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Jussila J

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Japo Jussila, Al Vrezec, Tina Jaklič, Hobo Kukkonen, Jenny Makkonen, Harri Kokko

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***APHANOMYCES ASTACI* ISOLATE FROM LATENTLY INFECTED STONE CRAYFISH
(*AUSTROPOTAMOBIOUS TORRENTIUM*) POPULATION IS VIRULENT**

Running head: Latent infection and virulence of *A. astaci*

^{1*}Japo Jussila, ²Al Vrezec, ²Tina Jaklič, ¹Hobo Kukkonen, ¹Jenny Makkonen and ¹Harri Kokko

¹Department of Environmental and Biological Sciences, The University of Eastern Finland,
70211 Kuopio, Suomi-Finland; japo.jussila@uef.fi; hobo.kukkonen@uef.fi;
jenny.makkonen@uef.fi; harri.kokko@uef.fi

²Department of Organisms and Ecosystems Research, SI-1000 Ljubljana, Slovenia;
al.vrezec@nib.si; tina.jaklic@nib.si

*corresponding author; The Department of Environmental and Biological Sciences, The
University of Eastern Finland; P.O.Box 1627, 70210 Kuopio, Suomi-Finland

Abstract

Aphanomyces astaci infection is the cause of crayfish plague in European crayfish. Here the virulence of an *A. astaci* As strain isolated from apparently healthy stone crayfish (*Austropotamobius torrentium*) from Slovenia was compared to that of the Psl-Puujärvi *A. astaci* isolate in 3 crayfish species: noble crayfish (*Astacus astacus*), signal crayfish (*Pacifastacus leniusculus*) from Finland and

stone crayfish from Slovenia. All 3 crayfish species were challenged with Psl-Puujärvi *A. astaci* and succumbed to crayfish plague, with both noble crayfish and stone crayfish showing 100 % mortality, while 25 % of the signal crayfish died during the challenge. In comparison, the As-Slovenia *A. astaci* isolate was pathogenic for noble crayfish but not for signal crayfish or stone crayfish. This finding suggests that *A. astaci* virulence could be species specific and a strain from latent *A. astaci* infection in one native European crayfish species could be detrimental to other native European crayfish species.

Keywords: crayfish plague; experimental infection; As-genotype; Psl-genotype; wild population; Slovenia

1 Introduction

The crayfish plague disease agent, the oomycete *Aphanomyces astaci*, Schikora, was introduced to Europe during the 1860s and quickly spread throughout the continent (Alderman, 1996; Jussila et al., 2015). After widespread crayfish plague epidemics in Europe, the once lively crayfisheries and wider crayfish trade collapsed (Alderman, 1996; Holdich et al., 1999; Souty-Grosset et al., 2006). There are indications that some of the native European crayfish stocks might have survived the crayfish plague epidemics and became permanent reservoirs of the pathogen (Makkonen et al., 2010; Jussila et al., 2011a; Viljamaa-Dirks et al., 2011; Schrimpf et al., 2012; Kušar et al., 2013; Maguire et al., 2016) or that the outbreaks of the crayfish plague were a result of interactions among different oomycete genotypes (Jussila et al., 2015b). Regardless of the history, *A. astaci* has so far been shown to be the only causal agent of the crayfish plague-like mass mortalities among

native European crayfish stocks (Alderman, 1996; Souty-Grosset et al., 2006; Kozubikova et al., 2008; Grandjean et al., 2014). However, there is evidence that different *A. astaci* isolates show considerable virulence variation, a sign of co-evolution with its European crayfish hosts (Makkonen et al., 2012a, 2014; Jussila et al., 2015a, 2015b), and that native European crayfish could have adapted to resist the infection pressures caused by different *A. astaci* genotypes in some cases (Jussila et al., 2011a; Viljamaa-Dirks et al., 2011; Kokko et al., 2012; Svoboda et al., 2012; Kušar et al., 2013; Svoboda, 2015; Jussila et al., 2015a, 2015b). These speculations on host-parasite co-evolution are based on molecular evidence with the assumption that the diagnostic tools utilised are specific enough (e.g., Vrålstad et al., 2009; Makkonen et al., 2012b; Grandjean et al., 2014).

The stone crayfish (*Austropotamobius torrentium*) is the smallest and the slowest growing indigenous crayfish in Europe with limited distribution endemic to Central and South-Eastern Europe (Kouba et al., 2015), though with high genetic diversity between populations (Trontelj et al., 2005; Klobučar et al., 2013). The species is listed in the European Union Habitats Directive (Annex II and V) as a species requiring high-priority conservation. Many populations of stone crayfish are at serious risk of extinction due to the growing number of threats to their integrity (Souty-Grosset et al., 2006). These mostly derive from various forms of anthropogenic pressures such as habitat loss and degradation, pollution and introductions of non-indigenous crayfish species, which act as competitors and crayfish plague agent vectors (Machino and Füreder, 2005; Pöckl and Streissl, 2005; Füreder, 2009). The stone crayfish mostly inhabits small forest streams and brooks from lowlands up to 1700 m a.s.l., (Kouba et al., 2015). Thus, in addition to being threatened by environment alterations, it can be taken as a flagship species within its natural distribution.

Although the stone crayfish is regarded as a crayfish plague susceptible species, latently infected populations were discovered in Slovenia (Kušar et al., 2013), and later also in Croatia, where an *A. astaci* isolate from these stone crayfish was determined as As-genotype and differed by 1 microsatellite allele from the As-genotype found from noble crayfish (*Astacus astacus*) (Maguire et al., 2016). In Slovenia stone crayfish are rather widely distributed, but predominantly inhabit the Danube drainage basin rather than the Adriatic drainage basin (Machino and Füreder, 2005; Govedič et al., 2007, 2011; Vrezec et al., 2013). Historical data indicates that the stone crayfish is within its natural distribution limits in Slovenia, while coexistence with the white-clawed crayfish (*Austropotamobius pallipes*) and noble crayfish has been recorded (Govedič, 2006; Souty-Grosset et al., 2006; Govedič et al., 2007, 2011). There are no documents indicating that stone crayfish would have been farmed for stocking purposes, but it is possible that it has been translocated together with noble crayfish (Govedič, 2006; Govedič et al., 2007, 2011). During the first crayfish plague epidemics of the late 19th century, the documented mass mortalities of the native crayfish in Slovenia were observed primarily among noble crayfish populations and very occasionally among stone crayfish populations (Šulgaj, 1937; Kušar et al., 2013).

In Slovenia in southern Europe, the first wave of crayfish plague epidemics was reported quite early in 1880 and lasted until 1935 (Franke, 1889; Šulgaj, 1937; Kušar et al., 2013; Maguire et al., 2016). The disease spread from the River Danube drainage basin and affected crayfish populations in all the main rivers draining into the River Danube (Jussila et al., 2015b). No outbreaks of crayfish plague were reported in western Slovenia or close to northern Italy even though the first European epidemics were observed in the River Po in

1859 (Cornalia, 1860; Alderman, 1996). This may not necessarily reflect a limited disease spreading from Italy, but it may be a result of poor understanding and documentation due to national boundaries in this region (Jussila et al., 2015b).

After the outbreaks of crayfish plague, there were collapses in almost all of the crayfish populations in the major rivers in Slovenia, especially among the noble crayfish, which had previously been an important and heavily fished species (Šulgaj, 1937). Numerous attempts at restocking noble crayfish populations were largely unsuccessful (Budihna, 1996).

However the first alien crayfish and *A. astaci* vector recorded in Slovenia in 2003 was the signal crayfish (*Pacifastacus leniusculus*), followed later in 2015 by the discovery of the spiny-cheeked crayfish (*Orconectes limosus*) (Bertok et al., 2003; Vrezec et al., 2013; Govedič et al., 2015). Signal crayfish have been confirmed to be carriers of *A. astaci* (Kušar et al., 2013), this was notably the first incident of a crayfish plague epidemic being identified in Slovenia since 1935.

The aim of our experiments was to investigate the cause of the latent *A. astaci* infection in stone crayfish (*A. torrentum*). To achieve this, we aimed to isolate the infecting strain in order to study its properties, virulence and resistance in different host species. This, together with the various studies already carried out on the virulence of *A. astaci*, would allow for a platform for further studies on the host-parasite adaptations of European crayfish and *A. astaci* isolates from different regions and observations on the possible macro level evolutionary trends.

2 Materials and Methods

2.1 *A. astaci* isolation from latently infected Slovenian stone crayfish *A. torrentium*

The Slovenian stone crayfish (n=20) were obtained from the river Borovniščica, Borovnica near Ljubljana, central Slovenia (45°54'08"N 14°22'48"E) in 2014. In Slovenia, the crayfish were collected under license no. 35601-75/2012-8 issued by the Slovenian Environmental Agency for use by National Institute of Biology. This population has been previously reported to be latently infected with *A. astaci* with a high prevalence of 55.6 % (Kušar et al., 2013) and with signs of micromelanisation, an indication of a possible latent or chronic infection with *A. astaci* (Cerenius et al., 2003; Oidtmann et al., 2006). This isolated population is located in an upper region of the river and has thus not been in contact with alien crayfish, but exists within area of known historical crayfish plague outbreaks at the end of the 19th and beginning of the 20th centuries (Kušar et al., 2013).

After collection, the Slovenian crayfish were transported live to the University of Eastern Finland in Kuopio, Finland. They were **initially** stored individually in **1 L** climate chambers for up to 6 months (10°C), **with a water exchange happening once a week**. Cultures for *A. astaci* isolation were prepared and monitored as described in Makkonen et al. (2010). The isolates originated from two small females that died in transit and presented with melanised hyphae. The remaining stone crayfish were stored live for the *A. astaci* challenge experiments.

2.2 Genetic characterisation of the isolates

DNA extractions from the obtained isolates were conducted using the EZNA Fungal DNA Isolation Kit (Omega Bio-Tek) following the manufacturer's extraction protocol for fresh or frozen specimens. PCR amplification was conducted with primers ITS1 and ITS4 (White et al., 1991), AaChiF and AaChiR (Makkonen et al., 2012b) as described previously. The amplicons were sequenced in LGC Genomics (Cologne, Germany) or GATC Biotech (Konstanz, Germany) and the results were compared to existing data in GenBank with Megablast searches, in addition to the sequences of our existing culture collection. Our sequences were submitted to NCBI GenBank with the access numbers KY449409 and KY449410.

2.3 Experimental crayfish

Crayfish specimens were obtained from three different sources. The wild noble crayfish (*Astacus astacus*) were acquired from a commercial trapper at Lake Rytiky (62°51'22"N, 27°25'06"E), the signal crayfish (*Pacifastacus leniusculus*) were from a Lake Saimaa commercial catch (61°13'29"N, 28°20'03"E) and the Slovenian stone crayfish (*A. torrentium*) from the river Borovniščica as mentioned previously.

Before the experiment, populations were kept separate to prevent cross infections and all the crayfish were acclimated for two weeks at the experimental system temperature in filtered Lake Kallavesi water. All of the crayfish were mature adults, and were sexed and weighed before being transferred into individual chambers in the experimental system.

The Lake Rytiky noble crayfish used in the experimental setup were uninfected by *A. astaci*.

The population experienced an *A. astaci* epidemic in the early 1980s, but recovered with the

aid of stockings and is currently producing commercial catches. Since the 1980s there have been no detections of *A. astaci* carriers in wild Lake Rytty stocks. Our stone crayfish were latently infected, and our signal crayfish chronically infected with *A. astaci*. The signal crayfish (*Pacifastacus leniusculus*) population in Lake Saimaa was originally stocked in the early 1990s, but experienced an *A. astaci* epidemic in 2007 that resulted in a population decline. A highly pervasive *A. astaci* infection (Strand et al., 2012; Jussila et al., 2013a, 2016) of the Psl-genotype (i.e., up to 90 % prevalence) is present in the population, which was recently found to be also hosting eroded swimmeret syndrome (Edsman et al., 2015).

2.4 Experimental setup

The experimental system consisted of individual interconnected 2 L tanks with recirculating filtered water from Lake Kallavesi (Jussila et al., 2011b) at a flow rate that ensured full turnover of the 2 L tanks every hour (Jussila et al., 2013b). In this experiment we were using **one common** set of three consecutive 5 µm filters (Spunflow QN, Dominic Hunter Technologies Ltd., England) followed by two 5 µm absolute filters (Pleatflow II, Prosep Filter Systems Ltd., England) side by side to ensure maximum filtration efficiency. The purpose of the filtration was to ensure that the *A. astaci* zoospores would be removed from the recirculating water **and** that the original challenge doses would be the sole source of the *A. astaci* infections. The crayfish were given sweet corn kernels (two each, Rainbow®) as food every second day. The experimental system was monitored daily and notes on crayfish behaviour, moults, mortalities and other relevant features were made. Water quality (DO-%, pH and temperature) was measured once a week (WTW Multi 3430 meter). **The sizes of the experimental groups were from 5 to 8 individual crayfish (see Figures 1 and 2).**

The day and night rhythm were 12 hours with lights on (fluorescent lights) and 12 off. The water temperature was kept stable by room air conditioning at 18 ± 1 °C. The water quality, DO-%, temperature and pH were monitored twice a week throughout the duration of the experiment. Gravel containing calcium was added to the sump tanks to maintain the pH close to the optimum of pH 7 in the recirculating water. Water quality parameters remained within the optimal ranges for crayfish throughout the study. The dissolved oxygen (DO-%) was 97.9 ± 0.6 % (min – max, 96.7 – 98.7 %), pH was 7.6 ± 0.2 (min – max, 7.3 – 7.8) and water temperature was 19.7 ± 1.1 °C (min – max, 18.1 – 22.0 °C).

2.5 *A. astaci* isolates, production of the zoospores and the challenge

We used a novel Slovenian *As*-isolate, UEFAt1D (from here on *As*-Slovenia) and Psl-Puujärvi isolate (UEF8866-2) from signal crayfish (Makkonen et al., 2011, 2012a,b, 2014) to infect the experimental crayfish. The latter has been reported to be very virulent (Jussila et al., 2013b) and thus a good candidate for *A. astaci* isolate virulence comparisons. Both of these isolates had been isolated by the University of Eastern Finland crayfish research group and then maintained on PG1-agar (Unestam, 1965).

Details of the methods for zoospore production, modified after Cerenius et al. (1988), are explained in Makkonen et al. (2012b). The challenge dose for the infection groups was 1000 zoospores mL⁻¹, **the *A. astaci* spore challenge been administered for all challenged crayfish within an hour. The control group crayfish were treated similarly to the experimental group crayfish, except for the *A. astaci* challenge.**

2.6 Statistics

The statistical package used was SPSS v.21 and the statistical differences were estimated using Kaplan-Meier (Log-Rank) with criteria for the statistically significant difference being $p < 0.05$. The means are expressed as mean \pm SD.

3 Results

Two *A. astaci* isolates (UEFAt1D, UEFAt1H) were obtained from a single Slovenian stone crayfish individual. They were isolated from different crayfish tissues. The amplicon sequenced with the ITS-primers was 100 % identical to the *A. astaci* sequences in GenBank. The chitinase sequences grouped the isolates into the As-group of *A. astaci*. In the genetic regions studied, no differences to the previous As-genotype isolates found from Finland were detected.

In the infection experiment, we observed a statistically higher mortality among the As-Slovenia *A. astaci* isolate challenged noble crayfish compared to the control group (Kaplan-Meier (Log-Rank), $\chi^2 = 8.2$, $p = 0.004$) and a lower mortality compared to the Psl-Puujärvi *A. astaci* isolate challenged group (Kaplan-Meier (Log-Rank), $\chi^2 = 14.0$, $p < 0.001$) (Fig. 1). The cumulative mortality of the noble crayfish challenged with both of *A. astaci* isolates was 100 % while all the control group crayfish survived. The average day of death for the Psl-Puujärvi *A. astaci* isolate challenged group was 5.0 ± 0.0 days, As-Slovenia *A. astaci* isolate challenged group 15.3 ± 1.5 days and the control group >60.0 days.

The stone crayfish had significant higher mortality when challenged with the Psl-Puujärvi *A. astaci* isolate compared to the control group (Kaplan-Meier (Log-Rank), $\chi^2 = 6.1$, $p = 0.013$), with all crayfish in the Psl-Puujärvi *A. astaci* isolate challenged group dying while the control group (infected only with As-Slovenia *A. astaci* isolate) also showed an elevated mortality of 40 % (Fig. 2). The average day of death for the Psl-Puujärvi *A. astaci* isolate challenged group was 7.4 ± 1.5 days and for the control group 57.4 ± 12.6 days.

The Slovenian stone crayfish challenged with the Psl-Puujärvi *A. astaci* isolate expressed an indicatively slower death rate compared to the similarly treated Lake Rytty noble crayfish (Kaplan-Meier (Log-Rank), $\chi^2 = 3.1$, $p = 0.079$), with a difference of 2.4 days in the average day of death.

Signal crayfish showed no mortality when challenged using the As-Slovenia *A. astaci* isolate, while the Psl-Puujärvi *A. astaci* isolate challenge caused a slightly elevated 25 % mortality (Fig. 2). There were no statistical differences in mortality among different experimental groups of signal crayfish.

4 Discussion

The *Aphanomyces astaci* responsible for the latent infection of the Slovenian stone crayfish population in the River Borovniščica (Kušar et al., 2013) was identified as belonging to the As-genotype. This *A. astaci* infection has caused no elevated mortality in this stone crayfish population in Slovenia, nor during the holding of them in Kuopio for a 6 month period. The

finding of a latent *A. astaci* infection bears similarities to several reports from other native European crayfish populations in recent years (Jussila et al., 2011a; Viljamaa-Dirks et al., 2011; Kokko et al., 2012; Svoboda et al., 2012; Manfrin and Pretto, 2014; Maguire et al., 2016). The results from this study also indicate that the As-Slovenia *A. astaci* isolate could be expressing lower virulence against tested stone crayfish, or the stone crayfish population's resistance towards this isolate could be considerably elevated. This speculation is based on the fact that all the Finnish Lake Rytiky noble crayfish died when challenged with this As-Slovenia *A. astaci* isolate in our study.

In our study, the As-Slovenia *A. astaci* isolate killed the Finnish Lake Rytiky noble crayfish efficiently, but to a lesser extent than the highly virulent Finnish Psl-Puujärvi *A. astaci* isolate. The observed stone crayfish death rate in the Psl-Puujärvi *A. astaci* isolate challenge was slightly slower compared to that of the Finnish noble crayfish, although the difference is not relevant from a practical view point as all of the crayfish died. The fact that stone crayfish were latently infected with the As-genotype *A. astaci* could also have affected their death rate when challenged with the Psl-Puujärvi *A. astaci* isolate, but this remains to be further studied. Alas, this finding verifies the frequently discussed matter that the Psl-genotype is a very potent killer of native European crayfish (e.g., Jussila et al., 2013b, 2015b) and further spreading of the alien *A. astaci* carrying crayfish must be prevented in the interest of native European crayfish conservation (Holdich et al., 2009; Kozák, 2015).

It should be noted that experimental conditions were prone to cause stress in already latently *A. astaci* infected stone crayfish and thus they may have been more susceptible to infection under the study conditions. This assumption is based on observation of elevated

mortality also among the control crayfish. This mortality, due to causes other than the experimental *A. astaci* challenge, could indicate that there might still be capacity for resisting *A. astaci* infection in these stone crayfish, similar to what has been observed in signal crayfish (Unestam, 1969; Unestam and Weiss, 1970; Kirjavainen and Westman, 1999; Aydin et al., 2014). This result together with our previous studies indicate that there are differing susceptibilities to As-genotype *A. astaci* infections among European native crayfish species, and that crayfish from latently infected, and thus coevolved, populations might develop higher resistance against As-genotype *A. astaci* infections (e.g., Makkonen et al. 2014). Further investigations are needed to understand the scope of the possible immune competence of stone crayfish against different *A. astaci* genotypes and isolates which occur and interact in European waters (Grandjean et al., 2014; Jussila et al., 2015b).

The latent *A. astaci* infection in the stone crayfish, detected first using molecular techniques (Kušar et al., 2013), was later confirmed by successful isolation of the pathogen strain in our study. We then used the obtained *A. astaci* isolate for spore production and successfully infected Finnish noble crayfish with this As-Slovenia *A. astaci* isolate. This protocol verifies that the molecular detection was correct, while some of the previous latent crayfish plague infections in the native European crayfish are lacking this confirmation and seem to be based solely on molecular detection (e.g., Jussila et al., 2011a; Kokko et al., 2012; Svoboda et al., 2012; Grandjean et al., 2014; Maguire et al., 2016). Although there appears to be no doubt of the existence of latent *A. astaci* infections among native European crayfish, caused by the As-genotype, the phenomena of the latent *A. astaci* infection requires further study to fully understand its relevance and role in the co-evolution of the native European crayfish host and *A. astaci* itself (Jussila et al., 2015b).

The remaining wild native crayfish stocks in Central and Southern Europe are all considered endangered (Kozak, 2015). A few of them are latently infected with *A. astaci* and some are still being eradicated by acute crayfish plague epidemics (Hefti and Stucki, 2006; Kozubíková et al., 2008, 2010, 2011; Hochwimmer et al., 2009; Cammà et al., 2010; Pârvulescu et al., 2012; Maguire et al., 2016). Developments in both analytic methods and knowledge of the relationship between *A. astaci* and its crayfish hosts give the opportunity to inform conservation planning, based on the latest evidence and understanding.

The noble crayfish challenged with the As-Slovenia *A. astaci* isolate in our study showed mortality comparable to that observed in some of the recent experiments carried out using virulent As-genotype *A. astaci* isolates (Makkonen, 2013). Often, but not every time, the As-genotype *A. astaci* kills all challenged noble crayfish under laboratory conditions (Makkonen et al., 2012a, 2014; Jussila et al., 2014). The virulence of the studied Slovenian As-genotype *A. astaci* isolate was thus high, but lower than that of the Psl-Puujärvi *A. astaci* isolate (Jussila et al., 2013b). **It remains to be studied, how would the Slovenian stone crayfish react to high dose of As-Slovenian *A. astaci* under experimental conditions.**

The signal crayfish showed indications of elevated mortality during the Psl-genotype *A. astaci* isolate challenge in this experiment, with a 25% mortality rate resulting from a moderate dose of 1000 *A. astaci* spores per mL (e.g., Makkonen et al., 2012a). It has previously been reported that signal crayfish could experience elevated mortality rates when infected with *A. astaci*, especially if the ambient conditions are challenging, or if individuals are stressed (Persson and Söderhäll, 1983; Thörnqvist and Söderhäll, 1993; Aydin

et al., 2014). The signal crayfish used in this study were chronic carriers of the Psl-genotype *A. astaci* (e.g., Strand et al., 2012; Jussila et al., 2014, 2016) and it remains to be studied whether the Psl-Puujärvi *A. astaci* isolate or the one already infecting Lake Saimaa signal crayfish was causing the mortality. The As-Slovenia *A. astaci* isolate challenge did not cause increased mortality in signal crayfish in this experiment, with the dose used in this study being significantly lower than in our previous comparable experiment (Aydin et al., 2014).

Our results have shown that Slovenian stone crayfish are latently infected with As-genotype *A. astaci* and are susceptible to Psl-genotype *A. astaci* infection. Furthermore, the studied As-Slovenia *A. astaci* isolate causes increased mortality among noble crayfish of Finnish origin. This verifies the assumptions that further spreading of *A. astaci* must be prevented, or the native crayfish still remaining in arc sites could be under threat. Therefore conservation management of endangered European crayfish species, especially of genus *Austropotamobius*, should extend to including monitoring of *A. astaci* (Grandjean et al. 2014) and measures for the limitation of disease spread, especially to isolated and still uninfected crayfish populations that are currently preserved at least in some parts of Central and Southern Europe (e.g., Kušar et al., 2013; Maguire et al., 2016).

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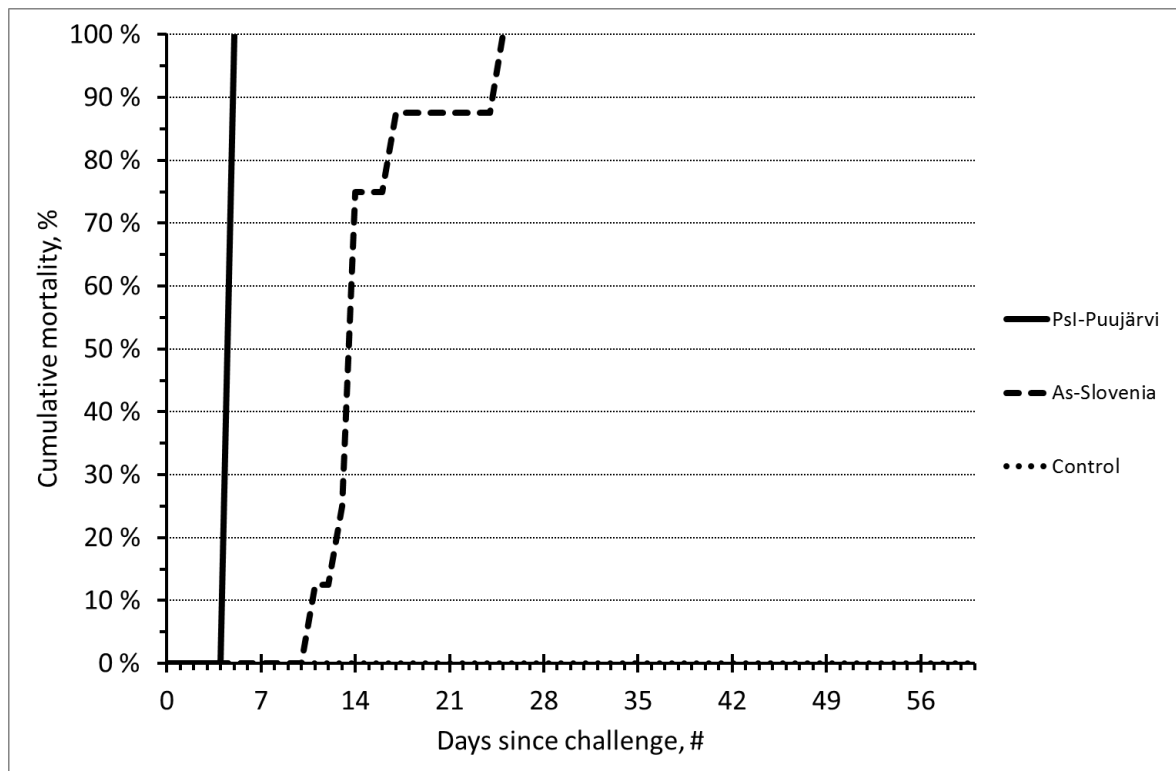


Figure 1. Lake Rytty noble crayfish (*A. astacus*) mortality during As-Slovenia and Psl-Puujärvi *A. astaci* challenge. Experimental group sizes were n=7 (Psl-Puujärvi and control group) and n=8 (As-Slovenia group).

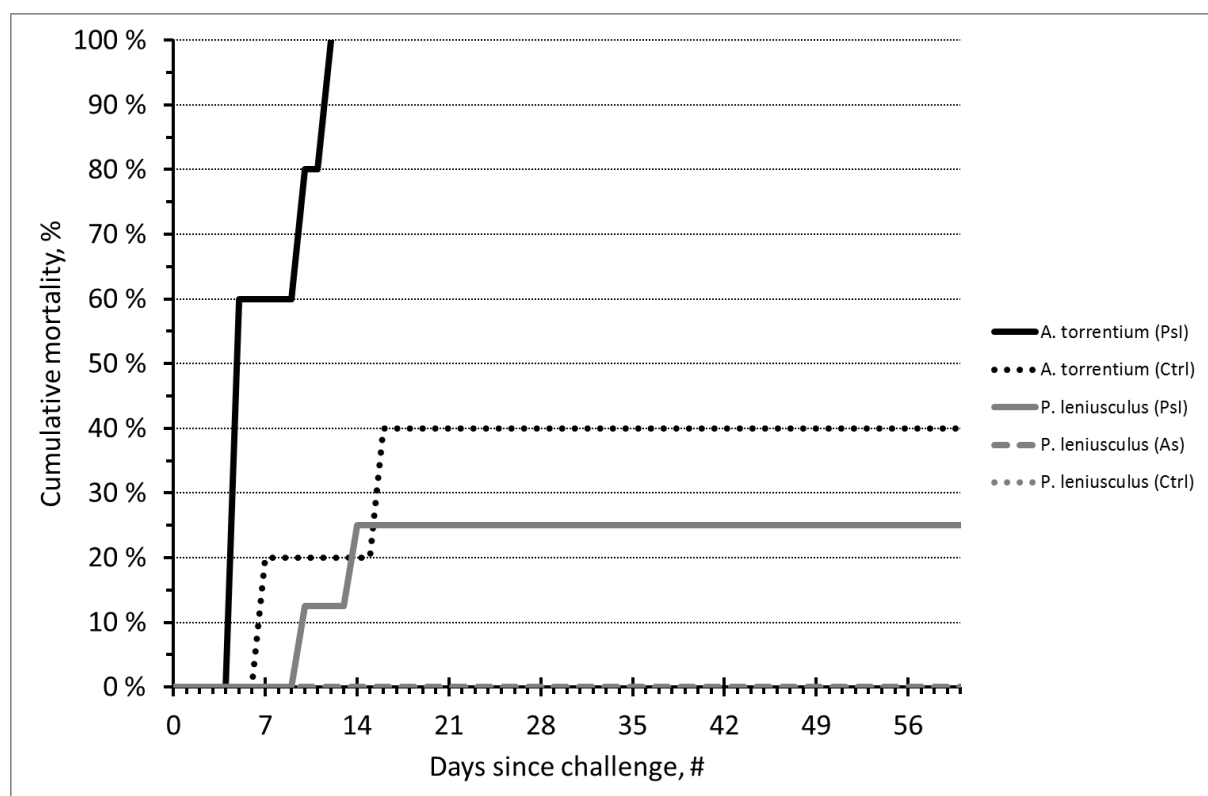
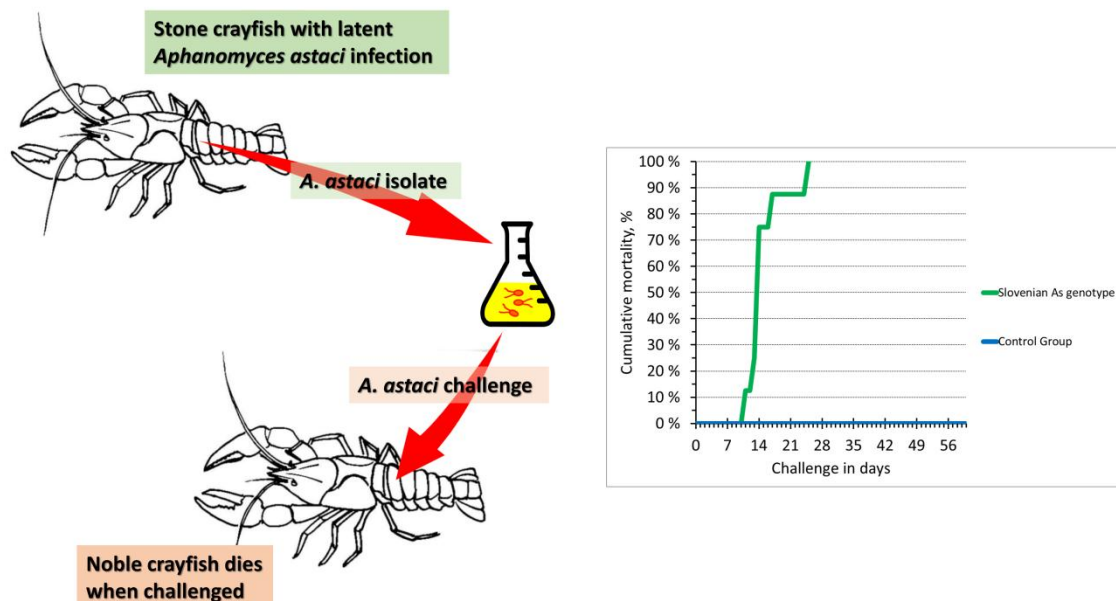


Figure 2. The Slovenian stone crayfish (*A. torrentium*) and Saimaa signal crayfish (*P. leniusculus*) mortality during As-Slovenia and Psl-Puujärvi *A. astaci* challenge. Abbreviations are as follows: Psl is Psl-Puujärvi *A. astaci*, As is As-Slovenia *A. astaci* and Ctrl is the control group. Experimental group sizes were n=6 (*P. leniusculus* (Psl), *P. leniusculus* (As)) and n=8 (*P. leniusculus* (Psl)) and n=5 (*A. torrentium* (Psl) and *A. torrentium* (control)).

Graphical abstract



Highlights

- ✓ Edemic isolated population of stone crayfish from Slovenia is latently infected with *Aphanomyces astaci* of As genotype
- ✓ This As isolate from these Slovenian stone crayfish kills Finnish noble crayfish efficiently
- ✓ The study shows variable resistance of native European crayfish to *A. astaci*
- ✓ The study highlights necessity to investigate health status of also native European crayfish before stockings