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Research Article

May *Cherax destructor* contribute to *Aphanomyces astaci* spread in Central Europe?

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Abstract

Transmission of the crayfish plague pathogen *Aphanomyces astaci* endangers native European crayfish. This pathogen, spread mainly by its natural hosts, North American crayfish, has also been detected in the aquarium trade in Europe. As the trade in ornamental crayfish is nowadays considered a key introduction pathway of non-European crayfish, it may contribute to crayfish plague spread. Non-American crayfish have been assessed as highly susceptible to the pathogen, and thus unlikely to participate in *A. astaci* spread from aquarium facilities. However, moderate resistance to this disease has been suggested for the Australian yabby *Cherax destructor*. This widely traded crayfish species exhibits high potential to establish in Central Europe, and has been assessed as a high-risk species with regards to its invasiveness. We investigated resistance of juvenile *C. destructor* towards three *A. astaci* strains differing in virulence (representing genotype groups A, B and E), present in Central European waters. *Cherax destructor* was exposed to two doses of *A. astaci* zoospores (10 and 100 spores ml^{-1}) and its mortality was further compared with that of the juvenile European noble crayfish *Astacus astacus*. While some survival among *C. destructor* individuals was observed after exposure to the least virulent *A. astaci* strain (genotype group A), total mortality of Australian crayfish was noted after infection with the two more virulent strains. However, in contrast to *A. astacus*, the mortality of *C. destructor* was significantly delayed. These results suggest that under favourable conditions *C. destructor* may contribute to crayfish plague spread in Central Europe.

Key words: *Astacus astacus*, temperate zone, crayfish plague, aquarium trade, survival test, Australian crayfish, non-indigenous species

Introduction

Increasing numbers of commodities traded all over the world result in deliberate or unintentional introductions of non-native species outside of their natural ranges (Hulme 2009). Besides such impacts as predation, competition, hybridization, and habitat modification, these non-native species may threaten native competitors through transmission of pathogens, parasites and parasitoids (Daszak et al. 2000; Peeler et al. 2011). The disease emergence driven by non-native species introductions may happen in a twofold manner, either by expanding the geographic range of pathogenic agents or by facilitating host-switching (Peeler et al. 2011). In other words, non-native organisms may bring new diseases to their novel ranges or may act as reservoirs of existing parasites (Strauss et al. 2012).

Freshwater ecosystems are particularly vulnerable to biological invasions (Ricciardi and Rasmussen 1999; Shea and Chesson 2002), with the key drivers of non-native species introductions being aquaculture and the associated trade of live organisms for direct consumption, ornamental purposes, or even research (Copp et al. 2007; Gozlan 2008; Peeler et al. 2011). Consequently, all these pathways also contribute to the introduction of exotic pathogens (Peeler et al. 2011; Rodgers et al. 2011). The crayfish plague agent, an oomycete *Aphanomyces astaci* Schikora, is an example of such introduced exotic pathogens. It is undoubtedly one of the most devastating emerging diseases in European freshwaters, also listed among worst invasive species in Europe as well as globally

(Lowe et al. 2004; DAISIE 2009). Its unintentional introduction from North America to Europe resulted in substantial declines and local extinctions of native crayfish populations (Holdich et al. 2009). Although the origin of *A. astaci* involved in the first mass mortalities of European crayfish populations remains unknown, further spread of this pathogen has been, to a large extent, facilitated by stocking and subsequent expansion of three North American crayfish species: the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817), the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), and the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Holdich et al. 2009). Natural dispersal and human-aided translocations of these crayfish have resulted in a wide spread of the crayfish plague infection in Europe. Even though import and stocking of North American crayfish are nowadays illegal in many European countries, additional non-indigenous crayfish species have been introduced through illegal introductions, garden pond escapes, and releases of aquarium or bait specimens (Chucholl 2013 and references therein).

Import, trade and transport of ornamental non-indigenous crayfish species are forbidden or restricted in many European regions (Svobodová et al. 2010). Nevertheless, the market for ornamental crayfish has grown rapidly in some Central European countries in the recent decade, and keeping crayfish as pet species became a popular hobby (Chucholl 2013; Patoka et al. 2014). Consequently, the trade in ornamental crayfish has recently gained in importance as a key introduction pathway of non-European species (Peay 2009; Chucholl 2013). In particular, populations of the marbled crayfish *Procambarus fallax* f. *virginalis* Martin, 2010, introduced through this pathway, have already established across Central Europe and the number of invaded countries is gradually increasing (Kouba et al. 2014; Samardžić et al. 2014; Lipták et al. 2016; Lőkkös et al. 2016; Novitsky and Son 2016). In addition, specimens of other popular ornamental crayfish species including the yabby *Cherax destructor* Clark, 1936, the redclaw *Cherax quadricarinatus* (von Martens, 1868), and the Florida crayfish *Procambarus alleni* (Faxon, 1884) have been discovered in the wild in Europe (Souty-Grosset et al. 2006; Holdich et al. 2009; Jaklič and Vrezec 2011; Gross 2013). Moreover, the recent report of *A. astaci*-infected crayfish in the German aquarium trade (Mrugała et al. 2015) highlighted that the ornamental trade may not only act as an introduction pathway for non-indigenous crayfish species, but also as a reservoir of the crayfish plague agent. The pathogen may be introduced from household aquaria, aquarium facilities, and garden ponds either with discarded water, or with infected crayfish. Although most of the *A. astaci* infections were detected in North American crayfish, other crayfish species such as Australian *C. quadricarinatus*, with infection acquired through horizontal transmission from other species, may also be purchased (Mrugała et al. 2015). This finding clearly demonstrates that releases of any non-European crayfish species, even those considered vulnerable to crayfish plague, may potentially contribute to the spread of *A. astaci*.

Thanks to a long co-evolutionary history with *A. astaci*, North American crayfish species have evolved defence mechanisms against growth of *A. astaci* mycelium in their cuticles (Cerenius et al. 2003). In contrast, crayfish of European, Asian and Australian origin that lack efficient immune responses are considered highly susceptible (Unestam 1969, 1972, 1975; reviewed in Svoboda et al. 2016). However, a differential susceptibility towards *A. astaci* has been also observed in populations of the European noble crayfish, *Astacus astacus* (Linnaeus, 1758), and has been linked to differences in *A. astaci* virulence (Makkonen et al. 2012, 2014; Becking et al. 2015). Four different *A. astaci* genotype groups (A, B, D and E), at least some of them differing in virulence, are known at present in Europe (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011) but the actual variation of this pathogen is probably higher (see Grandjean et al. 2014). A lowered virulence towards European crayfish species was observed in some strains from genotype group A isolated from infected European crayfish and implicated in latent *A. astaci* infections carried by *A. astacus* (Viljamaa-Dirks et al. 2011, 2013). The other three groups apparently exhibit substantially higher virulence and have been involved in numerous crayfish plague outbreaks in European crayfish populations (Filipová et al. 2013; Kozubíková-Balcarová et al. 2014; Rezinciuc et al. 2014).

In addition to variation in the pathogen's virulence, a variation in susceptibility towards *A. astaci* may be apparently present in non-American crayfish host species. Early studies by Unestam (1969, 1975) indicated that two crayfish species, the narrow clawed crayfish *Astacus leptodactylus* Eschscholtz, 1823 and *C. destructor,* seem less susceptible to *A. astaci* than the noble crayfish. Chronic *A. astaci* infections were indeed observed in various populations of the former species (Kokko et al. 2012; Pârvulescu et al. 2012; Schrimpf et al. 2012; Svoboda et al. 2012), and even the pathogen strain from genotype group B has been reported from infected specimens in Turkey (Svoboda et al. 2014a). The strain used by Unestam (1975) in the experimental exposure of *C. destructor* to *A. astaci* also belonged to genotype group B (see Huang et al. 1994). However, a strain

from genotype group D was used in a successful eradication of established populations of *C. destructor* in Spain (Souty-Grosset et al. 2006), suggesting that a substantial variation may exist in susceptibility of this crayfish to various *A. astaci* genotypes.

Cherax destructor, endemic to south-eastern Australia, has successfully spread outside of its native range throughout the whole continent (Coughran and Daly 2012), and its presence in Western Australia poses a threat to the endemic crayfish species (Beatty et al. 2005). It seems likely that it may also spread rapidly and impose a wide range of negative impacts on native species and freshwater ecosystems in other continents. In Europe, established populations of this Australian crayfish are already known from Spain and Italy, where this species is farmed (Holdich et al. 2009; Scalici et al. 2009; Kouba et al. 2014). Its survival in European temperate climate was believed to be constrained by low winter temperatures. However, a recent study revealed that it is capable of surviving Central European winters (Veselý et al. 2015). *Cherax destructor* is a common ornamental crayfish in these regions and some specimens probably originating from aquarium releases have already been reported from the wild (Hefti and Stucki 2006; Souty-Grosset et al. 2006). Its wide availability in the pet trade coupled with biological characteristics of a successful invader have resulted in its assessment as a high-risk species (Chucholl 2013; Papavlasopoulou et al. 2014; Patoka et al. 2014). In this context, the trade in ornamental crayfish should be considered a potential entry pathway of *C. destructor* to Central European open waters.

Cherax destructor released from household aquaria and/or aquarium facilities may not only threaten the native fauna as a prominent predator and competitor, but may also contribute to *A. astaci* spread in a twofold manner 1) through an introduction of already infected *C. destructor* individuals into the natural environment, and 2) through an increase in *A. astaci* prevalence if crayfish populations come into contact with pathogen zoospores. For these reasons, we tested whether this Australian crayfish species indeed shows a decreased susceptibility towards *A. astaci* infection. Juvenile *C. destructor* were exposed to *A. astaci* strains representing three genotype groups involved most often in crayfish plague outbreaks in Central Europe (Kozubíková-Balcarová et al. 2014), including two highly virulent strains and one of lower virulence (Becking et al. 2015), and patterns of its mortality were compared with similarly-aged *A. astacus* highly susceptible to crayfish plague.

Methods

Studied crayfish and Aphanomyces astaci *strains*

The yabby, *Cherax destructor*, originated from an experimental culture and were kept at the Research Institute of Fish Culture and Hydrobiology in Vodňany, Czech Republic. The noble crayfish, *Astacus astacus*, were caught with permission of the nature conservancy authorities from the pond Pařez (Kaliště, Czech Republic; 49º36′N, 15º19′E). Before the experiment, *A. astacus* were adapted to the communal rearing conditions in the laboratory for 3 weeks. All crayfish were approximately 4 months old at the beginning of the experiment; their total length ranged from 20 to 40 mm.

The crayfish were exposed to three *A. astaci* strains (Al7, Pec14 and Evira4805a/10; as in Becking et al. 2015), representative of genotype groups A, B and E present in Central European freshwaters (for discussion on nomenclature of *A. astaci* genotype groups, see Svoboda et al. 2016). These strains are kept in Petri dish cultures with RGY agar (Alderman 1982) at the Department of Ecology, Charles University in Prague, Czech Republic.

Experimental design

The infection trial was conducted in an experimental facility of the Research Institute of Fish Culture and Hydrobiology in Vodňany between November 2014 and February 2015. The crayfish were kept separately in glass dishes with 400 ml of aged tap water, which was changed every week. Water temperature (mean ± SD: 15.6 ± 0.4°C) was registered hourly using a data logger (Minikin, Environmental Measuring Systems, Brno, Czech Republic). No aeration was provided to prevent airborne pathogen cross-contamination among vessels; to check for possible oxygen depletion, oxygen content (8.0 ± 0.7 mg l^{-1}) was measured in two additional dishes with crayfish that were managed in an identical manner. Each glass dish was further covered with an aluminium foil. Feeding with two pellets (Biomar Inicio plus 1.5) took place three times per week. The crayfish were monitored daily; dead crayfish and exuviae were removed immediately and stored in 96% ethanol. The experiment was terminated after 100 days. All crayfish that survived the trial were euthanized and also stored in 96% ethanol.

In total, 60 individuals of *C. destructor* and 30 of *A. astacus* were exposed to three *A. astaci* strains with two different zoospore concentrations of 10 and 100 spores ml^{-1} in six different treatments (i.e. spore concentration/*A. astaci* strain combinations). *Astacus astacus*, due to their confirmed high susceptibility to

crayfish plague pathogen (Unestam 1969; Holdich et al. 2009), were used as a positive control to evaluate *A. astaci* virulence and infectiveness. Production of *A. astaci* zoospores was induced according to Cerenius et al. (1988). The motility of spores was checked, and spores were counted using the Bürker counting chamber. Appropriate volumes of the zoospore suspension were then added to the glass dishes with crayfish. For two *A. astacus* individuals (from treatments with Al7 and Evira4805a/10 strains and a dose of 10 spores ml^{-1}) the spore addition was accidentally omitted. Consequently, in each experimental trial 10 *C. destructor* and 4–5 *A. astacus* were used. In addition, 10 *C. destructor* and 6 *A. astacus* were treated as a pathogen-free control group.

DNA extraction and A. astaci *detection*

All crayfish used in the experiment were analysed for the presence of *A. astaci* infection. Additionally, 20 specimens of *C. destructor* from the same source as the experimental animals were tested before the experiment to rule out a chronic presence of the crayfish plague pathogen in this stock. Prior to dissection, total length (from the tip of the rostrum to the end of the telson) of each specimen was noted. Furthermore, the crayfish specimens were carefully examined for any presence of melanized spots as melanization is a common immune defence mechanism in crustaceans (Cerenius et al. 2008) and may indicate crayfish immune reaction to *A. astaci*. From each crayfish, the DNA was extracted using the DNeasy tissue kit (Qiagen) from up to 50 mg subsamples of mixed tissues (containing the soft abdominal cuticle, legs with basal joints, whole tail fan and any melanized tissues) ground in liquid nitrogen (as in Mrugała et al. 2015). The same procedure was also used for DNA isolation from the whole crayfish exuviae.

The detection of *A. astaci* infection was performed with TaqMan MGB quantitative PCR (qPCR) on the iQ5 BioRad thermal cycler as described in Vrålstad et al. (2009); with minor modifications of the original protocol to reduce likelihood of false positive results (as in Svoboda et al. 2014a).

Statistical analyses

The statistical analyses were performed in R version 3.2.2 (R Development Core Team 2015), with the package "survival" (Therneau and Grambsch 2000). To evaluate the differences in mortality rate between both crayfish species as well as two zoospore doses after exposure to each *A. astaci* strain the "survdiff" function was used. The significance level was set at 0.05. Non-parametric Kaplan-Meier survival analyses were performed using the "survfit" function. In addition, for graphical visualisation the packages "GGally" (Schloerke et al. 2014) and "ggplot2" (Wickham 2009) were employed.

Results

Aphanomyces astaci DNA was not detected in any crayfish used as a negative control, and in *C. destructor* individuals tested prior to the beginning of the experiment.

In comparison to *A. astacus*, the experimental exposure to all three *A. astaci* strains indicated higher resistance of *C. destructor* to the crayfish plague pathogen (Figure 1). Considerable differences in mortality rates were observed between the two tested species after infections with each *A. astaci* strain (Al7, group A: χ^2=22.1, df=3, p≤0.001; Pec14, group B: χ^2=43.3, df=3, p≤0.001; Evira4805a/10, group E: χ^2=90.2, df=3, p≤0.001). The detailed information about mortality of both crayfish species is provided in Table 1.

Infection with the least virulent *A. astaci* strain (Al7, genotype group A) resulted in deaths of two and six *C. destructor* individuals challenged with 10 and 100 spores ml^{-1}, respectively (Figure 1A). No statistical difference in mortality rate was found between the groups (χ^2=2.4, df=1, p=0.122). The first dead crayfish were found on the 34th day post-inoculation in both treatments. In the treatment with the lower spore concentration, crayfish died either during moulting or a few days afterwards. Similarly, two crayfish individuals died several days after moulting in the other treatment. Moderate to exceptionally high agent levels (A4–A7) were detected in the dead crayfish. In some crayfish individuals that survived the experimental infection, a higher pathogen load was detected in exuviae in comparison to crayfish bodies (Table 2).

The infection with the two more virulent *A. astaci* strains (Pec14 and Evira4805a/10) resulted in a total mortality of *C. destructor* individuals; without a statistical difference in mortality rate between the two spore concentrations (χ^2=0, df=1, p>0.8 for both strains). In each treatment, *A. astaci* infections reached very high to exceptionally high agent levels (A6 and A7) except for two individuals in which the pathogen loads were moderate and high (A4 and A5). In the treatment with the Pec14 (group B) strain, the first dead crayfish were recorded four to six days post-inoculation, but *C. destructor* died on average 46.4 ± 17.1 days (mean ± SD) after exposure to 10 spores ml^{-1} and 15.4 ± 12.5 days when challenged with 100 spores ml^{-1} (Figure 1B). While no

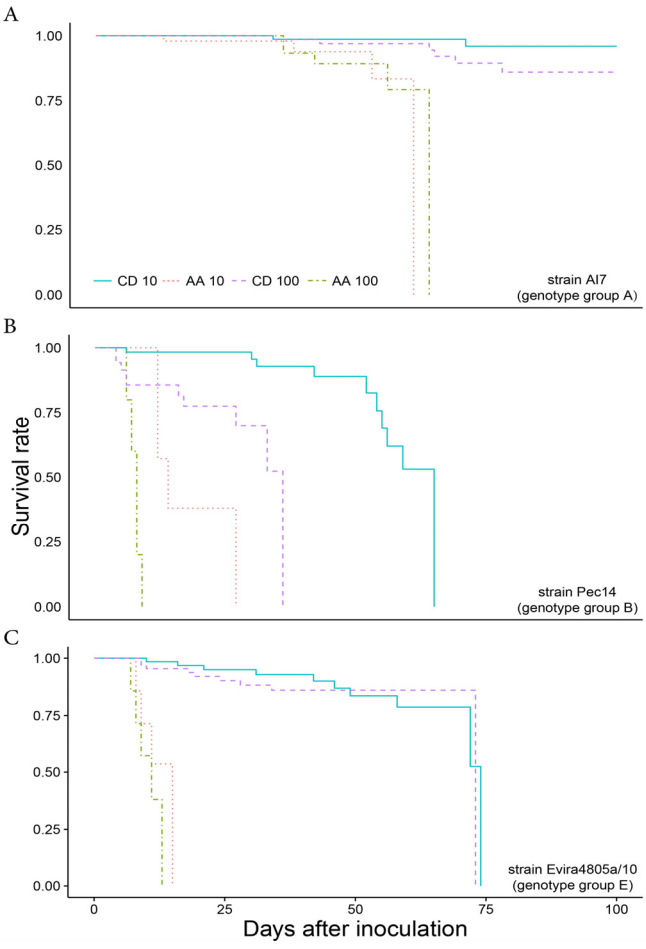

Figure 1. Kaplan-Meier survival analyses for both crayfish species: *Astacus astacus* (AA) and *Cherax destructor* (CD) after infection with three *A. astaci* strains (Al7, Pec14 and Evira4805a/10 representing genotype groups A, B and E, respectively) in two zoospore doses of 10 and 100 spores ml^{-1}. Curves are marked accordingly (e.g., AA10 standing for the crayfish species *A. astacus* and 10 spores ml^{-1}).

crayfish moulted after exposure to 10 spores ml^{-1}, one individual had moulted in the treatment with the higher spore concentration; this most likely contributed to its death two days later.

In the treatment with *C. destructor* individuals infected with the Evira4805a/10 strain (group E), mortality occurred on average 41.9 ± 22.4 and 24.0 ± 19.1 days post-inoculation with spore doses 10 and 100 spores ml^{-1}, respectively (Figure 1C). Whereas no moulting was observed in crayfish exposed to 100 spores ml^{-1}, five *C. destructor* moulted and died shortly afterwards in the treatment with 10 spores ml^{-1}. External body examination indicated that the remaining crayfish exposed to 10 spores ml^{-1} might have died prior to moulting.

All *A. astacus* individuals infected with *A. astaci,* died. After exposure to the zoospores of the Al7 strain, mortality occurred on average 41.0 ± 20.9 and 46.8 ± 12.6 days post-inoculation in the treatment with 10 and 100 spores ml^{-1}, respectively (Figure 1A). No statistical difference in mortality rate was observed between the treatments (χ^2=0.2, df=1, p=0.648). The molecular detection of crayfish plague pathogen indicated high and very high infection levels (A5–A6). In the treatment with 10 spores ml^{-1}, all crayfish died either on the same day or a few days after they moulted, which most likely contributed to their overall faster mortality. In the treatment with 100 spores ml^{-1}, no exuviae were collected but two crayfish individuals died during moulting.

Similarly, a total mortality was observed after exposure to the two more virulent *A. astaci* strains. Very high and exceptionally high agent levels (A6–A7) were detected. No crayfish had moulted during the experiment. The first dead *A. astacus* were recorded on the 12th and 6th day post-inoculation with the zoospores of the Pec14 strain, and 100% mortality was reached on the 27th and 9th day, in concentrations of 10 and 100 spores ml^{-1}, respectively (Figure 1B). Furthermore, exposure to the Evira4805a/10 strain resulted in the first crayfish deaths on 8th and 7th day; no *A. astacus* survived longer than 15th or 13th day of the experimental trial (Figure 1C). Whereas no difference in mortality rate

Table 1. Results of experimental infection with three *A. astaci* strains. N: number of crayfish individuals exposed to zoospores. Semi-quantitative agent levels based on the estimated amount of PCR-forming units (PFU) in the reaction (according to Vrålstad et al. 2009) are provided: A0 no *A. astaci* DNA, A1 (PFU < 5), A2 (5 ≤ PFU < 50), A3 (50 ≤ PFU < 10^3), A4 (10^3 ≤ PFU < 10^4), A5 (10^4 ≤ PFU < 10^5), A6 (10^5 ≤ PFU < 10^6, A7 (PFU ≥ 10^6).

Species	Treatment (spore ml^{-1})	N	Agent level in dead/surviving crayfish	Days to death	
				Average (days)	Mortality rate
Astacus astacus	Al7 (10)	4	A5-A6	41.0 ± 20.9	100%
	Al7 (100)	5	A5-A6	46.8 ± 12.6	100%
	Pec14 (10)	5	A6	15.4 ± 6.5	100%
	Pec14 (100)	5	A6-A7	7.6 ± 1.1	100%
	Evira4805a/10 (10)	4	A6-A7	10.8 ± 3.1	100%
	Evira4805a/10 (100)	5	A6-A7	10.0 ± 3.2	100%
Cherax destructor	Al7 (10)	10	A4-A6/A0-A2	52.5 ± 18.5	20%
	Al7 (100)	10	A4-A7/A0-A3	58.8 ± 16.8	60%
	Pec14 (10)	10	A6-A7	46.4 ± 17.1	100%
	Pec14 (100)	10	A6-A7	15.4 ± 12.5	100%
	Evira4805a/10 (10)	10	A5-A7	41.9 ± 22.4	100%
	Evira4805a/10 (100)	10	A4-A7	24.0 ± 19.1	100%

Table 2. Results of the qPCR analysis after an experimental infection with the least virulent Al7 strain. The *A. astaci* infection levels detected in *C. destructor* individuals that survived the 100-day long exposure and their exuviae sampled during the experiment are presented.

Concentration (spore ml^{-1})	Crayfish	Agent level in crayfish body	Moulting 1		Moulting 2	
			Day of moulting	Agent level in exuviae	Day of moulting	Agent level in exuviae
10	1	A0	24	A3	98	A0
	2	A0	23	A1	80	A0
	3	A0	25	A0	79	A0
	4	A0	8	A0		
	5	A0	3	A4	57	A0
	6	A0	2	A3	80	A0
	7	A2	68	A4		
	8	A0	33	A0	98	A0
100	1	A0	39	A0		
	2	A1				
	3	A3	4	A6		
	4	A0	99	A4		

was observed between the treatments with the two spore concentrations after infection with the Evira4805a/10 (χ^2=1.3, df=1, p=0.258), the difference in mortality rate after exposure to the Pec14 strain was highly significant (χ^2=20, df=1, p≤0.001).

Prior to death, individuals of both crayfish species tended to lose their limbs (claws and legs) after the challenge with the two more virulent *A. astaci* strains. Infection with the Al7 strain was followed by limb loss only in challenged *A. astacus* (regardless of the spore dose) and one *C. destructor* from the treatment with 100 spores ml^{-1}. Molecular analyses revealed exceptionally high pathogen load (more than 10^6 PFU) in that crayfish individual.

The external examination of crayfish bodies revealed the presence of macroscopic melanized spots in 73% of challenged *A. astacus.* These spots were mainly present on the soft abdominal cuticle, basal joints, legs, and on the tail fan. In contrast, only seven out of 60 *C. destructor* individuals were found with melanized spots on their body, associated with broken limbs and injured uropods. No visible melanization was present in the control animals.

Discussion

The potential interactions of *Cherax destructor* with three *Aphanomyces astaci* genotype groups occurring in Central European freshwaters were assessed for the first time. As suggested by Unestam (1975), we confirmed an elevated resistance of *C. destructor* to the crayfish plague pathogen in comparison to European *Astacus astacus*. Depending on the pathogen virulence, this may lead to chronic infections or delayed mortalities in *C. destructor* populations. Therefore, it seems possible that under certain conditions this Australian crayfish species may contribute to *A. astaci* spread in Central Europe.

Long co-evolutionary history between pathogens and their hosts often results in lowered virulence of pathogens and higher resistance of hosts (May and Anderson 1990), a mechanism that explains balanced host-pathogen relationship between North American crayfish species and the crayfish plague pathogen (Cerenius et al. 2003). Recent field observations, however, provided evidence of latent *A. astaci* infections in most European native crayfish species,

including *A. astacus* in Finland (Viljamaa-Dirks et al. 2011), *A. leptodactylus* in Turkey and Romania (Svoboda et al. 2012; Pârvulescu et al. 2012), the stone crayfish *Austropotamobius torrentium* in Slovenia (Schrank, 1803) (Kušar et al. 2013), the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) in Italy (Manfrin and Pretto 2014) as well as several crayfish species in Croatia (Maguire et al. 2016). This confirms that even crayfish species generally considered highly susceptible may carry this pathogen without quickly progressing to acute infection. This phenomenon has been linked to a decreased virulence of some *A. astaci* strains belonging to genotype group A (Makkonen et al. 2012, 2014; Viljamaa-Dirks et al. 2013, 2016) but apparently other genotype groups may also be involved (see Svoboda et al. 2014a).

The variation in host resistance may contribute to chronic infections as well, as highlighted by considerably different survival rates of *C. destructor* and *A. astacus* after infection with the Al7 strain in our study. Only some *C. destructor* individuals died during the experimental trial, in contrast to a total mortality observed in infected *A. astacus*. In most *C. destructor* and *A. astacus* individuals, mortality occurred either during or shortly after moulting, with the possible reasons being 1) high physiological demands of this process and likely associated moulting-dependent variation in immune responses (Cheng et al. 2003; Liu et al. 2004), 2) an increased availability of a suitable substrate for colonization by zoospores (carapace with lower Ca^{2+} content of premoult or freshly moulted crayfish; Aydin et al. 2014), or 3) an intensive spore release during moulting of infected animals (Strand et al. 2012; Svoboda et al. 2013). Interestingly, however, most *C. destructor* individuals were able to substantially reduce *A. astaci* infection level through moulting. Makkonen et al. (2012) speculated that inefficient attachment and germination of *A. astaci* spores and/or an effective crayfish immune response after infection by less virulent crayfish plague strains may limit pathogen growth. Both mechanisms also likely contributed to *C. destructor* ability to withstand and limit infection of the *A. astaci* strain of genotype group A, as observed in our experimental trial.

In comparison to adult crayfish, juvenile individuals moult at a considerably higher rate (Reynolds 2002). In freshwater shrimps, frequent moulting was considered an important factor in their apparent resistance to *A. astaci* infection (Svoboda et al. 2014b). Similarly, it was suggested that frequent moulting of juvenile crayfish is a reason for decreased pathogen prevalence within this age class (Vrålstad et al. 2011), although selective mortality of infected individuals could result in the same prevalence patterns. Our results suggest that moulting may influence the progress of infection differently in hosts with varying levels of susceptibility. In *A. astaci* hosts exhibiting increased resistance (as North American crayfish species, freshwater shrimps or the *C. destructor* tested here) it seems that moulting may lead to reduction of infection levels, while in noble crayfish (and possibly other highly susceptible hosts), it contributes to extensive mortality.

The effect of differently virulent *A. astaci* strains on *A. astacus* resistance has been assessed in several laboratory experiments (Makkonen et al. 2012, 2014; Becking et al. 2015). Although differences were apparent between some of the Finnish strains used by Makkonen et al. (2012), on the whole their results confirm the generally lowered virulence of *A. astaci* strains from genotype group A. Moreover, the use of geographically distant *A. astaci* strains in different experimental studies, originating either from Fenno-scandian *A. astacus* (Makkonen et al. 2012, 2014; Viljamaa-Dirks et al. 2016) or from *A. leptodactylus* of Armenian origin (Becking et al. 2015), provides a further evidence that the long-term interactions between *A. astaci* and European crayfish may have resulted in the pathogen's decreased virulence (Jussila et al. 2014). Interestingly, although we have used the same *A. astaci* strain (Al7) as in the study by Becking et al. (2015), in contrast to results of that study, all *A. astacus* individuals died in the present one. This highlights that caution is needed when comparing results from different experiments, as many factors apart from the overall strain virulence may influence mortality of the same host species. These include, among others, design and length of experimental trials, spore concentrations of an infective agent, age and physiological state of tested crayfish, or their population of origin. Use of juvenile individuals, longer infection trials, and higher spore dosages could have contributed to the higher *A. astacus* mortality rate seen in the present experiment.

Although all *C. destructor* individuals exposed to *A. astaci* strains from the two more virulent genotype groups (B and E) died, the delayed mortality may be an indicator of its ability to slow down the progress of *A. astaci* infection. Unestam (1975) hypothesised that melanin deposition may be correlated with some degree of resistance to *A. astaci* infection in Australian yabby. In our study, the melanization on *C. destructor* individuals was sporadically observed and was mainly associated with broken limbs or injured uropods. This was most probably not directly associated with *A. astaci* infection, as melanization is a common invertebrate immune response towards any damage (Cerenius et al. 2008). Three non-exclusive reasons may explain the lack of observable

A. astaci-associated cuticle melanization: 1) intensive moulting of juvenile crayfish, 2) micromelanization of areas of hyphal penetration (Aquiloni et al. 2011) that could be missed by the naked eye, and 3) less expressed and thus less competent immune systems of the young crayfish used in our experiment in comparison to adults. We presume that the immune response towards penetrating hyphae, i.e., encapsulation of hyphae by haemocytes and subsequent inhibition of its growth by capsule melanization (Unestam and Weiss 1970; Unestam and Nylund 1972), may be less effective in juvenile than in adult crayfish (as already observed in other groups of invertebrates; e.g., Dikkeboom et al. 1985; Dyrynda et al. 1995). If that is true, it may be expected that adult *C. destructor* may even more efficiently inhibit growth of *A. astaci* mycelium in their cuticles. Research focusing on differences in immunological responses between juvenile and adult crayfish is, however, crucial to test this hypothesis. In any case, our results clearly demonstrate that a difference in resistance towards *A. astaci* exists between European *A. astacus* and Australian *C. destructor*, with the latter being able to slow down the infection progress even of the two more virulent *A. astaci* strains.

Cherax destructor that might successfully establish in Central European waters (Veselý et al. 2015) may become infected with crayfish plague via zoospores present in the ambient water. Crayfish survival will then depend not only on the virulence of the transmitted *A. astaci* strain, but also on the amount of zoospores an individual will be exposed to, as shown by the faster mortality rate of crayfish exposed to higher zoospore concentrations (observed in our study as well as in Alderman et al. 1987; Makkonen et al. 2014; Becking et al. 2015). *Aphanomyces astaci* monitoring in open waters revealed relatively small concentrations (usually not more than 1–50 spore l^{-1}) in lakes inhabited by North American crayfish species (Strand et al. 2014). Substantial increases in spore release were reported during episodes of moulting and crayfish death (Strand et al. 2012; Makkonen et al. 2013; Svoboda et al. 2013), or acute disease outbreaks in European crayfish species (up to 500 spore l^{-1}; Strand et al. 2014). Fluctuations in ambient spore concentration may be decisive for potential survival of *C. destructor* in the presence of *A. astaci*. However, we hypothesise that yabby may survive coexistence with American crayfish species, as our specific experimental conditions imposed much higher pathogen pressure on the tested *C. destructor* individuals than generally encountered in the wild.

Introduction of this popular ornamental crayfish into Central European freshwaters may pose a substantial risk to native European crayfish species. *Cherax destructor* potential to survive Central European winters (Veselý et al. 2015; Kouba et al. 2016), together with its environmental plasticity known from Australia (Beatty et al. 2005), indicate a high potential for crayfish to establish populations in temperate Europe. Bearing this in mind, the prevention of *C. destructor* establishment in Central Europe should be given priority, as this prominent invader from Australia may cause a wide range of negative impacts on whole ecosystems (Coughran and Daly 2012), and also likely contribute to the spread of *A. astaci* in Europe.

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