the number of PCR-forming units (PFU) determined by qPCR using TaqMan MGB probes and expressed using the semi-quantitative levels A0–A7 for adults (as described by Vrålstad *et al.*, 2009); with slight modification of the protocol as in Svoboda *et al.* (2014). For juveniles, infection intensity was expressed as number of PFU because a direct comparison cannot be made here between juvenile (whole body) and adult (sample body) infection levels.

### Juvenile crayfish infection

Here, we monitored the survival of juvenile signal crayfish that hatched in the laboratory when exposed either to *A. astaci* at 1, 10 or 100 zoospores  $\text{mL}^{-1}$  or to a sham treatment (control). All crayfish used in this experiment hatched within a 3-day period in May 2017. The infection was conducted twice in separate experiments, the first time approximately 4 weeks after the crayfish hatched ( $n = 25$  crayfish per zoospore treatment) and the second time after 10 weeks with different crayfish ( $n = 17$  individuals per zoospore treatment). When the experiment began, crayfish were housed individually in 1 L pots containing distilled water with a gravel substrate for 48 h. After this acclimatization period, the pots were spiked with 1, 10 or 100 zoospores  $\text{mL}^{-1}$  (the control treatment was given a 20% water change). After a 24 h infection period, 80% of the water in all pots was changed. The crayfish were fed crushed algae wafers and frozen *Tubifex* bloodworm

(Shirley Aquatics, Solihull, West Midlands, UK) once every 2 days. A 50% water change was conducted 1 h after feeding to maintain water quality. For 14 days, we recorded crayfish deaths and any moults daily. Crayfish and moulted carapaces were stored in ethanol at  $-20^{\circ}\text{C}$  until DNA was extracted.

### Adult crayfish behaviour

Male crayfish behaviour was tested in an arena (Fig. 1) consisting of a tank ( $L: 100\text{ cm} \times H: 53\text{ cm} \times W: 48\text{ cm}$ ) with access to a terrestrial area ( $L: 120\text{ cm} \times H: 20\text{ cm} \times W: 20\text{ cm}$ ). At the start of the experiment, crayfish were divided into two groups: those destined for 'high-infection' and those to be kept at 'low-infection' levels. Those destined for the 'high-infection' group ( $n = 15$ , mean carapace length  $52.2\text{ mm}$ ,  $s.d. = 4.44$ ) were individually exposed to a dose of  $1000\text{ zoospores mL}^{-1}$  in  $500\text{ mL}$  of water for 24 h. Simultaneously, the 'low-infection' crayfish ( $n = 17$ , mean carapace length  $53.1\text{ mm}$ ,  $s.d. = 4.66$ ) were sham-infected by adding the same amount of distilled water instead of spore-containing water. After the 24 h period, all crayfish were returned to their individual tanks, where they were held for 1 week before their behaviour was assessed. Individual crayfish were placed into the behavioural arena (Fig. 1) and left to acclimatize overnight. Then, at 09:00 h the next day, their behaviour was recorded using an infrared CCTV camera (Sentient Pro HDA DVR 8 Channel CCTV, Maplin, Rotherham, UK) for 24 h (09:00–21:00 light and 21:00–09:00 dark). During video analysis, the time spent engaged in each of the following four behaviours was recorded for each crayfish: actively walking in water, in a refuge, stationary out of the refuge and moving out of water.

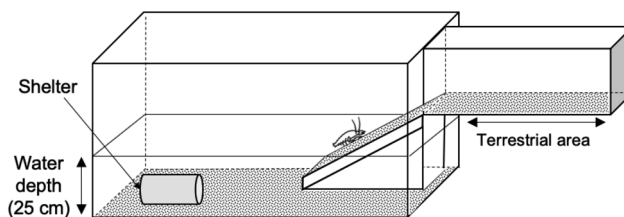
Following this, each crayfish was moved to an aquarium ( $W: 30\text{ cm} \times L: 61\text{ cm} \times D: 37\text{ cm}$ ) with covered sides and allowed to settle for 30 min before their response to being gently touched on the rostrum for 10 s was tested. Crayfish typically reacted by raising their chelae (an aggressive, threatening response) and/or retreating using a characteristic 'tail-flip' response. If a crayfish retreated, the glass rod was immediately moved again to touch the rostrum. This test was repeated three times with 5 min intervals. Whether the crayfish reacted with a 'tail-flip' and/or raised its chelae to attack was recorded. These responses were recorded since behavioural changes that affect a crayfish's ability to retreat or interact with conspecifics may have subsequent effects on competitive ability, resource acquisition, and ultimately, survival.

Following behavioural tests, crayfish were euthanized and *A. astaci* infection levels were quantified as described in the section 'Aphanomyces astaci culture and quantification'.

### Statistical analysis

All statistical analyses were performed in R version 3.5.1 (R Core Team, 2018). For the juvenile crayfish experiment, Kaplan–Meier survival analyses were performed using the 'survival' package in R (Terry and Therneau, 2018) with separate models run for both time points (4- and 10-weeks post-hatching). Both models included spore concentration and carapace length as independent variables. Model selection was based on Akaike information criterion (AIC). It was not possible to statistically assess the effect of moulting on mortality as an insufficient number of moulting events were recorded.

For the adult crayfish, the time spent moving (active), in shelter, stationary (outside of a shelter) or out of the water was quantified over 24 h for each individual. Generalized Additive Models for Location, Scale and Shape (GAMLSS) models (Stasinopoulos *et al.*, 2008) with appropriate distributions (see Table 1) were used to determine whether 'treatment' (i.e. high or low infection) or carapace length (mm) influenced the proportion of time



**Fig. 1.** Experimental arena used to assess crayfish behaviour. The tank was filled with water up to 3 cm below the level of the terrestrial area. The base of the arena, ramp (incline  $30^{\circ}$ ) and bridge were coated in 1–2 cm of pea gravel.

crayfish spent moving, in shelter, out of water or stationary. In the GAMLSS with beta-inflated or beta zero-inflated distributions, the  $\mu$  parameter refers to the average amount of time spent engaging in a particular behaviour, whilst  $\nu$  relates to the likelihood of a behaviour not occurring (Stasinopoulos *et al.*, 2008). To assess the response of crayfish to a touch stimulus, threatening or tail flip escape responses were scored separately. The crayfish were tested three times, and it was noted whether they retreated by tail flipping and/or threatened by raising the chelae at least once during the three tests. These data were analysed in binomial models (i.e. threat/no threat, tail flip/no tail flip), using GAMLSS. Treatment group and carapace length were included as independent variables.

## Results

### Juvenile crayfish infection

At 4-weeks old, zoospore concentration significantly affected survival of juvenile signal crayfish ( $z = 5.971$ ,  $P < 0.001$ ), with almost all crayfish dying in both the 10 and 100 zoospore  $\text{mL}^{-1}$  treatments after the 14-day experimental period (Fig. 2). Around half of the crayfish died in the 1 zoospore  $\text{mL}^{-1}$  treatment, whilst 92% of control treatment crayfish survived. Carapace length also had a significant effect on the survival of these crayfish, with larger individuals surviving longer ( $z = -4.387$ ,  $P < 0.001$ ). In contrast, survival of crayfish exposed to the same infection doses at 10 weeks of age was not significantly affected by the zoospore treatment; all crayfish in the control and 1 zoospore  $\text{mL}^{-1}$  treatments survived, whilst 88 and 82% of those in the 10 and 100 zoospore  $\text{mL}^{-1}$  treatments survived. All juvenile crayfish that were tested (Fig. 2) were previously infected (as they were descended from infected females), although those that were exposed to zoospores exhibited significantly elevated infection levels (subset tested for *A. astaci* infection using qPCR; Kruskal–Wallis  $\chi^2 = 9.7534$ ,  $D.F. = 3$ ,  $P = 0.021$ ; Fig. 2).

### Adult crayfish behaviour

All crayfish in the 'high-infection' group displayed agent levels A4–A6 (median number of PFU = 23 050;  $n[A4] = 3$ ;  $n[A5] = 13$ ;  $n[A6] = 1$ ), whilst all crayfish from the 'low-infection' group remained at very low ( $n[A2] = 9$ ) to low ( $n[A3] = 6$ ) infection levels (median number of PFU = 43). As such, for all analyses, crayfish behaviour was compared in terms of high and low infection.

Adults exposed to *A. astaci* zoospores (high-infection:  $1000\text{ zoospores mL}^{-1}$ ) were significantly less likely to leave the water and spent on average 1.3% (range: 0–3.8%) of the 24 h period in the terrestrial arena compared to those in the low-infection group (sham-infected), which spent 3.5% (range: 0.3–9.2%) out of water (GAMLSS,  $\nu$ ,  $LRT = 5.671$ ,  $P = 0.017$ ). In terms of the other behaviours, there was no significant difference between



**Table 1.** Results of GAMLSS statistical analyses and mean proportion of time crayfish spent engaged in different behaviours over 24 h

Response variable	Infection treatment group	Mean (%)	Range (%)	GAMLSS parameter	Family	Variable	DF	LRT	<i>P</i> value
Proportion of time active	Low	31	21–49	$\mu$	BE	Treatment	1, 28	0.016	0.898
	High	32	18–48			CL		0.024	0.876
Proportion of time out of water	Low	4	0–9	$\mu$	BEZI	Treatment	1, 25	2.095	0.147
	High	1	0–4			CL		0.075	0.784
				$\nu$		Treatment		5.671	<b>0.017</b>
						CL		0.125	0.724
Proportion of time in shelter	Low	21	0–55	$\mu$	BEINF	Treatment	1, 24	0.046	0.830
	High	17	0–75			CL		2.478	0.116
				$\nu$		Treatment		0.043	0.835
						CL		5.514	<b>0.019</b>
Proportion of time stationary	Low	44	11–76	$\mu$	BEINF	Treatment	1, 26	0.383	0.536
	High	50	0–76			CL		5.730	<b>0.017</b>
Proportion of crayfish that tail flipped	Low	75% <sup>a</sup>	na	$\mu$	BI	Treatment	1, 26	4.036	<b>0.045</b>
	High	35% <sup>a</sup>	na			CL		1.607	0.205
Proportion of crayfish that exhibited threat behaviour	Low	67% <sup>a</sup>	na	$\mu$	BI	Treatment		0.177	0.674
	High	65% <sup>a</sup>	na			CL		4.758	<b>0.029</b>

BE, beta; BEZI, beta zero-inflated; BEINF, beta-inflated; BI, binomial; s.d., standard deviation.

Significant results ( $P < 0.05$ ) are highlighted in bold.

<sup>a</sup>Denotes proportion of crayfish, not the mean.

crayfish from both the high- and low-infection groups, which spent 31.8 (s.d.  $\pm$  9.1%) of the time active,  $47.2 \pm 25\%$  stationary outside of a shelter and  $18.6 \pm 26\%$  in a shelter (Table 1; Appendix Table A2).

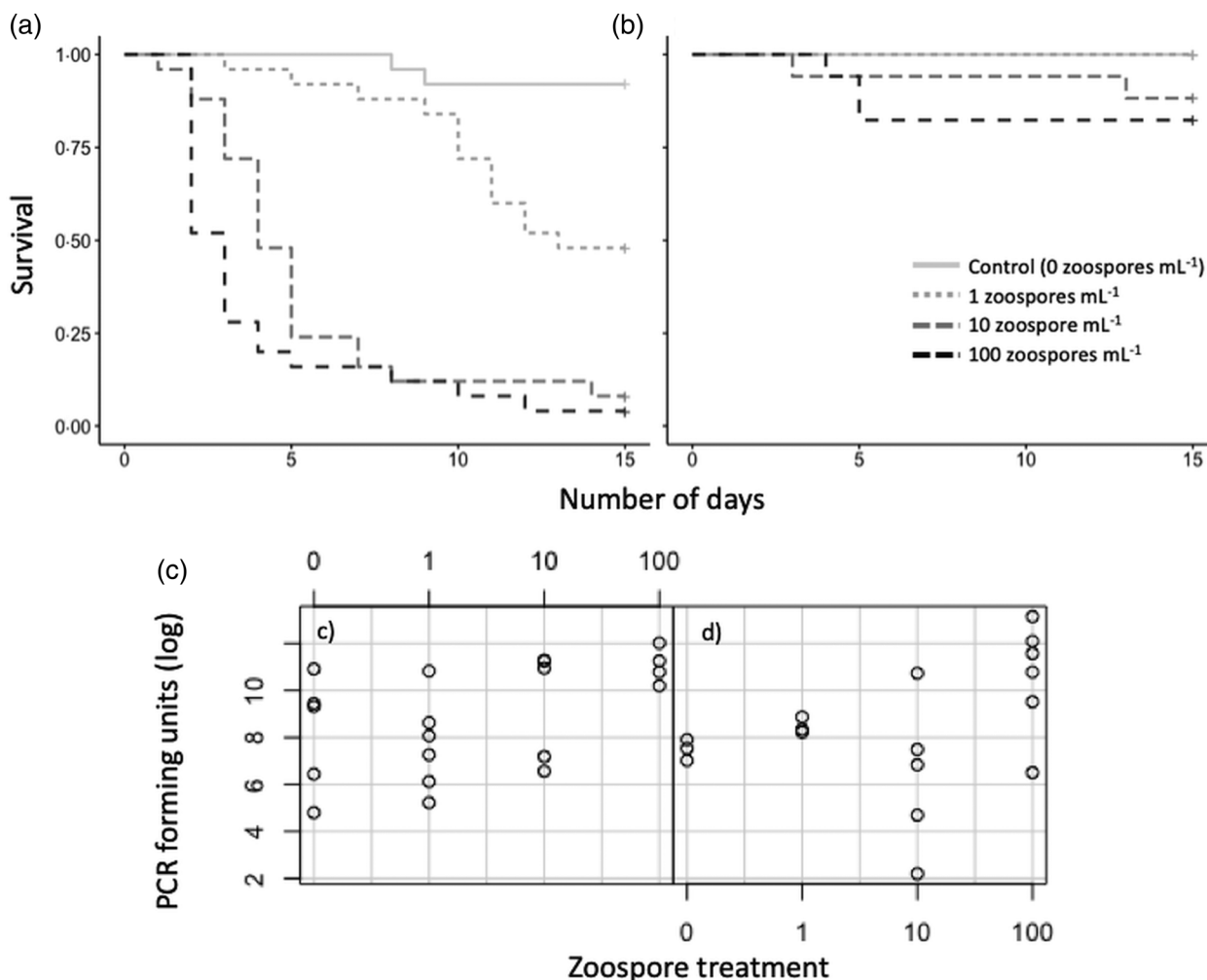
Crayfish from the high-infection group were also significantly less likely to mount a tail-flip response to tactile stimulation (GAMLSS,  $\mu$ , LRT = 4.036,  $P = 0.045$ ), where 35% of those in the high-infection group initiated a tail-flip response at least once compared to 75% of those from the low-infection group. Overall, though there was no significant difference between the two treatment groups, larger crayfish were more likely to display a threat response (GAMLSS,  $\mu$ , LRT = 4.758,  $P = 0.029$ ), spend less time in a shelter (GAMLSS,  $\nu$ , LRT = 5.514,  $P = 0.019$ ) and more time stationary outside of a shelter (GAMLSS,  $\nu$ , LRT = 5.730,  $P = 0.017$ ) compared to smaller crayfish.

## Discussion

Here, we show that *A. astaci* can cause almost total mortality in juvenile signal crayfish at ecologically relevant zoospore densities (Strand *et al.*, 2012, 2014), though larger, older individuals were less affected. Additionally, we show that a high *A. astaci* burden affects the behaviour of adult crayfish, making them almost half as likely to spend time on land and to escape from tactile stimulation compared to less infected individuals. The low-infection levels of our control crayfish did not differ from those frequently observed in *P. leniusculus* populations across Europe (Kozubíková *et al.*, 2011; Filipová *et al.*, 2013; Tilmans *et al.*, 2014) and in Japan (Mrugała *et al.*, 2017); although slightly higher infection levels (A2–A5) were reported in the UK (James *et al.*, 2017). Thus, the high-infection group in our study represents the outbreak of a highly virulent strain. Whilst signal crayfish are a highly successful invasive species in Europe that continue to spread (Peay *et al.*, 2010; Holdich *et al.*, 2014; James *et al.*, 2014), the negative impacts of crayfish plague reported here, especially in terms of

juvenile mortality, could have consequences for commercially harvested stocks by reducing recruitment and possibly resulting in population crashes. This also supports previous studies which have shown that commercially harvested signal crayfish populations can decline when *A. astaci* is present (Jussila *et al.*, 2016). Furthermore, these results add to growing evidence that *A. astaci* could play a more significant role in regulating invasive signal crayfish population dynamics than previously considered, which could play a role in determining invasion success (Jussila *et al.*, 2015).

In North American crayfish species, *A. astaci* can grow within the carapace, though constant host melanization of new hyphae prevents spore penetration to soft tissues (Unestam and Weiss, 1970; Nyhlén and Unestam, 1975; Cerenius *et al.*, 2003). In the current study, juvenile signal crayfish suffered extensive dose-dependent mortality when exposed to *A. astaci* zoospores around 4-weeks post-hatching. Slightly older (and therefore larger) crayfish, however, avoided this cost. Many juvenile crayfish studied here probably became infected rapidly after hatching, having acquired an infection from their mothers *via* horizontal transmission. Older and larger crayfish possibly have a better-developed immune response, capable of efficiently melanizing hyphae. It has been suggested that the immune response of juvenile crayfish to *A. astaci* infection is generally reduced compared to adults (Mrugała *et al.*, 2016), which seems to be the case in the current study. In other invertebrates too, younger individuals exhibit lower immune responses, for instance, snails showing greater susceptibility to schistosome parasites (Dikkeboom *et al.*, 1985). It has also been hypothesized, however, that juvenile crayfish could be less affected due to their higher moulting frequency compared to adults (Reynolds, 2002), allowing them to shed the growing hyphae and lower their *A. astaci* burden. Further research comparing the immunological capacity of juvenile and adult crayfish is required to confirm this. By the 10-week time-point, particularly susceptible individuals may have already succumbed to



**Fig. 2.** Survival (a, b) and infection levels (c, d) of juvenile signal crayfish infected with *A. astaci* for 2 weeks. Infection treatments were sham-infection, 1, 10 and 100 zoospores mL<sup>-1</sup>; (a, c) 4 weeks after hatching; (b, d) 10 weeks after hatching. Note in (b) sham-infection treatment is identical to infection treatment 1 (grey/dashed grey). A subset of juvenile crayfish from each treatment was tested using qPCR, (c) sham-infection, 0 zoospores mL<sup>-1</sup> ( $n=5$ ), 1 ( $n=6$ ), 10 ( $n=5$ ), 100 ( $n=4$ ); (d) 0 ( $n=3$ ), 1 ( $n=3$ ), 10 ( $n=5$ ), 100 ( $n=6$ ). See Appendix Table A1 for absolute values.

infection and therefore those used in the current experiment would have been more resistant to the pathogen. This appears unlikely though, since high levels of mortality were not observed in the communal holding tanks. Ecologically, the finding that relatively young crayfish hatchlings are highly susceptible to high doses of zoospores could have significant implications for signal crayfish recruitment and survival, especially in lentic environments, where zoospores are less likely to be washed away from the maternal crayfish.

Adult crayfish suffering from higher *A. astaci* infection levels during the current study exhibited a reduced tendency to leave the water. Although crayfish spend little time out of water in general, this finding suggests that populations of invasive signal crayfish with high burdens of *A. astaci* could be less likely to disperse overland to reach new aquatic habitats, a behavioural trait that can contribute to the spread of invasive crayfish (Grey and Jackson, 2012; Holdich *et al.*, 2014; Puky, 2014; Ramalho and Anastácio, 2014). Other invertebrates are less active when infected by parasites, potentially to avoid the associated fitness costs of dispersal. Flat back mud crabs (*Eurypanopeus depressus*) infected with rhizocephalans, for example, spend more time in shelter and are less active than uninfected crabs (Belgrad and Griffen, 2015), whilst sponge-dwelling snapping shrimp (*Synalpheus elizabethae*) infected by bopyrid isopods show 50% lower activity levels compared to uninfected individuals (McGrew and Hultgren, 2011). In

other invertebrates, many studies have shown that parasites can influence dispersal, though these studies focus on direct host manipulation, which does not seem to be the case here as there is no evidence of *A. astaci* actively manipulating the host. In terms of native European crayfish management, a lower tendency of infected individuals to disperse overland might be beneficial, by reducing the transmission of *A. astaci* to new waterbodies.

Highly infected crayfish were also less likely to respond to tactile stimulation by retreating in a characteristic 'tail-flip' manner. This reduced ability to escape could lead to increased predation of highly infected crayfish. *A. astaci* zoospores largely penetrate soft abdominal tissue (Vrålstad *et al.*, 2009), and it is possible that the reduced escape response is directly due to the general pathological effects of the parasite (Unestam and Weiss, 1970). Other parasites, such as *Thelohania contejeani*, also penetrate crayfish tissues, parasitizing the muscles and reducing the ability of crayfish to predate and feed (Haddaway *et al.*, 2012). It is also possible that highly infected crayfish exhibit a reduced tendency to move on land to reduce the risk of predation. In the same way, crustaceans become less active and tend to stay in a refuge when moulting, during which they are vulnerable to predators and largely unable to escape (Thomas, 1965; Cromarty *et al.*, 2000).

The crayfish used in the current study were from a population previously considered to be below the detection limit [ $n=30$  tested by James *et al.* (2017) exhibited A0–A1 levels]. However,

given that infection levels A2–A3 were found both among crayfish tested before the experiment began, as well as among those in the group not exposed to zoospores, it is evident that this population has either become infected since 2014, that a previously very low prevalence of *A. astaci* has since increased, or that crayfish present with A2–A3 infection levels in 2014 were just not trapped by James *et al.* (2017). Signal crayfish in Europe are generally associated with the group B strain of *A. astaci* (see Huang *et al.*, 1994; Grandjean *et al.*, 2014), which has also been found infecting another Welsh population, approximately 45 miles away from the population studied here (James *et al.*, 2017). Although not confirmed, the crayfish used in the current study were most likely initially infected with a group B strain and subsequently exposed to another strain from the same group. It is also possible that the tested crayfish were locally adapted to their original *A. astaci* infection (Gruber *et al.*, 2014; Jussila *et al.*, 2015), and the observed behavioural effects resulted from the exposure to the new *A. astaci* strain. As observed by Jussila *et al.* (2013), even assumed identical *A. astaci* strains may differently affect their crayfish hosts; therefore, the experimental crayfish in the current study likely dealt with multiple infections of closely related *A. astaci* strains. Further research is required, to explicitly compare the behaviour and survival of infected and uninfected signal crayfish, as well as investigate the effects of different *A. astaci* strains (both in single and multiple infections) on the behaviour and survival of infected crayfish.

In summary, we have shown that high levels of *A. astaci* cause severe mortality in young juveniles and affect the behaviour of adult signal crayfish. Mounting evidence suggests that signal crayfish may succumb to *A. astaci* more often than previously considered, which could be having an impact on commercially harvested populations (Aydin *et al.*, 2014; Edsman *et al.*, 2015). The crayfish exposed to zoospores in the current study displayed relatively high plague agent levels of A4–A6 (A7 being the highest level of infection; Vrålstad *et al.*, 2009). A longer period of infection, or higher infection dose, may induce further behavioural responses beyond those reported here, and in some cases even cause mortality as observed by Aydin *et al.* (2014), where signal crayfish were exposed to 10 000 zoospores mL<sup>-1</sup>. Female crayfish suffering from ESS carry far fewer fertilized eggs than uninfected females (Edsman *et al.*, 2015) which, coupled with the high juvenile mortality documented in the current study, could drastically reduce juvenile recruitment and result in population crashes. Similarly, crayfish plague could also have implications for the further spread of signal crayfish by affecting population dynamics, though this species has already successfully colonized large parts of Europe (Holdich *et al.*, 2014) and so the ecological impact may be negligible. Anecdotally, it was assumed that most North American crayfish are infected with *A. astaci*, though molecular methods have demonstrated that it is less prevalent than once thought. In France, for example, just over half of the signal crayfish populations tested were found to be positive for crayfish plague (Filipová *et al.*, 2013), and in the UK the prevalence was 56.5% (James *et al.*, 2017). It is possible, therefore, that the population dynamics of uninfected invasive populations may be affected when infected individuals are translocated and introduced.

**Financial support.** This project was funded by Coleg Cymraeg Cenedlaethol (JRT) and the Welsh Government and Higher Education Funding Council for Wales through the Sêr Cymru National Research Network for Low Carbon, Energy and the Environment (NRN-LCEE) AquaWales project.

**Conflict of interest.** None.

**Ethical standards.** The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

## References

- Alderman DJ (1982) *In vitro* testing of fisheries chemotherapeutants. *Journal of Fish Diseases* 5, 113–123.
- Aydin H, Kokko H, Makkonen J, Kortet R, Kukkonen H and Jussila J (2014) The signal crayfish is vulnerable to both the As and the PsI-isolates of the crayfish plague. *Knowledge and Management of Aquatic Ecosystems* 3.
- Bakker TCM, Mazzi D and Zala S (1997) Parasite-induced changes in behaviour and color make *Gammarus pulex* more prone to fish predation. *Ecology* 78, 1098–1104.
- Barber I, Mora AB, Payne EM, Weinersmith KL and Sih A (2017) Parasitism, personality and cognition in fish. *Behavioural Processes* 141, 205–219.
- Becking T, Mrugała A, Delaunay C, Svoboda J, Raimond M, Viljamaa-Dirks S, Petrussek A, Grandjean F and Braquart-Varnier C (2015) Effect of experimental exposure to differently virulent *Aphanomyces astaci* strains on the immune response of the noble crayfish *Astacus astacus*. *Journal of Invertebrate Pathology* 132, 115–124.
- Belgrad BA and Griffen BD (2015) Rhizocephalan infection modifies host food consumption by reducing host activity levels. *Journal of Experimental Marine Biology and Ecology* 466, 70–75.
- Cable J, Barber I, Boag B, Ellison AR, Morgan ER, Murray K, Pascoe EL, Sait SM, Wilson AJ and Booth M (2017) Global change, parasite transmission and disease control: lessons from ecology. *Philosophical Transactions of the Royal Society B: Biological Sciences* 372, 20160088.
- Cerenius L and Söderhäll K (1984) Repeated zoospore emergence from isolated spore cysts of *Aphanomyces astaci*. *Experimental Mycology* 8, 370–377.
- Cerenius L, Söderhäll K, Persson M and Ajaxon R (1987) The crayfish plague fungus *Aphanomyces astaci* – diagnosis, isolation, and pathobiology. *Freshwater Crayfish* 7, 131–143.
- Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I and Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to crayfish plague fungus, *Aphanomyces astaci*. *Cellular Microbiology* 5, 353–357.
- Cromarty SI, Mello J and Kass-Simon G (2000) Molt-related and size-dependent differences in the escape response and post-threat behavior of the American lobster, *Homarus americanus*. *Biological Bulletin* 199, 265–277.
- Dikkeboom R, Knaap WPWVD, Meuleman, EA and Sminia, T (1985) A comparative study on the internal defence system of juvenile and adult *Lymnaea stagnalis*. *Immunology* 55, 547–553.
- Edsman L, Nyström P, Sandström A, Stenberg M, Kokko H, Tiitinen V, Makkonen J and Jussila J (2015) Eroded swimmeret syndrome in female crayfish *Pacifastacus leniusculus* associated with *Aphanomyces astaci* and *Fusarium* spp. infections. *Diseases of Aquatic Organisms* 112, 219–228.
- Ercoli F, Ruokonen TJ, Koistinen S, Jones RI and Hämäläinen H (2015) The introduced signal crayfish and native noble crayfish have different effects on sublittoral macroinvertebrate assemblages in boreal lakes. *Freshwater Biology* 60, 1688–1698.
- Filipová L, Petrussek A, Matasová K, Delaunay C and Grandjean F (2013) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in populations of the signal crayfish *Pacifastacus leniusculus* in France: evaluating the threat to native crayfish. *PLoS One* 8, e70157.
- Grandjean F, Vrålstad T, Diéguez-Urbeondo J, Jelić M, Mangombi J, Delaunay C, Filipová L, Rezinciuc S, Kozubíková-Balcarová E, Guyonnet D, Viljamaa-Dirks S and Petrussek A (2014) Microsatellite markers for direct genotyping of the crayfish plague pathogen *Aphanomyces astaci* (oomycetes) from infected host tissues. *Veterinary Microbiology* 170, 317–324.
- Grey J and Jackson MC (2012) ‘Leaves and eats shoots’: direct terrestrial feeding can supplement invasive red swamp crayfish in times of need. *PLoS One* 7, e42575.
- Gruber C, Kortet R, Vainikka A, Hyvärinen P, Rantala MJ, Pikkarainen A, Jussila J, Makkonen J, Kokko H and Hirvonen H (2014) Variation in resistance to the invasive crayfish plague and immune defence in the native noble crayfish. *Annales Zoologici Fennici* 51, 371–389.
- Haddaway NR, Wilcox RH, Heptonstall REA, Griffiths HM, Mortimer RJG, Christmas M and Dunn AM (2012) Predatory functional response and prey choice identify predation differences between native/invasive and parasitised/unparasitised crayfish. *PLoS One* 7, e32229.



- Hatcher MJ, Dick JTA and Dunn AM (2014) Parasites that change predator or prey behaviour can have keystone effects on community composition. *Biological Letters* **10**. <https://doi.org/10.1098/rsbl.2013.0879>.
- Holdich DM, James J, Jackson C and Peay S (2014) The North American signal crayfish, with particular reference to its success as an invasive species in Great Britain. *Ethology Ecology & Evolution* **26**, 232–262.
- Huang TS, Cerenius L and Söderhäll K (1994) Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. *Aquaculture* **126**, 1–9.
- Hudson PJ, Dobson AP and Lafferty KD (2006) Is a healthy ecosystem one that is rich in parasites? *Trends Ecology & Evolution* **21**, 381–385.
- James J, Cable J and Slater F (2014) A.L.I.E.N. databases: addressing the lack in establishment of non-natives databases. *Crustaceana* **87**, 1192–1199.
- James J, Nutbeam-Tuffs S, Cable J, Mrugała A, Viñuela-Rodríguez N, Petrusek A and Oidtmann B (2017) The prevalence of *Aphanomyces astaci* in invasive signal crayfish from the UK and implications for native crayfish conservation. *Parasitology* **144**, 411–418.
- Jussila J, Kokko H, Kortet R and Makkonen J (2013) *Aphanomyces astaci* Ps1-genotype isolates from different Finnish signal crayfish stocks show variation in their virulence but still kill fast. *Knowledge and Management of Aquatic Ecosystems* **411**, 10.
- Jussila J, Vrežec A, Makkonen J and Kortet R (2015) Invasive crayfish and their invasive diseases in Europe with the focus on the virulence evolution of the crayfish plague. In Canning-Clode J (eds), *Biological Invasions in Changing Ecosystems Vectors, Ecological Impacts, Management and Predictions*. Warsaw/Berlin: DeGruyter Open Ltd, pp. 183–204.
- Jussila J, Tiitinen V, Edsman L, Kokko H and Fotedar R (2016) Signal crayfish in Lake Saimaa could be maladapted to the local conditions due to *Aphanomyces astaci* infection: a seven-year study. *Freshwater Crayfish* **22**, 53–60.
- Kozubíková-Balcarová E, Koukol O, Martín MP, Svoboda J, Petrusek A and Diéguez-Uribeondo J (2013) The diversity of oomycetes on crayfish: morphological vs. molecular identification of cultures obtained while isolating the crayfish plague pathogen. *Fungal Biology* **117**, 682–691.
- Kozubíková-Balcarová E, Beran L, Ďuriš Z, Fischer D, Horká I, Svoboda J and Petrusek A (2015) Status and recovery of indigenous crayfish populations after recent crayfish plague outbreaks in the Czech Republic. *Ethology Ecology & Evolution* **26**, 299–319.
- Kozubíková E, Vrálstad T, Filipova L and Petrusek A (2011) Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR. *Diseases of Aquatic Organisms* **97**, 113–125.
- Longshaw M, Bateman KS, Stebbing P, Stentiford GD and Hockley FA (2012) Disease risks associated with the importation and release of non-native crayfish species into mainland Britain. *Aquatic Biology* **16**, 1–15.
- Macnab V and Barber I (2012) Some (worms) like it hot: fish parasites grow faster in warmer water, and alter host thermal preferences. *Global Change Biology* **18**, 1540–1548.
- Makkonen J, Kokko H, Henttonen P and Jussila J (2010) Crayfish plague (*Aphanomyces astaci*) can be vertically transferred during artificial incubation of crayfish eggs: preliminary results. *Freshwater Crayfish* **17**, 151–153.
- Makkonen J, Jussila J, Kortet R, Vainikka A and Kokko H (2012) Differing virulence of *Aphanomyces astaci* isolates and elevated resistance of noble crayfish *Astacus astacus* against crayfish plague. *Diseases of Aquatic Organisms* **102**, 129–136.
- Makkonen J, Strand DA, Kokko H, Vrálstad T and Jussila J (2013) Timing and quantifying *Aphanomyces astaci* sporulation from the noble crayfish suffering from the crayfish plague. *Veterinary Microbiology* **162**, 750–755.
- McGrew M and Hultgren KM (2011) Bopyrid parasite infestation affects activity levels and morphology of the eusocial snapping shrimp *Synalpheus elizabethae*. *Marine Ecology Progress Series* **43**, 195–204.
- Mrugała A, Veselý L, Petrusek A, Viljamaa-Dirks S and Kouba A (2016) May *Cherax destructor* contribute to *Aphanomyces astaci* spread in Central Europe? *Aquatic Invasions* **11**, 459–468.
- Mrugała A, Kawai T, Kozubíková-Balcarová E and Petrusek A (2017) *Aphanomyces astaci* presence in Japan: a threat to the endemic and endangered crayfish species *Cambaroides japonicus*. *Aquatic Conservation: Marine and Freshwater Ecosystems* **27**, 103–114.
- Nyhlén L and Unestam T (1975) Ultrastructure of the penetration of the crayfish integument by the fungal parasite, *Aphanomyces astaci*, Oomycetes. *Journal of Invertebrate Pathology* **26**, 353–366.
- Peay S, Holdich DM and Brickland J (2010) Risk assessments of non-indigenous crayfish in Great Britain. *Freshwater Crayfish* **17**, 109–122.
- Preston DL, Mischler JA, Townsend AR and Johnson PTJ (2016) Disease ecology meets ecosystem science. *Ecosystems* **19**, 737–748.
- Puky M (2014) Invasive crayfish on land: *Orconectes limosus* (Rafinesque, 1817) (Decapoda: Cambaridae) crossed a terrestrial barrier to move from a side arm into the Danube River at Szeremle, Hungary. *Acta Zoologica Bulgarica* **66**, 143–146.
- Ramalho RO and Anastácio PM (2014) Factors inducing overland movement of invasive crayfish (*Procambarus clarkii*) in a ricefield habitat. *Hydrobiologia* **746**, 135–146.
- R Core Team (2018) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. <https://www.R-project.org/>.
- Reynolds JD (2002) Growth and reproduction. In Holdich DM (ed.), *Biology of Freshwater Crayfish*. Oxford: Blackwell Science Ltd, pp. 152–191.
- Söderhäll K, Svensson E and Unestam T (1978) Chitinase and protease activities in germinating zoospore cysts of a parasitic fungus, *Aphanomyces astaci*, oomycetes. *Mycopathologia* **64**, 9–11.
- Stasinopoulos M, Rigby RA and Akantziliotou C (2008) Instructions on how to use the GAMLSS package in R, second edition. Available at <http://www.gamlss.com/wp-content/uploads/2013/01/gamlss-manual.pdf>.
- Strand DA, Jussila J, Viljamaa-Dirks S, Kokko H, Makkonen J, Holst-Jensen A, Viljugrein H and Vrálstad T (2012) Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. *Veterinary Microbiology* **160**, 99–107.
- Strand DA, Jussila J, Johnsen SI, Viljamaa-Dirks S, Edsman L, Wiik-Nielsen J, Viljugrein H, Engdahl F and Vrálstad T (2014) Detection of crayfish plague spores in large freshwater systems. *Journal of Applied Ecology* **51**, 544–553.
- Svoboda J, Kozubíková-Balcarová E, Kouba A, Buřič M, Kozák P, Diéguez-Uribeondo J and Petrusek A (2013) Temporal dynamics of spore release of the crayfish plague pathogen from its natural host, American spiny-cheek crayfish (*Orconectes limosus*), evaluated by transmission experiments. *Parasitology* **140**, 792–801.
- Svoboda J, Strand DA, Vrálstad T, Grandjean F, Edsman L, Kozák P, Kouba A, Fristad RF, Koca SB and Petrusek A (2014) The crayfish plague pathogen can infect freshwater-inhabiting crabs. *Freshwater Biology* **59**, 918–929.
- Svoboda J, Mrugała A, Kozubíková-Balcarová E and Petrusek A (2017) Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *Journal of Fish Diseases* **40**, 127–140.
- Terry M and Therneau M (2018) Package 'survival'. Available at <https://CRAN.R-project.org/package=survival>.
- Thomas LR (1965) Moulting behaviour of the western Australian crayfish *Panulirus cygnus* George (Decapoda, Reptantia). *Crustaceana* **11**, 111–113.
- Tilmans M, Mrugała A, Svoboda J, Engelsma MY, Petie M, Soes DM, Nutbeam-Tuffs S, Oidtmann B, Rossink I and Petrusek A (2014) Survey of the crayfish plague pathogen presence in the Netherlands reveals a new *Aphanomyces astaci* carrier. *Journal of Invertebrate Pathology* **120**, 74–79.
- Tompkins DM, Dunn AM, Smith MJ and Telfer S (2011) Wildlife diseases: from individuals to ecosystems. *Journal of Animal Ecology* **80**, 19–38.
- Unestam T and Weiss DW (1970) The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. *Journal of General Microbiology* **60**, 77–90.
- Vilcinskas A (2015) Pathogens as biological weapons of invasive species. *PLoS Pathogens* **11**, 1–5.
- Vrálstad T, Knutsen AK, Tengs T and Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Veterinary Microbiology* **137**, 146–155.

## Appendix

**Table A1.** Number of PFU for subset of juvenile crayfish tested for *A. astaci*

Time point	Spore treatment	PFU
1	0	12 620
1	0	11 110
1	0	54 890
1	0	625
1	0	121
1	1	1422
1	1	453
1	1	50 290
1	1	185
1	1	3184
1	1	5585
1	10	1321
1	10	79 370
1	10	56 580
1	10	76 840
1	10	707
1	100	76 970
1	100	26 750
1	100	165 400
1	100	48 320
2	0	1868
2	0	1115
2	0	2720
2	1	7090
2	1	4182
2	1	3720
2	10	1781
2	10	928
2	10	45 790
2	10	9
2	10	109
2	100	13 590
2	100	510 300
2	100	106 100
2	100	665
2	100	48 050
2	100	178 500



**Table A2.** Time (in s) and proportion of time that crayfish spent engaged in behaviours over a 24 h period

Treatment	PFU	Carapace length (mm)	Active (s)	Active (%)	Out of water (s)	Out of water (%)	In shelter (s)	In shelter (%)	Stationary (s)	Stationary (%)
Low	416	42.6	34 250	0.396	2970	0.034	39 287	0.455	9893	0.115
Low	43	58.5	28 596	0.331	4877	0.056	0	0.000	52 927	0.613
Low	136	45.3	24 936	0.289	4180	0.048	47 332	0.548	9952	0.115
Low	31	53.8	31 939	0.370	3132	0.036	0	0.000	51 329	0.594
Low	170	53.7	22 232	0.257	1454	0.017	0	0.000	62 714	0.726
Low	51	51.8	21 532	0.249	5099	0.059	0	0.000	59 769	0.692
Low	38	53	33 427	0.387	2277	0.026	0	0.000	50 696	0.587
Low	51	49.9	21 681	0.251	205	0.002	47 577	0.551	16 937	0.196
Low	35	53.2	42 763	0.495	2074	0.024	39 903	0.462	1660	0.019
Low	31	55	19 073	0.221	2006	0.023	0	0.000	65 321	0.756
Low	14	46.9	31 990	0.370	1439	0.017	43 046	0.498	9925	0.115
Low	11	57	21 681	0.251	205	0.002	47 577	0.551	16 937	0.196
Low	16	54.7	20 400	0.236	5265	0.061	0	0.000	60 735	0.703
Low	1008	51.7	35 243	0.408	7945	0.092	4664	0.054	38 548	0.446
Low	373	56.6	18 312	0.212	2813	0.033	0	0.000	65 275	0.755
High	16 580	46	19 076	0.221	2216	0.026	65 108	0.754	0	0.000
High	23 050	47	31 848	0.369	1056	0.012	0	0.000	53 496	0.619
High	10 040	59	17 854	0.207	1358	0.016	28 070	0.325	39 118	0.453
High	7942	49	32 383	0.375	3278	0.038	24 993	0.289	25 746	0.298
High	30 680	46.4	24 435	0.283	330	0.004	0	0.000	61 635	0.713
High	26 060	55.1	18 332	0.212	1981	0.023	0	0.000	66 087	0.765
High	2136	50.3	20 502	0.237	0	0.000	63 969	0.740	1929	0.022
High	14 800	50	18 630	0.216	1771	0.020	46 372	0.537	19 627	0.227
High	88 430	53.7	34 845	0.403	0	0.000	2933	0.034	48 622	0.563
High	29 120	58.1	31 795	0.368	1032	0.012	0	0.000	53 573	0.620
High	23 580	51.8	32 499	0.376	0	0.000	0	0.000	53 901	0.624
High	29 310	52.9	38 940	0.451	2071	0.024	0	0.000	45 389	0.525
High	16 890	56.5	41 447	0.480	800	0.009	0	0.000	44 153	0.511
High	9480	54	15 890	0.184	0	0.000	7901	0.091	62 609	0.725
High	27 370	54	24 003	0.278	1541	0.018	6643	0.077	54 213	0.627
High	151 900	57.5	40 344	0.467	1233	0.014	0	0.000	44 823	0.519
High	14 110	62.2	28 783	0.333	1151	0.013	0	0.000	56 466	0.654