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Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*

Hostitelé a přenos původce račího moru *Aphanomyces astaci*

Ph.D. Thesis

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I declare that this thesis has not been submitted for the purpose of obtaining of the same or another academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and manuscripts. All literature sources I used when writing this thesis have been properly cited.

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Jiří Svoboda

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Contents

Attached publications and manuscripts

1. **Svoboda, J.**, Kozubíková, E., Kozák, P., Kouba, A., Bahadir Koca, S., Diler, Ö., Diler, I., Policar, T., Petrusek, A., 2012. PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eğirdir in Turkey. *Diseases of Aquatic Organisms* **98**, 255-259.
2. **Svoboda, J.**, Strand, D.A., Vrålstad, T., Grandjean, F., Edsman, L., Kozák, P., Kouba, A., Fristad, R.F., Bahadir Koca, S., Petrusek, A., 2014. The crayfish plague pathogen can infect freshwater-inhabiting crabs. *Freshwater Biology* **59**, 918-929.
3. **Svoboda, J.**, Mrugała, A., Kozubíková-Balcarová, E., Kouba, A., Diéguez-Uribeondo, J., Petrusek, A., 2014. Resistance to the crayfish plague pathogen, *Aphanomyces astaci*, in two freshwater shrimps. *Journal of Invertebrate Pathology* **121**, 97-104.
4. **Svoboda, J.**, Kozubíková-Balcarová, E., Kouba, A., Buřič, M., Kozák, P., Diéguez-Uribeondo, J., 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.
5. **Svoboda, J.**, Fischer, D., Kozubíková-Balcarová, E., Šťástková, A., Brůčková, M., Kouba, A., Petrusek, A. (*unpublished manuscript*). Experimental evaluation of the potential for crayfish plague transmission through the digestive system of warm-blooded predators.

Appendices

6. Tilmans, M., Mrugała, A., **Svoboda, J.**, Engelsma, M.Y., Petie, M., Soes, D.M., Nutbeam-Tuffs, S., Oidtmann, B., Roessink, I., 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.
7. Kozubíková-Balcarová, E., Koukol, O., Martín, M.P., **Svoboda, J.**, Petrusek, A., 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.

Preface

I started studying crayfish plague as early as in the second year of my B.Sc. studies, so I have spent more than seven years with one of the worst threats to European crayfish species, a disease caused by one of the 100 worst invasive species, *Aphanomyces astaci*. Those who know me might wonder why I have kept working on such a morbid topic for so long. There have been three key factors that have prevented me from changing my work. First, crayfish plague itself is not a particularly bright issue, but it certainly is interesting. To me, the most amazing thing about life are interactions, either between individuals or species, and the crayfish plague gives opportunity to study both. Second, most of the people I cooperated, studied or just kept meeting with were not only very good colleagues, but also very inspirational personalities. Third, I have a quality which Richard Feynman, the famous Noble Prize laureate, expressed in these words: "I always do that, get into something and see how far I can go". I am happy to believe that I share this quality. Unfortunately for the knowledge of humankind, that is probably all we two have in common.

Nonetheless, a man trying to go as far as possible must either devote his whole life to a challenge, or stop at some point and try another. Since I am one of the most fortunate men, I could choose my own challenge. For me, there have always been two most appealing challenges, scientific research and teaching at a secondary school. The three factors mentioned above have kept me following the path of scientific research for quite a time. However, teaching did not stop appealing me. Eventually, I came to the conclusion that teaching is the right work for me. Therefore, I do not consider this Ph.D. thesis as another step in my scientific career, but rather as a final one. Nevertheless, I believe that it is not a dead end. I hope that I will be able to profit from my short scientific experience during my lessons at secondary schools. And I would like to consider this work a minor, but decent contribution to the research of the crayfish plague pathogen.

Acknowledgements

Naturally, I would not be able to finish this thesis if it were not for the support of my family, my colleagues, my friends, and many others. In the following lines, I have included the names of only some of them, since many of those who deserve my gratitude are not going to read this thesis, and I will have to find a different way to thank them.

I am most grateful to Adam, who led my work in the first years and has remained the most important advisor during my Ph.D. studies. It was a great opportunity to cooperate with such a hardworking man with so broad knowledge, who still manages to enjoy his time with his family. Much of the work presented in this thesis was planned, done or analysed in cooperation with my colleagues at the Department of Ecology, especially Eva Kozubíková-Balcarová and Agata Mrugała. Feeling the warmth of their optimism, I could even keep crushing crayfish tissues in liquid nitrogen.

I would like to highlight also the pleasant cooperation with Pavel Kozák and Antonín Kouba from South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses in Vodňany. It was in Vodňany, where I worked on my first experiments with the crayfish plague, and where I enjoyed more than one morning with fresh cherries for breakfast. I am also grateful to Javier Diéguez-Uribeondo for teaching me the techniques for *A. astaci* cultivation, isolation and sporulation during my four-week stay in Madrid. Thanks to him, I admired not only the beauty of the famous Museo del Prado, but also of oomycetes and their sporulation.

The cooperation with David Strand and Trude Vrålstad was also very pleasant. I appreciate mostly the simple fact that we decided to join our efforts instead of competing for the first confirmation of the crayfish plague pathogen growth in crabs. Some of the work presented in the papers, especially with the agar plates, was done at the Department of Botany with the permission and help of Ondřej Koukol. There were many other people involved in the projects that are included in my thesis. Those who contributed most are either directly among the authors of the papers or thanked to in the acknowledgements.

Abstrakt (in Czech)

Račí mor decimuje populace evropských druhů raků již více než 150 let, a proto je jeho původce, oomycet *Aphanomyces astaci*, považován za jednoho ze 100 nejhorších invazních druhů na světě. Původce račího moru je silně přizpůsobený parazitickému způsobu života. Přesto jej lze, podobně jako mnohé další oomycety, izolovat z nemocných raků a pěstovat na agarových médiích (**kapitola 7**). Životní cyklus *A. astaci* zahrnuje tři základní stádia: mycelium rostoucí v tkáních hostitelů a zoospory a cysty, což jsou infekční stádia vyskytující se volně ve vodě.

Všechny dosud testované severoamerické druhy raků jsou vůči patogenu račímu moru do značné míry odolné, tj. navzdory infekci přežívají poměrně dlouho a nevykazují akutní příznaky nemoci. Proto mohou tyto druhy raků sloužit jako dlouhodobí přenašeči tohoto patogenu. K masivní tvorbě a uvolnění spor z infikovaných severoamerických raků dochází v době svlékání, nebo když jsou raci vystaveni nepříznivým podmínkám či hynou (**kapitola 4**). Ve svých experimentech jsem však prokázal, že ke sporulaci ze severoamerických raků dochází i mimo období svlékání, a to i když raci nejeví žádné zjevné známky nemoci. Proto musejí být infikovaní severoameričtí raci považováni za stálý zdroj nákazy (**kapitola 4**). Známé kmeny račího moru byly na základě genetické variability rozděleny do pěti skupin. Každá skupina sdružuje kmeny, které pravděpodobně pocházejí z téhož severoamerického druhu raka. To však nebrání jejich horizontálnímu přenosu na jiné druhy hostitelů (např. **kapitoly 2, 4 a 6**).

Všechny dosud testované druhy raků pocházející z Eurasie či Austrálie byly vůči račímu moru mnohem citlivější než severoameričtí raci. Nicméně, nalezeny byly i populace evropských raků, v nichž je původce račího moru přítomen, ale k hromadným úhynům nedochází. Takové latentní infekce byly dosud hlášeny z několika států včetně Turecka (**kapitola 1**). Ačkoliv už byly dokumentovány i latentní infekce kmenem pocházejícím ze severoamerického raka signálního (např. **kapitola 2**), latentní infekce jsou obvykle připisovány kmenům ze skupiny, která byla do Evropy introdukována dříve.

Kromě raků byl za hostitele *A. astaci* označen v minulosti i katadromní krab čínský (*Eriocheir sinensis*), což jsme nedávno potvrdili i pomocí molekulárních a mikroskopických metod (**kapitola 2**). Dále jsme prokázali, že infikován může být i semiterestrický krab *Potamon potamios*, a tak by měli být za potenciální hostitele považováni všichni krabi vyskytující se ve sladkých vodách (**kapitola 2**). Výsledky experimentů se sladkovodními krevetami, které jsou příbuzné rakům a krabům, naznačily, že k mírnému růstu původce račího moru v některých jedincích a svlečkách pravděpodobně došlo. Žádná kreveta po vystavení sporám však neuhynula (**kapitola 3**). Ostatní živočichové se zdají být vůči moru odolní. Ani data z naší pilotní studie, která zkoumala několik korýšů nepatřících mezi desetinožce (Decapoda), nenaznačila růst *A. astaci* v tkáních těchto korýšů, ačkoliv sdíleli jednu lokalitu s infikovanými raky (**kapitola 2**). Přesto však stále nelze považovat za zcela vyloučenou možnost, že někteří další korýši by se mohli příležitostně stávat hostiteli *A. astaci*, byť například jen při nepříznivých podmínkách.

Klíčovou roli v introdukci a šíření račího moru Evropou sehrály lidské aktivity. První severoameričtí raci byli do Evropy dovezeni za účelem chovu v akvakulturách. Za nedávné introdukce dalších druhů, z nichž některé prokazatelně mohou přenášet račí mor, jsou však nejspíše zodpovědní akvaristé (např. **kapitola 6**). Obezřetně musí být přistupováno i k vysazování původních evropských druhů raků, a to i v případě, že nejeví známky nemoci (**kapitola 1**). A zamezeno by mělo být i přesunům a vysazování kraba čínského (**kapitola 2**).

Uvážíme-li existenci latentních infekcí evropských raků a zejména možnost přenosu *A. astaci* kraby čínskými, mohlo by být šíření račího moru aktivním pohybem nakažených hostitelů významnější, než se donedávna předpokládalo (**kapitola 2**). Račí mor může být šířen i mrtvými těly hostitelů či jejich částmi; takový přenos byl prokázán i trávicí soustavou ryb. Přenos trávicí soustavou savců a ptáků je však velmi nepravděpodobný (**kapitola 5**).

Další výzkum račího moru bude pravděpodobně často využívat molekulární metody, které by však vždy měly být testovány i vůči dalším oomycetům, které se na nemocných racích vyskytují (**kapitola 7**). Ve své práci představuji i několik hypotéz, jež by mohly být v budoucnu testovány.

Abstract

The crayfish plague pathogen, the oomycete *Aphanomyces astaci*, has been decimating populations of European crayfish species for more than 150 years, and is therefore considered one of the 100 worst world's invasive species. *A. astaci* is highly specialised for a parasitic life, but it can be isolated from moribund crayfish and grown on synthetic media, as it is the case also for several other oomycetes (**chapter 7**). The life of *A. astaci* includes three basic forms: mycelium in host's tissues, and the infective units occurring in water, zoospores and cysts.

All North American crayfish species tested so far have shown some resistance to *A. astaci*, i.e., they could carry the infection for long, serving as vectors of the pathogen. Massive sporulation from infected North American crayfish starts when the host is moulting, stressed, or dying (**chapter 4**). However, I could show in my experiments that some sporulation occurs even from apparently healthy and non-moulting American crayfish hosting *A. astaci*, so infected North American crayfish must be considered a permanent source of the infection (**chapter 4**). Five genotype groups of *A. astaci* have already been distinguished. Strains from a particular genotype group probably share the same original host crayfish species of North American origin. Nevertheless, they can be transmitted horizontally to other hosts (e.g., **chapters 2, 4 and 6**).

In contrast to North American crayfish, all crayfish species of Eurasian and Australian origin so far exposed to *A. astaci* spores were more susceptible. Nevertheless, some populations of European crayfish with latent infection of *A. astaci* have recently been reported from several countries (including Turkey, **chapter 1**). Although some chronic infections caused by an *A. astaci* strain originating from the North American signal crayfish have been reported (e.g., **chapter 2**), latent infections are usually assumed to be a result of infection with a strain from the first genotype group that had been introduced to Europe.

Apart from crayfish, only the catadromous Chinese mitten crab *Eriocheir sinensis* was reported to host the crayfish plague pathogen, which we have recently confirmed by molecular and microscopic methods (**chapter 2**). In addition, we have shown that the semi-terrestrial crab, *Potamon potamios*, can also be infected with the pathogen, so all freshwater-inhabiting crabs should be considered as potential hosts (**chapter 2**). The experiments with freshwater shrimps, crustaceans related to crabs and crayfish, suggested minor growth of the pathogen in some individuals and exuviae. However, none of the shrimps exposed to *A. astaci* spores died (**chapter 3**). Other animals seem to be resistant to the pathogen. Even the data from our pilot research did not suggest any *A. astaci* growth in non-decapod crustaceans coexisting with infected crayfish (**chapter 2**). Nevertheless, the possibility that some other crustaceans may become accidental hosts of *A. astaci*, e.g., when stressed, has still not been entirely rejected.

Human activities had a key role in the introduction and dispersal of *A. astaci* in Europe. While the first North American crayfish have been introduced for aquaculture purposes, more recent introductions of new American crayfish species, some of which are proven *A. astaci* carriers, have probably been caused by hobbyists (e.g., **chapter 6**). Close attention must also be paid to the disease status of the crayfish during stocking, even when apparently healthy European crayfish are used (**chapter 1**). In addition, human-mediated dispersal of the crab *E. sinensis* should also be prevented (**chapter 2**).

With respect to the recent data on the latent infections of European crayfish, and particularly to the transmission of *A. astaci* by *E. sinensis*, the long-distance dispersal by the locomotion of the infected hosts might be more important than it was anticipated (**chapter 2**). Crayfish plague may be spread also by dead hosts and their body parts, the transmission has been proven even through the digestive tract of fish. In contrast, such a transmission through mammals and birds is highly unlikely (**chapter 5**).

Future research of *A. astaci* will probably gain from molecular methods. Their specificity, however, should always be tested against other oomycetes that may be present on moribund crayfish (**chapter 7**). In this thesis, I have also brought several hypotheses that might be tested in future.

Outline of publications and manuscripts

My thesis consists of an introduction, five first-author studies (**chapters 1-5**), and two studies in the appendices (**chapters 6 and 7**). In the introduction, I present my view of the current state of art and future perspectives concerning the crayfish plague transmission and the crayfish plague pathogen hosts, discussing published literature as well as my own research put into a general context. In addition, I have included some other issues concerning the life cycle of the crayfish plague pathogen, *Aphanomyces astaci*. Eventually, this introductory chapter will be supplemented by ideas of other colleagues, transformed to a separate manuscript and submitted for publication. The five first-author thesis chapters include four peer-reviewed papers published in international periodicals (**chapters 1-4**), and one as yet unsubmitted manuscript (**chapter 5**). The appendices contain two studies led by my colleagues. To those studies I contributed mostly in the form of laboratory work, e.g., isolation of oomycetes from crayfish, isolation of DNA and quantitative PCR. However, as a co-author of the studies, I also provided feedback on the manuscript texts, and approved their final versions.

Chapters 1 and 6 report on the presence of *A. astaci* in natural populations of *A. astaci* hosts. **Chapter 1** investigates the population of the narrow-clawed crayfish *Astacus leptodactylus* in the Turkish Lake Eğirdir. According to literature (Harlıoğlu, 2004, Harlıoğlu, 2008), the local crayfish population declined drastically in the mid-1980s due to introduction of crayfish plague, but partly recovered in the following years. Most interestingly, *A. leptodactylus* has been suspected to persist despite the presence of *A. astaci* (Harlıoğlu, 2004, Harlıoğlu, 2008), although the species was supposed to die when infected with the pathogen (Unestam, 1969b). To test the hypothesis that the European crayfish species coexists with the crayfish plague pathogen in the lake, we isolated DNA from 34 healthy-looking crayfish from the lake and tested their tissues by both conventional and quantitative PCR using *A. astaci*-specific primers. The presence of the crayfish plague pathogen was revealed in 5 individuals. From the current point of view, the study is one of the first reports of a long-term coexistence of *A. astaci* with European crayfish that confirmed the pathogen presence unambiguously.

While the first chapter focused on a crayfish population in one Turkish lake, **chapter 6** was a large scale study covering several localities in the Netherlands, the aim of which was to evaluate *A. astaci* prevalence in Dutch populations of alien crustaceans. Using *A. astaci*-specific quantitative PCR, we evaluated this pathogen's prevalence in Dutch populations of three confirmed crayfish carriers (*Orconectes limosus*, *Pacifastacus leniusculus*, *Procambarus clarkii*), two recently introduced crayfish (*Orconectes* cf. *virilis*, *Procambarus* cf. *acutus*), and the invasive catadromous crab *Eriocheir sinensis*. The infection with *A. astaci* was detected in some populations of *O. limosus*, *P. leniusculus*, *O*. cf. *virilis* and *E. sinensis*. Dutch *P. clarkii* seem only sporadically infected, and the pathogen was not detected in *P*. cf. *acutus* despite substantial sampling efforts. Our study was the first confirmation of crayfish plague infections in the Netherlands, the first confirmation of the crayfish *O*. cf. *virilis* as another *A. astaci* carrier, and demonstrated substantial variation in *A. astaci* prevalence among potential hosts within a single region.

Chapters 2 and 3 focus on the host range of *A. astaci*. As early as in the 1970s, Unestam (1972) suggested that the parasite host range may include not only crayfish but also other freshwater decapods. The hypothesis was based mostly on an old experimental study by Benisch (1940), which reported the infection of the Chinese mitten crab *E. sinensis* with *A. astaci.* However, the then determination of the pathogen could be considered doubtful, and the ability of *A. astaci* to grow in freshwater crabs had never been evaluated further. Therefore, we decided to test for the presence of *A. astaci* in a population of freshwater crabs coexisting with known carriers of the crayfish plague pathogen. We chose the population of *Potamon potamios* from Lake Eğirdir in Turkey, which is in contact with the infected population of the crayfish *A. leptodactylus*. At the International Association of Astacology conference in Innsbruck, we found out that our colleagues from Norway were evaluating the *A. astaci* infection in *E. sinensis* from the Swedish lake Vänern. We decided to join our efforts and this fruitful cooperation resulted in the paper presented here as **chapter 2**. The paper has

brought both molecular and microscopic evidence for *A. astaci* infection of both studied crab species. In contrast, a pilot small-scale screenings of benthopelagic mysids, amphipods and benthic isopods did not suggest any infection by *A. astaci* in non-decapod crustaceans.

However, we did not test freshwater shrimps in the study summarised in chapter 2. The main reason was that we did not manage to find any shrimps that had been exposed to zoospores of *A. astaci*. Naturally, our next step was to carry out transmission experiment with some freshwater shrimps in laboratory conditions (**chapter 3**). We exposed individuals of two unrelated Asian shrimp species, *Macrobrachium dayanum* and *Neocaridina davidi*, to *A. astaci* zoospores. Shrimp bodies and exuviae were tested for *A. astaci* presence by a species-specific quantitative PCR. We did not observe mortality of shrimps, and the amount of *A. astaci* DNA was decreasing in *N. davidi* faster than in *M. dayanum*, probably due to more frequent moulting of the former species. The shrimps were more resistant to the crayfish plague pathogen than European crayfish species, but the high pathogen DNA levels detected in some non-moulting individuals of *M. dayanum* suggest that *A. astaci* growth may be possible in tissues of that species.

Chapters 4 and 5 focus on the transmission of the crayfish plague pathogen. In **chapter 4**, we presented the data from our experiments with infected carriers, North American crayfish *O. limosus*. We evaluated changes in *A. astaci* spore release rate from infected individuals of this species by experiments investigating the pathogen transmission to susceptible noble crayfish, *Astacus astacus*, and by quantification of *A. astaci* spores caught by filters. The filters and tissues were then tested for the presence of *A. astaci* DNA by species-specific quantitative PCR. The experiments confirmed that *A. astaci* can be transmitted to susceptible crayfish during intermoult periods. The pathogen spore concentrations substantially varied in time, and significantly increased during moulting of infected hosts. The experiment summarized in this chapter was performed already during my MSc. study. During the PhD studies, I performed additional analyses, and transformed the undergrad thesis written in Czech into a peer-reviewed publication.

Chapter 5 focuses on the potential crayfish plague pathogen dispersal through mammalian and bird digestive systems. Such a transmission has mostly been considered unlikely because of high body temperature of warm-blooded vertebrate predators, but the experimental support that has been published so far is not convincing. Our study included a small-scale transmission experiment with the European otter (*Lutra lutra*) and the American mink (*Neovison vison*) fed with infected crayfish, and experiments testing survival of different *A. astaci* strains on agar plates at temperatures corresponding to those inside mammal and bird bodies. The pathogen was not isolated from predator excrements nor was it transmitted to susceptible crayfish through them. On agar, the pathogen usually died when incubated in bird and mammal body temperatures for relevant time. Nevertheless, the pathogen persistence varied and sporadic survival of *A. astaci* thus cannot be excluded entirely. With respect to our data, we consider the pathogen transmission through the digestive tract of warm-blooded predators less likely than the potential transmission on their surface.

Chapter 7 focuses on oomycetes colonising the crayfish cuticle. The chapter considerably differs from the others as it was part of the work led by my colleague Eva Kozubíková-Balcarová. In this project, I was included mostly to carry out some of the laboratory work with the cultures. Most importantly, I isolated the *A. astaci* strains from crayfish collected in the river Litavka during the crayfish plague outbreak in 2011, the first case when a strain of the genotype group E was isolated from infected European crayfish. In the study, cuticle of various crayfish was found to be colonised by numerous oomycetes (including the crayfish plague pathogen). Altogether, 95 oomycete isolates obtained during attempts to isolate *A. astaci* from presumably infected crayfish were analysed, and thirteen taxa were identified by molecular analysis. Morphological identification to species level was only possible for 15 % of isolates. Only seven isolates of *A. astaci* were obtained, all from the single disease outbreak in Litavka. We showed that oomycete cultures obtained as by-products of parasite isolation are valuable for oomycete diversity studies, but morphological identification may uncover only a fraction of their diversity.

INTRODUCTION

"*Aphanomyces astaci* has become known as the species causing mortalities in crayfish populations and it was a crayfish tissue where hyphae of *A. astaci* were found first..."

Hosts and transmission of *Aphanomyces astaci*

The first mass mortalities of crayfish, considered at present to have been caused by crayfish plague, were reported in Italy in 1859 (Alderman, 1996). Nevertheless, the mortalities in the Po basin were spatially separated and happened earlier than the outbreak from which crayfish plague started to spread further across Europe, which occurred in 1874 in France (Alderman, 1996). It took decades to prove that the causative agent of the disease is the oomycete *Aphanomyces astaci* (Söderhäll and Cerenius, 1999). More than 150 years from the first mass mortalities, *A. astaci* still threatens populations of European crayfish (Füreder, 2006, Holdich *et al.*, 2009). Furthermore, experiments have indicated that Asian and Australian crayfish species would also suffer if the pathogen was introduced to those areas (Unestam, 1975, Unestam, 1969b). The crayfish plague pathogen is therefore considered one of the 100 worst world's invasive species (Lowe *et al.*, 2004). Thanks to decades of the pathogen research, it is also one of the best studied invertebrate pathogens (Diéguez-Uribeondo *et al.*, 2006).

The aim of this chapter is to review and discuss the recent advances of *A. astaci* research with respect to its transmission, host range and life cycle, and to indicate possible directions for future research in these fields. The evolution of *A. astaci* virulence and resistance of its hosts are not reviewed in details since those issues have been discussed recently elsewhere (see Jussila *et al.*, 2014a, Gruber *et al.*, 2014). The life cycle of *A. astaci* has also been summarised in various previous reviews of *A. astaci* biology (e.g., Söderhäll and Cerenius, 1999, Cerenius *et al.*, 1988, Diéguez-Uribeondo *et al.*, 2006). However, brief summary of *A. astaci* life cycle is included to make the reading of the following detailed part clearer, and to discuss some hypothetical and controversial aspects of *A. astaci* life such as sexual processes, formation of gemmae-like structures, survival in brackish water, and partially saprophytic mode of life. The transmission of *A. astaci* was reviewed by Oidtmann *et al.* (2002b) over a decade ago. Since then, several studies have substantially enriched and altered the knowledge on the pathogen spread (e.g., **chapter 2**, Schrimpf, Schmidt and Schulz, 2014, Jussila *et al.*, 2011b, Strand, 2013). These recent findings on the pathogen transmission and hosts should be considered in conservation efforts targeting native European crayfish species, in particular when aiming to prevent the pathogen spread.

The life cycle and parasitism of *Aphanomyces astaci*

According to the published literature (e.g., Söderhäll and Cerenius, 1999, Cerenius *et al.*, 1988, Diéguez-Uribeondo *et al.*, 2006), there are three main forms of *A. astaci*: a hypha, a zoospore, and a cyst (Fig 1). The word *spore* is frequently used to denote both zoospores and cysts (e.g., Strand *et al.*, 2012) since they can turn one into another and both occur naturally on their own in water. In contrast, hyphae grow in the tissues of infected hosts, forming a mycelium. When hyphae protrude from the cuticle to the surrounding water, they can sporulate, i.e., form sporangia, each containing a row of primary spores. Primary spores extrude and turn into primary cysts which have a cell wall and attach to each other forming clusters called "spore balls". Each primary cyst releases one biflagellate zoospore, which actively searches for a new host, presumably benefiting from chemotaxis (Cerenius and Söderhäll, 1984a). This stage is terminated by the second encystment. During encystment, the spore drops or retracts its flagella and become encased in a cell wall covered with sticky substances. On a suitable substrate (host cuticle), the secondary cyst germinates, the emerging hypha penetrates the surface and grows into the host body, which completes *A. astaci* life cycle. Instead of germination, the secondary cyst may also release a new zoospore in a process known as repeated zoospore emergence (Cerenius and Söderhäll, 1984b, Cerenius and Söderhäll, 1985). This can help the spore to find a host because the zoospore encystation may occur also on unsuitable substrates or even in the water (or medium), in response to various stimuli such as change of temperature (Unestam, 1966b), shaking, and change of medium composition (Cerenius and Söderhäll, 1984b, Svensson and Unestam, 1975).

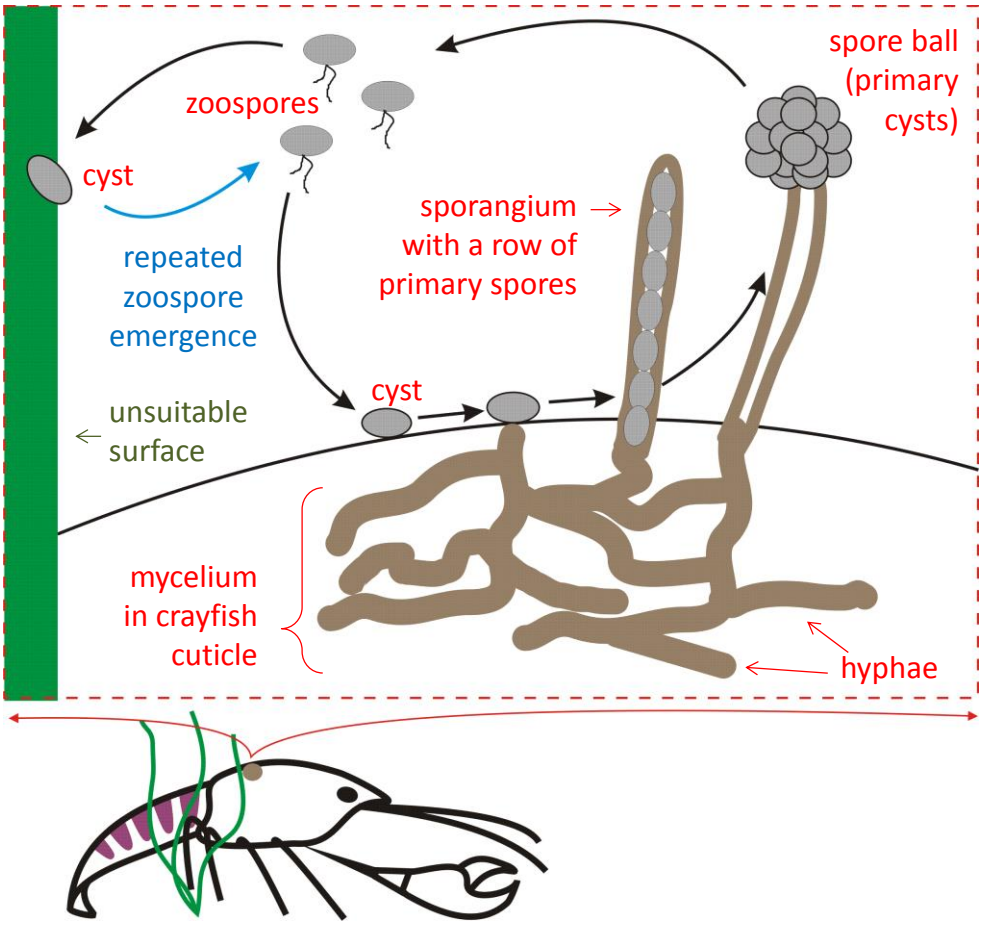

Figure 1: Summary of *A. astaci* life cycle, with a crayfish host and unsuitable substrate represented by an aquatic macrophyte. The figure was inspired by Cerenius *et al.* (1988) and Diéguez-Uribeondo *et al.* (2006).

A sexual apparatus of *A. astaci* has been reported at least twice (Rennerfelt, 1936, Schäperclaus, 1935), but none of the evidence is persuasive (Johnson, Seymour and Padgett, 2002). In addition, high similarity of RAPD (Random Amplified Polymorphic DNA) patterns and low variability of ITS (Internal Transcribed Spacer) sequences as well as microsatellite multilocus genotypes suggest clonal propagation of *A. astaci* (Cerenius and Söderhäll, 1996, Diéguez-Uribeondo *et al.*, 2007, Grandjean *et al.*, 2014). In contrast to saprophytic species, sexual reproduction has also not been found for most congeners belonging to the same animal parasitic lineage of the genus *Aphanomyces* as *A. astaci* (Diéguez-Uribeondo *et al.*, 2009). It has been hypothesized that asexual reproduction leads to a more effective selection of a particular genotype with enhanced parasitic abilities for a specific host (Diéguez-Uribeondo *et al.*, 2007), e.g., asexual reproduction preserves well-adapted combinations of genes that might be lost during sexual recombination (Nielsen and Heitman, 2007).

However, a conserved feature of microbial pathogens is that they limit sexual reproduction and thereby generate clonal populations with rare bursts of parasexual or sexual reproduction, likely as a response to novel selective pressures (Heitman, 2006). Recent population genetic studies suggest that also some human pathogenic fungi which were considered asexual may have some form of genetic exchange between individuals. For many of these fungi, it remains to be seen whether this genetic exchange is due to a classical sexual cycle or by other means such as same-sex mating (via selfing or outcrossing) or parasexual reproduction (Nielsen and Heitman, 2007). It is noteworthy that parasexual process, i.e., fusion of different hyphae, and subsequent genetic exchange, has also been reported in an oomycete, *Plasmopara halstedii* (Spring and Zipper, 2006). An evidence for recombination of chitinase genes within a single *A. astaci* genotype has been reported (Makkonen,

Jussila and Kokko, 2012a), but that could be a consequence of intragenomic recombination rather than of sexual process. Thus, although the possibility that the sexual apparatus might be formed under very particular environmental conditions (Johnson *et al.*, 2002) cannot be entirely excluded, we still assume that *A. astaci* life cycle does not include any sexual process.

Aphanomyces astaci apparently does not produce oospores, which in a typical oomycete life cycle serve as stages able to resist dry periods and extreme temperatures (Diéguez-Uribeondo *et al.*, 2009). Nevertheless, such absence does not necessarily mean that *A. astaci* is unable to produce any other resistant forms. Species of the genus *Aphanomyces* have not been reported to produce any gemmae (segments of hyphae, asexual propagules) or gemmae-like structures but for two exceptions, *A. astaci* and *A. pisci* (Johnson *et al.*, 2002). Srivastava (1979) described gemmae-like structures in his cultures isolated from aphanomycosis of an Indian fish, and Unestam (1969a) found that *A. astaci* may form thick walled as well as gemmae-like hyphal portions in a synthetic medium. When I cultivated *A. astaci* in the same medium as recommended by Unestam to induce these unusual structures, I also observed a few round structures which morphologically resembled those described by Unestam (J.S., unpublished data). To the best of my knowledge, no-one has investigated if these structures may play any specific role in the life cycle of the species, e.g., if they are more resistant to stressful conditions.

Although some *Aphanomyces* species can withstand even salinity of 20 ppt (Dykstra *et al.*, 1986), *A. astaci* is more sensitive to higher salinities (Unestam, 1969a). According to Unestam, the results of his experiments gave no evidence that *A. astaci* could survive in sea or brackish water. Indeed, the mineral salt mixture drastically reduced zoospore production and prevented the spore release into the medium, although the concentrations of minerals were lower than in sea water (Unestam, 1969a). However, the concentrations of salts in the mixtures tested by Unestam (1969a) did not correspond to those found in brackish water (for example, the relative concentration of calcium ions to other minerals in the tested salt mixtures was higher). As *A. astaci* reactions to the same concentrations of different cations vary (Cerenius and Söderhäll, 1984b), and the concentration of calcium cations might alter the negative effect of magnesium cations (Söderhäll and Cerenius, 1987), it would be prudent to support the assumption that *A. astaci* cannot survive in brackish water with further data, and test at which salt concentrations the pathogen may still spread.

Aphanomyces astaci has become known as the species causing mortalities in crayfish populations (Alderman, 1996) and it was a crayfish tissue where hyphae of *A. astaci* were found first (Söderhäll and Cerenius, 1999). Unestam (1969a) summarised a lot of evidence of parasitism in the physiology of *A. astaci*, such as the facts that the hyphae of *A. astaci* were able to penetrate the soft cuticle of crayfish, they grew in a crayfish serum, and the species survived after being injected into the crayfish body (Unestam, 1969a). The species produces great amounts of chitinase and prefers glucose as the source of carbon (Unestam, 1965, Unestam, 1966a). Repeated zoospore emergence is also considered an adaptation to parasitism (Cerenius and Söderhäll, 1985), common to several parasitic species of the genus *Aphanomyces* (Diéguez-Uribeondo *et al.*, 2009). In addition, there are also indications of co-evolution between crayfish and the pathogen both in the very specific level such as extracellular proteinases of *A. astaci* and their inhibitors produced by crayfish (Diéguez-Uribeondo and Cerenius, 1998), and in the very general one: differences between the rather high resistance of North American crayfish species (assumed to be the original *A. astaci* host) and the low resistance of crayfish from Europe, Asia and Australia (Unestam, 1969b, Unestam, 1975).

Apart from the traits of parasitism mentioned above, there are further indications that the species is specialised for a parasitic life: *A. astaci* can be easily outcompeted by other microbes in synthetic media (Cerenius *et al.*, 1988) and it generally does not survive in nature in the absence of hosts (Oidtmann, 2012). This does not exclude the ability to complete the life cycle in dead bodies or exuviae occasionally, nevertheless there seems to be no convincing evidence that the pathogen survives in the environment for a longer time once hosts have been eliminated. Johnson *et al.* (2002) searched for *A. astaci* in bottom sediments and shoreline waters known to harbour infected crayfish but have not once collected it by the usual gross culture technique. Although other studies reported that *A. astaci* was isolated from dead crustaceans other than decapods, e.g., amphipods and isopods

(Czeczuga, Kozlowska and Godlewska, 2002, Czeczuga, Kozłowska and Godlewska, 1999), such cultures were determined as *A. astaci* according to their morphology only, although *A. astaci* cannot be distinguished by such traits from its congeners (see Oidtmann, 2012). Therefore, it is likely that the species isolated from the crustaceans and reported as *A. astaci* by Czeczuga et al. were actually some of its saprophytic congeners (see e.g., Diéguez-Uribeondo *et al.*, 2009).

A. astaci obviously does not meet the definition of a facultative parasite, i.e., species living as a saprophyte, unless accidentally eaten or entering a wound or other body orifice (Roberts *et al.*, 2013, Zinsser *et al.*, 1988). Nevertheless, since the species can be isolated to synthetic media (e.g., Unestam, 1965, Alderman and Polglase, 1986), it is not an obligate parasite either (Oidtmann, 2012). The species might rather meet the definition of an "ecologically obligate parasite", i.e., a species invariably occurring in nature as parasite, but which can be grown in synthetic media (Sharma, 2008). That term, however, is used only sporadically. The phrase "near-obligate hemibiotrophic pathogen" has been used to characterise several plant pathogens, including the oomycete *Phytophthora infestans* causing late (potato) blight (e.g., Fry, 2008, Goodwin, 1997, Kobayashi *et al.*, 2012). A hemibiotroph is a species living partly as a biotroph (whose exclusive, natural growth environment is in or on living host cells), and which is partly associated with later stages of infection as a necrotroph or a saprophyte (Agrios, 2005). Since massive sporulation of *A. astaci* occurs around the death of a host (**chapter 4**, Makkonen *et al.*, 2013, Strand *et al.*, 2012), and *A. astaci* sometimes covers some body parts of dead crayfish with a dense mycelium (Fig. 2), *A. astaci* meets the definition and can be characterised as hemibiotrophic. In case *A. astaci* should be classified using the scale from facultative to obligate pathogens (parasites), it might probably be considered a "near-obligate pathogen" to suggest the dependence of *A. astaci* on its hosts, despite the ability to grow on synthetic media.

Even when not considering the obvious benefit of North American crayfish from the infection in the competition with susceptible European crayfish species (see e.g., Schrimpf *et al.*, 2013b), the impact of the infection with *A. astaci* in natural conditions does not have to be purely negative for all hosts (Cerenius *et al.*, 2003). An infected individual of a relatively highly resistant host (presumably a North American crayfish species) could benefit from this particular infection also by avoiding being infected by other parasites or pathogens due to the increased capacity to synthesise prophenoloxidase (one of the key components of the immune reactions of crayfish) and competition from the primary parasite towards other parasites trying to become established (Cerenius *et al.*, 2003). Similarly, *A. astaci* strains might influence the competition of native crayfish species in North America. However, to the best of my knowledge, no support for these hypotheses has been published yet.

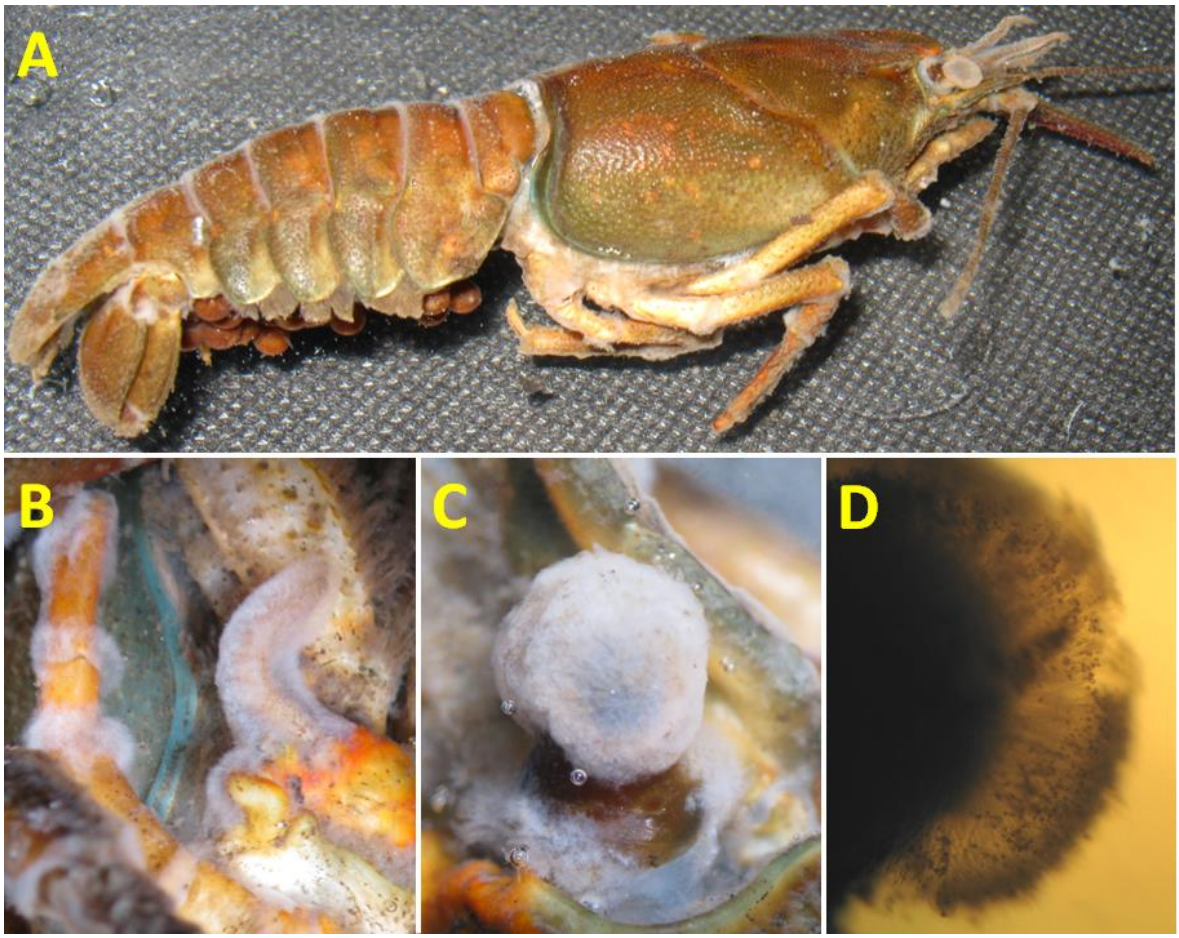

Figure 2: A dead individual of the noble crayfish *Astacus astacus* collected during a crayfish plague outbreak in the brook Černý near the village Pec (Czech Republic). Cotton-like mycelium of *A. astaci* can be seen on the soft cuticle between the segments of abdomen, legs and antennae (A). Detailed view (10x) of the mycelium on legs (B) and eye (C). Microscopic view (40x) of the mycelium on the eye with numerous spore-balls (D). The mycelium was determined as *A. astaci* not only according to its morphology, but it was also isolated from the individual on an agar plate, and determined as *A. astaci* by sequencing of an ITS DNA fragment as recommended by Oidtmann (2012). The extensive growth of *A. astaci* in the crayfish collected during the outbreak was also illustrated by exceptionally high levels of *A. astaci* DNA in samples of their tissues tested by a quantitative PCR (according to Vrålstad *et al.* 2009).

Hosts of *Aphanomyces astaci*

While the presence of *A. astaci* can be fatal to European crayfish, other animals in a locality with the crayfish plague outbreak do not seem harmed (Oidtmann, 2012). However, absence of harmful impact does not mean that a particular species cannot serve as a non-symptomatic host. Furthermore, the pathogen might not have met all potential hosts so far. Since *A. astaci* can apparently be transmitted to new hosts only by zoospores that are restricted to freshwater environments (Unestam, 1969a), all its hosts must live there, at least temporarily, so that they can get infected and spread the disease. There are some characteristics indicating that *A. astaci* parasitizes arthropods (Unestam, 1969a) such as the production of chitinase even in chitinless media, but no apparent amount of cellulases and pectinases (Unestam, 1966a). Nevertheless, it can hardly be assessed which of the vast number of arthropod groups living in freshwaters are potential hosts for *A. astaci* unless experimentally tested.

Crayfish non-indigenous to Europe

All North American crayfish species tested so far show high resistance to the crayfish plague pathogen (Tab. 1), i.e., they can be infected but they can restrict the pathogen growth to the cuticle (Cerenius *et al.*, 1988, Cerenius *et al.*, 2003). As a result, North American crayfish can act as chronic carriers of the disease (Söderhäll and Cerenius, 1999). Nevertheless, even they can suffer from the infection (Edsman *et al.*, 2015), and show an increased mortality after exposure to *A. astaci* if the immune system is suppressed, which may happen in natural conditions during moulting or attacks by other parasites or during bad environmental conditions (Cerenius *et al.*, 2003). The resistance has probably evolved independently and in a parallel fashion in both North American crayfish lineages, i.e., the genus *Pacifastacus* and the species-rich family Cambaridae (Unestam, 1972). Therefore, the crayfish plague pathogen most probably originates in North America and all North American crayfish species are supposed to share the resistance to *A. astaci* (Unestam, 1969b, Unestam, 1972).

Table 1 includes only the crayfish species, which were experimentally exposed to *A. astaci* spores. It does not include the non-indigenous crayfish species, which have been found in European waters or bought as aquarium pets and tested positive for the crayfish plague pathogen using *A. astaci*-specific molecular methods or isolation of *A. astaci*. The reason is that one cannot be sure about their resistance; it is not clear if they all were infected (*A. astaci* might have been present only in the form of spores), or how long they had been infected (if they are really able to survive with the infection for long).

Eleven non-indigenous crayfish species have been found in European waters so far (Tab. 2) (Holdich *et al.*, 2009, Kouba, Petrusek and Kozák, 2014). The pathogen has been detected in natural populations of six of them, and in captive individuals of two more species (Tab. 2). It is possible that many North American crayfish species originally carry their own *A. astaci* strains, and the apparent absence of the pathogen in tested individuals and populations of some species might result from a founder effect. Such pathogen-free individuals can probably get infected with *A. astaci* in the facilities of breeders and sellers upon contact with other crayfish species such as the red swamp crayfish *Procambarus clarkii*, well-known and widely spread carrier of *A. astaci* (Mrugała *et al.*, 2015). Similarly, the detection of *A. astaci* in the Australian red claw crayfish *Cherax quadricarinatus* from ornamental trade almost certainly resulted from such horizontal transmission within shop facilities, or during handling and packing (Mrugała *et al.*, 2015).

Table 1: Crayfish species tested for the resistance to *A. astaci*.
Susceptible – individuals frequently die after exposure to *A. astaci* spores; the class includes the species classified as of low and moderate resistance by Unestam (1969b). Resistant – individuals usually do not die after exposure to *A. astaci* spores; the class includes species classified as of high resistance by Unestam (1969b). The regions of origin are characterised according to Holdich *et al.* (2006).
References: 1 - Alderman, Polglase and Frayling (1987), 2 - Diéguez-Uribeondo and Söderhäll (1993), 3 - Persson and Söderhäll (1983), 4 - Roy (1993) after Stephens (2005), 5 - Unestam (1969b), 6 - Unestam (1969a), 7 - Unestam (1972), 8 - Unestam (1975), 9 - Vey, Söderhäll and Ajaxon (1983), 10 - Vorburger and Ribi (1999).

Species	Region of origin	Resistant or susceptible to *A. astaci*	References
Orconectes limosus	North America	resistant	9
Pacifastacus leniusculus	North America	resistant	3, 7, 8
Procambarus clarkii	North America	resistant	5
Procambarus hayi	North America	resistant	5
Cambarus bartoni	North America	resistant	5
Cambarus sp. (close to *C. extranius*)	North America	resistant	5
Cambarus latimanus	North America	resistant	5
Cambarus longulus	North America	resistant	5
Cambarus acuminatus	North America	resistant	5
Orconectes propinquus	North America	resistant	5
Orconectes erichsonianus	North America	resistant	5
Orconectes virilis	North America	resistant	5
Faxonella clypeta	North America	resistant	5
Astacus astacus	Europe	susceptible	2, 5, 6, 7, 8, 9
Austropotamobius torrentium	Europe	susceptible	10
Astacus leptodactylus	Europe, Asia	susceptible [M]	1, 5
Austropotamobius pallipes	Europe	susceptible	1, 5
Cambaroides japonicus	Japan	susceptible	5
Cherax papuanus	Papua New Guinea	susceptible	8
Cherax destructor	Australia	susceptible [M]	8
Cherax quinquicarinatus	Australia	susceptible [M]	8
Cherax quadricarinatus	Australia	susceptible	4
Geocherax gracilis	Australia	susceptible [M]	8
Astacopsis gouldi	Tasmania	susceptible [M]	8
Astacopsis fluviatilis	Tasmania	susceptible	8
Euastacus kershawi	Australia	susceptible	7
Euastacus clydensis	Australia	susceptible	8
Euastacus crassus	Australia	susceptible	8

[M] The species was classified as of moderate resistance by Unestam (1969b).

Table 2: Non-indigenous crayfish species in European waters and the results of *A. astaci* detection. References: 1 - Huang, Cerenius and Söderhäll (1994), 2 - Diéguez-Uribeondo *et al.* (1995), 3 - Kozubíková *et al.* (2011a), 4 - Mrugała *et al.* (2015), 5 – chapter 6, 6 - Schrimpf *et al.* (2013a), 7 - Keller *et al.* (2014), 8 - Rezinciuc *et al.* (2014), 9 - Marino *et al.* (2014). There have been published many reports presenting the evidence of *A. astaci* infections in some of the crayfish species, particularly in the first three American crayfish species introduced to Europe, *O. limosus, P. leniusculus, P. clarkii*. From these I have included only some of them, preferentially those presenting the genotype group of the *A. astaci* strain living in their tissues.

Species	Region of origin	*A. astaci* detected/not detected in nature; genotype group (reference)	*A. astaci* detected/not detected in pet trade or aquaculture; genotype group (reference)
Cherax destructor	Australia		
Cherax quadricarinatus	Australia		yes, ? (4, 9)
Orconectes immunis	North America	yes; ? (5, 6)	
Orconectes juvenilis	North America		
Orconectes limosus	North America	yes; E (3)	yes, ? (4)
Orconectes virilis	North America	yes; ? (5)	
Pacifastacus leniusculus	North America	yes; B, C (1)	
Procambarus cf. *acutus*	North America		
Procambarus alleni	North America		yes, D (4)
Procambarus clarkii	North America	yes, D (2, 8)	yes, D (4)
Procambarus fallax f. *virginalis*	North America	yes, ? (7)	yes, D (4, 7)

Genotype groups of *A. astaci*

Using RAPD, five genotype groups of *A. astaci* have been recognised so far: A, B, C, D and E (Huang *et al.*, 1994, Diéguez-Uribeondo *et al.*, 1995, Kozubíková *et al.*, 2011a). Strains from each genotype group probably share the same original host species: the signal crayfish *P. leniusculus* (B and C), the red swamp crayfish *P. clarkii* (D) and the spiny-cheek crayfish *O. limosus* (E) (Huang *et al.*, 1994, Diéguez-Uribeondo *et al.*, 1995, Kozubíková *et al.*, 2011a), which are the most widely spread North American crayfish species in Europe (Holdich *et al.*, 2009, Kouba *et al.*, 2014). Strains of different genotype groups may differ in their virulence (Viljamaa-Dirks *et al.*, 2013, Makkonen *et al.*, 2014) and climate requirements (Diéguez-Uribeondo *et al.*, 1995, Rezinciuc *et al.*, 2014).

The first genotype group to invade Europe (A) was isolated from infected noble crayfish *A. astacus* and its original host is not known (Huang *et al.*, 1994). So far, strains from genotype groups A, B, D, and E have been detected in natural populations of European crayfish species (e.g., Kozubíková-Balcarová *et al.*, 2014, Grandjean *et al.*, 2014, Rezinciuc *et al.*, 2014, Viljamaa-Dirks *et al.*, 2013, Vennerström, Söderhäll and Cerenius, 1998). New data on the presence of *A. astaci* in the aquarium trade (Mrugała *et al.*, 2015) support also the hypothesis that *A. astaci* strains can be horizontally transmitted between various North American crayfish species, since for example the marbled crayfish *Procambarus fallax* f. *virginalis* hosted a strain from the genotype group D, i.e., the group originally isolated from *P. clarkii*.

A. astaci strains of different genotype groups can also be differentiated by AFLP (amplified fragment length polymorphism) analysis (Rezinciuc *et al.*, 2014), and by the recently developed microsatellite genotyping (Grandjean *et al.*, 2014). The latter method uses nine microsatellite markers that allow unambiguous separation of all known RAPD-defined genotype groups of *A. astaci* (originally characterized from axenic cultures). In contrast to RAPD, however, microsatellite genotyping can be used also to analyse mixed-genome samples isolated directly from infected host tissues (Grandjean *et al.*, 2014). This allows pinpointing the sources of *A. astaci* infection

(Kozubíková-Balcarová *et al.*, 2014, Vrålstad *et al.*, 2014), and to decide whether *A. astaci* is transmitted horizontally between coexisting hosts (**chapter 2**). In addition, the method can recognise new *A. astaci* genotypes, even those that would be characterized as belonging to the same genotype group (Grandjean *et al.*, 2014).

The genotype groups of *A. astaci* have also been referred to as strains belonging to Genotypes 1, 2, 3 and 4 (Andersson and Cerenius, 2002), or as *Astacus* strain, *Pacifastacus* strain I, *Pacifastacus* strain II and *Procambarus* strain (Oidtmann *et al.*, 2002a), or in the abbreviated forms as As, PsI, PsII, Pc, and Or (e.g., Viljamaa-Dirks *et al.*, 2013). In contrast to the letters A, B, C, D and E, the abbreviations As, PsI, PsII, Pc, and Or include the information about the species from which a strain belonging to the group was isolated (e.g., As stands for *Astacus*). However, the crayfish plague pathogen can be transmitted horizontally among different crayfish species, so describing a group of strains using this system may eventually become confounding. Moreover, Huang *et al.* (1994) and Diéguez-Uribeondo *et al.* (1995) described the genotype groups using the letters A, B, C and D (though Huang *et al.* (1994) used the letters only referring to clusters in a dendrogram). In comparison, the abbreviations "PsI, PsII" do not appear in the study of Huang *et al.* (1994) at all, and "Pc" was originally used as a name for a strain, not a genotype group (Diéguez-Uribeondo *et al.*, 1995). As a result, there is a strain Pc representing the genotype group Pc, while there is no strain called PsI representing group PsI. In my opinion, the first system of names (A, B, ...) for *A. astaci* genotype groups should be preferred to keep the system consistent and simple. Whatever the nomenclature is used, however, it is important to differentiate between specific strains (i.e., genotypes) and genotype groups (that may comprise multiple genetically distinct strains, which might also differ in their biology).

European crayfish species and latent infections

In contrast to North American crayfish species, the immune response to *A. astaci* in European and Australasian crayfish species is so weak that the crayfish usually die soon after infection (Cerenius *et al.*, 2003, Tab. 1). However, some variation in susceptibility has been observed under laboratory conditions: not all individuals of the narrow-clawed crayfish *A. leptodactylus* died due to *A. astaci* during some experiments (Unestam, 1969b), while all individuals exposed to *A. astaci* spores died in those by Alderman *et al.* (1987). Similarly, latent infections, i.e., individual crayfish being positive for *A. astaci* for long periods of time without the crayfish population suffering mass mortalities nor showing gross symptoms (Jussila *et al.*, 2014a), have recently been reported in some populations of *A. leptodactylus* in Turkey (**chapter 1**, Kokko *et al.*, 2012) and Romania (Pârvulescu *et al.*, 2012, Schrimpf *et al.*, 2012). Since the taxon *A. leptodactylus* is assumed to be a species-complex (Holdich *et al.*, 2006), and indeed phylogenetic analyses revealed presence of at least two evolutionary lineages (Maguire *et al.*, 2014), the results of the infection with *A. astaci* might vary because individuals belonging to different lineages show different level of resistance. However, latent infections were found also in some populations of *A. astacus* in Finland (Viljamaa-Dirks *et al.*, 2013, Jussila *et al.*, 2011b, Viljamaa-Dirks *et al.*, 2011), and of the stone crayfish *A. torrentium* in Slovenia (Kušar *et al.*, 2013). The ability of *A. astacus* to survive for months with the infection by some *A. astaci* strains for several weeks has been confirmed also in laboratory conditions (Makkonen *et al.*, 2014, Makkonen *et al.*, 2012b).

Some populations of the crayfish species which were originally classified as of low and moderate resistance (see Unestam, 1969b) can even be as productive as to be under commercial exploitation despite latent infections with the pathogen (Jussila *et al.*, 2011b, **chapter 1**). I therefore believe that the sorting of hosts to three categories, of low, moderate and high resistance, suggested by Unestam (1969b), should be simplified: crayfish species can be considered either as resistant or susceptible to the crayfish plague pathogen (Tab 1). The word "resistant" describes those species which usually do not die after the exposure to *A. astaci* spores (i.e., the North American crayfish species), whereas the crayfish species that frequently die (i.e., crayfish from Europe, Asia, Australia, Tasmania and New Guinea) are classified as susceptible. There are no crayfish species from South

America and Madagascar included in Table 1 since I have not found any study reporting on their resistance to *A. astaci.*

Theoretically, the mechanism enabling latent infections can lie on both sides of the host-parasite interaction between crayfish and *A. astaci.* It has been reported that the result of infection depends on the virulence of the particular *A. astaci* strain (Jussila *et al.*, 2013, Makkonen *et al.*, 2012b). In the literature reporting on latent infections, these are usually assumed to result from infection with the *A. astaci* strain(s) from the genotype group A (Caprioli *et al.*, 2013, Kušar *et al.*, 2013) though only in some cases the genotype group of the particular strain was recognised (e.g., Viljamaa-Dirks *et al.*, 2011, Jussila *et al.*, 2011b, Viljamaa-Dirks *et al.*, 2013). However, latent infections with strain(s) of the genotype group B have also been reported (**chapter 1**, Viljamaa-Dirks *et al.*, 2013). Similarly, some noble crayfish individuals apparently survived with the infection of an *A. astaci* strain from the genotype group B for weeks in laboratory experiments (Jussila *et al.*, 2011a, 2014a). In addition, even within the same *A. astaci* genotype group, some variation in virulence may occur (Makkonen *et al.*, 2014). Likewise, different genotype groups may have similar impacts. Kozubíková-Balcarová *et al.* (2014) did not observe any apparent differences among crayfish plague outbreaks caused by different genotype groups of the pathogen (A, B and E), nor any differences in subsequent recovery of the affected crayfish populations.

The result of infection depends also on the pathogen load (Makkonen *et al.*, 2014), water temperature (Alderman *et al.*, 1987), and may vary according to the current state of the crayfish immune system, i.e., according to stress and physiological condition of the host (Jussila *et al.*, 2011b) and the presence of other pathogens (Jussila *et al.*, 2013). Crayfish immune system depends on the innate immune system, which includes coagulation, melanization by activation of the prophenoloxidase activating system, phagocytosis, encapsulation of foreign material, and nodule formation (Vazquez *et al.*, 2009). The key factor responsible for the resistance of North American crayfish against *A. astaci* seems to be high level of expression of prophenoloxidase (Cerenius *et al.*, 2003) – North American crayfish continuously produced high levels of prophenoloxidase transcripts, which could not be further increased, while in susceptible crayfish the transcription of prophenoloxidase and resistance to *A. astaci* were augmented by immunostimulants. However, the experiments by Gruber *et al.* (2014) indicated that survival time after experimental crayfish plague infection was not associated with phenoloxidase (the active form of prophenoloxidase). I assume that experiments with individuals from populations of European crayfish species with latent *A. astaci* infection might probably help finding the key factor(s) enabling latent infections.

Crabs, shrimps and non-decapod crustaceans

Apart from crayfish, a few other taxa have been tested for the resistance to *A. astaci* (Tab. 3). Chinese mitten crabs *Eriocheir sinensis* were reported to be infected and killed by the pathogen in 1940 (Benisch, 1940). However, the then determination of the oomycete could not be considered convincing. In 2014, the infection with *A. astaci* was confirmed in two crab species from multiple localities (**chapters 2 and 6**, Schrimpf *et al.*, 2014). The crayfish plague pathogen was detected by microscopic and molecular methods in *E. sinensis* coexisting with crayfish plague-infected signal crayfish *P. leniusculus* in lake Vänern (Sweden), and in *Potamon potamios* coexisting with infected narrow-clawed crayfish *A. leptodactylus* in the Turkish lake Eğirdir (**chapter 2**). The infection of *E. sinensis* was detected by molecular methods in specimens from three localities in the river Rhine in Germany where they coexist with spiny-cheek crayfish *O. limosus* and calico crayfish *O. immunis* (Schrimpf *et al.*, 2014), and in the Netherlands where these crabs coexist with *A. astaci*-infected *O. limosus* (**chapter 6**). It is likely that young crabs get infected from local crayfish population, as suggested also by results of microsatellite genotyping of the pathogen strain following Grandjean *et al.* (2014) in samples from lakes Vänern and Eğirdir (**chapter 2**).

In contrast, results of the first laboratory exposure of freshwater shrimps *Neocaridina davidi* and *Macrobrachium dayanum* to the pathogen spores indicated that freshwater shrimps are resistant to *A. astaci* (Tab. 3); however, the results also suggested some growth of the pathogen in

some individuals and exuviae of *M. dayanum* (**chapter 3**). Further experiments are needed to confirm the assumed growth and to test if *A. astaci* can sporulate from shrimp hosts, and thus spread the infection further. It might also be interesting to test for the pathogen colonization and growth in dead bodies or their parts such as exuviae, i.e., test the ability of *A. astaci* to live partly as a saprophyte.

The abovementioned studies can be regarded as a test of Unestam's hypothesis that *A. astaci* host range may include not only crayfish but freshwater decapods in general (Unestam, 1972). Apart from the closest relatives of crayfish, i.e., crabs and shrimps, several species from other taxa have been exposed to *A. astaci* (Tab. 3). The result was always the same – no *A. astaci* mycelium growth was proven and the mortality after the exposition to *A. astaci* was similar as in control tanks without the exposure to the pathogen. Although molecular methods for screening of the crayfish plague pathogen presence in non-symptomatic hosts have already been available for several years (Vrålstad *et al.*, 2009, Oidtmann *et al.*, 2006), no study has focused in detail on potential non-decapod crustacean hosts. Nevertheless, some pilot results have been included in our study focusing on crabs (**chapter 2**): several individuals of the benthopelagic mysid *Mysis relicta*, the amphipod *Pallasea quadrispinosa* and the benthic isopod *Asellus aquaticus* were not found to be infected with *A. astaci* despite the presence in the coexisting crayfish populations. This corresponds with the fact that other aquatic animals coexisting with infected crayfish in natural localities are not affected by the pathogen (Oidtmann, 2012). In addition, the crayfish plague pathogen usually does not survive for long in the absence of a suitable host; any exceptions can be explained through other mechanisms such as latent infections or re-introduction of the pathogen (Oidtmann, 2012). However, the possibility that some other crustaceans may become accidental hosts of the crayfish plague pathogen, e.g., when stressed, has still not been rejected.

Table 3: Other animals tested for the resistance to *A. astaci*.
References:1 - Benisch (1940); 2 – Unestam (1969b); 3 – Unestam (1972), 4 – chapter 3. Resistant – individuals usually do not die after exposure to *A. astaci* spores. Therefore, this class may include both species which can and which cannot be infected with the crayfish plague pathogen.

Species	Taxon	Resistant or susceptible to *A. astaci*	Reference
Eriocheir sinensis	Decapoda: Brachyura	resistant?*	1
Macrobrachium dayanum	Decapoda: Caridea	resistant	4
Neocaridina davidi	Decapoda: Caridea	resistant	4
Mysis relicta	Mysida	resistant	3
Daphnia longispina	Branchiopoda: Cladocera	resistant	2
Leptodora kindtii	Branchiopoda: Cladocera	resistant	2
Chydorus sphaericus	Branchiopoda: Cladocera	resistant	2
Bytotrephes longimanus	Branchiopoda: Cladocera	resistant	2
Bosmina sp.	Branchiopoda: Cladocera	resistant	2
Cyclops strenuus	Maxillopoda: Cyclopoida	resistant	2
Mesocyclops leuckarti	Maxillopoda: Cyclopoida	resistant	2
Eudiaptomus graciloides	Maxillopoda: Calanoida	resistant	2
Asplanchna priodonta	Rotifera: Monogononta	resistant	2

* The species can be infected and can transmit the pathogen (Schrimpf *et al.*, 2014); according to Benisch (1940), the infection may even be accompanied by crab mortality.

Transmission of *Aphanomyces astaci*

The only known infectious forms of *A. astaci* are spores, i.e. zoospores and cysts (Oidtmann *et al.*, 2002b), which can survive only in freshwater (Unestam, 1969a). Spores of *A. astaci* transmit the disease horizontally among distinct host individuals. Vertical transmission, in which disease is spread from one generation to the next by infected eggs, was supposed not to be a mode of transmission for *A. astaci* (Stephens, 2005). However, Makkonen *et al.* (2010) detected *A. astaci* DNA in the eggs of infected females and in one of the tested groups of artificially incubated newly-hatched juveniles using a molecular detection targeting *A. astaci* chitinase. In contrast, the crayfish plague infection in the samples was not detected by the quantitative PCR according to Vrålstad *et al.* (2009) (Makkonen *et al.*, 2010). This suggests that amount of *A. astaci* DNA was extremely low, the chitinase-based PCR might not have been species specific enough, or that the qPCR was not sensitive enough. Since the qPCR according to Vrålstad *et al.* (2009) can detect even one zoospore (Tuffs and Oidtmann, 2011), the former two explanations seem to be more likely. In addition, crayfish plague infection was not detected in samples of artificially incubated juveniles in a previous study at Evira (Viljamaa-Dirks, 2008, personal communication in Makkonen *et al.*, 2010). Furthermore, *A. astaci* spores and their DNA can persist for several weeks (**chapter 3**) so the detection of *A. astaci* DNA in eggs taken from infected females does not necessarily mean an infection, especially if the amount of *A. astaci* DNA might have been very low. Thus, the vertical transfer through eggs cannot be considered proven, though crayfish juveniles might still be infected with *A. astaci* from their mother in natural conditions because they hatch and remain attached to her abdomen until at least the first moult (Reynolds, 2002). Nonetheless, even when we assume that transmission of *A. astaci* is limited only to spores in freshwater environments, there are still many possible pathways of the pathogen dispersal (see Oidtmann *et al.*, 2002b). Generally, the crayfish plague pathogen might disperse from a locality to another either in the form of spores independently on the host, or in the tissues of infected hosts (from which the spores are released at the new locality).

Introductions and human-mediated transfer of live hosts

Human activities have had the most important role in the crayfish plague pathogen dispersal. The pathogen itself was most probably introduced to Europe due to transoceanic shipping (Alderman, 1996). During the first decades of the pathogen spread, wholesale trade of European crayfish and transport of contaminated crayfishing equipment substantially facilitated the dispersal of the disease (Alderman, 1996). Moreover, people have introduced several North American crayfish species to Europe. The first three American crayfish species introduced to Europe, *O. limosus*, *P. leniusculus*, and *P. clarkii*, were released intentionally to boost stocks of crayfish decimated by crayfish plague (Holdich *et al.*, 2006). Although it has been later shown that all the three species frequently carry and transmit the crayfish plague pathogen (e.g., Diéguez-Uribeondo, 2006, Kozubíková *et al.*, 2011b), they are still sometimes spread by people both legally and illegally (Holdich *et al.*, 2006). In addition, *A. astaci* hosts might be transported unintentionally, e.g., during transport of fish or shipping.

While the first crayfish species have been introduced to Europe for aquaculture purposes, recent discoveries of new non-indigenous crayfish species in Europe are the result of illegal stocking activities, one possible live fishing bait introduction and, more recently, garden pond escapes and aquarium releases (Chucholl, 2013). Two Central European countries, Germany and the Czech Republic, seem to be the leaders in crayfish imports nowadays (Chucholl, 2013, Patoka, Kalous and Kopecký, 2014). In total, 120 non-indigenous crayfish species have been available on German ornamental crayfish trade, 87 % of which are of North or Central American origin, and are, therefore, suspected to be crayfish plague vectors (Chucholl, 2013). For some of these, this has been confirmed by a pilot screening of aquarium trade (Mrugała *et al.*, 2015).

Close attention must be paid to the disease status of crayfish during stocking even if apparently healthy European species are used (Makkonen *et al.*, 2012b); the infection might not be noticed due to incubation period and latent infections. For example, an *A. astaci* strain (genotype

group A) has been isolated from narrow clawed crayfish *A. leptodactylus* imported alive without any permits to the Czech Republic from Eastern Europe for consumption (JS, unpublished data). Moreover, freshwater-inhabiting crabs have been confirmed as potential long-term hosts and vectors of *A. astaci* (Schrimpf *et al.*, 2014, **chapter 2**). The world aquaculture production, i.e., the production in China and the Republic of Korea, of Chinese mitten crabs has risen to ca 700,000 tonnes in 2012 (FAO, 2012). The infectious status of the crabs in the aquacultures is not known – a potential source of *A. astaci* in this region is the red swamp crayfish *P. clarkii*, which is intensively farmed and invades some open waters there (Hobbs, Jass and Huner, 1989, Yue *et al.*, 2010). Although the aquaculture, and therefore even intentional transport and stocking, of *E. sinensis* is not common in Europe, the crabs may be occasionally released to open waters in spite of legislation forbidding such introductions, as happened for example in the Czech river Litavka (Kozubíková-Balcarová *et al.*, 2014).

Locomotion of infected hosts and transmission through tissues of dead individuals

Until recently, the active long-distance dispersal of infected hosts seemed relevant only for the North American crayfish species, as they were the only known long-term reservoirs of *A. astaci*. However, infected individuals in populations of European crayfish species with latent *A. astaci* infection can probably serve as a long-term source of *A. astaci* spores as well. Furthermore, the catadromous crab *E. sinensis* has already invaded many European waters (Herborg *et al.*, 2003, Herborg *et al.*, 2007, Dittel and Epifanio, 2009). The crayfish plague pathogen apparently cannot be transmitted among *E. sinensis* vertically, since they have marine larvae (Kobayashi and Matsuura, 1995) and *A. astaci* cannot survive in sea water (Unestam, 1969a). However, the crabs can get infected when they migrate to freshwater, which might take even hundreds of kilometres upstream and then back (Herborg *et al.*, 2003, Dittel and Epifanio, 2009). During such migration, they could spread the pathogen even further and much faster than dispersing crayfish hosts.

Crayfish plague may be spread also by dead hosts or their body parts; it has been shown that a dead crayfish body might serve as a source of infection for at least 5 days at 21 °C, and probably longer in lower temperatures (Oidtmann *et al.*, 2002b). Nearly 600,000 tonnes of the confirmed *A. astaci* carrier American *P. clarkii* is produced and sold every year (FAO, 2012) for culinary purposes. Fortunately, the pathogen can be eliminated by low and high temperatures, e.g., one-week freezing at -5°C or one minute at 100 °C is lethal for *A. astaci* (Alderman, 2000, Oidtmann *et al.*, 2002b). In contrast, the amount of crayfish used as fishing bait is much lower, but the crayfish are usually not exposed to extreme temperatures, so they may serve as vectors of the crayfish plague pathogen as well.

As far as the transport of dead crayfish or their body parts by other animals is concerned, the transmission of *A. astaci* through the digestive tract of fish has already been proven (Oidtmann *et al.*, 2002b). The transmission through the digestive tract of warm-blooded predators, in contrast, seems to be very unlikely (**chapter 5**). In a pilot exposure experiment, the pathogen was not transmitted through the excrements of one European otter *Lutra lutra* and one American mink *Neovison vison* to susceptible stone crayfish *A. torrentium*. In addition, the experiments testing *A. astaci* survival in body temperatures of mammals and birds have shown that the sole effect of temperature should usually prevent the pathogen spread through their digestive tracts. Therefore, the pathogen transmission through the digestive tract of warm-blooded predators is very unlikely, probably even less likely than the potential transmission of *A. astaci* spores on their surface.

Dispersal of *A.astaci* spores

Since both *A. astaci* spores are sensitive to desiccation (Alderman and Polglase, 1986, Smith and Söderhäll, 1986), the dispersal of *A. astaci* spores among watersheds on the surface of animals is mostly limited. The transmission of *A. astaci* on the surface of fish seems to be unlikely because of continuous production and anti-infectious properties of fish mucus (Oidtmann *et al.*, 2002b). Therefore the dispersal of *A. astaci* spores in natural conditions, i.e., not including the transport of

water by man, seems to be limited mostly by water currents transporting the microscopic spores on long-distances within a watershed.

The success of *A. astaci* infection depends on the number of spores the host is exposed to (Unestam and Weiss, 1970, Alderman *et al.*, 1987, Diéguez-Uribeondo *et al.*, 1995, Makkonen *et al.*, 2014). However, the estimation of LD_{50} for *A. astaci* (Lethal Dose, 50%, i.e., the amount of spores required to kill 50% of the tested individuals), which was presented for example by Unestam and Weiss (1970), faces problems in experimental design (Alderman *et al.*, 1987). Furthermore, the LD_{50} probably varies with respect to the virulence of the particular *A. astaci* strain and resistance of the particular crayfish species population (see e.g., Jussila *et al.*, 2013, Makkonen *et al.*, 2012b).

Naturally, high *A. astaci* prevalence in a crayfish population and high pathogen load in the infected crayfish generally lead to a higher spore density in the water (Strand *et al.*, 2014), and very high concentrations may be found in tanks where large numbers of crayfish per water volume are kept (Strand *et al.*, 2011). The concentrations of *A. astaci* spores can be several hundred spores L^{-1} in a river with crayfish plague outbreak, while they did not usually exceed 1 spore L^{-1} in water bodies hosting infected populations of North American crayfish (Strand *et al.*, 2014). However, the results obtained in localities with North American crayfish varied from no detection of *A. astaci* to ca 100 spores L^{-1}. These results correspond to previous laboratory studies, which had revealed that massive sporulation from infected crayfish starts when the host is dying or moulting, but some sporulation still occurs even from apparently healthy and non-moulting American crayfish hosting *A. astaci* (Strand *et al.*, 2012, **chapter 4**, Makkonen *et al.*, 2013). The concentrations also vary among different microhabitats in a water body (Strand *et al.*, 2014, Strand *et al.*, 2012).

Unestam (1969a) found that his spore suspension kept at 14 °C infected all crayfish placed in the spore water 6 days after spore addition, but not after 15 days. In sterile laboratory conditions, *A. astaci* zoospores remain motile for up to 5 days at 2 °C (Unestam, 1966b), and the spores usually remain encysted only for several hours before they germinate or release new zoospores (Alderman and Polglase, 1986, Svensson and Unestam, 1975). However, the periods may probably be substantially longer, since a spore suspension of *A. astaci* stored for two months at 2 °C still contained viable spores (Unestam, 1966b), while the number of consecutive zoospore generations rarely exceeds three, apparently being limited by the initial stock of proteins present in a released spore (Cerenius and Söderhäll, 1984b). To my knowledge, the decrease in viable spore number in time has never been properly quantified. The problem is that the quantitative PCR cannot distinguish the *A. astaci* DNA isolated from viable spores from the DNA isolated from other sources such as the extracellular DNA or dead spores. Nevertheless, an exponential curve would fit the data on the amount of *A. astaci* DNA isolated from inert substrates immersed in a spore suspension (**chapter 3**). The half-life of the DNA calculated from the exponential regression was 3.1 days, suggesting that the half-life of the spores at 20 °C might be no more than three days (likely less, as short fragments of DNA used for qPCR-based detection in that study should be detectable even some time after spore death). However, the experiment was run in aged tap water; the survival of spores in more natural conditions, e.g., including other microorganisms, remains to be investigated.

Prevention of *A. astaci* dispersal

I would like to conclude the part about the transmission of *A. astaci* with a brief list of measures to prevent the pathogen dispersal, especially those that have been discussed recently. Obviously, any stocking of hosts infected with *A. astaci*, especially North-American crayfish species, into the wild should be avoided if possible. Similarly, the activities that might lead to escape or release of a carrier of *A. astaci* from captivity, such as using North American crayfish as fishing bait and ornamental crayfish trade in general, should be minimized. The implementation of mitigation and remediation measures might be applied if a crayfish plague carrier appears in a locality (Gherardi *et al.*, 2011). However, preventing the introduction of non-indigenous crayfish species is far more cost-effective and environmentally desirable than measures taken after their introduction and establishment (Gherardi *et al.*, 2011).

One of the factors that may prevent the spread of non-indigenous crayfish species are barriers such as waterfalls (Gherardi *et al.*, 2011). Similarly, the spread of the crayfish plague outbreak in a population of a susceptible species might sometimes be eliminated by physical and electric barriers (e.g., Frings *et al.*, 2013, Benejam *et al.*, 2015, Kozubíková-Balcarová *et al.*, 2014). These facts should be considered also generally in the comparison of the benefits and costs of barriers in aquatic systems inhabited by crayfish (Rahel, 2013). To prevent the transfer of *A. astaci* spores on the surface of fishing, crayfishing gear and any other things that have been in contact with water from a locality with plague-infected hosts, the items should be cleaned of organic matter first (Jussila *et al.*, 2014b), preferentially with hot water. Subsequently, the disinfectants Proxitane®5:14, Virkon®S (Jussila *et al.*, 2014b), sodium hypochlorite (Alderman and Polglase, 1985), or iodophors (Alderman and Polglase, 1985, Lilley and Inglis, 1997) may be applied, or the items should be thoroughly dried at least (see Smith and Söderhäll, 1986, Alderman *et al.*, 1987). Water can be decontaminated using peracetic acid in the concentration of 10 mgL^{-1} (Jussila, Makkonen and Kokko, 2011a).

Crayfish plague pathogen can be dispersed through the transport of fish (Alderman *et al.*, 1987, Oidtmann *et al.*, 2002b). Any fish movements from the site of a current epidemic of crayfish plague carries a high risk of spread and should generally be avoided (Oidtmann, 2012). However, that could hardly be applied to fish transport from all sources containing plague-infected North American crayfish. The ways crayfish plague could be transmitted during fish transport are: (1) spores in the transport water; (2) spores and mycelium on or in the skin of fish; (3) mycelium and spores in the gastrointestinal tract of fish; and (4) crayfish accidentally transported with the fish (Oidtmann *et al.*, 2002b).

The transmission through the fish gastrointestinal tract is possible (Oidtmann *et al.*, 2002b). Nevertheless, if transported fish are kept a few days without access to crayfish, so they can empty their gastrointestinal tract before stocking into new water courses, they should not be a source of the infection with *A. astaci* (Oidtmann *et al.*, 2002b). In addition, chemical disinfection of water where the fish were kept was sufficient to prevent the transmission of *A. astaci* in the experiments by Alderman *et al.* (1987), suggesting that the likelihood of successful transmission inside fish is low (though probably higher for predators of crayfish). Despite some indications from *in vitro* experiments (Häll and Unestam, 1980), transmission via fish skin was not observed during *in vivo* experiments (Oidtmann *et al.*, 2002b). Furthermore, any forms of *A. astaci* present on fish surface will be partially exposed to chemical disinfectants used for water decontamination (Häll and Unestam, 1980). Therefore, the prevention of *A. astaci* dispersal during transport of fish should mainly focus on precautions against accidental co-transport of crayfish and on the elimination of *A. astaci* spores in transport water.

It has been shown that malachite green could prevent the transmission of *A. astaci* through transport water (Alderman *et al.*, 1987, Lilley and Inglis, 1997). However, the use of this dye has been banned in several countries because of its potential carcinogenicity, mutagenicity and teratogenicity, e.g., the European Council imposed a strict ban on the use of malachite green in all age categories of fish intended for human consumption (Sudová *et al.*, 2007, Srivastava, Sinha and Roy, 2004). Unfortunately, the concentration of peracetic acid tested and found effective against the *A. astaci* spore germination and practical disinfection of water containing *A. astaci* spores would not be suitable in the presence of fish (Jussila *et al.*, 2011a). The potential of some other disinfectants to eliminate *A. astaci* has already been tested: formaldehyde and potassium permanganate (Häll and Unestam, 1980), sodium chloride, hydrogen peroxide, sodium hypochlorite and FAM30®, acetic acid and povidone iodine (Lilley and Inglis, 1997, Fuangsawat, Abking and Lawhavinit, 2011). However, further studies should determine the most appropriate concentrations and immersion time, focus on the toxicity of these chemicals to the transported fish, and eventually deliver a protocol for routine decontamination of water during transport of fish intended for human consumption.

Future perspectives

I would like to finish this chapter with a short list of hypotheses that may be tested by the future research. I give these as testable statements, which may or may not turn out to be true:

The life cycle and parasitism of *A. astaci*

- The formation of the gemmae-like and thick walled structures is not relevant for the pathogen persistence and transmission.
- The genome of the crayfish plague pathogen in its original region (North America) does not indicate any sexual or parasexual processes in the life cycle of the species.
- *A. astaci* can complete the whole life cycle in dead bodies or exuviae of its host.
- The North American crayfish species can benefit from the infection with *A. astaci* in natural conditions of their original habitats in North America.

Hosts of *A. astaci*

- *A. astaci* is not present in Asian aquacultures of the Chinese mitten crab *E. sinensis.*
- The crab *E. sinensis* can succumb under some conditions to the infection of *A. astaci*, so the pathogen might cause considerable losses to the aquaculture production of this species if it appeared there.
- Adult (less-frequently moulting) freshwater shrimps can be infected by *A. astaci* and transmit it.
- *A. astaci* does not infect any crustaceans but for crayfish, crabs (and possibly shrimps), even when they are stressed.
- The crayfish species from South America and Madagascar are susceptible to *A. astaci*.
- The enhanced resistance of some European populations enabling the latent infections with *A. astaci* is caused by high levels of expression of prophenoloxidase.
- Latent infections of European crayfish species are also possible with *A. astaci* strains from the genotype group D and E.
- Latent infections may be responsible for *A. astaci* persistence and dispersal in regions where North American crayfish species are not present.
- Different "new" non-indigenous crayfish species recently introduced to Europe from North America originally carry their own genetically distinct *A. astaci* strains.
- Such distinct *A. astaci* strains vary in their virulence and pathogenicity to the European crayfish, and in their climate requirements.
- The likelihood of transmission of an *A. astaci* strain to a North American crayfish species depends on the original host species.
- The pathogenicity of *A. astaci* strains from different genotype groups to a specific North American crayfish species may vary.

Transmission of *A. astaci*

- *A. astaci* spores cannot survive in brackish water long enough to infect a new host there (in conditions relevant for, e.g., Black, Caspian and Baltic Sea).
- *A. astaci* spores survive in water from natural localities shorter than in sterile conditions.
- The transmission of *A. astaci* spores in water during fish transport can be prevented by a chemical disinfection that may be applied to fish intended for human consumption.

There is no doubt that research on crayfish plague will continue, hopefully exploring at least some of the hypotheses outlined above. Further research will probably gain from the recently developed molecular tools, e.g., sensitive detection and quantification of *A. astaci* DNA by species-specific quantitative PCR (Vrålstad *et al.* 2009), and direct genotyping of *A. astaci* from DNA samples of infected host tissues (Grandjean *et al.* 2014). Perhaps, further techniques, such as fluorescence in situ hybridization, will be developed for *A. astaci.* Any detection methods, however, must be tested also against the other oomycetes living in or on the crayfish cuticle (**chapter 7**). I hope my successors are successful, and their results useful both in research and crayfish conservation.

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ATTACHED PUBLICATIONS AND MANUSCRIPTS

The crayfish plague outbreak in the population of *Astacus astacus* in the Černý brook near Pec pod Čerchovem (Western Bohemia) on 15 January 2014.

Chapter 1

Svoboda, J., Kozubíková, E., Kozák, P., Kouba, A., Bahadir Koca, S., Diler, Ö., Diler, I., Policar, T., Petrusek, A., 2012. PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eğirdir in Turkey.
***Diseases of Aquatic Organisms* 98, 255-259.**

"Although the mechanisms allowing the long-term coexistence of *Aphanomyces astaci* with crayfish in Turkey remain to be identified, the data presented here support other recent evidence of crayfish in Europe coexisting with this parasite over extended periods of time in chronic rather than acute infection states..."

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NOTE

PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eğirdir in Turkey

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ABSTRACT: Many populations of the narrow-clawed crayfish *Astacus leptodactylus* in Turkey, including those inhabiting Lake Eğirdir, declined drastically in the mid-1980s due to introduction of crayfish plague *Aphanomyces astaci*. However, unlike many other localities, there has been some recovery in the *A. leptodactylus* population inhabiting this lake even though crayfish plague has been suspected to have persisted since then. In support of this, DNA from 5 of 34 healthy-looking crayfish sampled recently from the lake tested positive by both conventional and real-time PCR using species-specific primers targeting the rDNA internal transcribed spacer region, and product sequence analysis confirmed the identification of *A. astaci*. This complies with other recent reports of coexistence of native European crayfish with this pathogen, and further research is now needed to identify the key mechanisms allowing it.

KEY WORDS: *Aphanomyces astaci* · *Astacus leptodactylus* · Host–pathogen coexistence · Melanisation · rDNA-internal transcribed spacer sequence · Lake Eğirdir

INTRODUCTION

Beginning in the 1860s, the *Aphanomyces astaci* oomycete that causes crayfish plague has spread across Europe decimating native crayfish species (Alderman 1996). Although the disease has the potential to completely annihilate populations of native European crayfish (Reynolds 1988, Alderman 1993, Diéguez-Uribeondo 2006, Kozubíková et al. 2008), in some lakes in Finland (Jussila et al. 2011b, Viljamaa-Dirks et al. 2011) and elsewhere (Sweden: Fürst 1995; Turkey: Harlioğlu 2004, 2008) such crayfish seem to coexist with the pathogen. Specific local water chemistries, low host population densities, variations in strain virulence and variable crayfish resistance have been suggested as potential reasons for this.

Narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) are native to Eastern Europe and adjacent regions and have been harvested commercially in Turkey since the 1960s (Rahe & Soylu 1989). In 1985 and 1986, crayfish catch numbers collapsed catastrophically due to crayfish plague (Fürst & Söderhäll 1987, Baran & Soylu 1989, Rahe & Soylu 1989, Timur 1990). Almost all populations affected by this disease either disappeared completely or were

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dramatically reduced. However, there were exceptional cases where crayfish numbers either remained continuously high or recovered enough to sustain commercial harvesting despite the presumed presence of the crayfish plague pathogen (Rahe & Soylu 1989, Harlioğlu 2004, 2008).

Crayfish resistance and/or unique water chemistries have been assumed as reasons facilitating the long-term coexistence of *Aphanomyces astaci* in crayfish inhabiting such lakes (Rahe & Soylu 1989, Harlioğlu 2008). *A. astaci* was unambiguously proven as the cause of crayfish mortalities in the 1980s (Baran & Soylu 1989, Rahe & Soylu 1989, Huang et al. 1994). However, its later presence in Turkish lakes (e.g. Aydin & Dilek 2004, Harlioğlu 2004, 2008) has only been assumed from symptoms that are not specific for the crayfish plague pathogen (Cerenius et al. 1988, Oidtmann et al. 1999). As *A. astaci* should not survive at a locality when its crayfish hosts disappear (Söderhäll & Cerenius 1999) and its presence in Turkey after 1989 has not been proven definitively, its distribution in the country might now be more restricted than assumed.

The narrow-clawed crayfish population inhabiting Lake Eğirdir (Isparta Province; 38° N, 31° E, altitude 917 m above sea level [a.s.l.], area 482 km^2) collapsed in 1986, presumably due to crayfish plague (Fürst & Söderhäll 1987). By 1999, numbers recovered sufficiently to allow commercial harvesting, although catch sizes have been lower and prone to fluctuations (Fig. 1). The aim of this study was to examine Lake Eğirdir crayfish using PCR and rDNA sequence analysis to test for the contemporary presence of *A. astaci*.

MATERIALS AND METHODS

Over 2 occasions in 2009, a total of 32 crayfish (mean body length ± SD: 111 ± 22 mm) were caught in fyke-net traps in the southern part of Lake Eğirdir (37° 53′ N, 30° 53′ E). In March, 2 egg-bearing females were caught and kept in a tank until June, when samples of their tissues and 1 juvenile from each of their broods were collected for analysis. In November, 30 more crayfish were captured and their tissues sampled shortly after capture. Both crayfish captured in March as well as their offspring and 13 of the 30 crayfish captured in November displayed melanised spots of various sizes (1 to 10 mm diam.), which might develop in response to *Aphanomyces astaci* hyphae (Unestam 1969, Fürst & Söderhäll 1987, Baran & Soylu 1989) but also to other pathogens (Söderhäll & Cerenius 1998). One uropod, soft abdominal cuticle, eye stalk, walking leg joint, and prominent melanised cuticle regions of each crayfish were dissected aseptically and preserved in 96% ethanol. Up to 50 mg of this material crushed in

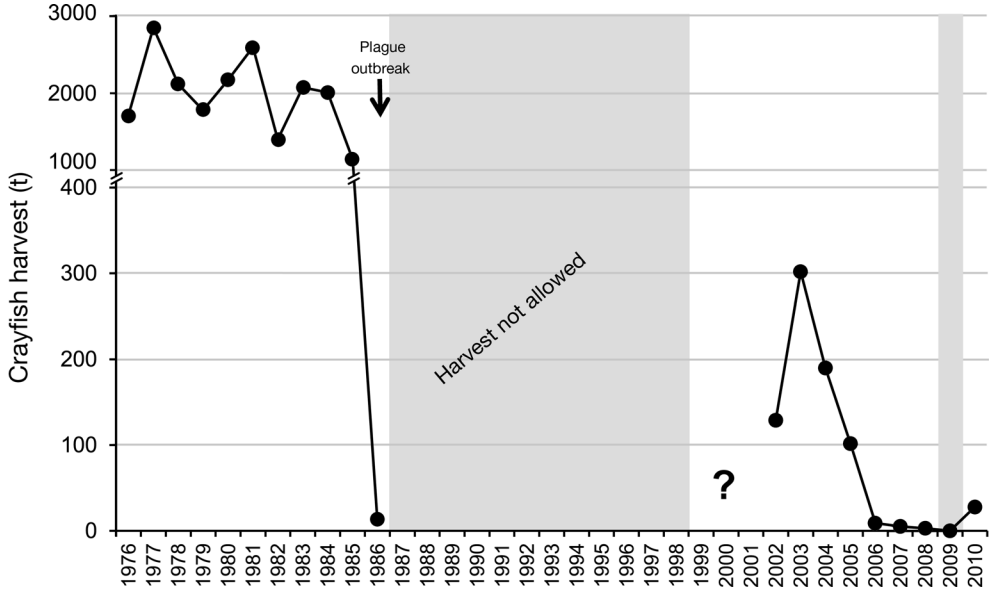

Fig. 1. *Astacus leptodactylus.* Annual crayfish harvest from Lake Eğirdir between 1976 and 2010. Periods when capture of crayfish was banned are shaded, and the 1986 crayfish plague outbreak is indicated. Data from 1976 to 1986 (Gülle et al. 2008) and from 2002 to 2010 (Eğirdir Town Food, Agriculture and Livestock Directorate) reflect official catches reported by local fishermen. No reliable harvest data were found between 1999 and 2001

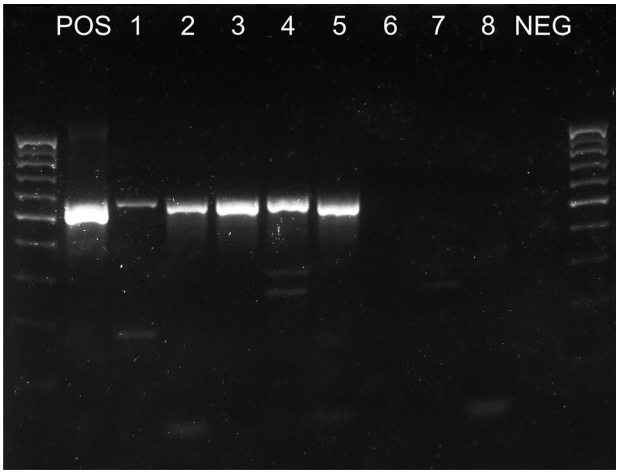

Fig. 2. *Astacus leptodactylus.* Agarose gel showing the ~550 bp rDNA internal transcribed spacer (ITS) region PCR product amplified using primers specific for *Aphanomyces astaci* and 10× diluted DNA from 8 selected narrow-clawed crayfish captured from Lake Eğirdir in 2009. Each lane corresponds to product from an individual crayfish. POS: DNA isolated from pure laboratory culture of *A. astaci*. NEG: no template control; 100 bp size markers were loaded to side lanes

liquid nitrogen was used to extract DNA using a DNeasy tissue kit (QIAGEN).

Undiluted DNA and DNA diluted 10× to overcome potential effects of PCR inhibitors (Vrålstad et al. 2009) were amplified using a PCR protocol targeting an internal transcribed spacer (ITS) region of the *Aphanomyces astaci* rDNA gene (Oidtmann et al. 2006). DNA isolated from a laboratory culture of this pathogen was used as a PCR positive control, and PCR products visualized by agarose gel electrophoresis were sequenced and compared to published *A. astaci* sequences. To validate putative detections, DNA isolates were also analysed using an *A. astaci*-specific TaqMan real-time PCR protocol (Vrålstad et al. 2009).

RESULTS

PCR products corresponding in size to that amplified from *Aphanomyces astaci* DNA were obtained from both females and 1 juvenile sampled in June and from 2 of the 30 crayfish captured in November (Fig. 2). DNA from these individuals also tested positive by real-time PCR. For 1 of the 2 crayfish captured in November, a PCR product was only amplified from 10× diluted DNA, suggesting the presence of inhibitors. All 5 PCR-positive crayfish came from the 17 exhibiting some degree of cuticle melanisation. Sequences determined for the 5 PCR products were identical (GenBank JN185321) and corresponded exactly to ITS-rDNA sequences published for all genetic groups of *A. astaci* known presently (Huang et al. 1994, Diéguez-Uribeondo et al. 1995, 2009, Kozubíková et al. 2011).

DISCUSSION

These data confirm unambiguously the existence of an *Aphanomyces* sp. with an ITS sequence corresponding exactly to the crayfish plague pathogen in some narrow-clawed crayfish inhabiting Lake Eğirdir in 2009, and we thus consider it a proof of contemporary presence of *A. astaci* in Turkey. However, as no PCR detections of *A. astaci* were made in 12 of 17 crayfish exhibiting cuticle melanisation, this does not seem to be a specific indicator of crayfish plague; such symptoms in Turkish crayfish should be interpreted with care (see Fürst & Söderhäll 1987, Rahe & Soylu 1989, Diler & Bolat 2001).

Aphanomyces astaci was detected in 2 *Astacus leptodactylus* crayfish that survived rearing in captivity for 3 mo. This finding supports recent reports of latent infections occurring in noble crayfish *A. astacus* (L. 1758) populations in Finland (Jussila et al. 2011b, Viljamaa-Dirks et al. 2011) and detections of *A. astaci* in apparently healthy *A. leptodactylus* inhabiting the Danube Delta in Romania (Pârvulescu et al. 2012). Considering the history of crayfish harvest in the lake and the time separating our study and previous unambiguous diagnoses of the pathogen in Turkey, we conclude that *A. astaci* may have indeed persisted in the lake for over 2 decades without eliminating the local crayfish population.

Several hypotheses may explain the continuing coexistence of crayfish plague in narrow-clawed crayfish inhabiting previously affected lakes in Turkey, although none are supported fully by available data. In lakes such as İznik and Akşehir where crayfish populations persisted, unique water chemistry has been suggested to have played some role (Fürst & Söderhäll 1987, Rahe & Soylu 1989, Harlioğlu 2004, 2008). However, the *Astacus leptodactylus* population disappeared completely from Lake Akşehir after 1990, apparently due to crayfish plague (Harlioğlu 2008), despite the presumed protective effect of elevated concentrations of magnesium ions (127 mg Mg^{2+} l^{-1}; Rahe & Soylu 1989). As magnesium ion concentrations are substantially lower in Lake Eğirdir (5 to 59 mg Mg^{2+} l^{-1}; Gülle et al.

2008), it does not seem to be the main factor responsible for sparing crayfish from acute disease and death.

More likely, the long-term coexistence with the crayfish plague pathogen may be due to increased resistance of local crayfish to the disease. However, whilst evidence of *Astacus leptodactylus* resistance has been found in some challenge experiments (Unestam 1969, Fürst & Söderhäll 1987, Fürst 1995), no evidence of resistance has been found in others (Alderman et al. 1987, Baran & Soylu 1989, Rahe & Soylu 1989). Whether these conflicting observations have been caused by variations in the susceptibility of crayfish stocks sourced from different locations, as found recently with noble crayfish (Jussila et al. 2011a), or variable exposure to other stress factors impairing crayfish defence responses has yet to be resolved. The long-term coexistence of noble crayfish with the crayfish plague pathogen across Fennoscandia has also been attributed to possibly reduced virulence of some *Aphanomyces astaci* strains as well as to inefficient pathogen dispersal in low-density populations of crayfish (Fürst 1995, Viljamaa-Dirks et al. 2011). Although differences in virulence among *A. astaci* strains are directly or indirectly supported by experimental data (Diéguez-Uribeondo et al. 1995, Viljamaa-Dirks & Torssonen 2008), the simultaneous impact of crayfish plague across Turkish lakes in the 1980s suggests that a highly virulent strain was introduced. Nevertheless, subsequent local adaptation of crayfish and/or the parasite cannot be excluded in the lakes in which crayfish populations avoided complete decimation.

While low population densities may limit pathogen spread and impact, available data on *Astacus leptodactylus* densities in Lake Eğirdir are scarce and limited to short time periods in different parts of the lake. However, crayfish densities estimated in 2 such local studies decreased from 1.9–5.1 to 0.5–1.2 ind. m^{-2} between 1999–2000 and 2005 (Bolat 2004, Bolat et al. 2011), which corresponds to decreases in harvests over the same period (Fig. 1). Harvests since 1999 have generally been 1 to 2 orders of magnitude lower than those in the early 1980s before crayfish plague appeared and dropped again steeply after 2004 prompting the introduction of a harvest ban in 2009. Crayfish plague seems to have persisted in lower-density crayfish populations in Turkish lakes decimated by disease in the mid-1980s (Fürst & Söderhäll 1987). The long-term data from Lake Eğirdir suggest that the presence of the pathogen may have prevented recovery of the populations to pre-exposure numbers and sporadically impacts remaining crayfish. However, while these observations suggest some *A. leptodactylus* crayfish might have adapted to better resist the pathogen and its impact might be limited by low host densities, future larger-scale studies will be needed to evaluate these hypotheses.

Although the mechanisms allowing the long-term coexistence of *Aphanomyces astaci* with crayfish in Turkey remain to be identified, the data presented here support other recent evidence of crayfish in Europe coexisting with this parasite over extended periods of time in chronic rather than acute infection states (Jussila et al. 2011b, Viljamaa-Dirks et al. 2011, Pârvulescu et al. 2012). As crayfish plague remains a major threat to indigenous Old World crayfish species both in the wild and in aquaculture (Souty-Grosset et al. 2006), further studies are needed to better understand its virulence, host interactions and epidemiology to devise strategies for recovery of native crayfish populations.

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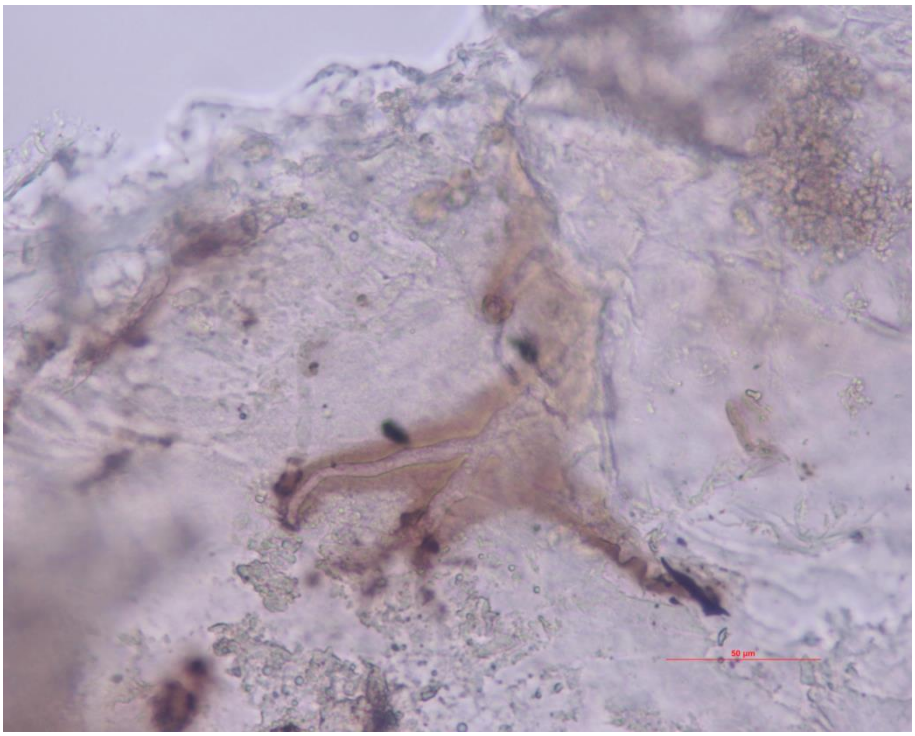
Chapter 2

Svoboda, J., Strand, D.A., Vrålstad, T., Grandjean, F., Edsman, L., Kozák, P., Kouba, A., Fristad, R.F., Bahadir Koca, S., Petrusek, A., 2014. The crayfish plague pathogen can infect freshwater-inhabiting crabs.
***Freshwater Biology* 59, 918-929.**



"Our study demonstrates that *Aphanomyces astaci*, the crayfish plague pathogen, was present in cuticles of the freshwater-inhabiting crabs *Potamon potamios* and *Eriocheir sinensis*..."

Freshwater Biology

Freshwater Biology (2014) **59,** 918–929 doi:10.1111/fwb.12315

The crayfish plague pathogen can infect freshwater-inhabiting crabs

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SUMMARY

1. The oomycete *Aphanomyces astaci* is generally considered a parasite specific to freshwater crayfish, and it has become known as the crayfish plague pathogen. Old experimental work that reported transmission of crayfish plague to the Chinese mitten crab *Eriocheir sinensis*, and the ability of *A. astaci* to grow in non-decapod crustaceans, has never been tested properly.

2. We re-evaluated the host range of *A. astaci* by screening for the presence of *A. astaci* in two crab species cohabiting with infected crayfish in fresh waters, as well as in other higher crustaceans from such localities. The animals were tested with species-specific quantitative PCR, and the pathogen determination was confirmed by sequencing of an amplified fragment of the nuclear internal transcribed spacer. Furthermore, we examined microscopically cuticle samples from presumably infected crab individuals for the presence of *A. astaci*-like hyphae and checked for the presence of pathogen DNA in such samples.

3. Screenings of benthopelagic mysids, amphipods and benthic isopods did not suggest infection by *A. astaci* in non-decapod crustaceans. In contrast, both studied lake populations of crabs (a native semiterrestrial species *Potamon potamios* in Turkey, and an invasive catadromous *E. sinensis* in Sweden) were infected with this parasite according to both molecular and microscopic evidence.

4. Analyses of polymorphic microsatellite loci demonstrated that *A. astaci* strains in the crabs and in cohabiting crayfish belonged to the same genotype group, suggesting crayfish as the source for crab infection.

5. The potential for *A. astaci* transmission in the opposite direction, from crabs to crayfish, and potential impact of this pathogen on populations of freshwater crabs require further investigations, because of possible consequences for crayfish and freshwater crab conservation and aquaculture.

Keywords: *Aphanomyces astaci*, *Eriocheir sinensis*, host range, invasive species, *Potamon potamios*

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Introduction

The oomycete *Aphanomyces astaci* (Oomycetes, Saprolegniales) has caused and still causes heavy losses of indigenous European freshwater crayfish populations (Alderman, 1996; Holdich *et al*., 2009). Due to its devastating impacts, it has been included among 100 of the worst invasive alien species in Europe and the whole world (Lowe *et al*., 2004; DAISIE, 2009). *A. astaci* has become one of the best-known pathogens of invertebrates (Alderman, 1996; Diéguez-Uribeondo *et al*., 2006), and it is usually considered as a parasite specific to freshwater crayfish (Decapoda, Astacidea) (e.g. Alderman, 1996; Söderhäll & Cerenius, 1999; Diéguez-Uribeondo *et al*., 2006).

A few studies have tried to evaluate the host range of this pathogen outside the group of freshwater crayfish. The growth of *A. astaci* on fish scales reported by Häll & Unestam (1980) was not confirmed by experiments *in vivo* (Oidtmann *et al*., 2002), and several planktonic crustaceans and one rotifer did not die after they had been exposed to *A. astaci* (Unestam, 1969b, 1972). One study, however, stands out among those evaluating the potential of crustaceans other than crayfish to be hosts of *A. astaci*. Benisch (1940) reported experimental transmission of the presumed crayfish plague pathogen from moribund individuals of the European noble crayfish *Astacus astacus* to the Chinese mitten crab *Eriocheir sinensis*. The experiments resulted in moderate death rates for the crabs. However, while some pathogen had indeed been transmitted to *E. sinensis*, it remains uncertain whether it was *A. astaci*, since the pathogen was not isolated in culture for direct tests of pathogenicity and species identification (see Cerenius *et al*., 1988; Oidtmann *et al*., 1999; Oidtmann, 2012). Considering Benisch's experiment with crabs, Unestam (1972) in his work on *A. astaci* specificity suggested that the parasite host range may include not only crayfish but freshwater decapods in general (i.e. higher crustaceans including crabs, crayfish and shrimps).

Surprisingly, no work evaluating the ability of *A. astaci* to parasitise decapods other than crayfish has been published since Benisch (1940), although the potential of *A. astaci* to infect other freshwater decapods would have important consequences for management of susceptible crayfish populations, especially in Europe and adjacent regions. Moreover, freshwater crabs and shrimps play important ecological roles in aquatic habitats (De Grave, Cai & Anker, 2008; Yeo *et al*., 2008), and they are important in the global aquaculture industry. The 2010 annual harvest of freshwater shrimps (Decapoda, Caridea) and Chinese mitten crabs was about 500 000 tons each, with a total value of over 6.4 billion USD (FAO, 2012). Reductions in yield or changes in population characteristics of freshwater decapods may thus impact ecosystem functioning as well as aquaculture and fisheries.

Apart from crayfish and possibly freshwater-inhabiting crabs, there has been no reliable evidence for other hosts of *A. astaci* (Unestam, 1969b, 1972; Oidtmann *et al*., 2002). Occasional reports of the occurrence of *A. astaci* in dead freshwater crustaceans (e.g. Czeczuga, Kozlowska & Godlewska, 2002; Czeczuga, Kiziewicz & Gruszka, 2004) were based on morphology only, and they seem unreliable since *A. astaci* morphological features are not specific enough (see Cerenius *et al*., 1988; Oidtmann *et al*., 1999; Oidtmann, 2012). For such screening, molecular detection, particularly species-specific quantitative PCR (qPCR), is more appropriate due to its high specificity and sensitivity (see Vrålstad *et al*., 2009; Tuffs & Oidtmann, 2011; Oidtmann, 2012).

We tested the hypothesis that freshwater crabs can serve as alternative hosts of the crayfish plague pathogen when cohabiting with infected crayfish. We used recently developed molecular methods allowing species-specific detection of *A. astaci* in host tissues (Oidtmann *et al*., 2006; Vrålstad *et al*., 2009) to analyse individuals representing two genera of crabs that may come into contact with *A. astaci*-infected crayfish in natural habitats. In the Western Palaearctic, such taxa include (i) the invasive catadromous Chinese mitten crab *E. sinensis* (Varunidae), one of the 100 worst invasive species in the world (Lowe *et al*., 2004), and (ii) several strictly freshwater to semiterrestrial species of a native crab genus *Potamon* (Potamidae), which are found in southern Europe and the Middle East (Brandis, Storch & Türkay, 2000). We obtained and screened samples of both crab genera from populations known to be in contact with *A. astaci*-infected crayfish: *E. sinensis* from a Swedish lake inhabited by North American signal crayfish *Pacifastacus leniusculus*, a natural vector of *A. astaci*, and *Potamon potamios* from a Turkish lake inhabited by infected narrow-clawed crayfish *Astacus leptodactylus*, a native Western Palaearctic species relatively susceptible to crayfish plague. In addition, we analysed samples of three benthic or benthopelagic crustacean species, representing other orders of higher crustaceans frequently found in fresh waters (Amphipoda, Isopoda, and Mysida), coexisting with infected North American crayfish.

Methods

Crustacean samples

Altogether seven crustacean species were tested in this study (Table 1). A total of 30 individuals of *P. potamios*

Table 1 General overview of *A. astaci* detection in tested crustaceans. Results of *A. astaci*-specific qPCR in tested tissues of crabs (*Eriocheir sinensis*, *Potamon potamios*), coexisting crayfish (*Astacus leptodactylus*, *Pacifastacus leniusculus*), benthopelagic crustaceans *Mysis relicta* and *Pallasea quadrispinosa* coexisting with *A. astaci*-positive *P. leniusculus*, and benthic isopod *Asellus aquaticus* coexisting with *A. astaci*-positive *Orconectes limosus*. Countries are abbreviated as follows: CZ: Czech Republic, NO: Norway, SE: Sweden, TR: Turkey

Locality (country code)	Vänern (SE)		Eğirdir (TR)		Øymarksjøen (NO)		Smečno (CZ)
Species	*Eriocheir sinensis*	*Pacifastacus leniusculus*	*Potamon potamios*	*Astacus leptodactylus*	*Mysis relicta*	*Pallasea quadrispinosa*	*Asellus aquaticus*
No. individuals tested	6	20	30	30	10	10	8
No. individuals positive	6	12	13	2	0	1‡	0
Prevalence	100%	60%	43%	7%	0%	10%	0%
95% confidence interval	42–100%	36–81%	25–63%	1–22%	0–41%	0–45%	0–48%
Agent levels*							
Negative (A0)	–	8	17	28	10	9	8
Very low (A2)	–	7	1	–	–	1‡	–
Low (A3)	1	3	2	1	–	–	–
Moderate (A4)	2	1	7	–	–	–	–
High (A5)	1	–	2	1	–	–	–
Very high (A6)	2	1	1	–	–	–	–

*Results of *A. astaci* detection using *A. astaci*-specific qPCR according to Vrålstad *et al.* (2009) are given as semiquantitative categories. The scale is logarithmic; thus, each category usually represents one order of magnitude higher level of pathogen DNA than the previous one (for details, see Vrålstad *et al.*, 2009). For those individuals of which more than one sample of tissues was tested (*E. sinensis*, *P. potamios*), only the highest value found in any analysed tissue is listed (results of all tested samples are in the Tables S2 and S3).
‡This result is not considered as the evidence for the host infection, because it may have been caused by occasionally attached *A. astaci* spores (see Discussion).

crabs were caught in Lake Eğirdir (Turkey; 37.9°N, 30.9°E) where they coexist with the native population of the narrow-clawed crayfish (*Astacus leptodactylus*) recently shown to be infected by the crayfish plague pathogen (Svoboda *et al.*, 2012). The thirty *Potamon* individuals (14 males and 16 females; mean carapace length ± SD: 39 ± 5 mm) were captured in May 2010 and kept for 10 days in a common tank before being killed and dissected. Selected body parts from each individual were preserved in 96% ethanol. Thirty individuals of *A. leptodactylus* from Lake Eğirdir captured in November 2009 were already analysed for *A. astaci* presence in a previous study (Svoboda *et al.*, 2012).

Six individuals of the Chinese mitten crab (*Eriocheir sinensis*) were captured from the south-eastern part of Lake Vänern (Sweden; 58.8°N, 13.3°E) that is colonised by the invasive signal crayfish (*Pacifastacus leniusculus*), a natural host of *A. astaci* (Unestam, 1969b, 1972). The crabs (five males and one female; mean carapace length ± SD: 63 ± 4 mm) were captured in August 2009 for a behavioural experiment that lasted for 24 h, and then frozen at −20 °C. Samples of 20 *P. leniusculus* from this lake were captured in September 2011 and stored in 96% ethanol prior to testing for *A. astaci* infection.

Two benthopelagic crustacean species, *Mysis relicta* and *Pallasea quadrispinosa*, representing two orders (Mysida and Amphipoda) of higher crustaceans (Malacostraca), were collected in Lake Øymarksjøen (Norway; 59.33°N, 11.65°E) where they coexist with confirmed *A. astaci*-positive *P. leniusculus* (Vrålstad *et al.*, 2011). Ten individuals of each species were captured at 10 m depth in September 2012. Eight individuals of the benthic isopod *Asellus aquaticus* (Isopoda, Malacostraca) coexisting in a pond in Smečno (Czech Republic, 50.188°N, 14.047°E) with strongly infected *A. astaci*-positive *Orconectes limosus* (Kozubíková *et al.*, 2011b; Matasová *et al.*, 2011) were captured in May 2013. These crustacean samples were stored in 96% ethanol prior to further analyses.

Sample processing and DNA extraction

Sample processing and DNA isolation differ slightly because samples from the involved localities were processed independently in two laboratories. *Eriocheir sinensis*, *P. leniusculus*, *M. relicta* and *P. quadrispinosa* were analysed at the Norwegian Veterinary Institute (NVI) in Oslo, and *P. potamios*, *A. leptodactylus* and *A. aquaticus* at the Charles University in Prague.

Tissues of *P. potamios* individuals were processed in two stages. At first, soft abdominal cuticle, soft cuticle from two joints, the second gonopods from every male, three endopods of pleopods from every female and any melanised pieces of cuticle (found in 24 of 30

individuals) were sampled. These tissues were pooled and ground in liquid nitrogen. A separate sterile mortar was used for tissues of each individual. DNA from up to 40 mg of ground tissues was extracted with the DNeasy tissue kit (Qiagen, Venlo, the Netherlands) by following the manufacturer's instructions to obtain one DNA isolate for each specimen. Additional tissue samples (telson, two joints of walking legs and either a gonopod in males or two endopods of pleopods in females) were processed separately for those *P. potamios* individuals that tested positive for *A. astaci* presence in the pooled DNA isolate. Individuals of *A. aquaticus* were analysed whole, using the same DNA extraction method as described above. For *A. leptodactylus*, pooled DNA isolates had previously been prepared (Svoboda *et al.*, 2012) from one uropod, soft abdominal cuticle, one eye stalk, one walking leg joint and prominent melanised cuticle regions of each crayfish. An environmental control and DNA extraction control to account for potential contamination were prepared during each isolation batch.

From each of the six *E. sinensis*, seven to ten pieces of tissue were dissected: the telson, the soft abdominal cuticle, soft cuticle from two leg joints, setae from the claw, two of the maxillipeds and up to three pieces of melanised tissues (which were observed in all six sampled specimens). Each tissue sample was subsequently processed separately. For *P. leniusculus*, the telson and two uropods were dissected as one tissue sample from each of the 20 individuals. Melanised spots were sampled if present, which was the case for three crayfish individuals. *Mysis relicta* and *Pallasea quadrispinosa* individuals were analysed whole. For all samples processed at the NVI in Oslo, DNA was extracted following the CTAB protocol provided by Vrålstad *et al.* (2009). An environmental control and DNA extraction control was included as above.

Quantitative real-time PCR

All samples were analysed with *A. astaci*-specific qPCR (Vrålstad *et al.*, 2009), with minor modifications to increase the assay specificity (Strand, 2013). These included increased annealing temperature (from 58 to 62 °C) and decreased synthesis time (from 60 to 30 s). The TaqMan Environmental Master Mix (Life Technologies, Carlsbad, CA, U.S.A.) was used to reduce the potential PCR inhibition (see Strand *et al.*, 2011). The qPCR was performed on an iQ5 (Bio-Rad, Hercules, CA, U.S.A.) system for *P. potamios*, *A. leptodactylus* and *A. aquaticus* samples and a Mx3005 QPCR (Stratagene, La Jolla, CA, U.S.A.) system for *E. sinensis*, *P. leniusculus*, *P. quadrispinosa* and *M. relicta* samples. Undiluted and 10× diluted DNA isolates were used as templates for each sample, and an environmental control, DNA extraction control and a PCR blank control were included in each run. Four *A. astaci* calibrants were prepared and used to generate a standard curve to estimate the number of PCR-forming units (PFU), and then designate the semiquantitative agent level (A0-A7) for each analysed sample (for details, see Vrålstad *et al.*, 2009; Kozubíková *et al.*, 2011b). In the absence of inhibition, a mean PFU value per sample was estimated from both the undiluted and diluted DNA sample, while in the case of inhibition, only the diluted sample value was used (Kozubíková *et al.*, 2011b). We roughly estimated the number of *A. astaci* genomic units in the isolates from the PFU values, using conversion factors of PFU per spore previously obtained in each laboratory (for details, see Strand *et al.*, 2011; Svoboda *et al.*, 2013).

Considering the number of analysed specimens and the number of positive *A. astaci* detections, we estimated the prevalence of *A. astaci* in the studied populations. We then calculated its 95% confidence interval as in Filipová *et al.* (2013), using the function 'epi.conf' included in the library epiR (Stevenson *et al.*, 2013) for the statistical package R, v. 3.0 (R Core Team, 2013).

Sequencing

The presence of *A. astaci* DNA in representative crab samples that yielded positive qPCR results was confirmed by sequencing of a 569-bp-long amplicons including parts of internal transcribed spacers (ITS) 1 and 2 and 5.8S rDNA according to Oidtmann *et al.* (2006) and as recommended by the World Organisation for Animal Health (Oidtmann, 2012). Purified PCR products of one *E. sinensis* and three *P. potamios* DNA isolates were sequenced in both directions on the ABi 3130 Genetic Analyser (Life Technologies). The resulting sequences representing the pathogen from both host species (GenBank accession numbers KF748131 and KF748132) were compared with publicly available sequences of *A. astaci*.

Microsatellite analyses

We used a recently developed set of microsatellite markers (F. Grandjean, T. Vrålstad, J. Diéguez-Uribeondo, M. Jelić, J. Mangombi, C. Delaunay, L. Filipová, S. Rezinciuc, E. Kozubíková-Balcarová, D. Guyonnet, S. Viljamaa-Dirks, A. Petrusek, unpublished data) that distinguishes the five known genotype groups of *A. astaci* (A–E; Huang, Cerenius & Söderhäll,

1994; Diéguez-Uribeondo *et al.*, 1995; Kozubíková *et al.*, 2011a) and can be applied directly on mixed genome samples, that is, DNA isolates obtained from tissues of infected hosts. Nine microsatellite loci (Table 2, primer sequences are provided in Table S1) were selected out of a larger panel of candidate loci identified from 454 pyrosequencing of a library enriched with repetitive sequences. These loci showed variation among at least some of the reference strains representing *A. astaci* genotype groups (Table 2) and at the same time showed little cross-amplification with *Aphanomyces* species related to *A. astaci* and other oomycete taxa isolated from crayfish (F. Grandjean, T. Vrålstad, J. Diéguez-Uribeondo, M. Jelić, J. Mangombi, C. Delaunay, L. Filipová, S. Rezinciuc, E. Kozubíková-Balcarová, D. Guyonnet, S. Viljamaa-Dirks, A. Petrusek, unpublished data). Based on the protocol developed by the above team, we analysed the variation of these polymorphic loci to genotype the pathogen in *A. astaci*-positive isolates showing high agent level (A5–A6) from *E. sinensis* (three individuals), *P. potamios* (3), *A. leptodactylus* (1) and *P. leniusculus* (2) from the studied lakes. The resulting allele sizes were compared with those observed in axenic cultures of reference *A. astaci* strains (Table 2).

Microscopic examinations

To support results obtained by the molecular detection methods described above, we searched for hyphae corresponding morphologically to *A. astaci* in tissues of *A. astaci*-positive *P. potamios* and *E. sinensis* specimens. For this purpose, we dissected small pieces of soft cuticle from abdomen and joints from each of the six *E. sinensis*, and one piece of soft abdominal cuticle from every *P. potamios* whose pooled sample of selected tissues tested positive in qPCR. The pieces of cuticle were cut with sterilised tools, cleaned of attached muscles and connective tissues with a scalpel, and immersed in distilled water. At 100× and 400× magnification, we searched for hyphae corresponding to features of *A. astaci* (for details, see Alderman & Polglase, 1986; Cerenius *et al.*, 1988; Oidtmann *et al.*, 1999). Such hyphae were documented by digital cameras attached to the microscopes. All examined pieces of *Eriocheir* cuticle and the pieces of *Potamon* cuticle in which *A. astaci*-like

Table 2 Microsatellite analyses. The table compares allele sizes of nine microsatellite markers for reference strains of *A. astaci* genotype groups A–E and studied *A. astaci*-positive crabs and crayfish. The matching allele combinations between a reference strain and infected crabs and crayfish are highlighted by bold font

A. astaci strain*	Host species	Origin and reference†	Fragment sizes at microsatellite loci								
			Aast2	Aast4	Aast6	Aast7	Aast9	Aast10	Aast12	Aast13	Aast14
VI03557 (group A)	*Astacus astacus*	Sweden (1962); H94	160	103	157	207	180	142	–	194	246
VI03555 (group B)	*Pacifastacus leniusculus*	U.S.A. (1970); H94	**142**	**87**	**148**	**215**	**164/182**	**132**	**226/240**	**202**	**248**
VI03558 (group C)	*Pacifastacus leniusculus*	Sweden (1978); H94	154	87	148	191	164/168	132	226	202	248
VI03556 (group D)	*Procambarus clarkii*	Spain (1992); D95	138	131	148	203	180	142	234	194	250
Evira4605 (group E)	*Orconectes limosus*	Czech Republic (2010); K11a	150	87/89	148/157	207	168/182	132/142	234/240	194/202	248
Crab and crayfish species (and no. of individuals) analysed											
	Eriocheir sinensis (3)	Sweden (2009)	**142**	**87**	**148**	**215**	**164/182**	**132**	**226/240**	**202**	**248**
	Potamon potamios (2)	Turkey (2010)	**142**	**87**	**148**	**215**	**164/182**	**132**	**226/240**	**202**	**248**
	Pacifastacus leniusculus (2)	Sweden (2011)	**142**	**87**	**148**	**215**	**164/182**	**132**	**226/240**	**202**	**248**
	Astacus leptodactylus (1)	Turkey (2009); S12	**142**	**87**	**148**	**215**	**164/182**	**132**	**226/240**	**202**	**248**

*VI numbers refer to assigned strain numbers in the culture collection of the Norwegian Veterinary Institute where the isolates are maintained. Evira numbers refer similarly to assigned strain numbers in the culture collection of the Finnish Food Safety Authority Evira (OIE reference laboratory for crayfish plague). Original codes for reference strains VI03557 (A), VI03555 (B), VI03558 (C) and VI03556 (D) are L1, P1, Kv and Pc, respectively (Huang *et al.*, 1994; Diéguez-Uribeondo *et al.*, 1995).

†References are abbreviated as follows: D95: Diéguez-Uribeondo *et al.* (1995), H94: Huang *et al.* (1994), K11a: Kozubíková *et al.* (2011a), S12: Svoboda *et al.* (2012).

hyphae were found were then tested for the presence of *A. astaci* DNA by qPCR as described above.

Results

Molecular confirmation of A. astaci presence in crab tissues

Tissues from all six examined individuals of *E. sinensis* and 13 of 30 individuals of *P. potamios* yielded qPCR results indicating *A. astaci* presence. Table 1 lists the highest agent level of *A. astaci* detected in any analysed tissue from each specimen together with results of *A. astaci* detection in cuticles of coexisting crayfish species. Positive DNA isolates from all species contained low to very high agent levels (A2–A6 according to Vrålstad *et al*., 2009). Levels A2 and A6 corresponded to approximately 1–10 and 20 000–200 000 genome units in the original sample, since *c*. 100 PFU corresponds to one genomic unit (Strand *et al*., 2011; Svoboda *et al*., 2013 and unpublished data). The ITS sequences acquired to confirm the qPCR results (one from *E. sinensis*, three from *P. potamios*) were identical to publicly available reference sequences of *A. astaci*. The negative controls included in qPCR analyses remained negative for all runs.

Aphanomyces astaci DNA was found in all body parts tested in both crab species, but its distribution was heterogeneous and did not match between the two crab hosts. Of the tissues tested separately, 75 % of *P. potamios* and 83 % of *E. sinensis* samples yielded positive *A. astaci* detection (see Tables S2 and S3 in Supporting Information). For *E. sinensis*, the highest concentrations of the pathogen DNA were quantified in the soft abdominal cuticle, walking leg joints and melanised tissues. In contrast, the lowest agent levels of *A. astaci* was found in joints of *P. potamios*, while the highest concentrations were quantified in the mixture of different tissues from this species (soft abdominal cuticle, joints, melanised spots and gonopods or pleopod endopods).

No trace of *A. astaci* DNA was detected in mysids *Mysis relicta* or isopods *Asellus aquaticus* (Table 1). Only one sample of an amphipod *Pallasea quadrispinosa* was weakly positive, just above the limit of detection (level A2). Due to the low levels of *A. astaci* DNA in this apparently positive sample, it was not possible to conduct sequencing or microsatellite analyses, so the result cannot be regarded as a reliable confirmation of an *A. astaci*-carrier status for this amphipod. However, due to the modest number of individuals analysed, the 95% confidence intervals of prevalence remain wide (up to 48 %; Table 1), and thus the negative results also cannot be considered conclusive at the whole-population level.

Microsatellite analysis

The *A. astaci* genotype group B, corresponding to the genotype isolated from the signal crayfish *P. leniusculus* (Huang *et al*., 1994), was identified in all the tested tissue samples from the crabs *E. sinensis* and *P. potamios* and crayfish *A. leptodactylus* and *P. leniusculus*. The genotype found in all four species was strictly identical with the reference strain of *A. astaci* genotype B (Pl isolated from *P. leniusculus*; Table 2), without any allele variation at all nine microsatellite loci analysed (Table 2).

Microscopic examinations

Microscopic screening of soft cuticles from presumably infected crab hosts resulted in observation of characteristic oomycete hyphae (Fig. 1) in two of 13 (*Potamon*) and in one of six (*Eriocheir*) examined crab individuals. The observed hyphae were aseptate, with rounded tips and a diameter of *c.* 4–13 μm (Fig. 1a–d). The tissue immediately adjacent to the hyphae was melanised in some areas of the cuticle from one *Potamon* individual (Fig. 1a, the outer edge of the melanised area is indicated by an arrow), while elsewhere in the same sample and in the cuticle of *Eriocheir*, melanisation was not observed (Fig. 1b–d). In some areas of the *Potamon* cuticle, the hyphae were frequently branching, forming a three-dimensional net (Fig. 1b). Despite their relatively small area (*c.* 3 × 3 and 5 × 5 mm), the two pieces of *Potamon* cuticle with observed hyphal growth contained high and moderate levels of *A. astaci* DNA (agent levels A5, A4) corresponding to *c.* 15 000 and 1000 genomic units, respectively. The DNA isolate obtained from the cuticle of *Eriocheir* with detected hyphae (Fig. 1c,d) also tested *A. astaci*-positive, with high level of the pathogen DNA (A5, i.e. *c.* 15 000 genomic units in the original sample). Most other pieces of *Eriocheir* cuticle (10 of 13) examined also tested positive for *A. astaci* DNA (agent levels from A2–A4, corresponding to 1–2000 genomic units), although we had not succeeded in observing any *A. astaci*-like hyphae in them.

Discussion

Our study demonstrates that *A. astaci*, the crayfish plague pathogen, was present in cuticles of the freshwater-inhabiting crabs *P. potamios* and *E. sinensis*, both coexisting with *A. astaci*-positive crayfish. Substantial proportions of

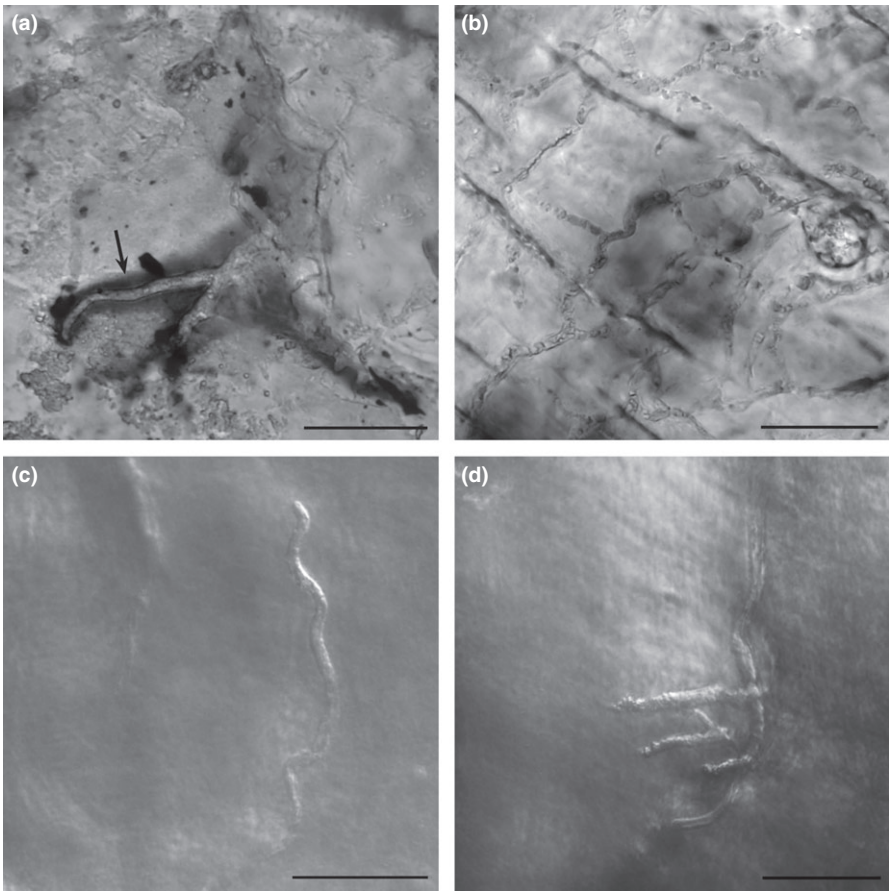

Fig. 1 Photomicrographs of *Aphanomyces astaci*-like hyphae in the cuticle of freshwater-inhabiting crabs. Hyphae corresponding to morphological features of *A. astaci* were found in the soft abdominal cuticle of both tested species, *Potamon potamios* (a, b) and *Eriocheir sinensis* (c, d). The darker area adjacent to hyphae in (a) (indicated by an arrow) is likely due to melanin deposition. In contrast, no such melanisation was observed along hyphae shown in (b), *c.* 1 mm from the location of (a), as well as along hyphae from *E. sinensis* tissues (c, d). Hyphae in some parts of the cuticle of *P. potamios* formed dense three-dimensional net (b). Scale bars in all photos indicate 50 μm.

crab individuals within the affected populations (100 % and 43 % of the analysed *E. sinensis* and *P. potamios* specimens, respectively) were apparently infected. The analyses were carried out by comparable methods in two independent laboratories, no analysis of control samples indicated laboratory contamination, and the results were consistent for different crab species coexisting at two distant localities with different crayfish species. In both crab species, the pathogen load found in certain tissues exceeded in many cases any level that could be regarded as a chance attachment of pathogen zoospores on the body surface. Instead, the highest observed levels, corresponding to several thousands of genomic units, suggested an extensive infection.

Furthermore, microscopic evaluations of the soft abdominal cuticle of one *E. sinensis* and two *P. potamios* specimens revealed aseptate hyphae matching the morphological features of *A. astaci* (for details, see Alderman & Polglase, 1986; Cerenius *et al.*, 1988; Oidtmann *et al.*, 1999). In some areas of a *Potamon* cuticle, these hyphae were apparently melanised as observed in North American carrier crayfish (Cerenius *et al.*, 1988; Söderhäll & Cerenius, 1999; Aquiloni *et al.*, 2011) or in native European crayfish with a persistent infection (Viljamaa-Dirks *et al.*, 2011). Although the cuticle pieces with visible hyphae were small and their surface was thoroughly cleaned, they contained high and moderate *A. astaci* DNA levels. This strongly supports the conclusion that we indeed observed hyphae of *A. astaci*.

With respect to the infection of *E. sinensis* reported by Benisch (1940), Unestam (1972) suggested that *A. astaci* might be limited to freshwater decapods in general. However, Benisch's study only describes infection of the crabs under laboratory conditions and the identification of the pathogen as *A. astaci* would not be considered convincing based on current state of the art (see Cerenius *et al.*, 1988; Oidtmann *et al.*, 1999; Oidtmann, 2012). Thus, no alternative crustacean hosts have recently been considered when the pathogen transmission pathways and natural reservoirs were reviewed (see Oidtmann *et al.*, 2002; Small & Pagenkopp, 2011; Oidtmann, 2012). However, our results confirm that *A. astaci* can infect crabs in freshwater habitats. Moreover, the match of the pathogen genotype groups between coexisting crayfish and crabs strongly suggests that the pathogen was transmitted between these taxa. In experiments by Benisch (1940), the crayfish plague was apparently transmitted to *E. sinensis* from moribund

crayfish. However, that study did not reveal whether *A. astaci* is able to complete its life cycle in crabs, that is, to sporulate and infect additional hosts. As far as crayfish are concerned, conditions resulting in high *A. astaci* sporulation apparently occur after moulting (presumably in exuviae) or soon after death of infected North American crayfish host species (Strand *et al.*, 2012; Svoboda *et al.*, 2013) as well as after death of infected susceptible European crayfish *A. astacus* (Makkonen *et al.*, 2013). Nevertheless, sporulation from *A. astaci* hyphae does not depend on interactions with crayfish tissues and can be induced (by washing with water) even from mycelia cultivated on artificial media (Cerenius *et al.*, 1988). Since the amount of infection in some crabs was as high as in susceptible crayfish dying from the crayfish plague (see Vrålstad *et al.*, 2009), *A. astaci* spore release from such hosts seems likely, at least when their immune system is impaired. The possibility of zoospore release from infected crabs, their exuviae, or cadavers, thus warrants further attention.

If infected crabs are indeed able to release zoospores, crabs should be considered true hosts of *A. astaci*. More important, however, are potential consequences for susceptible crayfish species that may get in contact with those crabs, especially in Europe where *E. sinensis* has invaded numerous regions (for details, see Herborg *et al.*, 2003, 2007; Dittel & Epifanio, 2009). Despite spending most of its lifetime in fresh water, adult *Eriocheir* reproduce and die in the sea, and their larval stages are found in marine zooplankton (Kobayashi & Matsuura, 1995). Since *A. astaci* does not survive in marine or brackish water (Unestam, 1969a), the crab's planktonic larvae should not be infected. However, juvenile crabs can become *A. astaci* carriers if they enter watersheds with *A. astaci* reservoirs, such as infected crayfish (or possibly crabs). Since they can migrate hundreds of kilometres upstream and then back (Herborg *et al.*, 2003; Dittel & Epifanio, 2009), infected specimens might spread the pathogen faster and further (up to two orders of magnitude) in comparison with invasive American crayfish species (see Holdich, Haffner & Noël, 2006), which are the most important carriers of *A. astaci* in Europe (Diéguez-Uribeondo *et al.*, 2006; Oidtmann, 2012).

Potamon potamios is independent of the sea for completion of its life cycle (Cumberlidge *et al.*, 2009), though as a semiterrestrial species, it does not spend entire life in fresh waters either (Warburg & Goldenberg, 1984). We have presented evidence that infected population of the crab coexists with *A. leptodactylus* crayfish in Lake Eğirdir (Turkey). Since these freshwater crabs and crayfish are widespread in Turkey (Brandis *et al.*, 2000; Harlıoğlu, 2008; Bolat *et al.*, 2010) and at least some Turkish populations of *A. leptodactylus* are persistently infected with *A. astaci* (Kokko *et al.*, 2012; Svoboda *et al.*, 2012), other populations of *P. potamios* are probably infected as well. This particular species is restricted to the Middle East and some Greek islands but its congeners are distributed in other parts of the Western Palearctic such as Italy, Turkey, Iran and the Pontocaspian region (Brandis *et al.*, 2000), where they may possibly get into contact with crayfish (see Holdich *et al.*, 2006). It is not presently clear whether *Potamon* populations in other countries also coexist with infected crayfish, for example with *Procambarus clarkii*, which is widespread in southern Europe (see Holdich *et al.*, 2006). Nevertheless, while these crabs might serve as local reservoirs of *A. astaci*, their potential for long-range transmission of the pathogen seems much more limited than for catadromous *E. sinensis*, since *Potamon* do not perform long-distance migrations.

Some of the tested tissues of both crab species contained *A. astaci* DNA at levels corresponding to infected tissues of susceptible crayfish that died from crayfish plague (see Vrålstad *et al.*, 2009). Despite that, crabs tested in our study were captured alive. Similarly, mortalities of *E. sinensis* were spread over months from the first exposure of the crabs to crayfish infected with *A. astaci* (Benisch, 1940). The present data thus correspond with Unestam's (1969b) suggestion that *E. sinensis* is a species of moderate resistance to the pathogen. As the two crab species included in our study belong to different higher taxa (*Potamon*: family Potamidae, subsection Heterotremata; *Eriocheir*: Varunidae, Thoracotremata; De Grave *et al.*, 2009), and have different geographic origins and life cycles, the moderate level of resistance to *A. astaci* might be shared by freshwater-inhabiting crabs in general.

The resistance of crabs to *A. astaci* might also depend on the particular strain of *A. astaci*. As was shown for crayfish, the virulence of *A. astaci* strains can differ, especially when strains from different genotype groups are compared (Makkonen *et al.*, 2012; Jussila *et al.*, 2013). According to our analyses, both crab species were infected with a strain from the genotype group B. Although strains from this group are highly virulent to European crayfish (Makkonen *et al.*, 2012; Jussila *et al.*, 2013), we did not notice any signs of a serious disease of the tested crabs before they were killed. In the first half of the 20th century, when Benisch (1940) performed his experiments, the genotype group B had probably not yet been introduced to Europe, and the strain most likely belonged to the group A (see Huang *et al.*, 1994). This

means that crabs that died in Benisch's experiment were probably exposed to the genotype group A, which has been recently reported to show lower virulence to crayfish (Makkonen *et al*., 2012). Nonetheless, the virulence of different *A. astaci* strains to crayfish and crabs can hardly be compared across studies separated by decades, especially as the virulence of the pathogen is likely to evolve through time (Makkonen *et al*., 2012) and depends on many factors, such as the spore dose and temperature (e.g. Alderman, Polglase & Frayling, 1987). Thus, any potential negative impact of *A. astaci* on crab population dynamics remains to be assessed by further studies, which should also consider variability in virulence of different *A. astaci* strains. Considering the extent and value of *E. sinensis* aquacultures (see FAO, 2012), such a study is highly desirable particularly for that species, even though it shows at least some resistance to *A. astaci*.

Astacus leptodactylus has also been classified as a species of moderate resistance to *A. astaci* by Unestam (1969b), and its populations do coexist with the crayfish plague pathogen in several Turkish lakes (Kokko *et al*., 2012; Svoboda *et al*., 2012) and apparently also in the Danube (Pârvulescu *et al*., 2012; Schrimpf *et al*., 2012). It has been supposed that *A. astaci* had been present in *A. leptodactylus* populations in some Turkish lakes since the first outbreaks in the 1980s (Harlıoğlu, 2008; Kokko *et al*., 2012; Svoboda *et al*., 2012). However, an *A. astaci* strain of the genotype group A was isolated from a crayfish in Turkey in the 1980s (Huang *et al*., 1994), whereas we detected an *A. astaci* strain of the group B in crayfish and crabs from Lake Eğirdir. This suggests that the history of the crayfish plague pathogen in Turkish lakes may be more complex, involving more than one introduction, and that the massive crayfish plague outbreaks in Turkey in the 1980s (see Harlıoğlu, 2008) might have been caused by a different strain from the recent one.

Samples of benthopelagic and benthic crustaceans representing other malacostracan orders (mysids, amphipods and isopods) remained negative in *A. astaci*-specific qPCR tests, except for one sample with a very low agent level (A2) corresponding to <10 genomic units. Samples analysed from zooplankton hauls from a lake with confirmed *A. astaci*-infected signal crayfish (Strand, 2013) were also mostly negative, and the few positive samples had only very low agent level. In our opinion, the few cases of detection of low levels of *A. astaci* DNA in non-decapod crustaceans (i.e. *Pallasea quadrispinosa* and crustacean zooplankton) are not an evidence of infections. They may rather represent traces of spores released from coexisting infected crayfish that were either randomly attached to animal bodies or ingested by filter feeders. This view is further supported by analyses of water samples collected at the same time, which contained *A. astaci* spore concentrations coinciding with the highest levels detected in the plankton samples (Strand, 2013). Our data therefore do not suggest that the tested species were parasitised by *A. astaci* at the time of their capture. The results also correspond with the experiments of Unestam (1969b, 1972), who observed that the mortality rates of *Mysis relicta*, several planktonic crustaceans (cladocerans and copepods) and a rotifer did not increase after exposure to *A. astaci*. However, lack of increased mortality does not exclude the presence of non-lethal *A. astaci* infections, and we tested only moderate number of individuals of benthopelagic and benthic crustaceans from a few localities. As the wide 95% confidence intervals (Table 1) clearly show, much more thorough screening or experimental work is needed to conclude whether these crustaceans can or cannot be parasitised by *A. astaci*.

It also remains to be explored if *A. astaci* has a potential to infect other freshwater decapods, as Unestam (1972) suggested. Apart from crabs and crayfish, the order Decapoda includes two other infraorders (Caridea and Anomura) with some freshwater species (De Grave *et al*., 2008, 2009; Cumberlidge *et al*., 2009). Unlike the relatively unimportant freshwater anomurans, freshwater shrimps are highly diverse, are present in all biogeographical regions except Antarctica (De Grave *et al*., 2008), and some have substantial economic value (FAO, 2012). As the early detection and control of diseases and pathogens is vital for freshwater shrimp aquaculture (Kutty, 2005), experimental work evaluating the susceptibility of these species to *A. astaci* is highly desirable.

Our results clearly demonstrate that the freshwater-inhabiting crab species *E. sinensis* and *P. potamios* can be infected by *A. astaci*. This is not only a rehabilitation of the conclusions from Benisch (1940), who considered *E. sinensis* as a species susceptible to *A. astaci*, but may also suggest that such crabs can serve as long-term, symptom-free carriers of the pathogen. Hence, both conservation and fishery management of susceptible crayfish species in Europe should consider that not only crayfish, but also crabs may serve as *A. astaci* hosts. The screening of other crustacean orders does not support such a conclusion for non-decapod crustaceans. Our work has also re-opened numerous questions that are important from conservational, parasitological and even economic points of views. These include the real ranges of decapod hosts and symptom-free carriers of *A. astaci*, the carrier status of invasive *E. sinensis* populations

across Europe, and the potential impact of different *A. astaci* genotype groups to a broader range of freshwater Decapoda in nature and aquaculture.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Additional characteristics of the analysed microsatellite loci for *Aphanomyces astaci*: primer sequences and repeat motifs.

Table S2. Results of the *A. astaci*-specific qPCR analyses of different tissues of *Eriocheir sinensis*.

Table S3. Results of the *A. astaci*-specific qPCR analyses of different tissues of *Potamon potamios*.

(Manuscript accepted 12 December 2013)

Supporting information: Table S1:

Additional characteristics of the analysed microsatellite loci for *Aphanomyces astaci*: primer sequences and repeat motifs.

Locus	Primer sequence	Repeat motif
Aast2	F: 6-FAM-GCTTGATTCGTGTTCTGGGT	AT_8
	R: CAAAACGCTCATCCAATGCTA	
Aast4	F: TAGTGGTTCCTTTATTCTAAATTGC	TA_8
	R: CAGCACCATGGGTGTTATGT	
Aast6	F: ACACCTTGAACAAGCCAGTG	CGA_8
	R: CAAGTCGACGGAGGTACTCG	
Aast7	F: CTGAATGGTGCACTAGTACAATCTG	TA_8
	R: GCCCAATGCGGATAGACTC	
Aast9	F: TCGGAAAGTTTGTCCACTTG	AT_7
	R: GCCAGCGTATTACGTTCACA	
Aast10	F: CATTGACGACAGCATGAACC	AT_7
	R: CGTCCAAATCAACGAAAGAA	
Aast12	F: CCATGGCAACATCATCAAAG	CA_6
	R: GGATGATATCGACGCTTGTG	
Aast13	F: TCGACGTGTTGTTTGGCTTA	TG_{10}
	R: GCGTTGCCTAGTTGTTGTGA	
Aast14	F: CATTTCAGCGGAATGCTACA	TG_6
	R: GTCGAGTGGTCATTCAGTGC	

Supporting information: Table S2:

Results of the *A*. *astaci* specific qPCR analyses of different tissues of *Eriocheir sinensis*. A0 to A6 reflect the detected agent levels in the analysis (for details, see Vrålstad *el al*. 2009). NA indicates that a particular tissue sample was either unavailable or not analysed. Darker shading reflects higher agent levels. Note that the A1 level is not considered a positive *A. astaci* detection.

Individuals	1	2	3	4	5	6	No. of positive
Tissue							detections in tissues
Telson	A3	A1	A3	A3	NA	A5	4/5
Abdomen - Soft	A3	A2	A4	A4	A6	A5	6/6
Joint 1 - Soft cuticle	A3	A2	A5	A3	A6	A3	6/6
Joint 2 - Soft cuticle	A3	NA	A5	A4	A6	A3	5/5
Setae on Claw	A2	A3	A3	A3	A3	A1	5/6
Maxillipeds Left	A1	NA	A0	A4	A0	A0	1/6
Maxillipeds Right	A1	A4	A2	A2	A3	A0	4/6
Melanised tissue*	A2	A2	A3	A3	A6	A6	6/6
Melanised tissue*	NA	A3	A3	A2	A2	A0	4/5
Melanised tissue*	NA	NA	NA	NA	A5	A2	2/2

* Up to three additional tissue pieces with visible melanized spots were dissected from each crab in addition to the mentioned tissue.

Supporting information: Table S3:

Results of the *A*. *astaci* specific qPCR analyses of different tissues of *Potamon potamios*. Mixture stands for pooled tissues of soft abdomen cuticle, soft cuticle from two joints, the second gonopods from every male, 3 endopods of pleopods from every female, and any melanised pieces of cuticle (found in 20 individuals). Darker shading reflects higher agent levels (for details, see Vrålstad *el al*. 2009). Note that the A1 level is not considered as positive *A. astaci* detection.

Individuals	1	2	3	4	5	6	7	8	9	10	11	12	13
Tissue													
Telson	A3	A4	A2	A4	A0	A4	A4	A0	A3	A4	A0	A5	A4
Two joints	A2	A0	A0	A1	A0	A1	A0	A0	A3	A0	A2	A3	A3
Endopod/Gonopod	A0	A0	A2	A2	A0	A3	A3	A1	A3	A2	A0	A4	A2
Mixture	A3	A3	A4	A3	A2	A2	A3	A5	A6	A4	A3	A5	A3

Chapter 3

Svoboda, J., Mrugała, A., Kozubíková-Balcarová, E., Kouba, A., Diéguez-Uribeondo, J., Petrusek, A., 2014. Resistance to the crayfish plague pathogen, *Aphanomyces astaci*, in two freshwater shrimps.
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"...high pathogen DNA levels were detected in some non-moulting individuals of *Macrobrachium dayanum*, suggesting that *Aphanomyces astaci* growth may be possible in tissues of this species..."

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Resistance to the crayfish plague pathogen, *Aphanomyces astaci*, in two freshwater shrimps

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ABSTRACT

Aphanomyces astaci, the causal agent of the crayfish plague, has recently been confirmed to infect also freshwater-inhabiting crabs. We experimentally tested the resistance of freshwater shrimps, another important decapod group inhabiting freshwaters, to this pathogen. We exposed individuals of two Asian shrimp species, *Macrobrachium dayanum* and *Neocaridina davidi*, to zoospores of the pathogen strain isolated from *Procambarus clarkii*, a known *A. astaci* carrier likely to get into contact with shrimps. The shrimps were kept in separate vessels up to seven weeks; exuviae and randomly chosen individuals were sampled throughout the experiment. Shrimp bodies and exuviae were tested for *A. astaci* presence by a species-specific quantitative PCR. The results were compared with amounts of *A. astaci* DNA in an inert substrate to distinguish potential pathogen growth in live specimens from persisting spores or environmental DNA attached to their surface. In contrast to susceptible crayfish *Astacus astacus*, we did not observe mortality of shrimps. The amount of detected pathogen DNA was decreasing steadily in the inert substrate, but it was still detectable several weeks after zoospore addition, which should be considered in studies relying on molecular detection of *A. astaci*. Probably due to moulting, the amount of *A. astaci* DNA was decreasing in *N. davidi* even faster than in the inert substrate. In contrast, high pathogen DNA levels were detected in some non-moulting individuals of *M. dayanum*, suggesting that *A. astaci* growth may be possible in tissues of this species. Further experiments are needed to test for the potential of long-term *A. astaci* persistence in freshwater shrimp populations.

1. Introduction

The causal agent of crayfish plague, the oomycete *Aphanomyces astaci*, is one of the most intensively studied pathogens of freshwater invertebrates. Due to its devastating impact on populations of European crayfish, it has been included among the 100 worst invasive species in the world (Lowe et al., 2004). The species seems restricted to freshwater environment (Unestam, 1969a) and highly adapted to a parasitic mode of life (e.g., Unestam, 1965, 1969a). It has a very limited host range, similarly as *A. invadans-piscicida*, a congener which also lacks sexual reproduction (see Diéguez-Uribeondo et al., 2009). *A. astaci* is well known for its ability to infect and kill crayfish (Decapoda, infraorder Astacidea) (Diéguez-Uribeondo et al., 2006). The resistance to *A. astaci* infection varies among crayfish species: European, Asian and Australian crayfish are much more susceptible to this pathogen than those from North America (e.g., Unestam, 1969b, 1975). However, infection by the pathogen was recently confirmed also in freshwater-inhabiting crabs (infraorder Brachyura) (Schrimpf et al., 2014; Svoboda et al., 2014), which corresponds to the assumption of Unestam (1972) that even decapods other than crayfish might become infected with *A. astaci*.

In addition to crayfish and crabs, the order Decapoda includes two other infraorders (Caridea and Anomura) comprising some freshwater species (Bond-Buckup et al., 2008; De Grave et al., 2008). Freshwater-inhabiting anomurans are rare: one genus restricted to South America (Bond-Buckup et al., 2008) and a single island species of hermit crab (McLaughlin and Murray, 1990). In contrast, freshwater shrimps account for approximately a quarter of all described carideans and are present in all biogeographical regions except of Antarctica (De Grave et al., 2008). Some

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freshwater shrimps, particularly from the genus *Macrobrachium* (family Palaemonidae), are farmed in an extensive aquaculture industry. With a harvest exceeding 450,000 tons, they accounted for a value of more than 2 billion USD in 2010 (FAO, 2012). The key region for the freshwater shrimp industry as well as the centre of their biodiversity is South-East Asia (De Grave et al., 2008; FAO, 2012). A potential source of *A. astaci* in this region is the red swamp crayfish *Procambarus clarkii*, which is a known carrier of the pathogen (Diéguez-Uribeondo and Söderhäll, 1993; Diéguez-Uribeondo et al., 1995), and is not only intensively farmed but also invades some open waters there (Hobbs et al., 1989; Yue et al., 2010). Nevertheless, freshwater shrimps, crayfish and *A. astaci* might get into complex interactions also in Europe, where *A. astaci* is widespread (Diéguez-Uribeondo et al., 2006), and both susceptible and carrier crayfish species may get into contact with shrimp populations. Moreover, shrimps are locally used as fish bait, and if capable of at least temporarily hosting *A. astaci*, they might introduce this pathogen to new localities. However, no data are available on resistance (or potential susceptibility) of freshwater shrimps to *A. astaci* infection.

The purpose of this study was to evaluate the resistance of two selected species of freshwater shrimps (*Macrobrachium dayanum* and *Neocaridina davidi*) to *A. astaci* in a laboratory experiment. These Asian shrimp species represent the two most numerous families of freshwater shrimps, Palaemonidae and Atyidae, respectively (De Grave et al., 2008). The shrimps were exposed to *A. astaci* zoospores, the only infectious stage of *A. astaci* (Söderhäll and Cerenius, 1999). Later, the pathogen presence in the shrimps was tested with species-specific quantitative PCR.

2. Material and methods

2.1. Origin of crustaceans and A. astaci strain

The shrimps *M. dayanum* and *N. davidi* originated from pet trade. Both species are widespread among hobby breeders in Europe and they have even been found in a thermally polluted stream in Germany (Klotz et al., 2013). The animals were exposed to *A. astaci* strain SAP880 belonging to a genotype group D, i.e., its original host was *P. clarkii* (Diéguez-Uribeondo et al., 1995; Rezinciuc et al., 2013). The strain is kept at the culture collection of the Department of Mycology, Royal Botanical Garden CSIC, Madrid. It has been isolated during a crayfish plague outbreak in a population of *Austropotamobius pallipes*, which proves its original high virulence. As a control for its infectiveness under our experimental conditions, four individuals of the susceptible noble crayfish *Astacus astacus* were exposed to zoospores of this strain. The noble crayfish were obtained from experimental facilities of the Faculty of Fisheries and Protection of Waters, University of South Bohemia.

2.2. Experimental design

The experiments took place at the Department of Ecology, Charles University in Prague. More individuals of *M. dayanum* than *N. davidi* were used (Table 1) because the results of a pilot experiment had suggested that long-term persistence of the pathogen is more likely in the former species. Several individuals of each species were euthanized by freezing and tested for the presence of *A. astaci* before the experiment to confirm that animals from each source had not been already infected with *A. astaci* or contaminated with its DNA. Other individuals were exposed to *A. astaci* zoospores and kept for a certain period in the experiment. The remaining ones were treated in the same way, but no zoospores were added to their vessels. These served as negative controls to rule out laboratory cross-contamination, and to control for background mortality.

Every individual used in the experiment was kept in a separate vessel at 20 °C in aged tap water (aerated for at least 24 h before the addition to the vessel). The shrimps were kept in glass beakers with approx. 200 ml of water with no aeration. *A. astacus* individuals were kept in 5 l glass jars with approx. 500 ml of water to ensure sufficient room and oxygen supply for substantially larger-bodied crayfish. Water in these jars was aerated with an airstone during the experiment to avoid oxygen depletion; except for the first six hours after zoospore additions to allow for spore attachment. One uropod was cut from each crayfish before the experiment for additional tests to support the assumption that the experimental crayfish were originally free of infection.

2.3. Zoospore preparation and exposure

Zoospore suspensions were produced according to Cerenius et al. (1988), counted in a haemocytometer and appropriate volumes of the suspension were added to randomly chosen vessels in order to reach a concentration of 1000 zoospores ml^{-1}. The same amount of zoospores was added to every vessel once more, 4 days after the first one, to increase the likelihood of infection. Thus, altogether 4×10^5 zoospores were added to every vessel with *N. davidi* (15 ind.) and *M. dayanum* (43 ind.) at the beginning of the experiment, and 1×10^6 zoospores to every vessel with *A. astacus* (4 ind.). Since we cannot estimate how many zoospores had remained active from the first addition to the second, we can only conclude that the maximal concentration of zoospores found at a time in a vessel ranged between 1000 and 2000 zoospores ml^{-1}. Cross-contamination among vessels was prevented by appropriate measures such as use of sterile tools for all manipulations in a vessel, and use of lids on the vessels with airstones to prevent aerosol-borne contamination.

2.4. Experimental conditions and sampling

Water in the vessels of both negative controls and zoospore-exposed individuals was changed every week, first time five days after the second addition of zoospores. Each time, the whole volume was carefully poured out of the vessel so that only the crustacean remained inside, and aged tap water was added immediately. The animals were fed with granular fish feed (Sera vipagran), first time one week after the second addition of zoospores and then

Table 1
Numbers of animals and filters tested for presence of *A. astaci* DNA, and other details of experimental design. Range of body lengths (from rostrum to the end of tail fan) of individuals exposed to zoospores, volume of vessels with each individual, and presence or absence of active aeration during the experiment is noted for each species and filters.

Species	Individuals tested before experiment	Negative controls (no zoospores added)	Individuals exposed to zoospores	Body length range (mm)	Volume (ml)	Aeration
Neocaridina davidi	12	6	15	10–15	200	No
Macrobrachium dayanum	6	13	43	30–35	200	No
Astacus astacus	6	4	4	75–85	500	Yes
Filters (inert substrate)	0	8	63	25	200	No

once a week, two days before water renewal. Most individuals were kept in the experiment for 27 days (*N. davidi*) or 49 days (*M. dayanum* and *A. astacus*) after the second zoospore addition; some individuals were sampled during the course of the experiment to collect data about temporal changes in the quantity of *A. astaci* DNA detected in their bodies. Experiment with *N. davidi* was terminated earlier due to a more frequent moulting of this species, as we assumed that this process could reduce the infection. Date of moulting or animal death was always noted. The exuviae were left in the vessel for three days to allow for possible sporulation of *A. astaci* in the cuticle shed by the shrimp, and re-infection of the host. After that, the exuviae were removed unless eaten by the shrimp. Sampled shrimps and their exuviae were transferred from experimental vessels to 2 ml Eppendorf tubes with 96% ethanol; sampled crayfish and their exuviae were transferred to plastic bags. All the samples were kept at −80 °C until processed further.

2.5. Inert control

A. astaci spores (a term encompassing both the motile zoospores and cysts that are formed from them) may survive in the environment for up to several weeks (CEFAS, 2000; Unestam, 1969a). Data from the experiment with shrimps may be biased by the persistence of added *A. astaci* zoospores or their DNA because molecular-based approach cannot distinguish hyphae growing in a host from other sources of *A. astaci* DNA (i.e., spores attached on its cuticle or present in its gastrointestinal tract, and extracellular DNA). Because the distinction between *A. astaci* growth and residuals from added zoospores was crucial for our study, we included in the experiments also an inert substrate to control for the DNA from the zoospores.

To estimate the amount of isolated *A. astaci* DNA, which could originate from added zoospores only, we set up fourteen beakers (identical with those in which shrimp individuals were kept) with several non-overlapping circular polycarbonate filters (Whatman Nucleopore, diameter 25 mm, pores 2 μm) placed on the beaker bottom (diameter 60 mm). These filters were chosen as they are made of a material suitable for DNA isolation from attached or filtered spores (see Svoboda et al., 2013), but they should not be a suitable substrate for the growth of the parasitic *A. astaci* (see e.g., Unestam, 1969a). Preliminary tests involving a five-day experiment (J. Svoboda, unpublished data) confirmed that the presence of the polycarbonate filters did not change the amount of *A. astaci* DNA isolated from *A. astaci* spore suspensions.

Zoospores were added to beakers containing the filters in the same way as to the vessels with tested animals, and the beakers were treated as the vessels with shrimps (no airstone, 20 °C, water changed once a week). Altogether ca 4×10^5 zoospores were added to every vessel at the beginning of the experiment, 2×10^5 on the first day and 2×10^5 four days later. Filters were sampled regularly in one-week intervals. As a control for cross-contamination, no *A. astaci* zoospores were added to two beakers with four filters each.

2.6. Microscopic examination of shrimp cuticle

A. astaci cannot be determined based on its morphology (Cerenius et al., 1988; Oidtmann, 2012), and microscopic examination may not reveal the hyphae even in moribund susceptible crayfish infected by *A. astaci* (see e.g., Kozubíková et al., 2008). However, microscopic examination of tissues can serve as a strong supporting evidence of *A. astaci* growth when combined with subsequent molecular detection (see Svoboda et al., 2014). We searched for hyphae corresponding morphologically to *A. astaci* in selected tissues of *M. dayanum*. Uropods and telsons were dissected from all individuals exposed to *A. astaci* spores and cleaned of muscles and connective tissues with a scalpel. The cuticle was immersed in distilled water and examined at 100× and 400× magnification for the presence of hyphae showing features of *A. astaci* (for details, see Alderman and Polglase, 1986; Cerenius et al., 1988; Oidtmann et al., 1999). The likelihood of microscopic detection of hyphae might be possibly increased by staining. However, we preferred not to risk any bias for the subsequent molecular detection – all examined tissues were returned to 96% ethanol and used for DNA isolation with the rest of the body to obtain a single DNA isolate for each individual. To prevent any cross-contamination with *A. astaci* DNA, we cleaned all tools (scalpels, tweezers, slides, cover slips) with 15% hydrogen peroxide, and metallic tools were flame sterilized before use on another individual. Since the microscopic examination of *M. dayanum* was time-consuming and the results were poor, we did not apply it on *N. davidi*.

2.7. Molecular detection of A. astaci

From *A. astacus*, the following tissues were cleaned with cotton sticks and dissected: whole soft abdominal cuticle, two uropods, telson, two basal joints from walking legs, and any noticed melanised spots and wounds (found in all individuals). These body parts have been reported as being most often infected by the pathogen (Oidtmann et al., 2006). The tissues were crushed in liquid nitrogen, and up to 40 mg of the tissue mixture were used for subsequent DNA isolation with the DNeasy Animal Tissue kit (Qiagen) resulting in isolates with the volume of 200 μl. The same kit was used also for the DNA isolation from filters and exuviae.

In contrast, DNA was isolated from whole bodies of the tested shrimp species (*M. dayanum* and *N. davidi*), as we did not want to influence the results by a priori choice of only some body parts, and dissection of the whole shrimp cuticle would be impractical due to small size of the animals. To isolate DNA from shrimps with the body mass largely exceeding the limit for the isolation kit, we used a phenol-chloroform method (for details on the protocol, see Supplementary Information). DNA extracted from the whole shrimp body was dissolved in a volume of 100 μl. Negative controls, i.e., tubes containing Milli-Q water only and no crayfish tissues, were included in each DNA isolation batch. In every isolation batch, we also included one positive control, a tube containing 100 μl of water with approx. 1000 *A. astaci* spores and a few milligrams of crushed cuticle from a non-infected crayfish.

The pathogen DNA was quantified in all isolates by a well-established sensitive molecular method, a quantitative PCR (qPCR) according to Vrålstad et al. (2009). The original protocol (Vrålstad et al., 2009) has been modified to enhance the specificity of the reaction (Strand, 2013): increased annealing temperature (from 58 to 62 °C) and decreased synthesis time (from 60 to 30 s). TaqMan Environmental Master Mix (Applied Biosystems) was used to reduce potential PCR inhibition, as recommended by Strand et al. (2011). Negative controls were included in all qPCR runs. We tested for potential inhibition for each sample by a qPCR analysis of 10× diluted isolates (for details, see Kozubíková et al., 2011). Neglecting variation of up to 15%, we detected only eight DNA isolates with minor signs of PCR inhibition. For these samples, numbers of PCR forming units were calculated using the results of three replicates with 10× dilutions.

In this study, we focus mostly on the amount of *A. astaci* DNA quantified for the whole volume of DNA isolates. Therefore, the numbers of PFU in 5 μl were multiplied by 20 or 40, depending on the DNA isolation method (phenol-chloroform and DNeasy Animal tissue kit, respectively). Subsequently, the numbers of PFU were converted to *A. astaci* genomic units (hereafter abbreviated as C), which should correspond to the number of *A. astaci* spores or nuclei in hyphae (for details, see Vrålstad et al., 2009). We used the conversion factor of 143 PFU per spore, relevant for the *A. astaci*

strain used and DNA isolation efficiency. This conversion factor was estimated from *A. astaci* DNA quantification from the twelve positive DNA isolation controls, i.e., isolates from samples including approx. 1000 of *A. astaci* spores. Results of the qPCR detection of *A. astaci* might also be expressed in semiquantitative agent values ranging from no agent (A0) to exceptionally high level of the agent (A7) (Vrålstad et al., 2009). These categories are based on the number of PCR forming units (PFU) quantified in a reaction, i.e., in 5 μl of a sample, and they were defined according to the amounts of *A. astaci* DNA isolated from susceptible crayfish (Vrålstad et al., 2009). We use those categories only when commenting on our data expressed in genomic units to make the comparison with other studies easier.

In accordance with Vrålstad et al. (2009) we set the limit of detection, considered as an unambiguous evidence of *A. astaci* DNA presence, to 5 PFU in 5 μl of a DNA isolate. This corresponded to 1.4 C for samples isolated by an isolation kit, and to 0.7 C for shrimp bodies isolated by the phenol-chloroform extraction (the difference being caused by different total volume of the isolates). The limit of reliable quantification of the qPCR analysis, recommended by Vrålstad et al. (2009), is ten times higher than the limit of detection, i.e., 50 PFU (corresponding in our study to 14 C and 7 C, respectively). In the presentation of our results, we show also quantitative values below these thresholds, although such results below the limit of quantification may be considered only approximate. Replacing them by surrogate values in the middle of the interval below the limit of quantification, however, did not affect any results of the data analyses substantially.

2.8. Data analysis

We calculated a half-life of detectable *A. astaci* DNA detected on the inert substrate (filters) from the respective parameter of an exponential regression of genomic units quantified in the filters over time. Furthermore, we expressed the rate of decrease of *A. astaci* DNA detected in filters and shrimp exuviae in the first four weeks of the experiment (i.e., period when all species were kept in the experiment) as the slope parameter of linear regressions of the relationship between log-transformed C-values and day of the experiment. The same way, we expressed the temporal trend in bodies of non-moulting *M. dayanum* throughout the experiment.

The amount of *A. astaci* genomic units detected in bodies of moulting and non-moulting *M. dayanum* at the end of the experiment was compared by the Mann–Whitney *U*-test calculated in Statistica 6.1 (Statsoft, Inc., Tulsa, USA). The same test was used to compare the quantification in positive controls isolated by the two different DNA isolation methods.

3. Results

3.1. Analysis of control samples and animals

All positive DNA isolation controls (i.e., isolates from samples including approx. 1000 of *A. astaci* spores) gave qPCR signals corresponding to the range of ca 500–1600 C. The difference between the two isolation methods was not significant (column chromatography: range in control samples 503–1494; phenol-chloroform: range 472–1624; Mann–Whitney U test, $N_1 = 7$, $N_2 = 5$, $U = 12$, $Z = 0.89$, $p = 0.37$). DNA extracts from all negative controls remained negative in qPCR. This included the filter controls, tissue from crayfish and shrimps used as negative controls, as well as the crayfish and shrimps tested before the experiment. Mortality of control animals was negligible, only one *M. dayanum* used as a negative control died during the experiment.

3.2. Detection of A. astaci DNA in inert substrate

The maximal amounts of *A. astaci* DNA were isolated from filters collected one and three days after the addition of *A. astaci* spores. The median reached 53,000 C (Fig. 1A), which corresponds to 13% of the added zoospores. Thus, about 52% of the added spores were found in this period at four filters covering ca 59% surface of the vessel bottom. Subsequently, the amounts of *A. astaci* DNA in filters were steadily decreasing (slope parameter of the linear regression log-transformed C values in the first four weeks: −0.11). The half-life of *A. astaci* DNA in filters calculated from the exponential regression was 3.1 days. Until 33 days from the second spore addition, the amount of *A. astaci* DNA in all isolates from the filters exceeded the limit of reliable detection (i.e., 5 PFU in the qPCR reaction). Even at the end of the experiment (49 days after the spore addition), two out of four analysed samples were above this limit (Fig. 1A).

3.3. Detection of A. astaci DNA in control crayfish

Two out of four *A. astacus* died after the exposure to *A. astaci* zoospores, in the third and fourth week of the experiment. Tissues of those individuals contained exceptionally high and very high agent levels corresponding to nearly 621,000 C and 54,000 C (Fig. 1B, squares). Two other individuals had survived until the end of the experiment when they were euthanized and their tissues sampled. In the tissues of these individuals, a very high agent level corresponding to ca 43,000 C and a moderate agent level corresponding to 640 C were detected.

3.4. Detection of A. astaci DNA in shrimps

No *N. davidi* individual died in the experiment. All individuals retained in the vessels until the end of the experiments moulted at least once, three of them moulted twice. The average moulting rate for *N. davidi* individuals exposed to zoospores was 0.31 moults per week (i.e., one moult in 23 days). The amount of *A. astaci* DNA quantified in the bodies and exuviae of *N. davidi* exposed to *A. astaci* zoospores was decreasing steadily during the experiment (Fig. 1C and E). This is particularly apparent on results from first exuviae of the respective animals; the respective slope of the regression line was −0.15. At the end of the first week of the experiment, low agent level corresponding to approx. 70 C was found on average in tissues and high agent level corresponding to approx. 7000 C was detected on average in exuviae of *N. davidi*. In contrast, in the fourth week of the experiment, neither exuviae nor any body of *N. davidi* contained *A. astaci* DNA exceeding the limit of quantification (7 C).

No specimen of *M. dayanum* exposed to *A. astaci* zoospores died during the experiment. Five individuals retained until the end of the experiments did not moult at all, 28 moulted once, and two moulted twice. The average moulting rate for individuals exposed to zoospores was 0.18 moults per week (one in 39 days). No hyphae corresponding to morphologic features of *A. astaci* were observed in microscopically examined shrimp body parts (i.e., uropods and telson). The amount of *A. astaci* DNA detected in bodies of individuals of this species (Fig. 1D) varied considerably. No decrease was observed for individuals that had not moulted during the experiment (slope: 0.004), and all yielded qPCR signal above the limit of *A. astaci* DNA detection. The quantified *A. astaci* DNA in *M. dayanum* bodies at the end of experiment ranged from no detection to high agent level corresponding to 2200 C. The qPCR signal did not reach the level of detection in 20 out of 28 individuals that had moulted once, and both individuals that had moulted twice. The difference in detected *A. astaci* DNA between individuals sampled at the end of the experiment that had not moulted ($N = 5$)

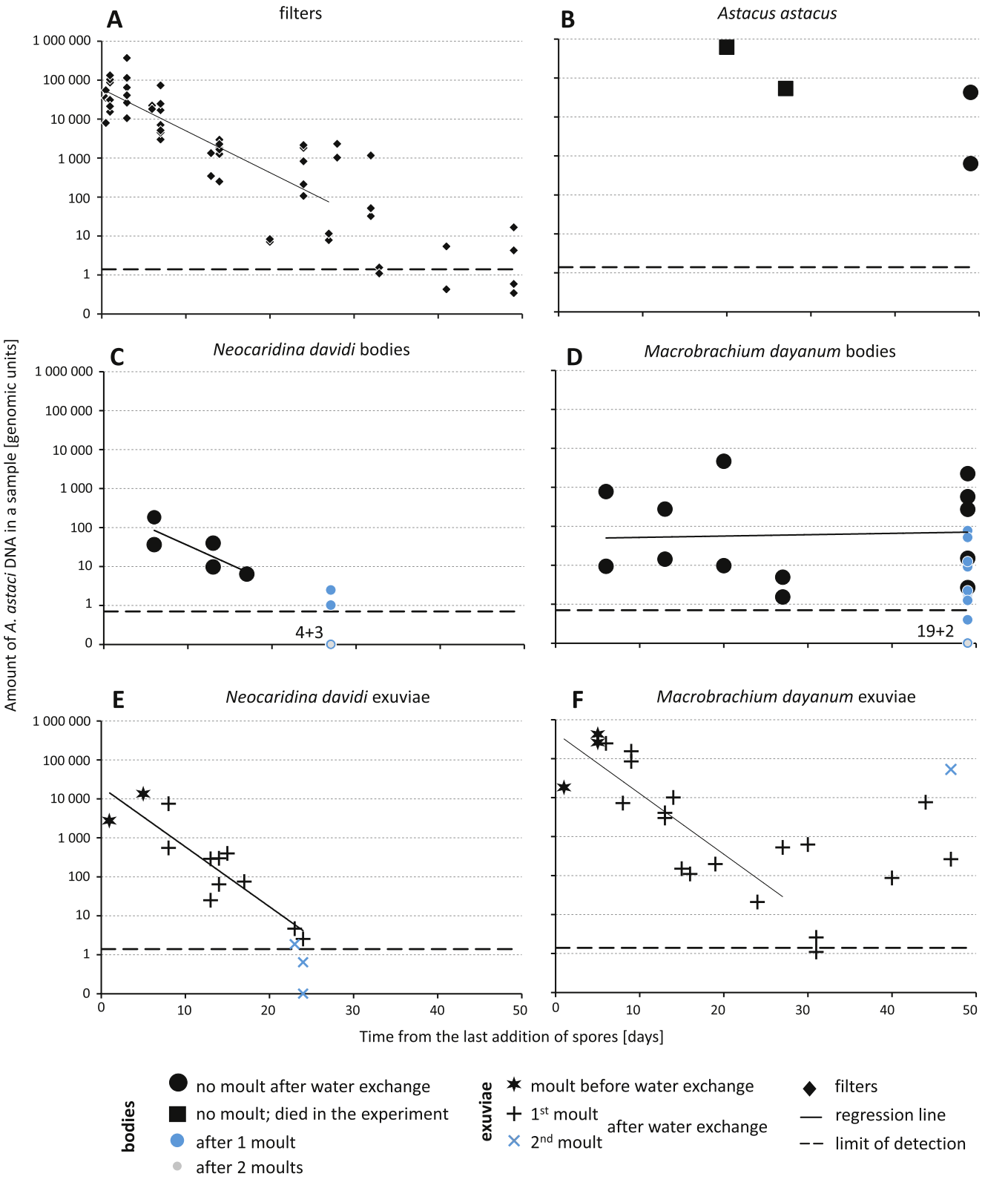

Fig. 1. Quantification of *A. astaci* DNA in samples exposed to *A. astaci* zoospores. The amount of *A. astaci* DNA isolated from filters (A), selected tissues of crayfish bodies (B), and whole bodies (C, D) and exuviae (E, F) of the shrimps *M. dayanum* and *N. davidi* is given in genomic units (C) calculated for the whole sample (i.e., volume of DNA isolate). Time "0" corresponds to the day of the second zoospore addition. Black symbols indicate samples that were exposed to the original zoospore suspension (i.e., before the first water exchange). Numbers next to symbols directly on the horizontal axes quantify the number of shrimp bodies whose DNA isolates gave no signal in qPCR (separately for different moult categories).

and those that had moulted ($N = 30$) was highly significant (Mann–Whitney U test, $U = 6$, $Z = 3.25$, $p = 0.001$).

Unlike *N. davidi*, the amounts of *A. astaci* DNA detected in exuviae of *M. dayanum* did not show a monotonous trend (Fig. 1F). After initial decrease in the first four weeks, comparable to the former species (slope: −0.16), some exuviae from the first moults of the respective animals yielded qPCR signals corresponding to moderate or high agent levels; even higher level was observed for one exuviae from the second moult of the respective individual.

4. Discussion

Our results showed that the tested strain of *A. astaci* did not cause mortality of studied freshwater shrimps and suggest that frequent moulting might be an important factor responsible for the apparent resistance of the shrimps. The results suggest that the pathogen may grow in shrimp tissues, but it is not clear whether it can complete its life cycle in such hosts. The amount of *A. astaci* DNA isolated from an inert substrate (polycarbonate filters) was decreasing except for the first few days after zoospore addition. We assume that the increase at the beginning was caused by gradual spore sedimentation and attachment. Then, the influence of spore loss due to water exchange, death of spores, and decay of *A. astaci* DNA prevailed. Therefore, the data from the analyses of the inert substrate can be considered an assessment of the rate of loss of *A. astaci* spores under our experimental conditions (likely a conservative one, as the DNA may be still detected for some period after the cell death).

The survival of *A. astaci* in water without a host is limited by the persistence of zoospores and cysts. More precisely, the limit is the total length of several consecutive generations of these, because if an *A. astaci* zoospore encysts, a new zoospore may be subsequently released in a process known as repeated zoospore emergence (Cerenius and Söderhäll, 1984b). It was shown that *A. astaci* spores can survive in experimental conditions for at least 14 days in temperatures up to 15 °C (CEFAS, 2000), and Unestam (1966) found surviving spores at 2 °C even after two months from their addition. Our study, in which we detected *A. astaci* DNA even after seven weeks at the temperature of 20 °C, thus do not contradict previous results. However, we do not know whether any active spores would be found in the treatments with inert substrate at the end of our experiment.

Even 2300 genomic units (C) of *A. astaci* were detected on some filters sampled four weeks since the zoospore addition. This confirms that persistence of spores may potentially bias the results of *A. astaci* molecular detection focusing on infection status of tested animals. The concentration of zoospores added in the beginning of our experiment (1000–2000 zoospores ml^{-1}) may be found in the vicinity of infected crayfish in experimental conditions soon after their moult or death (see Makkonen et al., 2013; Strand et al., 2012; Svoboda et al., 2013). In natural waters, however, a strong bias due to spore persistence is less likely since the concentrations of spores are usually lower, reaching tens of spores per ml during crayfish plague outbreaks, and substantially less in habitats inhabited by infected American crayfish (Strand et al., 2014). Nevertheless, our results confirm that molecular quantification of *A. astaci*, particularly if relatively low agent levels are detected in atypical hosts or substrates, should be interpreted with care. Whenever possible, such results should be supported by alternative methods, e.g., histology or transmission experiments (see also Schrimpf et al., 2014; Svoboda et al., 2014). Furthermore, protocols for processing samples should include steps reducing the likelihood of detection of spores attached at the surface; e.g., thorough rinsing in spore-free water and mechanical cleaning of surface of tested body parts.

Our study presents results of experiments with only one strain of *A. astaci* but different strains of *A. astaci* can vary in virulence to a crayfish species, especially when strains from different genotype groups are compared (Makkonen et al., 2012, 2014; Jussila et al., 2013). The *A. astaci* strain used in this study was able to infect and kill susceptible crayfish species under our experimental conditions. The two *A. astacus*, which had survived until the end of the experiment, were probably infected by the pathogen as well since moderate and very high agent levels were found in their cuticles (for comparison, see Vrålstad et al., 2009). Nevertheless, the virulence of the strain used in our experiment might be lower than the virulence of strains from the signal crayfish (genotype group B), which killed experimental noble crayfish within a week from the exposure to comparable concentrations of spores (Jussila et al., 2013; Makkonen et al., 2012, 2013, 2014). Actually, it might be as low as that of strains of the genotype group A isolated from infected *A. astacus* in Fennoscandia (see Makkonen et al., 2012, 2013, 2014). However, variation in *A. astaci* virulence evaluated by experiments with susceptible crayfish might not be relevant for other potential host taxa, so direct comparative experiments exposing the same hosts to various *A. astaci* strains should be eventually carried out.

The results indicate that the shrimps are more resistant to the *A. astaci* strain used in our study (genotype group D) than the susceptible host crayfish *A. astacus*. The minor differences in experimental design between small-bodied shrimps and large-bodied crayfish are unlikely to explain the difference. On the one hand, there were 2.5 times more zoospores in vessels with crayfish than in those with shrimps (due to higher volume in crayfish vessels), and thus more spores could chemotactically seek crayfish. On the other hand, water in the vessels with crayfish became aerated six hours after spore addition. This could influence the likelihood of successful infection of the crayfish, since agitation may induce zoospore encystment in *A. astaci* (Cerenius et al., 1988).

Uropods and telsons of *M. dayanum* individuals were chosen for microscopic examination because soft cuticle can be dissected easily from them and we presumed that the pathogen would start the infection in soft cuticle, as in crayfish (see e.g., Unestam and Weiss, 1970; Oidtmann et al., 2006). However, no hyphae were observed in the samples despite the subsequent qPCR indicating *A. astaci* DNA presence in some of the individuals. Thus, we either overlooked hyphae in the examined cuticle or the *A. astaci* DNA was present on or in other parts of the body (e.g., soft cuticle in joints). Both explanations are possible; according to our previous experience with infected crabs, it is very easy to overlook even a dense net of *A. astaci* hyphae in the cuticle (Svoboda et al., 2014). Similarly, microscopic examination of cuticle failed to detect *A. astaci* hyphae in moribund stone crayfish *Austropotamobius torrentium* collected during a crayfish plague outbreak (Kozubíková et al., 2008).

As we presumed, shrimps were able to remove any attached cysts and possibly shed hyphae in their old cuticle through moulting. We assume that different frequency of moulting (with values expectable under our experimental conditions; Jiří Patoka and Pavel Šablatura, pers. comm.) may be the reason for the difference in patterns observed for *M. dayanum* and *N. davidi*. Other differences between the two species may be related to different body size. Although zoospores were probably chemotactically attracted to both shrimp species (see Cerenius and Söderhäll, 1984a; Unestam, 1969a) regardless of their body size, the results indicate that body surface area might have mattered. The amount of *A. astaci* DNA quantified in exuviae of approx. 3× smaller *N. davidi* was consistently about an order of magnitude lower than in larger *M. dayanum* individuals (Fig. 1E vs. F), which had correspondingly larger body surface available for spore attachment.

The steady decrease of *A. astaci* DNA quantified in exuviae and bodies of *N. davidi* and in exuviae of *M. dayanum* during the first four weeks of the experiment, and the apparent purgatory effect of moulting, suggest that *A. astaci* DNA isolated from tested shrimps originated mostly from the zoospores added at the beginning of the experiment. These could be attached to body or exuvial surface, and in case of whole shrimp bodies, also in the intestinal tract. However, the patterns observed for the bodies and exuviae of *M. dayanum* sampled after the fourth week of the experiment suggest that growth of *A. astaci* might have occurred in some individuals. The amount of *A. astaci* DNA detected in bodies of *M. dayanum* that had not moulted in the experiment was not decreasing despite the passing time and repeated water exchange. Even at the end of the experiment, when the maximal amount of *A. astaci* DNA in inert substrate corresponded to approximately 17 C, one non-moulting *M. dayanum* individual carried more than 1000 C. The results, however, cannot be considered a conclusive evidence for *A. astaci* growth in the shrimps because the alternative that detected *A. astaci* DNA originated from the added zoospores cannot be completely rejected without longer-term experiments and/or additional evidence such as histology.

A. astaci growth seems also a likely explanation of very high and high agent levels corresponding to more than 7000 C, which were detected in some *M. dayanum* exuviae more than six weeks since the zoospore addition. Nevertheless, these results may be at least partly explained by colonization of shrimp exuviae and subsequent growth during the three days between the moult and exuviae sampling. As there are significant similarities in the immune systems of crayfish and shrimps (Sritunyalucksana and Söderhäll, 2000), and crayfish immune reactions against the crayfish plague pathogen involve haemocytes (Söderhäll and Cerenius, 1999), we assume that *A. astaci* may grow in exuviae, unrestricted by the immune system. Similarly, *A. astaci* can grow in artificial media not containing any crayfish tissues (see e.g., Alderman and Polglase, 1986). Furthermore, the pathogen can also grow in some other animal tissues with impaired immunity; it was observed even in fish scales *in vitro* but not *in vivo* (Häll and Unestam, 1980; Oidtmann et al., 2002).

5. Conclusions

The tested strain of *A. astaci* did not cause mortality of studied freshwater shrimps. Thanks to frequent moulting, they are apparently able to remove the attached spores or substantially reduce the infection (if present). However, high levels of *A. astaci* DNA detected from *M. dayanum* exuviae suggest at least short-term growth of *A. astaci* on this substrate, and it seems possible that some growth also occurred in non-moulting individuals of this species. Further experiments, preferably focusing on shrimps with lower moult frequency, are thus needed to investigate a potential of long-term *A. astaci* persistence in freshwater shrimp populations, and their potential ability to infect other hosts. Furthermore, other genotypes of *A. astaci* might be tested as well, as the virulence varies among different strains of this pathogen species. Our study also demonstrates that *A. astaci* DNA originating from the pathogen zoospores can be detected after several weeks on substrates not allowing its growth. Studies relying on molecular detection methods should take this into account, particularly if working with unusual host species and detecting relatively low agent levels.

Conflict of interest statement

The authors declare no conflict of interest. The study sponsors had no role in the study design, data collection, analysis and interpretation, writing of the manuscript, and the decision to submit the manuscript for the publication.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2014.07.004.

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Resistance to the crayfish plague pathogen, *Aphanomyces astaci*, in two freshwater shrimps: Electronic Supplementary Material:

Phenol-chloroform extraction protocol

Ethanol from Eppendorf tubes, in which the shrimp bodies were preserved, was poured away, and samples were dried for 30 min at 60 °C. Tissues were ground with a sterile micropestle until the material was completely crushed. Then, it was mixed with 400 µl of Queen's buffer (1.21 g Tris(hydroxymethyl)aminomethane hydrochloride, 0.58 g NaCl, 3.73 g EDTA, 10 g n-lauroylsarcosine sodium salt and ultrapure water were mixed to obtain 100 ml of the buffer) and 40 µl of proteinase K solution (10 $mg \cdot ml^{-1}$; Qiagen). After the addition of 100 µl of 10% sodium dodecyl sulfate (SDS) solution and mixing, the tubes were incubated for 12 hours at 37 °C. Then, 300 µl of phenol and 300 µl of chloroform were added and mixed. After centrifugation (2 min, 2000 g), 570 µl of the upper phase was transferred to a new Eppendorf tube, 600 µl of chloroform was added, mixed by vortexing and centrifuged (2 min, 2000 g). 470 µl of the upper phase was transferred to a new tube, and addition of chloroform, mixing and centrifugation was repeated. Then, 370 µl of the upper phase was transferred to a new tube, 180 µl of ice cold (−20 °C) 3M sodium acetate was added, and the rest of the tube volume was filled with ice cold 99.9% ethanol. After mixing and two-hour incubation at −20 °C, the tubes were centrifuged (13,000 g, 35 min) to pellet the DNA, and the ethanol was removed without disturbing the DNA pellet. The pellet was rinsed in 1 ml of ice cold 70% ethanol and centrifuged (13,000 g, 10 min). The supernatant was carefully discarded, and the DNA pellet was dried for one hour at 40 °C before re-suspension in 100 µl of AE buffer (Qiagen).

Chapter 4

Svoboda, J., Kozubíková-Balcarová, E., Kouba, A., Buřič, M., Kozák, P., Diéguez-Uribeondo, J., 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.**

"Experimental transmission of *Aphanomyces astaci* from infected *Orconectes limosus* to uninfected crayfish individuals was monitored by molecular detection in crayfish tissues and by molecular detection of spores filtered regularly from the water."

Temporal dynamics of spore release of the crayfish plague pathogen from its natural host, American spiny-cheek crayfish (*Orconectes limosus*), evaluated by transmission experiments

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SUMMARY

The crayfish plague pathogen, *Aphanomyces astaci*, is one of the most serious threats to indigenous European crayfish species. The North American invasive spiny-cheek crayfish, *Orconectes limosus*, is an important source of this pathogen in central and western Europe. We evaluated potential changes in *A. astaci* spore release rate from infected individuals of this species by experiments investigating the pathogen transmission to susceptible noble crayfish, *Astacus astacus*. We filtered defined volumes of water regularly to quantify spore concentration, and sampled crayfish tissues at the end of the experiment. The filters and tissues were then tested for the presence of *A. astaci* DNA by species-specific quantitative PCR. Additionally, we tested the efficiency of horizontal transmission to apparently uninfected *O. limosus*. The experiments confirmed that *A. astaci* can be transmitted to susceptible crayfish during intermoult periods, and that the pathogen was more frequently detected in noble crayfish recipients than in American ones. The pathogen spore concentrations substantially varied in time, and significantly increased during moulting of infected hosts. Our study strengthens the evidence that although the likelihood of crayfish plague transmission by water transfer from localities with infected American crayfish might increase when these are moulting or dying, no time-periods can be proclaimed safe.

Key words: *Aphanomyces astaci*, *Orconectes limosus*, *Astacus astacus*, crayfish plague transmission, real-time PCR, host moulting, sporulation.

INTRODUCTION

The oomycete *Aphanomyces astaci* Schikora, the causal agent of crayfish plague, is one of the most serious threats to European indigenous crayfish species (ICS) (Füreder, 2006; Holdich *et al*. 2009). Although a few cases of long-term co-existence of populations of ICS and *A. astaci* in nature have been documented recently (Jussila *et al*. 2011; Viljamaa-Dirks *et al*. 2011; Kokko *et al*. 2012; Svoboda *et al*. 2012), infected individuals of susceptible crayfish species die in most cases within a few days or weeks after getting infected with this parasite (e.g. Unestam, 1969*b*; Vey *et al*. 1983; Alderman *et al*. 1987). In contrast, individuals of the 3 most widespread non-indigenous crayfish species (NICS) in Europe, the signal crayfish *Pacifastacus leniusculus* (Dana), the spiny-cheek crayfish *Orconectes limosus* (Rafinesque), and the red swamp crayfish *Procambarus clarkii* (Girard), often host the crayfish plague pathogen as a chronic infection and transmit it to indigenous crayfish species (ICS) (Holdich *et al*. 2006). The transmission of the disease occurs when pathogen zoospores are released from infected crayfish (Söderhäll and Cerenius, 1999; Oidtmann *et al*. 2002), and these encyst and subsequently germinate on a new host's body surface. A few spores of *A. astaci* (motile zoospores or short-lived cysts formed by these, from which a new zoospores may re-emerge) may survive *in vitro* for at least 2 months at low temperatures (2 °C) (Unestam, 1966), and for at least 2 weeks at 15 °C (Cefas, 2000). However, susceptible crayfish could not be infected by spores kept for 15 days at 14 °C, or for 9 days at 10 °C (Unestam, 1969*a*; Matthews and Reynolds, 1990). Therefore, water containing *A. astaci* spores but no crayfish is a potential source of infection only for a few days. Consequently, the timing of zoospore release determines when the crayfish plague pathogen can be spread by water previously in contact with infected crayfish.

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Based on transmissions of the disease from American to susceptible crayfish hosts, it has been concluded that spores are released from infected NICS when the host crayfish is dying, moulting, stressed or has an impaired immunity (Persson and Söderhäll, 1983; Diéguez-Uribeondo and Söderhäll, 1993; Söderhäll and Cerenius, 1999; Vogt, 1999; Oidtmann *et al*. 2002; Cerenius *et al*. 2003). However, pathogen spores also may be released from NICS more or less continuously, although in varying amounts, as mentioned by, for example, Söderhäll and Cerenius (1999). As spores of *A*. *astaci* cannot be distinguished from related oomycete species by morphology (Cerenius *et al*. 1988; Oidtmann *et al*. 1999), their presence in water with crayfish could have been deduced only from infection and subsequent death of susceptible crayfish. Indeed, crayfish plague was transmitted to susceptible hosts even from apparently healthy and non-moulting NICS (Diéguez-Uribeondo and Söderhäll, 1993). However, the likelihood of infection depends not only on spore presence and concentration but also on other factors such as water temperature (Diéguez-Uribeondo *et al*. 1995), host species (Unestam, 1969*b*; Alderman *et al*. 1987) or population of host origin (Makkonen *et al*. 2012). Transmission experiments without direct quantification of pathogen spores could thus provide valuable but incomplete information regarding the actual rate and timing of spore release.

Molecular methods developed in the last several years that allowed direct detection of the presence of *A*. *astaci* by species-specific amplification of the pathogen DNA (e.g. Oidtmann *et al*. 2006; Vrålstad *et al*. 2009) opened new options for both field and experimental studies. In particular, the use of quantitative PCR facilitated quantification of *A*. *astaci* spores in water (Strand *et al*. 2011). Thanks to this possibility, and due to the lack of quantitative information about *A*. *astaci* spore release from NICS, our study and the one by Strand *et al*. (2012) were conducted. These 2 independent studies both focused on *A*. *astaci* spore release but differed in the experimental design and used crayfish hosts. In the case of infected signal crayfish *P*. *leniusculus* studied by Strand *et al*. (2012), continuous sporulation from a host individual and higher spore release from a dying host were confirmed; however, increased sporulation from a moulting host was not significant (Strand *et al*. 2012).

The main aim of our study was to evaluate, in aquarium transmission experiments, the changes in *A*. *astaci* spore release rate from infected spiny-cheek crayfish *O*. *limosus*. This NICS is one of the most widespread invasive crayfish in Europe (Holdich *et al*. 2009) and an important carrier of crayfish plague (Kozubíková *et al*. 2009; Pârvulescu *et al*. 2012). We focused not only on potential changes in spore release rate, but also on the success of pathogen transmission to susceptible noble crayfish co-habiting the experimental aquaria, with regard to death or moulting of the American host. Experimental transmission of *A*. *astaci* from infected *O*. *limosus* to uninfected crayfish individuals was monitored by molecular detection in crayfish tissues and by molecular detection of spores filtered regularly from the water. Furthermore, we also evaluated the success of experimental transmission of the pathogen from infected individuals of *O*. *limosus* to presumably uninfected ones.

MATERIALS AND METHODS

Experimental setup

This study consists of 3 experiments with transmission of *A*. *astaci* from *O*. *limosus*: (1) transmission to *A*. *astacus* (in 2008), (2) transmission to *A*. *astacus* supplemented by water sampling in order to quantify *A*. *astaci* spore concentrations (in 2009) and (3) transmission to uninfected *O*. *limosus* (in 2008). The transmission of *A*. *astaci* to *A*. *astacus* (Experiments 1 and 2) was evaluated in 25 experimental aquaria (10 in 2008 and 15 in 2009), transmission to *O*. *limosus* (Experiment 3) in 10 aquaria. At the beginning, each aquarium was filled with 5 L of aged tap water (left for over 24 h at room temperature in open containers). Then, it was stocked with 2 crayfish: 1 *O*. *limosus* infected with the crayfish plague pathogen ('donor') and 1 'recipient', i.e. an uninfected *A*. *astacus* (Experiments 1 and 2) or an uninfected *O*. *limosus* (Experiment 3).

The spiny-cheek crayfish were chosen as donors because the species seems to be the most important source of the crayfish plague pathogen in the Czech Republic, being the most widespread NICS in the country (Petrusek *et al*. 2006), and the pathogen prevalence in its populations is often high (Kozubíková *et al*. 2009, 2011*b*). The donor spiny-cheek crayfish were caught in a pond in Smečno (50°11′N, 14°03′E) where the prevalence of *A*. *astaci* consistently reached almost 100% over several years (Kozubíková *et al*. 2011*a*; Matasová *et al*. 2011). The presence of the pathogen in donors was verified by real-time PCR both before (by analysis of one half of a uropod taken from each donor) and after the experiment (by analysis of up to 50 mg of a homogenized tissue mixture containing soft abdominal cuticle, 1 uropod, 1 eye stalk and all visually noticed melanized spots). The presence of the pathogen DNA was unambiguously confirmed in all such samples of donor tissues sampled at the end of the experiment, and in all but 1 uropod samples. With regard to the low probability of *A*. *astaci* transmission among aquaria (see below), we conclude that all donors had been infected from the start of the experiments.

Noble crayfish used as recipients originated from the Světlohorská reservoir (49°00′N, 13°04′E). The source population may be considered plague-free, as it has had a relatively high and stable population

density since at least 2002 (unpublished data from a regular yearly monitoring by P. Kozák and co-workers), and no crayfish mass mortality has been observed at this locality or anywhere in adjacent regions in recent decades. All noble crayfish looked healthy at the beginning of the experiment. Spiny-cheek crayfish used as recipients were captured in the flooded quarry near Starý Klíčov (49°24′N, 12°58′E) where the prevalence of *A. astaci* seems to be extremely low (the pathogen DNA was detected only in 1 out of 132 tested individuals; Matasová *et al.* 2011). No pathogen DNA was detected in the uropods cut from any of the used recipient individuals before the experiments.

Recipients and donors were separated by use of a metal grid that allowed water exchange but prevented direct physical contact. The aquarium was aerated (which also stimulated water flow), and crayfish were fed with carrot once a week. Accidental transmission of pathogens among aquaria was prevented by using disposable gloves during any manipulation, cleaning tools with bleach after each use, and covering the aquaria with glass to eliminate the spread of aerosols. Furthermore, there were 4 control aquaria in each experimental setup containing 1–3 noble crayfish individuals handled in the same way as the experimental aquaria to test for such contamination. The experiments started in June and lasted either 72 or 87 days (in 2008 and 2009, respectively). At the end of this period, the surviving donors were removed and surviving recipients were kept in aquaria for 12 more days to allow potential infections to develop.

Every day, water temperature (fluctuating between 19 and 23 °C) was measured, and crayfish and water quality were checked visually. In the case of increased turbidity, half of the aquarium volume was replaced with aged tap water. Exuviae and cadavers of recipients were removed immediately after being found during daily inspections, whereas those of donors were kept in aquaria for a few days to enable possible spore release. An exuvia was left in an aquarium for 4 days, while cadavers were kept only for 1 (in 2008) or 2 days (in 2009). Eventually, all cadavers and exuviae were removed, their surface washed with tap water, and they were kept separately deep-frozen (at −80 °C) until dissection.

In 2008, we evaluated only the success of crayfish plague transmission and thus only crayfish tissues were analysed for the presence of *A. astaci* DNA (Experiments 1 and 3). In 2009, the sampling was extended to detect pathogen spores in the aquarium water (Experiment 2). Samples of water from all aquaria were filtered approximately once a week to test for the presence of *A. astaci* spores. Furthermore, a sample was filtered from an aquarium every day when there was a cadaver or an exuvia of the donor. These samples were taken to evaluate potential changes in spore release rate according to the donor's condition (death, moulting, inter-moult period). At each time-point for each aquarium, up to 100 ml of water collected several millimetres below the water surface was filtered using a syringe (Omnifix, volume 50 ml) through a polycarbonate filter (Whatman Nucleopore, diameter 25 mm, pores 2 μm) held in filter holders (Swin-Lok, Whatman). The total filtered volume (lower than 100 ml if filter clogging occurred) was always noted and used for subsequent calculations. Filters were handled by sterile tools and stored at −80 °C until molecular analysis; syringes and filter holders were sterilized by bleach after every use. In total, 11–14 filters were prepared from each aquarium.

Molecular detection of A. astaci

Both types of sample (crayfish tissues and filters) were tested for *A. astaci* presence by the real-time PCR detection of *A. astaci* DNA. Soft abdominal cuticle, 1 uropod, 1 eye stalk and all noticed melanized spots were dissected from each crayfish, as these body parts have been reported as being most often infected by the pathogen (Oidtmann *et al.* 2004, 2006; Vrålstad *et al.* 2011). All the tissues were crushed together in liquid nitrogen and up to 50 mg of the mixture were used for subsequent DNA isolation using the DNeasy Animal Tissue kit (Qiagen). DNA from one half of each filter was isolated as described by Strand *et al.* (2011). Negative controls, i.e. tubes containing distilled water only and no crayfish tissues, were included in every DNA isolation batch.

The pathogen DNA was quantified in all isolates by real-time PCR according to Vrålstad *et al.* (2009); any amount above the limit of detection was considered as a positive detection of *A. astaci* in the respective sample. To reduce potential PCR inhibition, TaqMan Environmental Master Mix (Applied Biosystems) was used as recommended by Strand *et al.* (2011). Negative controls were included in all real-time PCR runs. Moreover, we tested for the inhibition for each sample by the real-time PCR analysis of 10× diluted isolates. If there is no inhibition, the cycle threshold (Ct) (i.e. an extrapolated cycle number when the fluorescence signal in real-time PCR exceeds a threshold value), differs between undiluted and 10× diluted isolates by a value reflecting the dilution and efficiency of the PCR run (approaching a theoretical value of 3·32 in the case of 100% efficiency). When the PCR with undiluted samples is inefficient due to the presence of inhibitors, it improves with dilution, and the differences in Ct values become substantially smaller. Neglecting variation of up to 15% (see Kozubíková *et al.* 2011*b*), we did not detect any case of serious PCR inhibition in our study.

To estimate the number of spores in filters, and quantify the amount of pathogen genomic units in crayfish tissues, we assessed the number of

PCR-forming units per spore (PFU_{spore}) for 2 different strains of *A. astaci* (Evira4806a/07 and SAP880). The former strain was isolated from Smečno, i.e. the same population used as the source of donors (Kozubíková *et al.* 2011*a*), and is deposited at the OIE Reference Laboratory for Crayfish Plague, Finnish Food Safety Authority Evira, Kuopio, Finland. The latter was isolated from *Procambarus clarkii* and kept at the Department for Mycology, Royal Botanical Garden CSIC, Madrid, Spain. Spore suspensions were prepared, counted in a haemocytometer, diluted and suspension volumes containing *c.* 100, 1000 and 10000 spores were filtered in 9 replicates for each dilution and strain. DNA was isolated from the filters and quantified as described above. We pooled the results from both *A. astaci* strains, as the arithmetic mean and median of PFU_{spore} differed negligibly (2·5%) between them. The distribution of the data was positively skewed, so the median was used for subsequent calculations. A conservative estimate of the limit of detection (5 PFU per reaction) from the filters corresponded to a concentration of 254 spores L^{-1}.

Statistical analysis

We tested whether the probability of crayfish plague transmission (i.e. *A. astaci* detection in recipient tissues) was higher in those aquaria in which donors moulted or died if compared with the other aquaria. The hypothesis was tested using one-tailed Fisher's exact test comparing the number of cases when transmission to recipient was detected for aquaria in which the respective event occurred and in which it did not, separately for aquaria in which the donor moulted and in which it died. One aquarium in which the donor both moulted, and 9 weeks later died, could be included in both tests as no *A. astaci* was found in the recipient tissues. One-tailed Fisher's exact test was used also to test the hypothesis that the probability of pathogen transmission is higher for the noble crayfish than for the spiny-cheek crayfish recipients, using the data from all experimental aquaria together.

To discover whether the anticipated changes in spore release rate coincide with death or moulting of a host, the results from *A. astaci* detection in filter samples were divided according to death and moulting of the donor crayfish. For the purpose of statistical analyses (i.e. permutation tests described in the paragraph below), we defined the period of donor moulting as being 1 week before the exuvia was taken out of the aquarium (i.e. the day when the exuvia was found, and 3 days before and 3 days after). Data on spore counts on filters from this period were compared with those from periods when the donor was present in the respective aquarium but no crayfish (neither donor nor recipient) was dying. Similarly, the period of the donor death was defined as 1 week before the cadaver was removed (i.e. the day when the cadaver was found, 5 days before and 1 day after that), and this was compared with periods of donor presence, excluding its moulting and eventual recipients' death. The period of the recipient's death was defined as 1 week before the cadaver was removed (i.e. the day when the cadaver was found and 6 days before), and it was compared with other periods of donor presence, excluding its moulting or death, if relevant for the respective aquarium.

To test the hypotheses that either (i) the likelihood of a positive detection of *A. astaci* spores on filters or (ii) their number quantified by real-time PCR increases in the period of donor moulting, we used permutation tests designed for this purpose. The first test, evaluating only the likelihood that *A. astaci* DNA is detected from filters (i.e. only the positive/negative detection but not quantity), was designed as follows. For each aquarium in which the donor moulted, we calculated what proportion of filters with positive *A. astaci* detection came from the period of moulting, and these proportions were summed across all relevant aquaria. Subsequently, we randomly re-ordered the results of all tests within each aquarium, and calculated the same test statistics; this randomization was repeated 1000 times. The statistics obtained from real data were then compared with the distribution of results of randomized datasets to obtain the *P*-value. The test comparing the quantity of detected spores in the period of moulting and other periods was performed in a similar manner but instead of summing the numbers of positive detections, we summed the calculated spore concentrations (spores L^{-1}) from all filters. The hypotheses that spore concentrations (and likelihood of spore detections, respectively) increase during the periods of donor death or infected recipient death were tested the same way, with the time-periods of interest defined above.

RESULTS

Analysis of negative controls

We detected no *A. astaci* DNA during analyses of negative controls (from DNA isolation and real-time PCR), or from isolates from filters and tissues of *A. astacus* from control aquaria. We thus conclude that the results were not influenced by cross-contamination of experimental aquaria or by laboratory contamination.

Transmission of A. astaci *between crayfish*

Aphanomyces astaci was transmitted to a noble crayfish in 3 out of 4 aquaria (75%) in which the donor moulted, in 1 out of 4 aquaria (25%) in which the donor died, and in 8 out of 18 aquaria (44%) in

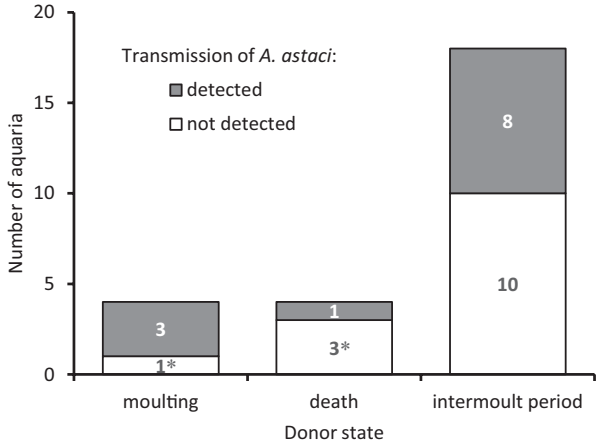

Fig. 1. Detection of *Aphanomyces astaci* DNA in tissues of recipients (*Astacus astacus*) at the end of the experiment, which was considered as the direct evidence of successful crayfish plague transmission. Numbers of aquaria in the respective categories are given in columns. Only those cases of moulting and death of donors that occurred while the recipient crayfish was present in the respective aquarium were included. An asterisk indicates one aquarium in which the donor moulted and 63 days later died; for the purpose of the analyses these two events were considered separately. Data from Experiments 1 and 2 (from 2008 and 2009) are pooled.

which the donor neither moulted nor died (Fig. 1). In 6 of these 8 aquaria the donors survived without moulting until the end of the experiment (i.e. 61, 57, 56, 41, 29 and 10 days after the death of the recipient). Nevertheless, neither in aquaria in which the donor moulted nor in those in which it died was the rate of crayfish plague transmission significantly higher than in the others (one-tailed Fisher's exact tests, $P > 0{\cdot}29$). In the experiment with transmission to *O. limosus*, the pathogen was found in the recipient's tissues only in 1 out of 10 aquaria, the only aquarium in which the donor moulted. The rate of transmission to *A. astacus* (48%) was thus significantly higher than to *O. limosus* (one-tailed Fisher's exact test; $P = 0{\cdot}039$).

Detection of pathogen spores from water samples

The release of *A. astaci* spores into aquarium water, as monitored by quantification of the pathogen DNA on the filters, was mostly consistent with the results of the transmission to susceptible hosts. The pathogen spores were detected in 12 aquaria and *A. astaci* was detected in the tissues of 9 out of the 12 recipients. Detailed results including the temporal dynamics of spore quantification and state of both donor and recipient crayfish in each aquarium are shown in Supplementary Fig. S1 (in Online version only).

Spore concentrations in water were below the detection limit (254 spores L^{-1}) for most of the time in all 15 aquaria where spore concentrations were measured, and no pathogen DNA was detected in 85% (154 of 181) of the filters obtained during the presence of donors. The pathogen DNA was detected in 27 filters, from which 14 (52%) came from the periods of moulting and death of donors and death of infected recipients. The detected concentrations of *A. astaci* DNA corresponded to less than 10^5 spores L^{-1} in all but 3 cases (1 from the period of recipient's death: 150 000 spores L^{-1}, 2 from the period of donor moulting: 961 000 and 131 000 spores L^{-1}).

The results of the detection *of A. astaci* DNA on filters in the 2 weeks preceding and following moulting of donor crayfish, or death of either donor or recipient, are shown in detail in Fig. 2. *Aphanomyces astaci* spores were detected in the period of donor moulting in all 3 aquaria in which this event occurred during Experiment 2, while no such detection was recorded in the same aquaria in the weeks before moulting, or more than 1 week after moulting (Fig. 2A). The frequency of positive detection as well as spore concentrations detected from filters in the moulting period were significantly higher than in other weeks of the experiment ($P < 0{\cdot}001$).

The results of *A. astaci* detection in filters prepared around the death of the 9 recipient noble crayfish in whose tissues *A. astaci* was detected (Fig. 2B) also confirm substantial release of pathogen spores in this period. The spore concentration in aquarium water exceeded the limit of detection in 7 out of 10 samples filtered within 1 week before the death of the recipient, or on the day it died (Fig. 2B and Supplementary Fig. S1 – in Online version only). Proportions of positive detections from filters, as well as of spore concentrations in the water, were significantly higher in this period than in the preceding weeks of the experiment ($P < 0{\cdot}006$).

In periods of donor deaths (i.e. 6 days before the death and 1 day afterwards), neither the rates of spore detection nor concentrations were significantly higher than in the preceding weeks ($P = 0{\cdot}35$; Fig. 2C). No spores were detected in this period in 2 of the 3 aquaria in which the donors died. This was despite the fact that the amounts of *A. astaci* DNA detected in the donor tissues in all 3 aquaria were similar (in the 2 aquaria in which spores were not detected, the pathogen loads in donor tissues reached 81 and 125% of that in the tissues of the donor in the third aquarium).

DISCUSSION

Our study shows that the molecular detection of *A. astaci* spores is useful in experimental work to further elucidate the dynamics of crayfish plague pathogen transmission. We provided clear evidence that *A. astaci* may be frequently transmitted from *O. limosus* to a susceptible crayfish during the intermoult period (i.e. when the infected host is neither moulting nor dying), and directly confirmed that the

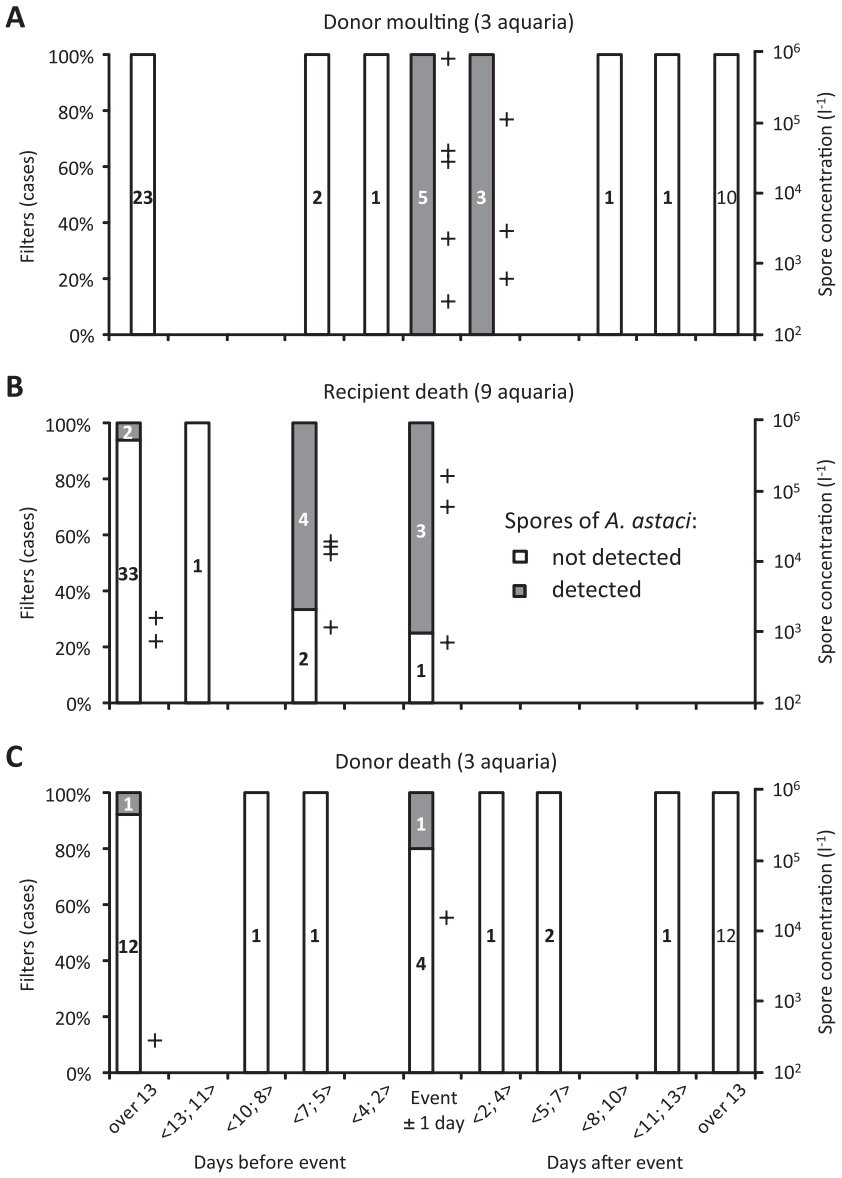

Fig. 2. Detection and quantification of *Aphanomyces astaci* spores on filters during Experiment 2. Numbers in the columns give the number of analysed filters (from each aquarium no more than 1 filter per day was prepared), spore concentrations assessed from filters on which *A. astaci* DNA was detected are shown next to the respective columns as crosses. Data are divided into intervals of 3 days from the day of: (a) moulting of donors, (b) death of infected recipients or (c) death of donors. Only results outside the other two evaluated periods are included in each graph (e.g. (a) does not include data from periods of infected recipient and donor deaths). The likelihoods of positive detections, as well as the detected spore concentrations, were significantly higher in periods of donor moulting (a) and infected recipient deaths (b) but not in periods of donor death (c).

moulting of infected host crayfish is accompanied by a significant increase in pathogen spore release. Thus, despite substantial variation in detected spore release rate, the results of infection experiments confirm that no period can be regarded as 'safe' when the potential for disease transmission to susceptible crayfish species is considered.

Aphanomyces astaci detection in recipient tissues confirms that the pathogen was transmitted from *O. limosus* to *A. astacus* during inter-moult periods. This is consistent with results of experimental studies of *P. leniusculus* and *P. clarkii* (Diéguez-Uribeondo and Söderhäll, 1993; Strand *et al.* 2012). As we were not able to detect spore concentrations below the detection limit of *c.* 250 spores L^{-1}, we cannot assess whether spores were released from infected donors continuously in small amounts or only intermittently. The source of the spores detected in water usually could not be unambiguously assigned, because the spores could have been released not only from the donor but also from an already infected recipient, which is particularly likely in the days immediately preceding their death (see Makkonen *et al.* 2013). However, in one aquarium (no. 1), the source of the detected spores (up to 3450 spores L^{-1}) must have been the donor because more than 1 month

separated the recipient's death and the filtration, and there were no spores detected in 10 filters prepared from this aquarium in the weeks before and after spore detection.

Despite the relatively high limit of detection, these results prove that the spore release rate may vary in time at least by 1 order of magnitude. Furthermore, we observed large changes in the number of quantified spores in short time-periods: for example, the detected concentrations dropped from more than 10^4 spores L^{-1} to below the detection limit within 2 days in aquarium no. 5, and *c*. 30-fold decrease in spore concentration was observed in samples from 2 consecutive days in aquarium no. 13. These results suggest that the period in which spores are detectable in water can be limited to less than a few days after their release. Therefore, while the number of detected spores on filters reflects the present concentration in water samples, it should not be considered as a sum of spores released in a longer period (as assumed by Strand *et al*. 2012). The decrease of spore concentration could have been partly caused by an uneven distribution of motile zoospores in the aquarium water (see Strand *et al*. 2012). However, the water in the aquaria was mixed by aeration, so this decrease should rather be attributed either to zoospore death or attachment to solid surfaces. This is in accordance with previous studies, in which active motility of zoospores at room temperatures lasted for about 2 days (Unestam, 1966; Alderman and Polglase, 1986).

Strand *et al*. (2012) documented that spores are released continuously or very frequently from infected *P. leniusculus*; they estimated that *c*. 2800 spores were weakly released by an infected crayfish individual that was neither moulting nor dying (which, given a weekly sampling interval, was likely to be a gross underestimate of the actual spore release). In our aquaria, the release of 2800 spores would correspond to concentrations of *c*. 560 spores L^{-1}, a number well above our limit of detection. However, we did not detect *A. astaci* in 92% of the filters from inter-moult periods (130 samples). While the apparent differences between our results and those of Strand *et al*. (2012) may be due to methodological issues (experimental design, efficiency of spore detection, etc.), it is not unlikely that to some extent the intensity of sporulation is also influenced by the biology of respective crayfish hosts and pathogen strains, and their interactions.

The results of the detection of *A. astaci* in recipients' tissues and in filters correspond well to each other; the results (positive or negative) were congruent in both types of samples from 12 out of 15 aquaria. In 1 aquarium (no. 8) spores were detected only after the infected recipient's death. Nevertheless, considering the length of the motile period of spores (see above), spores could have been present in the aquarium in a detectable concentration even before the death, in a period when no sample was taken. In 2 aquaria (nos 5 and 10), spores were detected in the water although DNA of *A. astaci* was not found in tissues of recipients, which lived for further 23 and 13 days, respectively. Nevertheless, the absence of *A. astaci* in samples from the 2 crayfish individuals does not necessarily mean that the pathogen had not been transferred in these cases. Alderman *et al*. (1987) have shown that even mortality of susceptible species can be delayed if the concentration of spores is low, so the pathogen could have grown in the 2 recipients' body parts that were not used for detection. Altogether, our data suggest that transmission to susceptible recipients occurred most likely during short-time bursts of spore release so intensive that spore concentrations exceeded the limit of detection. Such events could occur in periods when the pathogen growth is not sufficiently inhibited by the host's defence reactions, or in exuviae.

Statistical tests confirmed a significant increase in the spore release rate during moulting of infected American host crayfish. It is worth noting that moulting frequency is particularly high in fast-growing and relatively short-lived cambarid crayfish such as *P. clarkii* and *O. limosus*. These moult several times per season not only as juveniles but also as reproductively active males that, unlike *P. leniusculus*, exhibit cyclic dimorphism accompanied by moulting (Hobbs, 1974). On the contrary, *P. leniusculus* usually moults only once per season when aged 4 or more years (Lewis, 2002), an age rarely attained by invasive cambarids in Europe (Holdich *et al*. 2006; Chucholl, 2012). Thus, the dynamics of *A. astaci* spore release in populations of cambarid crayfish may be affected by frequent moulting events of infected individuals.

In 3 out of 4 aquaria in which donor *O. limosus* moulted, we also observed transmission of the crayfish plague to susceptible recipients. Nevertheless, the transmission rate in these aquaria was not significantly higher than in aquaria without moulting and death events, most likely due to the low number of replicates. In future studies, manipulations with the condition of infected hosts that would increase the chance that the studied event (i.e. moulting, death) occurs during the experimental period could increase efficiency of the laboratory work.

Detection in filters also suggested that spores are released in high concentrations from dying infected noble crayfish, as has been mentioned for ICS by previous studies (Söderhäll and Cerenius, 1999; Vogt, 1999; Nylund and Westman, 2000; Oidtmann *et al*. 2002) and experimentally quantified by Makkonen *et al*. (2013). Although the spores detected in the period of the recipient's death could have been released from donors, this seems unlikely because spontaneous release from non-moulting donors was rarely detected. Moreover, Makkonen *et al*. (2013) also observed a substantial release of *A. astaci*

zoospore in water tanks with infected noble crayfish in their pre-mortem phase.

Similarly, the crayfish plague transmission from dying NICS was mentioned by Oidtmann *et al.* (2002), and a significantly higher rate of sporulation from moribund and dead *P. leniusculus* was documented by Strand *et al.* (2012). In contrast, an increased sporulation rate was observed in only 1 out of 3 death events in our experiment (aquarium no. 5), and neither the probability of the pathogen transmission nor the detection of spores was significantly higher in the period of donor death. Nonetheless, even Strand *et al.* (2012) did not observe increased sporulation from over one-fifth of moribund individuals of *P. leniusculus* (4 out of 18 cases), so we do not consider the results of our study to contradict their study. Possibly, the observed variation in spore release among dying donors could be related to their health status or reason for death: *Aphanomyces astaci* spores are likely to be intensively released particularly from individuals with acute crayfish plague, which may occasionally develop also in NICS, for example under stress (Unestam and Södehäll, 1977; Persson and Söderhäll, 1983; Persson *et al.* 1987). Indeed, Vey *et al.* (1983) succeeded in transmitting crayfish plague from *O. limosus* only in a closed aquarium system where the pathogen rapidly caused their death. In our experiments, however, the amount of detected pathogen DNA was almost the same in the sampled tissues of all 3 donor cadavers while death of only 1 of them coincided with detection of spores. Thus, either acute plague was not the reason for the death of the one donor crayfish, or the increased growth of pathogen occurred only in tissues that were not analysed.

According to our results, the combination of spore filtration from water and subsequent real-time PCR quantification of spores seems to be very useful for experimental research. Nevertheless, it has already been pointed out that spores may not be detected in water samples even in their presence due to the concentrations being too low and the limitations of the sampling methods (Strand *et al.* 2011). Thus, the failure to detect *A. astaci* spores does not rule out the possibility that infected crayfish are present in the sampled water body. Indeed, in our experiment, spores were frequently not detected in water inhabited by infected spiny-cheek crayfish, despite the fact that spore concentrations exceeded the detection limit in the experimental aquaria in other periods. Therefore, when searching for spores released from NICS one should consider also possible changes in spore release rate. Our results certainly underestimate the frequency of spore release due to the relatively high limit of detection (that can be substantially influenced by the filtered volume of water). Thus, it is likely that an improved method combining filtering and molecular detection may be useful for environmental monitoring of *A. astaci* presence in the wild, as suggested by Strand *et al.* (2011, 2012). Nonetheless, while the detection of spores may not be successful in the case of their low densities in water, the analysis of crayfish tissue is not absolutely reliable either, especially if the prevalence of *A. astaci* in the host population is low (Kozubíková *et al.* 2011*b*). Thus, it would be useful to compare the sensitivity as well as the cost efficiency of both approaches in natural NICS populations.

Although our experiment was not specifically designed to test for the mechanisms allowing the persistence of *A. astaci* in infected NICS, the result from aquarium no. 13 showed that the pathogen could be detected in tissues of *O. limosus* sampled already 3 days after moulting (category A4, i.e. moderate pathogen level, according to Vrålstad *et al.* 2009). However, concentrations of 30 and 130 spores mL^{-1} were detected in the aquarium 1 and 3 days after the moulting, so we cannot distinguish whether the parasite remained in the body in spite of the moulting, the body was re-infected by spores released from exuvia after the moulting, or whether both processes played a role. In a natural *O. limosus* population (Pšovka Brook, Czech Republic), a decrease in prevalence of the pathogen during the season, explained as a potential influence of moulting, was detected (Matasová *et al.* 2011). However, such a decrease was not observed in the Smečno pond (Matasová *et al.* 2011), from which the donor crayfish originated, possibly because the average pathogen load at this locality was substantially higher, exceeding all other *O. limosus* populations that we have analysed so far (see Kozubíková *et al.* 2011*b*).

We detected transmission of the pathogen from infected to apparently uninfected *O. limosus* only in 1 out of 10 experimental aquaria. The rate of detectable transmission to spiny-cheek crayfish was significantly lower than to noble crayfish, which is in accordance with the relatively high resistance of North American and the high susceptibility of European crayfish species (Unestam, 1969*b*, 1975), as well as with previous experimental results (see Unestam, 1969*b*, 1975; Vey *et al.* 1983). However, our experiment is probably the first convincing experimental transmission among individuals of this species, because the recipients in previous studies (Schikora, 1916; Schäperclaus, 1935; Vey *et al.* 1983) may have already been infected before the experiments, and there was no reliable method to test for presence of the pathogen. Horizontal transmission of *A. astaci* among adult NICS may result not only in persistence of the pathogen within host populations, but possibly also in transmission of different strains of *A. astaci* upon contact of different populations or species of North American crayfish hosts (as has been suggested for example by Kozubíková *et al.* (2011*a*)).

Although our study focuses on the same topic, i.e. *A. astaci* spore release from NICS, as the recent study published by Strand *et al.* (2012), the design of

the experiments and the selected crayfish species and *A. astaci* strains differed. As the general patterns of sporulation of the two different *A. astaci* strains from the two NICS agree and are compatible with the previous results of crayfish plague transmission from *P. clarkii* (Diéguez-Uribeondo and Söderhäll, 1993), the conclusions seem to be valid for infected NICS in general. The pathogen spore release rate from NICS certainly changes substantially in time and elevated spore release frequently coincides with host moulting and sometimes with its death. The pathogen can be transmitted during inter-moult periods and therefore infected NICS should be regarded as a permanent source of infection. Further experiments focusing on *A. astaci* spore release from crayfish hosts may evaluate in more detail the key factors that affect its timing and magnitude.

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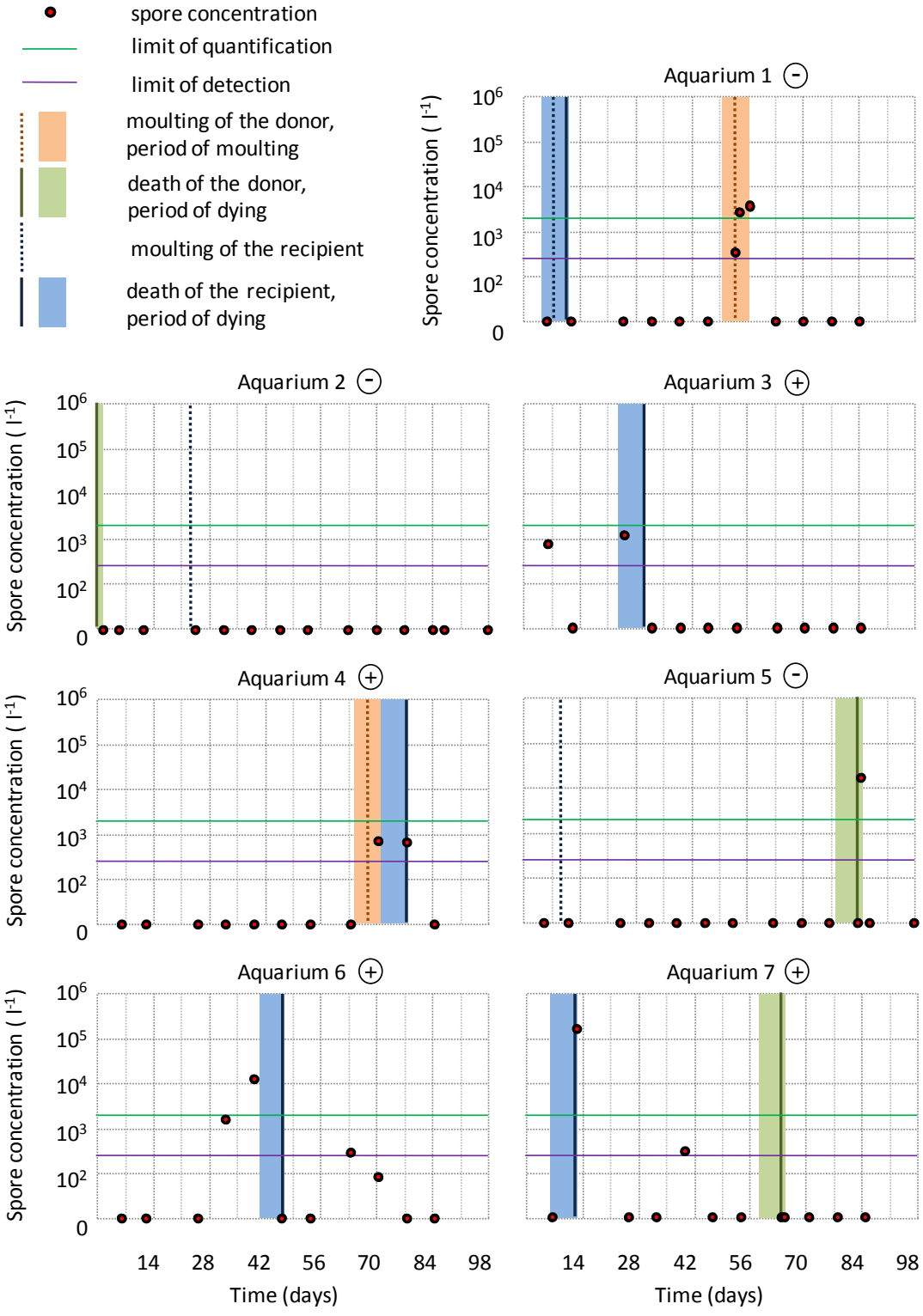
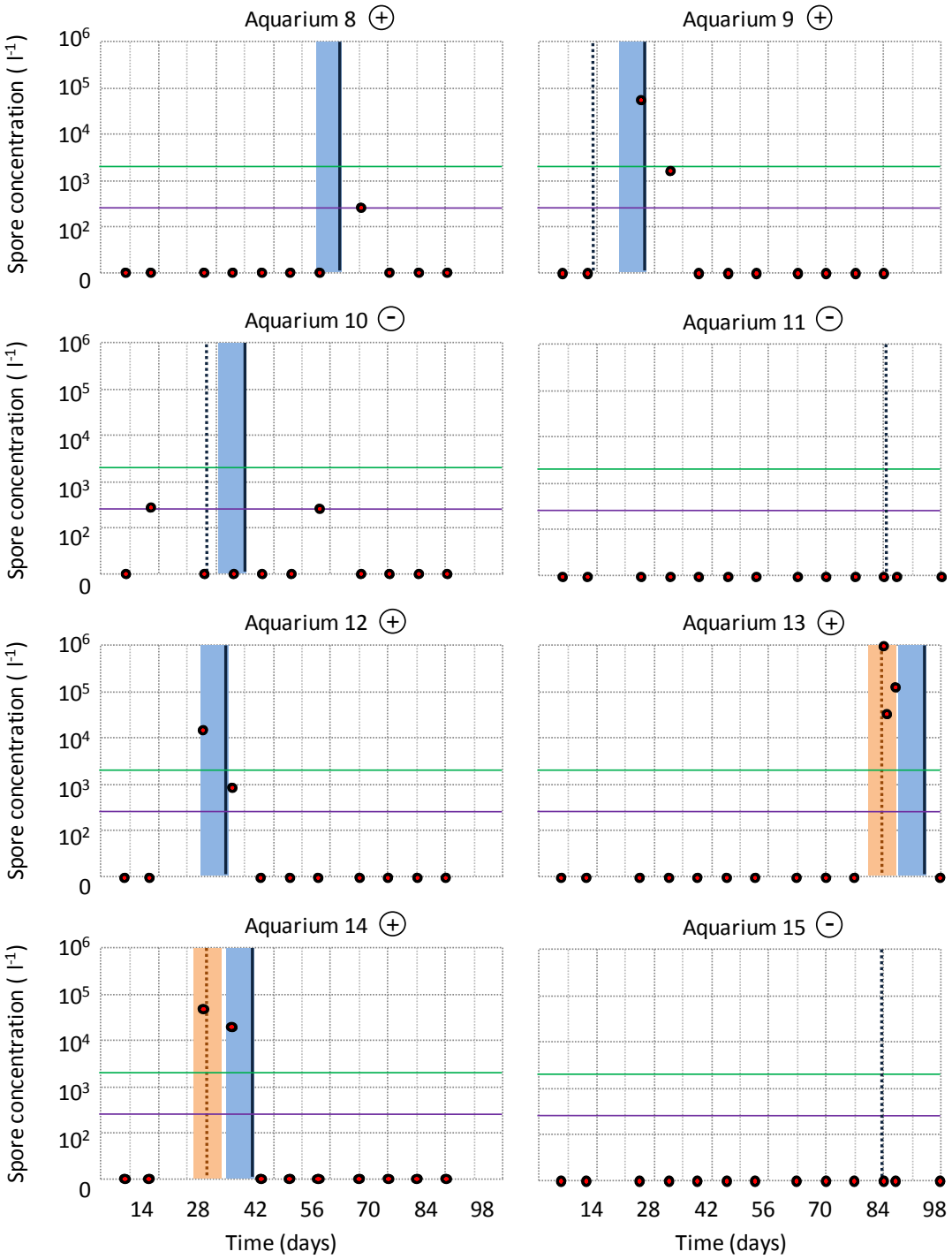
Supplementary Figure 1

Detection of *A. astaci* spores in water from experimental aquaria set up in the experiment 2 (in 2009). Detection limit and limit of quantification are indicated by horizontal lines, key events (deaths or moulting of donors or recipients) are indicated by vertical lines; coloured rectangles indicate time periods around these events considered for statistical anlayses (see Methods). Plus and minus sings in circles indicate for each aquarium whether *A. astaci* has been detected in tissues of recipients after their death or after the end of the experiment.



Continued on next page.

Supplementary Figure 1 (continued]



Chapter 5

Svoboda, J., Fischer, D., Kozubíková-Balcarová, E., Šťástková, A., Brůčková, M., Kouba, A., Petrusek, A. Experimental evaluation of the potential for crayfish plague transmission through the digestive system of warm-blooded predators.

unpublished manuscript

"The pathogen was neither isolated from predator excrements nor transmitted to susceptible crayfish exposed to the excrements."

Experimental evaluation of the potential for crayfish plague transmission through the digestive system of warm-blooded predators

(unpublished manuscript)

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Abstract

The crayfish plague pathogen (*Aphanomyces astaci*), is one of worst world invasive species. It has been shown that it can be transmitted through the digestive system of fish. The pathogen dispersal through mammalian and bird digestive tracts has mostly been considered unlikely but the available experimental evidence was not sufficiently convincing. Our study included a transmission experiment with a European otter (*Lutra lutra*) and an American mink (*Neovison vison*) fed with infected crayfish, and experiments testing survival of different strains of *A. astaci* on agar plates at temperatures corresponding to those inside mammal and bird bodies. The pathogen was neither isolated from predator excrements nor transmitted to susceptible crayfish exposed to the excrements. On agar, the pathogen occasionally survived for 15 min at 40.5 °C and for 45 min at 37.5 °C, but it always died when incubated at those temperatures for 45 min and 75 min, respectively. The five tested *A. astaci* strains differed in their resistance to high temperatures; a strain from the genotype group E and one strain from the group D were more susceptible than other tested strains (genotype groups A, B and D). Interestingly, survival at high temperatures to some extent varied when repeated after several weeks or months, suggesting that some yet unknown external or physiological factors may influence *A. astaci* temperature resistance. In general, we consider the pathogen transmission through the digestive tract of warm-blooded predators unlikely, most probably even less likely than the potential transmission on their surface.

Key-words: *Aphanomyces astaci*, bird, mammal, body temperature, endozoochory

Introduction

Crayfish plague caused by the oomycete *Aphanomyces astaci* is one of the most serious diseases of crayfish (Diéguez-Uribeondo et al. 2006), which drastically reduced European populations of these ecologically and economically important crustaceans (Alderman 1996). Although latent infections with the crayfish plague pathogen have been recently reported from some populations of several European indigenous crayfish species (e.g., Jussila et al. 2011, Svoboda et al. 2012, Kušar et al. 2013), mass mortalities caused by the pathogen are still widespread across Europe (e.g., Caprioli et al. 2013, Viljamaa-Dirks et al. 2013, Kozubíková-Balcarová et al. 2014), and crayfish plague is considered responsible for recent steep declines of native crayfish in various countries (Holdich et al. 2009). In addition, similar impact might be expected also in the populations of Asian and Australian crayfish species if the pathogen was introduced to the areas of their distribution (Unestam 1969c, 1972). Therefore, *A. astaci* has been included among the 100 worst invasive species not only in Europe (DAISIE 2009), but also in the world (Lowe et al. 2004). Since no effective treatments to enhance resistance or cure infected crayfish have been discovered, the only known way to protect susceptible crayfish from *A. astaci* is to prevent this pathogen's dispersal (Oidtmann 2012). Therefore, identification and evaluation of all possible dispersal pathways is of substantial importance.

The dispersal of *A. astaci* is limited by its adaptations to a parasitic life (see e.g., Unestam 1969a, 1972) and by the narrow host range, crayfish and freshwater crabs being the only proven hosts in nature (Unestam 1972, Schrimpf et al. 2014, Svoboda et al. 2014a, Svoboda et al. 2014b). The life cycle of *A. astaci* (for a summary see e.g., Söderhäll & Cerenius 1999) apparently does not include a sexual process, i.e., the species does not form any oospores resisting dry periods and extreme temperatures (Diéguez-Uribeondo et al. 2009). Thus, the parasite can reach a new locality either as hyphae, which grow only in the tissues of its hosts, or in the form of unicellular and relatively short-lived spores (i.e., zoospores and cysts), which are vulnerable to desiccation (Smith & Söderhäll 1986, Söderhäll & Cerenius 1999). Several pathways of the pathogen dispersal have been identified: 1) infected hosts, 2) spore-contaminated water and items that have been in recent contact with such water, 3) animals that either have been in contact with spore carrying water or animals that have been feeding on infected crayfish (Oidtmann et al. 2002). The last pathway might be facilitated by the fact that infected crayfish are progressively paralysed, lose their limbs and show abnormal behaviour such as daytime activity (Oidtmann et al. 2002, Jussila et al. 2013, Makkonen et al. 2014), which makes them an easy prey.

European crayfish are preyed upon by wide range of fishes, mammals, e.g., the European otter (*Lutra lutra*) and the American mink (*Neovison vison*), and birds, especially wading birds, waterfowl, cormorants, gulls and terns (Huner 2000, Holdich et al. 2006). The potential for transmission of *A. astaci* through the digestive tract of fish has been proven experimentally (Oidtmann et al. 2002). This transmission allows relatively long-range transport (in comparison with crayfish natural migratory activity), but mostly only within a watercourse. In contrast, the transmission through the digestive tracts of mammals and birds has not been tested, although if possible, it could result in overland dispersal to new watersheds. The American mink and the otter have also been suggested as possible vectors of the crayfish plague in Ireland by Reynolds (1988). However, Oidtmann et al. (2002) considered endozoochory by mammals and birds unlikely because *A. astaci* died after 12 h at 37°C. The temperature found in mammals and birds is indeed high, ca 37 °C for relevant groups of mammals and ca 41 °C for relevant groups of birds (Clarke & Rothery 2008). However, the food

passing time can be substantially shorter than 12 hours (Liers 1951, Malone 1965). Therefore, further evidence is needed to answer the question whether, or under which condition, *A. astaci* might be transmitted through the digestive system of warm-blooded predators.

This study reports on the results of several experiments on the potential of *A. astaci* transmission through the digestive tracts of mammals and birds. We focused on two questions: 1) Can mammal predators transmit *A. astaci* through their digestive tract? 2) Can *A. astaci* survive long enough in body temperatures of mammals and birds? Thus, we first tested in a small-scale experiment the following hypothesis: 1) The excrements of mammals that fed on plague-infected crayfish may be the source of infection with this pathogen. We fed two mammalian predators with infected crayfish and tested for the presence of viable forms of *A. astaci* in their excrements. Since the morphology of *A. astaci* is not species-specific enough (Oidtmann 2012), and molecular detection cannot determine whether the source of the detected DNA is viable, the excrements were tested in a transmission experiment, and we tried to isolate the pathogen from the predator faeces to agar plates. After failing to find evidence for the pathogen survival, we tested a follow-up hypothesis: 2) The effect of high temperature alone can prevent the pathogen transmission through the digestive tracts of mammals and birds. This was evaluated by testing for survival of *A. astaci* mycelium in laboratory cultures at the temperatures corresponding to the body temperatures of mammals and birds.

Methods

Feeding of predators

An otter (*Lutra lutra*) and an American mink (*Neovison vison*) kept in Zoo Ohrada in Hluboká nad Vltavou (the Czech Republic) were fed with plague-infected noble crayfish (*Astacus astacus*) in October 2011. For this purpose, twelve dead crayfish without any signs of decay were collected in the river Litavka, where massive mortality of noble crayfish was occurring at the time (Kozubíková-Balcarová et al. 2014). In the evening, the crayfish were served as the only food of the day to the predators, which had not been fed the preceding day.

Excrements laid in the following 48 hours were collected, for details see Supplementary table S1. First excrements were laid during the night, but collected in the morning, so the exact time of defecation was not known. Each excrement was split with sterile scalpel into halves. One half was used in the transmission experiment, i.e., put to a water tank with two individuals of the susceptible stone crayfish (*Austropotamobius torrentium*). From the other half, all visible pieces of crayfish cuticle were picked with tweezers and washed three times with tap water. Then, 20 pieces from every excrement were used in the isolation experiment (evaluating whether *A. astaci* culture could be obtained from them), while a few other cuticle pieces were used directly for DNA isolation. The summary of the experiment is provided in Fig. 1, and details of the follow-up procedures are given below.

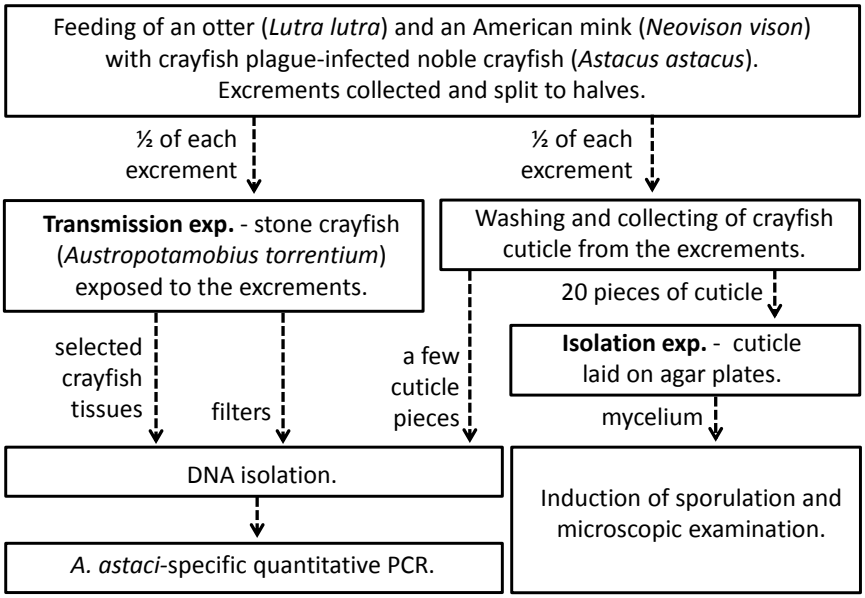

Figure 1: Feeding experiment and the following laboratory work including the transmission and the isolation experiments which were performed in 2011.

In 2013 and 2015, one otter and one mink individual were repeatedly fed with crayfish again, to measure the shortest time that the crayfish cuticle spends in the predators' digestive tracts (i.e., the shortest passing time). Altogether, forty adults of the signal crayfish (*Pacifastacus leniusculus*) were euthanized by freezing and served to predators. They were not tested for the presence of *A. astaci*, as we did not expect significant influence of *A. astaci* on the measured passing time. In contrast, we presumed that the amount of crayfish fed and any other food served with crayfish might have some effect (see Malone 1965). Therefore, the crayfish were served *ad libitum* (to simulate the conditions during a crayfish plague outbreak), and with or without the usual diet, for details see Supplementary table S2. Each time, the predators' pens were checked every 10 min, and any excrements found were collected to plastic bags and later examined for the presence of crayfish cuticle.

Transmission experiment

For the transmission experiment (Fig. 1), ten male stone crayfish (*A. torrentium*; 45 to 75 mm long from rostrum to the end of a tail fan) showing no signs of diseases were captured in the Bílý brook (49°37'15"N, 13°43'55"E), where no mass mortality has been reported in recent years. The crayfish were divided in pairs to five plastic tanks, each with ca 50 l of drinking groundwater. One tank with two crayfish was used as a control. To the other four tanks, halves of two or three excrements were added as a potential source of infection (we collected five excrements of the otter and four of the mink). 100 ml of water was filtered from each of the tanks 24 and 48 hours after the excrement addition to test for the presence of *A. astaci* spores following the methods described in Svoboda et al. (2013). Water was filtered through polycarbonate filters (Whatman Nucleopore, diameter 25 mm, pores 2 µm) in filter holders (Swin-Lok, Whatman) using 50 ml syringes (Omnifix). The filters were kept for six weeks at −20°C and then at −80°C before subsequent analyses. Disposable gloves, syringes, filter holders and other tools sterilised by bleach after every use, and covers of tanks eliminating spread of aerosols were used to prevent accidental pathogen transmission among water tanks. The tanks were kept outside to simulate natural conditions, so the water temperature was between 17 °C and 3 °C in the first five weeks of the transmission experiment. Then, the crayfish

were moved to new water tanks with aged tap water and kept at 21 °C for nearly 6 weeks (to let any potential infection develop). The crayfish were checked daily, dead individuals were removed and kept deep frozen (in −80 °C) till dissection. After 75 days from the excrement addition, all the surviving crayfish were euthanized and kept separately deep-frozen.

Soft cuticle from the abdomen and telson, two uropods, two pleopods, inner joints from three pereiopods and any noticed melanised parts of the cuticle were dissected from each stone crayfish, as these crayfish body parts have been reported as most often infected with the pathogen (Oidtmann et al. 2004, Oidtmann et al. 2006, Vrålstad et al. 2011). All the tissues from each crayfish were crushed together in liquid nitrogen and up to 50 mg of the mixture were used for subsequent DNA isolation. DNA from the mixture and from filters was isolated following the protocol for animal tissues for the DNeasy Animal Tissue kit (Qiagen). Negative controls, i.e., tubes containing only distilled water but no crayfish tissues, were included in each DNA isolation batch. The pathogen DNA was detected and quantified in all DNA isolates by the quantitative PCR according to Vrålstad et al. (2009). To reduce the potential PCR inhibition, Taq Man Environmental Master Mix (Applied Biosystems) was used as recommended by Strand et al. (2011). Negative controls were included in all qPCR runs. To quantify the amount of pathogen in crayfish tissues, we used the number of PCR-forming units (PFU) per spore. We set the limit of detection, an unambiguous evidence of *A. astaci* DNA presence, to 5 PFU in 5 µl of a DNA isolate in accordance with Vrålstad et al. (2009). This corresponded to a concentration of ca 14 spores l^{-1} for the filtered water from experimental tanks. The numbers of genomic units were calculated using the conversion factor 143 PFU per spore, which was estimated from *A. astaci* DNA quantification in twelve DNA isolates from samples including approx. 1000 of *A. astaci* spores (Svoboda et al. 2014a).

Isolation experiment

In the isolation experiment, we tried to isolate *A. astaci* on agar plates following the same protocol as in Kozubíková-Balcarová et al. (2013). Briefly, twenty pieces of cuticle (50 to 250 mg) chosen at random from those selected from each excrement were rinsed in 70% ethanol and in antibiotic solution (penicillin G solution at a concentration of 100 mg l^{-1}) and placed on sterilised river water-glucose-yeast (RGY) agar media containing penicillin G and oxolinic acid. The agar plates were then checked every day in the following two weeks and any observed mycelia were examined with the naked eye. The strains whose mycelia roughly corresponded to the morphology of *Aphanomyces* were washed with water to induce sporulation and examined at 100x magnification to search for the typical *Aphanomyces* spore-balls (for details, see Alderman & Polglase 1986, Cerenius et al. 1988, Oidtmann et al. 1999).

High-temperature tolerance of *A. astaci* cultures

Cultures of five *A. astaci* strains, representing various genotype groups known from European waters, were used in this experiment: Al7 (genotype group A), Pec14 (B), Li10 (E), 183 (D), 185 (D). The first three strains were isolated and kept in the stocks of the Charles University in Prague, the last two were kindly provided by Satu Viljamaa-Dirks from the Finnish Food Safety Authority Evira, OIE reference laboratory for crayfish plague, Kuopio. Strain Al7 was isolated from narrow clawed crayfish (*Astacus leptodactylus*) imported from Eastern Europe (JS, unpublished data); strains Pec14 and Li10 were responsible for two crayfish plague outbreaks in Czech watercourses, in the Černý brook and the river Litavka, respectively (Kozubíková-Balcarová et al. 2014); strains 183 and 185 were isolated from crayfish bought through the pet trade, 183 from *Procambarus fallax* f. *virginalis* and

185 from *P. clarkii* (Mrugała et al. 2015). The determination of all strains as *A. astaci* was confirmed by sequencing of a 569 bp long amplicons including parts of internal transcribed spacers (ITS) 1 and 2 and 5.8S rDNA according to Oidtmann et al. (2006), as recommended by OIE (Oidtmann 2012). All strains were characterized by nine microsatellite markers according to Grandjean et al. (2014) to determine the *A. astaci* genotype group.

Two types of agar media were used, RGY and PG1. The RGY (river water-glucose-yeast extract) medium were prepared according to Alderman (1982): 1 g of yeast extract, 5 g of D(+)glucose, 12 g of agar and 1 l of water (non-chlorinated drinking water was used instead of river water from the original protocol). The PG1 (peptone-glucose) medium followed the protocol in Unestam (1969b), i.e., consisted from 3 g of peptone, 6 g of D(+)glucose, 15 g of agar, 100 ml of sodium phosphate buffer (pH=7, final concentration 0.013 M), and 900 ml of drinking water (used instead of a weak inorganic salt solution in distilled water from the original protocol). All strains grew on these media in control laboratory conditions.

Axenic cultures of the strains grown at 4 °C on RGY media were inoculated on new agar plates and kept at 20 °C for a certain time (2-14 weeks) for acclimation to a higher temperature (corresponding to temperatures that infected crayfish may experience in summer). During the acclimation period, the strains were inoculated to new agar plates (usually every two weeks). Some cultures grown at 4 °C were used for the experiments directly. In the experiment itself, round pieces of agar with *A. astaci* mycelium (ca 20 mm^2 each) were inoculated on a new Petri dish with one of the agar media. Some of the agar plates served as controls, i.e., they were moved directly to 20 °C. Most plates, however, were incubated for a specific time at a higher temperature (36.5 °C, 37.5 °C or 40.5 °C) in an incubator (Q Cell 60). These temperatures were chosen to roughly represent temperatures which might be found in the digestive tracts of mammals (36.5 °C, 37.5 °C) and birds (40.5 °C). The temperature in the incubator was monitored every 30 s by a dual-channel temperature data logger (Comet S0121). To check for the difference in the temperature of the air in the incubator and in the agar plates, one of the two channels was put to a sealed agar plate. After exposure to high temperatures, all the plates were moved to 20 °C. In the following three weeks, they were examined weekly for the pathogen growth from the inoculated agar pieces.

The experiments were repeated with minor changes in the design to test for various factors that may possibly affect *A. astaci* survival, every time including controls. In total, 1580 pieces of agar with *A. astaci* mycelium were incubated at high temperatures. Then, their survival was checked, and compared with the results of 789 controls. More details including the number of replicates are provided in Supplementary table S3 (available only in the electronic version). Here, we briefly describe the variation in the design. In September 2014, we varied the length of the acclimation period at 20 °C – the cultures were acclimated for eight, four, and two weeks, or used without acclimation. In December 2014, we varied the incubation time at high temperature in order to find the limit for *A. astaci* survival more accurately. In addition, we tested for the survival in a slightly lower temperature (36.5 °C instead of 37.5 °C). In January 2015, seven-week old cultures of *A. astaci* were tested to evaluate possible influence of a culture age on the survival at high temperatures. At the beginning of March 2015, we tried to test the difference of *A. astaci* survival on plates with the two media, i.e., PG1 and RGY. At the end of March and in May 2015, additional experiments focused on two factors: 1) the difference between cultures grown on the two media, 2) possible influence of a specific part of the colony from which the tested piece of mycelium was cut, i.e., the age of the

tested mycelium. For the latter purpose, we cut the mycelium in three distances from the centre of the colony (the original place of inoculation), which approximately corresponded to the ages of two, six and ten days.

Ethical note

DF has permission for handling of crayfish species protected by the Czech legislation (*A. astacus* and *A. torrentium*) through the Nature Conservation Agency of the Czech Republic . The protocol of the feeding experiment was designed in accordance with the Czech legislation on the protection of animals used for scientific purposes, which was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Science, Charles University in Prague.

Results

Feeding of predators

Both predators consumed the crayfish readily; the otter ate whole crayfish while the mink often did not eat most of the hardest parts of the crayfish body (chelipeds, carapace, terga of abdomen). Even pieces as large as whole uropods, legs missing only two or three segments, and more than half of an antenna passed through the digestive tract of the otter, while only smaller cuticle pieces were found in the excrements of the mink. First pieces of crayfish cuticle were found in otter excrements 3 h 30 min and 4 h after feeding. The minimum time a piece of cuticle spent in the digestive tract of the mink was between 1 h 50 min and 4 h; for details see Supplementary table S2.

Transmission and isolation experiments

In these experiments, nine excrements were used. The shortest time which the cuticle spent in the digestive tracts is not known exactly, but it was less than 17 h for the otter, and less than 26 hours but probably more than 18 hours for the mink (no crayfish cuticle was found in the halves of the first two excrements). DNA of *A. astaci* was detected by the qPCR in all samples of crayfish cuticle taken from excrements. We detected no *A. astaci* DNA during analyses of negative controls of DNA isolation and qPCR, or in any samples from control water tank (i.e., in filters and in tissues of *A. torrentium*). Five stone crayfish, including both crayfish from the control tank, died during the transmission experiment. The mortalities occurred from 64 to 71 days from the day of excrement addition. However, no *A. astaci* DNA was found in any sample of *A. torrentium* cuticle and no *A. astaci* DNA was found in the 15 filters, which were obtained from the tanks 24 and 48 hours from excrement addition.

We did not succeed in isolation of *A. astaci* on agar plates using the cuticle extracted from the predator excrements. Many strains of various fungi or fungus-like species grew on the plates but none of them showed *Aphanomyces*-like characteristics, and thus could not be the crayfish plague pathogen.

High temperature tolerance

All 789 controls, i.e., plates inoculated with *A. astaci* but not incubated at temperature higher than 20° C, survived (Fig. 2). The temperature data logger showed that the temperature in the plates put to the incubator (set to a target temperature of 36.5 °C, 37.5 °C, or 40.5 °C) was rising quickly first, reaching 35 °C in ca 10 min. However, the temperature in a plate reached the target temperature as late as 20 min after placing into the incubator (see Supplementary figure S4). Thus, for all the

experiments, we conservatively estimated the time of incubation in the target temperature as the time spent in the incubator minus 20 minutes.

The survival rate of *A. astaci* varied substantially both within one batch of plates, i.e., plates inoculated and tested in a single day, and among different batches. *A. astaci* survived for 45 min at 37.5 °C in 15 % of replicates. Interestingly, none of 45 replicates survived the incubation at 36.5 °C for 30 min, but six replicates (of strain 187, genotype group D) survived the incubation in the same temperature for 75 min (Fig. 2). Half of the replicates in which the temperature just reached 40.5 °C resulted in *A. astaci* death. Incubation at 40.5 °C for 15 min gave different results in September and in December. In the first trial, *A. astaci* sometimes survived (26% out of 180 replicates), while none out of 60 replicates survived the incubation in December. 75 min at 37.5 °C and 45 min at 40.5 °C were always lethal for all five tested strains of *A. astaci* (Fig. 2).

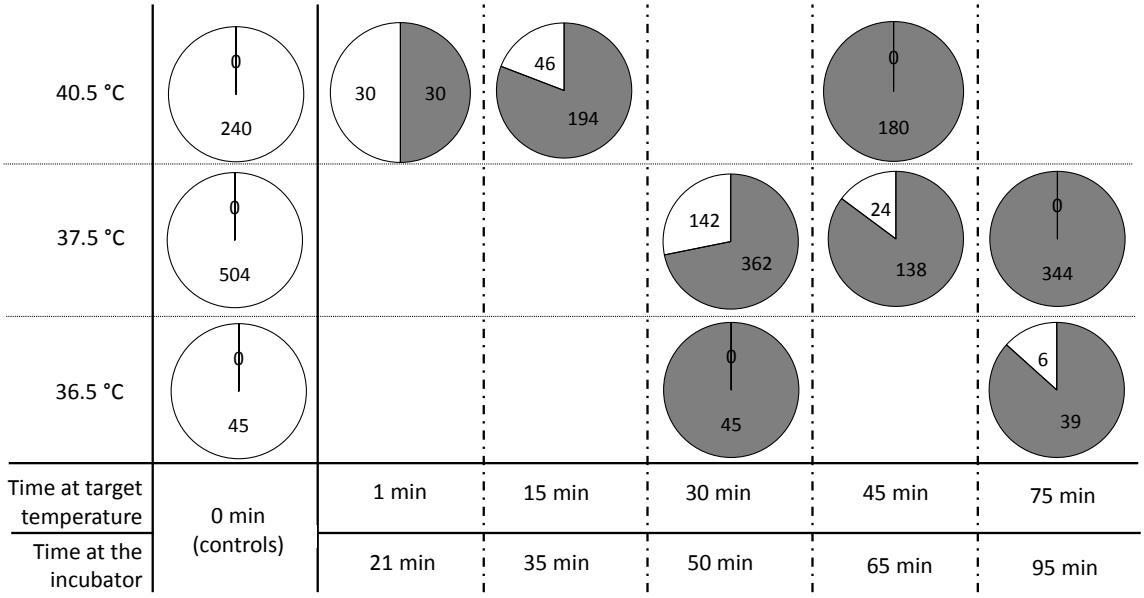

Fig. 2: Results of the experiments with the incubation of *A. astaci* at 40.5 °C, 37.5 °C and 36.5 °C. The figure summarises several experiments performed between September 2014 and May 2015, using all the five strains tested in this study. Numbers of replicates are given, grey colour indicates the cultures that were no longer viable after high-temperature exposure, white colour indicates surviving cultures.

The strains of *A. astaci* differed in their survival at higher temperatures (Fig. 3), the difference was significant for 15 min at 40.5 °C (X^2 test, $X^2 = 29.4$, df = 4, $p<0.001$) but not for 45 min at 37.5 °C ($X^2 = 4.85$, df = 4, p=0.3). The best surviving were the strains 187 (genotype group D), Pec 14 (group B) and Al7 (group A). In contrast, the strain Li08 (genotype group E) was substantially more susceptible to exposure to high temperatures than all other strains.

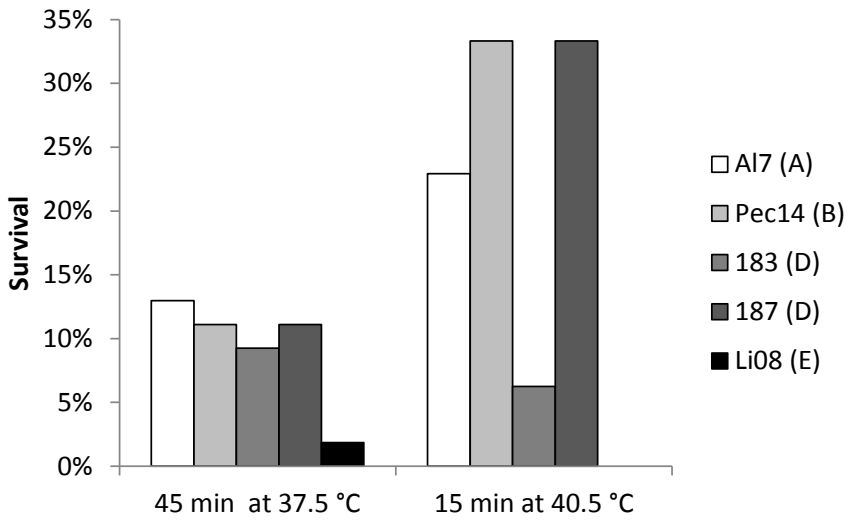

Fig 3: Survival of the tested *A. astaci* strains after selected threshold periods at high temperatures corresponding to bird and mammal digestive tract, respectively.

The experiments testing the influence of the length of acclimation period to the survival at higher temperatures resulted in heterogeneous pattern showing no clear trend (for details see Supplementary table S5). The results of the experiment testing the survival of relatively old mycelia (inoculated from plates grown for 49 days) did not differ substantially from the rest. Similarly, the survival of *A. astaci* mycelia did not vary with respect to the place in the plate from which the inoculated mycelium was cut, i.e., pieces with mycelium 2, 6, and 10 days old gave similar results. The first experiment testing the influence of the type of agar medium on the high-temperature survival suggested higher *A. astaci* persistence on the PG1 medium, but that was not confirmed in the second round of the experiment.

Discussion

The pathogen DNA was detected in all samples of their cuticle found in the excrements so the crayfish ingested by the otter and the American mink in our experiment were indeed infected with *A. astaci*. Some of the stone crayfish died during the transmission experiment, but *A. astaci* DNA was not detected in any of their tested body parts. No crayfish had died in less than 60 days, which exceeds or approaches the usual period between infection and death of susceptible crayfish even after an exposure to low concentration of *A. astaci* spores (see Alderman et al. 1987, Oidtmann et al. 2002). We therefore conclude that the crayfish plague pathogen was not transmitted to stone crayfish in our experiment.

Since the susceptible European crayfish species may occasionally withstand concentrations about 100 *A. astaci* spores per litre, apparently without getting infected (Unestam & Weiss 1970, Oidtmann et al. 2004), the absence of transmission cannot be taken as a conclusive evidence of lack of *A. astaci* sporulation. No *A. astaci* DNA was detected by filtration of water from the tanks with crayfish, although the same method was successfully used to detect *A. astaci* in water tanks with infected North American crayfish (Svoboda et al. 2013). Thus, it is apparent that at the time of filtration, no massive sporulation occurred in the experimental tanks. However, the filtration was done only twice, 24 and 48 hours from the excrement, and the detection limit was about 14 spores per litre. Therefore, our work included further experiments, results of which may be used to assess the

likelihood of *A. astaci* transmission more precisely, and to expand the discussion on all warm-blooded predators, i.e., to include birds.

The isolation of *A. astaci* from the excrements on agar plates was not successful, although the *A. astaci* strain causing the mass mortality in Litavka (i.e., the source of infected crayfish in our feeding experiment) had been isolated several times from freshly dead crayfish in our laboratory using the same method (Kozubíková-Balcarová et al. 2013). However, *A. astaci* isolation attempts may frequently fail due to competition with faster growing microorganisms (see Unestam 1965, Oidtmann et al. 1999, Kozubíková-Balcarová et al. 2013), which were certainly present in high numbers in predator excrements. To avoid the possible bias caused by such interactions, we continued in our work trying to focus on the key factors that may prevent the pathogen transmission in the digestive tract of warm-blooded vertebrate predators.

Any ingested food, including crayfish tissues with attached *A. astaci* spores or infected with *A. astaci* hyphae, faces various stressors during the passage through the predator digestive system, including mechanical disruption, chemical stress (low pH, enzymes) and high temperature. Since relatively large pieces of cuticle (e.g., whole uropods) passed through the digestive tract of the otter, the mechanical disruption is apparently not critical for the survival of *A. astaci* in mammalian digestive systems. In at least some birds, mechanical disruption is also not limiting, as viable propagules of various plants and aquatic invertebrates frequently pass through (Figuerola & Green 2002), and even living insect larvae were observed in Black-tailed Godwit (*Limosa limosa*) faeces (Green & Sánchez 2006).

Inside large intact pieces of crayfish cuticle, *A. astaci* mycelium can probably remain protected from the chemical stressors such as the low pH and digestive enzymes. In contrast, if a mammal or a bird swallows *A. astaci* spores attached on their prey, the spores must face the low pH in the stomach and several enzymes throughout the digestive tract. Therefore, we consider the survival in the digestive tract of warm-blooded animals less likely for *A. astaci* spores than for mycelium within the crayfish cuticle. Similarly, crayfish-plague transmission could not be achieved by force feeding of fish with *A. astaci* spore suspension, while it was performed after force feeding of fish with crayfish cuticle infected with *A. astaci* mycelium (Oidtmann et al. 2002).

The temperature, however, should reach the same level outside and inside any ingested crayfish body parts. Thus, it seems the most critical factor for the survival of *A. astaci* mycelia in the digestive system of warm-blooded predators. Oidtmann et al. (2002) did not consider the transmission of *A. astaci* through warm-blooded predators likely because the pathogen was neither transmitted nor cultivated on agar from crayfish cadavers after 12 hours at 37 °C. However, it has been published that food passing time can be only about 1 hour for mallards (Malone 1965) and European otter (Liers 1951), and about 3 hours for the American mink (Gugołek et al. 2013). Indeed, the shortest passing time of crayfish body parts through mammalian predators reached comparable values in our relatively small-scale experiments: 1 h 50 min for the American mink and 3 h 30 min for the European otter.

Nevertheless, even shorter incubations in relevant temperatures, 75 min at 37.5 °C and 45 min at 40.5 °C, were lethal for *A. astaci* in all our trials with monitored temperature. The bird body temperature, being higher than mammalian, was more lethal to tested *A. astaci* strains. However, it should be considered that gut passage times in birds may be even shorter than 45 min. For example,

eggs of *Artemia salina* can pass through killdeers (*Charadrius vociferus*) even as early as in 10 minutes following their ingestion (Proctor et al. 1967). Interestingly, *A. astaci* did survive 15 min at 40.5 °C. In addition, the type of food ingested with the propagules can strongly influence the rate of passage through the digestive tract (Malone 1965), and very short gut passage time might be expected if the predator is affected by some diseases.

Even minor decrease of temperature may prolong *A. astaci* survival, and strain 187 (genotype group D) survived the incubation at 36.5 °C for 75 min in six out of nine replicates. Body temperature of mammals and birds may fluctuate in response to several factors and rhythms (Prinzinger et al. 1991). Generally, the mean level of circadian rhythm of body temperature is usually in the range 37-39 °C in mammals and 40-42 °C in birds, with the amplitude ca 1-4 °C (Refinetti & Menaker 1992). The highest differences, about 4-6 °C, are between the body temperature of a resting and of a flying bird; the lowest temperatures during resting are about 38 °C for Pelecaniformes, 38.5 °C for Charadriiformes, and 39 °C for Anseriformes, Ciconiiformes, Gaviiformes and Podicipediformes (Prinzinger et al. 1991). Moreover, a decrease of body temperature by 1-3 °C below normal resting levels is presumably common in all birds and mammals (Prinzinger et al. 1991). There are species whose temperature might fall even substantially lower, but torpor, hibernation, and controlled hypothermia during food scarcity, do not seem relevant for the topic of *A. astaci* spread after feeding on crayfish.

The survival of *A. astaci* in body temperatures of mammals and birds varied so the exact time limits, i.e., the minimal time necessary for the pathogen elimination at the particular temperature, have not been found. The survival seems to be influenced by yet unknown factors. The potential factors that we have tested (i.e., the medium composition, the length of acclimation period, the age of mycelium in the tested range) apparently did not affect the survival consistently. Nevertheless, the combination of usual food passage time and temperature which challenges any particle in the digestive tracts of mammals is close to the limit of *A. astaci* survival. Thus, a certain (though relatively unlikely) combination of an unusually short passage time and rather low body temperature may make the internal transport of viable *A. astaci* through mammal or bird digestive tract possible.

However, the likelihood of such event should be compared with that of the transmission on predator body surface. Predators of crayfish may be exposed to relatively high concentrations of *A. astaci* spores when they feed on crayfish during crayfish plague outbreak. In fish, the transmission through the digestive tract seems more important than the transmission on fish surface, apparently due to antifungal properties of fish mucus (Oidtmann et al. 2002). Although *A. astaci* spores are sensitive to desiccation, they were transmitted by fine-mesh net drained for two hours in 15 °C (Alderman et al. 1987). Thus, the surfaces of warm-blooded predators may become substrates for occasional *A. astaci* transport. The occurrence of some water birds, such as cormorants, gulls and terns, in numerous flocks (see Huner 2000) might favour the transport of spores on their surface. Nevertheless, lakes only several kilometres from crayfish plague outbreaks have not been struck by the disease although water birds moved among these localities a lot (Unestam 1973), suggesting that even if spores were transported, successful transmission of the disease this way is unlikely.

Some of the results of our experiments with the survival of *A. astaci* at high temperatures are also interesting from a more general point of view. The results do not indicate that strains of the genotype group D generally show more tolerance to higher temperatures than strains from other genotype groups (Fig. 3), which was reported for less extreme temperatures from 27 °C to 29.5 °C

(Diéguez-Uribeondo et al. 1995). Interestingly, *A. astaci* mycelium sometimes did not start to grow noticeably until the third week after the exposure to high temperatures, while the growth of *A. astaci* was apparent after one week in all controls and in most of the cultures that have survived the incubation at high temperatures.

From the available evidence and our experiments, we can thus conclude that the likelihood of dispersal of *A. astaci* by warm-blooded predators is very low. However, neither the transmission on their surface nor the transmission through their digestive tract can be excluded entirely. Considering the resistance of *A. astaci* to physiologically relevant high temperatures, it is apparent that only very short passing times would allow for survival in mammal or bird digestive tracts. Although *A. astaci* spores are sensitive life stages, they might survive in water for days and even for weeks, especially in low temperatures (Unestam 1966). Therefore, spores contaminating wet body surfaces might survive longer than inside predator bodies. In any case, overland dispersal by such predators would be a very low-probability event, and management activities should primarily focus on other *A. astaci* dispersal pathways (particularly, preventing spread of American crayfish that serve as *A. astaci* carriers; Holdich et al. 2009).

Acknowledgements

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Supplements

Supplementary table S1: The excrements used in the transmission experiment. Feeding was preceded by a day without any food. Only the crayfish were served. Time of excrement sampling was measured from the time of feeding. NA means that the excrement was not collected.

Animal	mink	otter
Food fed	3 crayfish	5 crayfish
Excrement	Time of excrement sampling	
1st	17 h †*	17 h *
2nd	18 h †	18 h
3rd	26 h	26 h
4th	42 h	26 h
5th	NA	42 h

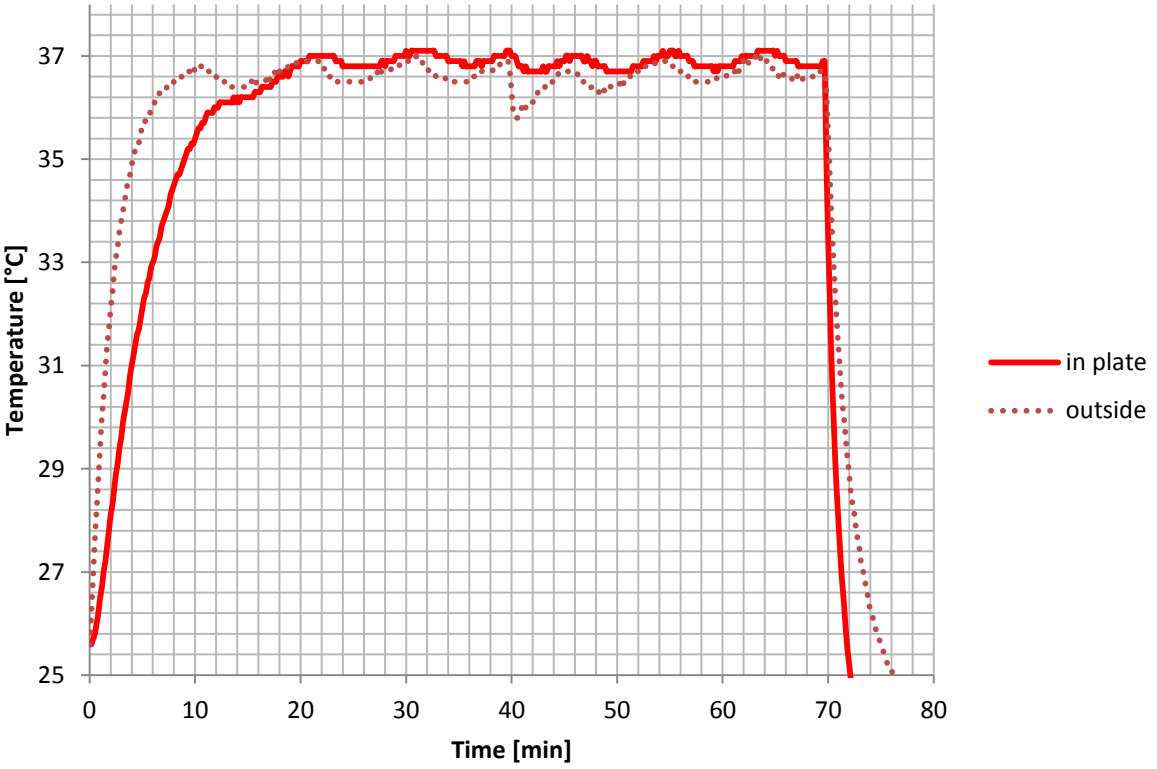
† No crayfish cuticle was found in the examined half of the excrement.
* The excrement might have been defecated earlier, but the pen was not checked before morning.

Supplementary table S2: Time of the first defecation of crayfish cuticle measured from the feeding. A row reports on the result of one experiment. The experiments were separated by a period of one week at the least, during which no crayfish were served. Crayfish cuticle was found in all the excrements.

Animal	Time of defecation measured from the feeding	Food
mink	4 h	3 crayfish + ordinary food
mink	3 h 30 min	3 crayfish + ordinary food
mink	1 h 50 min	4 crayfish
mink	3 h 15 min	6 crayfish *
mink	3 h 30 min	3 crayfish + ordinary food
otter	4 h	5 crayfish + ordinary food
otter	3 h 30 min	5 crayfish + ordinary food

*Feeding preceded by a day without any food.

Supplementary table S3 (details on the incubation experiment) is available only in the electronic version.
Genotype groups were determined according to Grandjean et al. (2014). RGY – river water-glucose-yeast extract, PG1 – peptone-glucose medium. A piece of mycelium (replicate) was considered alive, if growth was apparent in the three weeks following the incubation at high temperature. The sign "-" indicates that the experiment was not carried out, or that source plates were not labelled at the particular experiment.

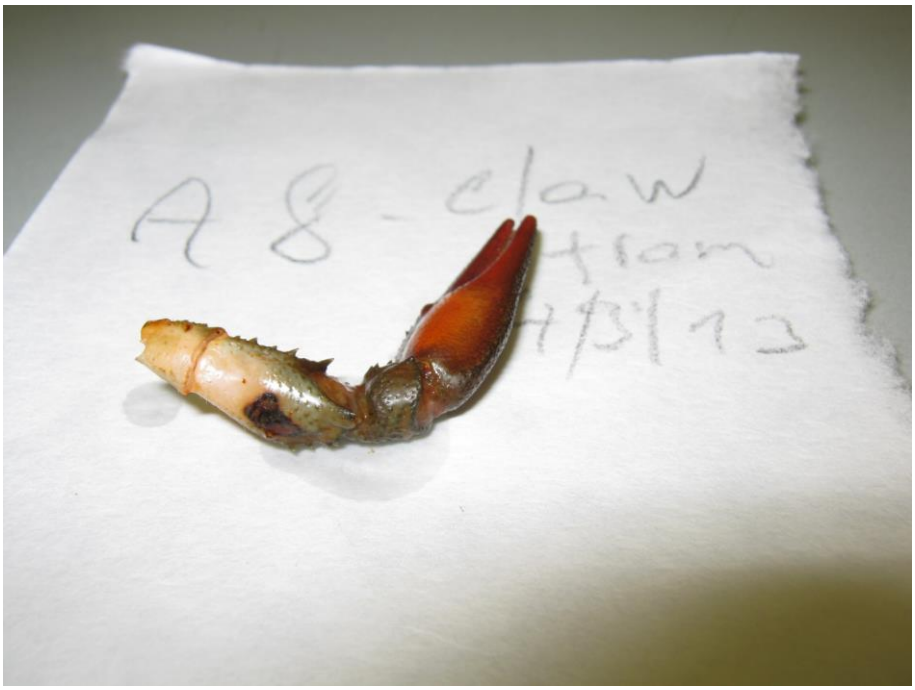


Supplementary figure S4: The temperature of the air in the incubator and in the agar plates. The data were obtained by a dual channel temperature data logger (Comet S0121). One of the two channels was put to a sealed agar plate.

Supplementary table S5: Experiments testing the role of acclimation period length. The table summarises experiments using all five strains tested in this study.

Period at 20 °C [weeks]	Survival after 30 min at 37.5 °C	Survival after 15 min at 40.5 °C
0	4% (2 out of 45)	40% (18 out of 45)
2	27% (12 out of 45)	0% (0 out of 45)
4	0% (0 out of 45)	58% (26 out of 45)
8	18% (8 out of 45)	4% (2 out of 45)

APPENDICES



The clinical signs of European crayfish infected with *Aphanomyces astaci* vary; infected crayfish may even lose their limbs as happened also during our transmission experiment.

Chapter 6

Tilmans, M., Mrugała, A., Svoboda, J., Engelsma, M.Y., Petie, M., Soes, D.M., Nutbeam-Tuffs, S., Oidtmann, B., Roessink, I., 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.

"In contrast with apparently thriving alien crustaceans, Dutch populations of indigenous noble crayfish *Astacus astacus* have disappeared at an alarming rate since the second half of the twentieth century...and presently only one residing population remains..."

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Survey of the crayfish plague pathogen presence in the Netherlands reveals a new *Aphanomyces astaci* carrier

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ABSTRACT

North American crayfish species as hosts for the crayfish plague pathogen *Aphanomyces astaci* contribute to the decline of native European crayfish populations. At least six American crayfish species have been reported in the Netherlands but the presence of this pathogenic oomycete with substantial conservational impact has not yet been confirmed in the country. We evaluated *A. astaci* prevalence in Dutch populations of six alien crustaceans using species-specific quantitative PCR. These included three confirmed crayfish carriers (*Orconectes limosus, Pacifastacus leniusculus, Procambarus clarkii*), two recently introduced but yet unstudied crayfish (*Orconectes* cf. *virilis, Procambarus* cf. *acutus*), and a catadromous crab *Eriocheir sinensis*. Moderate levels of infection were observed in some populations of *O. limosus* and *P. leniusculus*. Positive results were also obtained for *E. sinensis* and two Dutch populations of *O.* cf. *virilis*. English population of the latter species was also found infected, confirming this taxon as another *A. astaci* carrier in European waters. In contrast, Dutch *P. clarkii* seem only sporadically infected, and the pathogen was not yet detected in *P.* cf. *acutus*. Our study is the first confirmation of crayfish plague infections in the Netherlands and demonstrates substantial variation in *A. astaci* prevalence among potential hosts within a single region, a pattern possibly linked to their introduction history and coexistence.

1. Introduction

The oomycete *Aphanomyces astaci* Schikora is the causative agent of the crayfish plague, a disease responsible for high mortalities of indigenous crayfish species throughout Europe (e.g., Alderman, 1996). It was suspected as early as in the 1960s that non-indigenous crayfish species (NICS) play a crucial role in the transmission of the crayfish plague pathogen to populations of native European crayfish (Unestam, 1969). All three North American crayfish invaders widely established in Europe, *Orconectes limosus* (Rafinesque), *Pacifastacus leniusculus* (Dana), and *Procambarus clarkii* (Girard), are confirmed carriers of *A. astaci* (Diéguez-Uribeondo and Söderhäll, 1993; Unestam, 1972; Vey et al., 1983). These species had been imported to Europe before 1975 for stocking purposes and have become widespread since then (Holdich et al., 2009; Kouba et al., 2014).

At least seven other crayfish species of North American and Australasian origin have become established in Europe more recently, mainly thanks to introductions from aquarium trade and aquaculture (Holdich et al., 2009). Five of these "new NICS" are of North American origin, and thus potential carriers of *A. astaci* (see Oidtmann, 2012; Unestam, 1972, 1969). However, it has been shown that the prevalence of *A. astaci* may substantially vary among species, regions, and even local populations (e.g., Filipová et al., 2013; Kozubíková et al., 2011a; Schrimpf et al., 2013a). Thus, the potential to spread *A. astaci* cannot be assessed unless a particular species (population) is tested for the presence of the pathogen. So far, only one of the new NICS, the calico crayfish *Orconectes immunis* (Hagen), has been confirmed as a vector of this pathogen (Filipová et al., 2013; Schrimpf et al., 2013b). Nevertheless, these findings highlight the potential of other newly introduced North American crayfish species to spread the crayfish plague agent.

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To date, seven non-indigenous crayfish species have been reported in the Netherlands (although the taxonomic status of some of them is not entirely clear; see Filipová et al., 2010, 2011). These include: narrow-clawed crayfish *Astacus leptodactylus* (first reported in 1982), spiny-cheek crayfish *Orconectes limosus* (1973), virile crayfish *O.* cf. *virilis* (2006), signal crayfish *Pacifastacus leniusculus* (2005), white river crayfish *P.* cf. *acutus* (2006), red swamp crayfish *Procambarus clarkii* (1989), and marbled crayfish *P. fallax* f. *virginalis* (2006) (Adema, 1989, 1982; Geelen, 1978, 1975; Geelen and Oomen, 1973; Soes and van Eekelen, 2006; Soes and Koese, 2010). While *A. leptodactylus* originates from Eastern Europe, the other six alien crayfish species found in the Netherlands are of North American origin. Although the present status of the marbled crayfish population is unclear (Soes and Koese, 2010), the country still harbors one of the highest numbers of potential crayfish plague carriers in Europe (see Kouba et al., 2014). Moreover, since the early 1930s Dutch waters have been invaded by the Chinese mitten crab *Eriocheir sinensis* (Herborg et al., 2003; Kamps, 1937), which can also get infected by the pathogen from carrier crayfish (Svoboda et al., 2014).

In contrast with apparently thriving alien crustaceans, Dutch populations of indigenous noble crayfish *Astacus astacus* have disappeared at an alarming rate since the second half of the twentieth century. Whereas during the period from 1660 to 1947, 38 Dutch localities were still inhabited by *A. astacus*, their number gradually decreased over time (Geelen, 1978), and presently only one residing population remains (Ottburg and Roessink, 2012).

The presence of *A. astaci* in the Netherlands was never officially confirmed although epizootics of crayfish plague were implicated as one of the major reasons for the decline of native crayfish in the Netherlands. For example, this disease was the presumed cause of the mass mortality of some of the last Dutch populations of *A. astacus* in the Roosendaalse Brook in 2001 (Niewold, 2002), since when only a single population in the Netherlands remains in an isolated pond near Arnhem. Infection by this pathogen has only been studied for one Dutch population of *A. leptodactylus* so far, and the few screened individuals tested negative (Roessink and Ottburg, 2012). As a reintroduction program aiming to increase the number of noble crayfish populations in the Netherlands has been recently launched (Ottburg and Roessink, 2012), knowledge on the distribution of the crayfish plague pathogen is of paramount importance for its success.

In the present study, we screened populations of all five well established North American alien crayfish species as well as one population of the Chinese mitten crab with the OIE-recommended (Oidtmann, 2012) molecular diagnostic methods to confirm the infection by *A. astaci*. Based on experience from other European countries, we expected a widespread presence of *A. astaci* in populations of the well-known and common *A. astaci* carriers (*O. limosus*, *P. leniusculus, P. clarkii*). We also hypothesized that individuals of *E. sinensis* would test positive, since they are in contact with North American crayfish in Dutch waters, and thus can get infected. For the first time, we also provide results of testing of two recently introduced crayfish taxa, *Orconectes* cf. *virilis* and *Procambarus* cf. *acutus*, for which no data on *A. astaci* infections were previously available. We assumed that due to their North American origin, they may also host *A. astaci* in European waters.

2. Materials and methods

2.1. Sampling and DNA extraction

To evaluate the presence of *A. astaci* in Dutch waters, populations of five North American alien crayfish (spiny-cheek crayfish *Orconectes limosus*, virile crayfish *Orconectes* cf. *virilis*, signal crayfish *Pacifastacus leniusculus*, white-river crayfish *Procambarus* cf. *acutus*, and red swamp crayfish *Procambarus clarkii*) and one Asian crab species that gets into contact with potential *A. astaci* carriers in Dutch freshwaters (Chinese mitten crab *Eriocheir sinensis*) were sampled. The approximate locations of the sampled populations are presented in Fig. 1. Their exact position, sampling details, and number of individuals sampled per population are summarized in Table 1.

Sample storage, processing and DNA isolation slightly differed as samples from the involved localities were processed independently in two laboratories. Selected samples of all five crayfish taxa were analyzed at the Central Veterinary Institute in Lelystad, the Netherlands (CVI). In parallel, other samples of four of these taxa (all but *P. leniusculus*) and samples of *E. sinensis* were analyzed at the Department of Ecology, Charles University in Prague, Czech Republic (CUNI). To confirm correct detection of the pathogen and to compare the quantitative results obtained in the two laboratories, a selection of DNA isolates was analyzed both at CUNI and at CVI.

Upon sampling, specimens were stored in plastic bottles filled with 96% ethanol (CUNI), or frozen and stored at −20 °C (CVI). We dissected either soft abdominal cuticle, any melanization on the body visible by naked eye, and pieces of two uropods (CUNI) or exclusively soft abdominal cuticle (CVI) from each crayfish individual. From crab specimens we used soft cuticle from telson and abdomen, 4 joints from chelipeds, second pair of maxillipeds, and any melanized wounds after a pereiopod loss. Dissection tools were cleaned with UV-light and sodium hydroxide, or with hydrogen peroxide and flame sterilization after dissection of each individual to prevent cross-contamination. The dissected tissues were pooled together in order to obtain one DNA isolate for each specimen.

Prior to DNA extraction, the tissues were mechanically disrupted and homogenized. Grinding in sterile mortars with liquid nitrogen was used at CUNI. At CVI, the tissues were homogenized with TeSeE PRECESS 24 homogenizer (BioRad) in IDEXX tissue

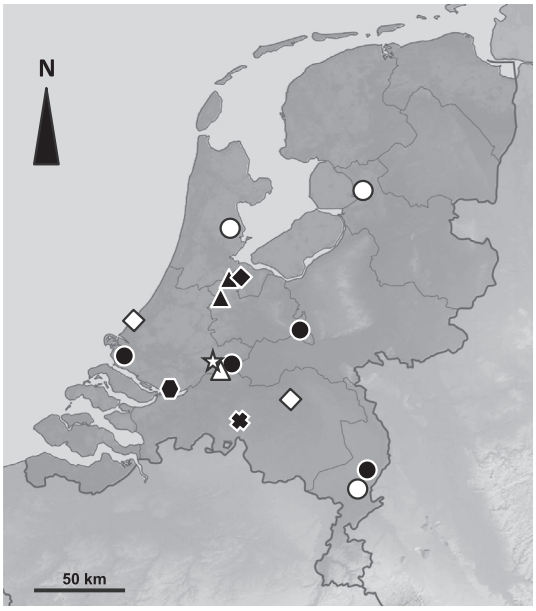

Fig. 1. Map of the Netherlands with approximate locations of analyzed populations of *O. limosus* (circle), *O.* cf. *virilis* (triangle), *P. leniusculus* (cross), *P. acutus* (star), *P. clarkii* (diamond) and *E. sinensis* (hexagon). Populations in which *A. astaci* infection was detected are indicated by black shapes, those without *A. astaci* detection by white shapes. In cases where sampled populations are in close vicinity to each other, only one location is marked in the map.

Table 1
Results of *Aphanomyces astaci* detection in populations of five North American crayfish and one Asian crab species occurring in the Netherlands. For positive detections, semi-quantitative agent levels are provided.

Sp.	Sampling site	River basin	Type of water body	Coordinates		Month of sampling	Individuals tested	*A. astaci* infected	Prevalence (95% CI)	Agent level			
				Latitude (N)	Longitude (E)					A2	A3	A4	A5
Orconectes limosus													
	Brielle	Rhine	Ditch	51°54′00″	4°10′00″	September 2012	6	1	16% (0.4–64%)	1			
	Gorinchem[a]	Rhine	River	51°50′08″	4°56′07″	May 2012	19	5	26% (9–51%)	1	3	1	
	Gouwzee	Rhine	Lake	52°26′21″	5°03′24″	February 2013	6	–	0% (0–58%)				
	Meuse	Meuse	River	51°17′32″	6°04′05″	October 2013	13	3	23% (5–54%)	2	1		
	Roermond	Meuse	River	51°11′23″	5°58′52″	June–August 2012	10	6	60% (26–88%)	3	3		
	Wageningen[a]	Rhine	Canal	51°57′58″	5°37′05″	October 2012	5	4	80% (28–99%)	1	3		
	Zwarte meer	Rhine	Lake	52°38′36″	6°00′17″	February 2013	7	–	0% (0–53%)				
Orconectes virilis													
	Boven-Hardinxveld[b]	Rhine	Ditch	51°50′01″	4°54′23″	May & December 2012	2 + 1	–	0% (0–80%)				
	Kanis[a]	Rhine	Canal	52°08′21″	4°53′35″	October 2012	7	4	57% (18–90%)	3		1	
	Oukoop[a]	Rhine	Ditch	52°13′07″	4°58′57″	September 2012	12	7	58% (28–85%)	4	3		
Pacifastacus leniusculus													
	Tilburg	Meuse	Brook	51°31′04″	5°04′43″	June 2012	5	4	80% (28–99%)	3	1		
Procambarus acutus													
	Alblasserwaard	Rhine	Ditch	51°52′01″	4°53′07″	September 2012	13	–	0% (0–34%)				
	Boven-Hardinxveld[a]	Rhine	Ditch	51°50′01″	4°54′23″	May 2012 & March 2013	20 + 20	–	0% (0–13%)				
	Giessenburg [a]	Rhine	Ditch	51°51′26″	4°54′18″	May 2012	20	–	0% (0–24%)				
Procambarus clarkii													
	Den Haag[a]	Rhine	Ditch	52°04′47″	4°15′34″	August 2012	20	–	0% (0–24%)				
	Schijndel[a]	Meuse	Ditch	51°38′09″	5°27′06″	October 2012	20	–	0% (0–24%)				
	'Terra Nova'	Rhine	Lake	52°13′09″	5°02′13″	October 2012	10	1	10% (0–45%)	1			
Eriocheir sinensis													
	Hollandsch Diep[a]	Rhine	River	51°41′55″	4°28′30″	September & November 2012	29	5	17% (6–36%)	2	1	1	1

[a] Populations analyzed at CUNI.
[b] Independently in both laboratories.

disruption tubes equipped with ceramic beads for 2 × 45 s, followed by freezing at −20 °C and a second disruption step of 2 × 45 s of the still frozen material.

Up to 40 mg of the homogenized tissues of each individual was used to obtain the DNA isolates with the DNeasy tissue kit (Qiagen) at both laboratories. A DNA extraction control (an Eppendorf tube containing Milli-Q water, treated as other sample-containing tubes) was prepared during each isolation batch to control for potential cross-contamination among samples. One control was included for every 10 samples. These remained negative in all cases. The DNA isolates were stored at −20 °C.

2.2. Aphanomyces astaci detection

To test for the presence of *A. astaci* DNA, the quantitative PCR assay was performed as described by Vrålstad et al. (2009), with minor modifications that differed at the two laboratories. At CVI, the qPCR reaction was carried out with 1× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems), 1.25 units of Uracil-DNA Glycosylase (New England BioLabs) were added per reaction to prevent carry-over contamination, and the final volume was 20 µl per reaction. The qPCR was carried out in an AB 7500 with fast block (Applied Biosystems) according to the program: 37 °C for 10 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 3 s and 58 °C for 30 s. At CUNI, the qPCR was performed on an iQ5 (Bio-Rad), the TaqMan Environmental Master Mix (Applied Biosystems) was used to reduce the potential PCR inhibition (see Strand et al., 2011) and annealing temperature was increased (from 58 to 62 °C) while synthesis time was decreased (from 60 to 30 s) to further increase the assay specificity (Strand, 2013).

At both laboratories, undiluted and 10× diluted original DNA isolates, a DNA extraction control, and a PCR blank control were included in each run. The number of PCR-forming units (PFU) added to each reaction was calculated using a standard curve, whose construction differed in detail between the two laboratories. At CVI, the method described in Kušar et al. (2013) was followed: dilution series of *A. astaci* DNA was prepared and analyzed in triplicates in three qPCR runs to give one standard curve, which was used to calculate the numbers of PFU in all samples according to their qPCR results. More details about this procedure are given as supporting information (Table S1, Fig. S1 in the Electronic Supplementary Material). At CUNI, the protocol published by Vrålstad et al. (2009) was followed; four *A. astaci* calibrants were prepared and used in every qPCR run to generate a standard curve. The number of PFU in an original DNA isolate was calculated according to Kozubíková et al. (2011a). Eventually, the quantitative results in PFU (obtained in either laboratory) were translated into more comprehensible and more robust semi-quantitative agent levels (A0–A7; Kozubíková et al., 2011a; Vrålstad et al., 2009). A test performed with identical DNA isolates in both laboratories indeed confirmed that both approaches gave comparable results despite the methodological differences described above. The isolates which did not contain any *A. astaci* according to CVI tested negative (agent level A0) also at CUNI. The quantitative data obtained in the two laboratories for other isolates (agent level A2–A7) mostly corresponded as well, resulting in different (but neighboring) agent levels only occasionally for samples containing *A. astaci* DNA in concentrations close to the limits of detection and quantification (see more details in Electronic Supplementary Material).

Since we tested for the presence of *A. astaci* in our samples using molecular methods only, the results could have been biased if DNA had not been isolated in sufficient quality and quantity from tested tissues (e.g., due to poor quality of the samples, or handling mistakes during DNA isolation). Thus, a few DNA isolates of apparently insufficient quality were excluded from the results to minimize the possible bias caused by false negatives, i.e., isolates from tissues parasitized by the pathogen but resulting in no detection of *A. astaci* DNA in the analyses. At CUNI, DNA concentration of all DNA isolates was estimated with the Nanodrop 1000

Spectrophotometer (Thermo Fisher Scientific). Two isolates out of 147 with outlying absorbance ratios (at 260/230 nm and 260/280 nm) indicating substantial presence of contaminants were excluded. Furthermore, the difference in PFU in undiluted and 10× diluted DNA isolates was used to check for potential inhibition of qPCR (for details, see Kozubíková et al., 2011a); no such inhibition was observed. At CVI, the Eukaryotic 18S rRNA Endogenous Control kit (Applied Biosystems) was used according to the manufacturer's protocol to check the integrity of the isolated crayfish DNA. Out of 75 analyzed samples, four were omitted from the dataset that yielded high Ct values (Ct > 26), i.e., suggesting low concentration or quality of host DNA, and at the same time tested negative for *A. astaci*.

The confirmation of *A. astaci* DNA in samples from representative infected populations that yielded positive qPCR results proceeded with sequencing of a 569 bp long amplicons including parts of internal transcribed spacers (ITS) 1 and 2 and 5.8S rDNA according to Oidtmann et al. (2006), as recommended by OIE (Oidtmann, 2012). As the conventional PCR is less sensitive than the qPCR approach (Kozubíková et al., 2011b; Tuffs and Oidtmann, 2011), the ITS sequences were obtained from infected individuals with agent levels A3 and higher according to qPCR analysis. The PCR products of these *A. astaci*-positive isolates were purified with ethanol precipitation and sequenced in both directions on the ABi 3130xl Genetic Analyzer (Applied Biosystems). Resulting sequences were compared to publicly available sequences of *A. astaci*, and the representative ones were deposited to GenBank (KF944440–KF944443, KJ710432–KJ710434).

To estimate prevalence in studied populations, we calculated 95% confidence intervals for the prevalence values obtained from the number of *A. astaci*-positive and total number of tested samples per population. This was conducted as in Filipová et al. (2013), using the function "epi.conf" from the library epiR (Stevenson et al., 2013) for R v. 3.0 (R Core Team, 2013).

3. Results

The presence of *Aphanomyces astaci* was detected in populations of four North American crayfish and one Asian crab species present in the Netherlands (*O. limosus*, *O.* cf. *virilis*, *P. leniusculus*, *P. clarkii* and *E. sinensis;* Table 1). Out of 216 examined crayfish and 29 crab individuals, 35 crayfish and five crabs tested positive for the pathogen. The isolates positive for *A. astaci* reached low (A2) to high (A5) agent levels (Table 1).

For confirmation of *A. astaci* infections, we obtained eight ITS sequences from representative populations of four host taxa: *O. limosus* (Gorinchem, Meuse, Roermond, and Wageningen), *O.* cf. *virilis* (Kanis and Oukoop), *P. leniusculus* (Tilburg), and *E. sinensis* (Hollandsch Diep). These were all identical to the *A. astaci* reference sequences available in GenBank. From a single apparently infected specimen of *P. clarkii*, no ITS sequence was obtained, presumably due to low level of pathogen infection.

The pathogen prevalence in all studied populations was highly variable, ranging from 0% to 80%. However, as the wide confidence intervals for the prevalence estimates indicate, the lack of detection in most populations (especially those with relatively low numbers of individuals analyzed) cannot be considered an evidence of absence of the crayfish plague pathogen in these populations.

Individuals infected with *A. astaci* were detected in four out of seven tested populations of *O. limosus*, the most widespread and common alien crayfish in the Netherlands (Table 1). In these populations, the prevalence ranged from moderate (16–25%) to high (80%). Moreover, high prevalence (80%) of *A. astaci*-positive crayfish was observed in the analyzed population of *P. leniusculus*. In contrast, in *P. clarkii,* the second most widespread alien crayfish in the Netherlands, only one individual out of 50 analyzed (from three populations) tested positive for the pathogen, with a very low agent level (A2).

Contrasting patterns of crayfish plague prevalence were also observed in populations of the recently introduced crayfish species, *P.* cf. *acutus* and *O.* cf. *virilis.* Despite an extensive sampling (73 individuals analyzed from three sampling sites) no pathogen was detected in specimens of the former, whereas moderate prevalence (57–58%) of *A. astaci* infection was detected in two out of three populations of the latter.

4. Discussion

The Netherlands harbors one of the highest diversity of nonindigenous crayfish in Europe, comprising established populations of six North American and one Eastern European species (Kouba et al., 2014). The first study focusing on the presence of *A. astaci* in Dutch crayfish (Roessink and Ottburg, 2012) did not detect any infection in *A. leptodactylus* population. Our study, however, unambiguously revealed the presence of the crayfish plague pathogen in the Netherlands in its natural hosts, i.e., North American crayfish species, and additionally in the Chinese mitten crab.

Relatively high prevalence of infection was repeatedly detected in *O. limosus,* the most widespread nonindigenous crayfish in Dutch waters (Soes and Koese, 2010). This species thus serves as an important *A. astaci* reservoir in the Netherlands. In addition, high prevalence was also observed in the studied *P. leniusculus* population. Although the range of this host species is still restricted to only two water bodies near the eastern and southern borders of the country (Soes and Koese, 2010), its further expansion is likely and the species may therefore contribute to spread of the crayfish plague pathogen in Dutch waters. Moreover, *A. astaci* was also detected in the tissues of some *E. sinensis*, a migratory crab species capable of covering long distances (Herborg et al., 2003; Kamps, 1937). The crabs likely acquired the infection from coexisting *O. limosus,* widespread in the rivers Rhine and Meuse (Soes and Koese, 2010), whose populations in these basins are infected by *A. astaci* (Fig. 1, Table 1).

Aphanomyces astaci infections were also detected for the first time in Dutch *Orconectes* cf. *virilis*, a recent North American invader shown to represent a distinct clade within the virile crayfish species complex (Filipová et al., 2010). The virile crayfish is the second new NICS found in European waters testing positive for this pathogen's presence, after *Orconectes immunis* in Germany and France (Filipová et al., 2013; Schrimpf et al., 2013b). To date, *O.* cf. *virilis* has a restricted distribution in Europe, limited to the Netherlands and the United Kingdom, and molecular analyses revealed that both populations belong to the same phylogenetic lineage (Filipová et al., 2010). This suggests a common introduction pathway for both populations. Interestingly, an independently conducted analysis (B. Oidtmann and S. Nutbeam-Tuffs, unpubl. data) confirmed that the English population of this species has also a high prevalence of *A. astaci.* Out of 21 specimens sampled in October 2009 from the River Lee (the Thames catchment) and analyzed with the same ITS-based qPCR detection method (Vrålstad et al., 2009), 18 individuals (86%) tested positive.

The identity of *A. astaci* infections in the virile crayfish deserves further consideration. Four different *A. astaci* genotype groups, associated with different host species, are known so far (Diéguez-Uribeondo et al., 1995; Huang et al., 1994; Kozubíková et al., 2011b) but it is not unlikely that additional *A. astaci* strains, differing in such properties as virulence (Jussila et al., 2011; Makkonen et al., 2014; Viljamaa-Dirks et al., 2011) or climate requirements (Diéguez-Uribeondo et al., 1995; Rezinciuc et al., 2013), may be

introduced with new host taxa. We consider likely that the virile crayfish had been already infected prior to its introduction. Similarly as for *O. immunis* (Schrimpf et al., 2013b), we may speculate that in such case, European populations of *O.* cf. *virilis* might carry their own specific strain of *A. astaci*. However, we cannot also exclude independent horizontal transmission of the pathogen from another infected species after establishment of the virile crayfish in both the Netherlands and the United Kingdom.

The confirmation that *O.* cf. *virilis* is another *A. astaci* carrier supports the assumption that North American crayfish species in general have the potential to carry latent *A. astaci* infections (Oidtmann, 2012). This is further supported by the observation that crayfish spread through the pet trade may occasionally be infected by *A. astaci* (A. Mrugała et al., unpubl. data). Interestingly, unlike for *Orconectes* spp. and *P. leniusculus*, only one very weakly infected individual was observed for *Procambarus clarkii,* an important *A. astaci* host elsewhere in Europe (Aquiloni et al., 2011; Rezinciuc et al., 2013), and no *A. astaci* infection was observed in examined Dutch specimens of *P.* cf. *acutus*. The three sampling sites of *P.* cf. *acutus* are geographically close to each other, likely originating from a single original source. The apparent absence (or very low prevalence) of the pathogen in studied population of this taxon might thus result from a founder effect. That would be in accordance with several studies investigating the presence of *A. astaci* infections in established populations of North American crayfish across Europe, which also did not detect the pathogen in at least some of the studied populations (e.g., Schrimpf et al., 2013a; Skov et al., 2011; but see Kozubíková et al., 2011a). In particular, the results of Schrimpf et al. (2013a) reveal that some European populations of these crayfish may be free of this pathogen.

The variability in *A. astaci* prevalence in populations of its natural carriers has been explained by several factors, including: age and size of sampled individuals (Vrålstad et al., 2011), temporal fluctuations in pathogen presence (Matasová et al., 2011), and the type of the water body inhabited by crayfish (Kozubíková et al., 2009). Additionally, pathogen prevalence may also be shaped by introduction history, i.e., infection levels and life-stage of founder individuals (Kozubíková et al., 2009; Torchin et al., 2003). In this context, however, it remains open whether the contrasting patterns of *A. astaci* prevalence in Dutch *Orconectes* and *Procambarus* populations are linked to their different origin and introduction pathways or whether other factors mentioned above played a key role.

It is worth attention that *P.* cf. *acutus* in the Netherlands gets into contact with several other North American crayfish (*O. limosus*, *O.* cf. *virilis*, and *P. clarkii*). As Dutch *Orconectes* spp. are frequently infected by *A. astaci*, pathogen transmission from these species to *P.* cf. *acutus* may be eventually expected. However, it is possible that horizontal transmission of a particular *A. astaci* genotype between different North American host taxa is limited by host-pathogen incompatibilities.

In the future, more detailed sampling, including in particular locations where multiple potential *A. astaci* carriers coexist, may provide better insights into the mechanisms responsible for this pathogen's distribution in North American crayfish populations, and may improve predictions of further spread of various *A. astaci* genotypes. Understanding of such processes may facilitate efforts to limit the impact of these exotic species. Additional introductions of new *A. astaci* hosts should also be prevented, particularly through informing the general public to avoid releases of species available through ornamental pet trade (apparently the most important entry pathway for exotic crayfish in Europe at present; Chucholl, 2013; Peay, 2009). In the Netherlands, however, where numerous exotic crayfish species already live in a small area characterized by numerous interconnected water bodies, management of these aquatic invaders is difficult and presents a particular challenge for conservation and reintroduction of indigenous crayfish populations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2014.06.002.

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Survey of the crayfish plague pathogen presence in the Netherlands reveals a new *Aphanomyces astaci* carrier: Electronic Supplementary Material

Details on the calibration of *A. astaci*-specific quantitative PCR at Central Veterinary Institute, Lelystad, the Netherlands (CVI)

For the in-house calibration of the quantitative PCR for detection of *A. astaci* (Vrålstad et al., 2009) the methodology of Kušar et al. (2013) was generally followed with some modifications. A standard 4-fold serial dilution was prepared using a DNA isolate from pure culture of *A. astaci* (SVA strain SVA56/2003). The DNeasy tissue kit (QIAGEN) was used for the DNA extraction. The starting concentration of the standard was determined to be 4.96 ng/µl (mean of 4 replicates) using a NanoDrop 1000 (NanoDrop products, Wilmington, DE, USA). The 4-fold serial dilution was tested in three independent qPCR runs in triplicates and the results are given in Ct values (Table S1).

Table S1. The mean Ct was calculated from nine Ct-values obtained for a particular standard tested in triplicates in 3 independent PCR runs. The following columns show corresponding standard deviation (SD), coefficient of variation (CV, calculated from positive results only) and the percentage of qPCR replicates yielding a Ct value (Detection %), i.e., detection of *A. astaci*. The estimation of number of PFU was based on the percentage as explained in detail in the text.

Standard	Dilution	Concentration of DNA (ng/µl)	Mean Ct	SD	CV (%)	Detection (%)	Estimated PFU
S1	undil.	4.96	16.67	0.71	4.24	100	1×4^{11}
S2	4^{-1}	1.24	17.92	0.09	0.49	100	1×4^{10}
S3	4^{-2}	$3{,}1 \times 10^{-1}$	19.96	0.09	0.44	100	1×4^{9}
S4	4^{-3}	$7{,}8 \times 10^{-2}$	22.35	0.42	1.86	100	1×4^{8}
S5	4^{-4}	$1{,}9 \times 10^{-2}$	24.56	0.60	2.42	100	1×4^{7}
S6	4^{-5}	$4{,}8 \times 10^{-3}$	26.69	0.61	2.30	100	1×4^{6}
S7	4^{-6}	$1{,}2 \times 10^{-3}$	28.51	0.37	1.31	100	1×4^{5}
S8	4^{-7}	$3{,}0 \times 10^{-4}$	30.55	0.40	1.31	100	1×4^{4}
S9	4^{-8}	$7{,}6 \times 10^{-5}$	32.72	0.53	1.60	100	1×4^{3}
S10	4^{-9}	$1{,}8 \times 10^{-5}$	34.85	0.70	2.00	100	1×4^{2}
S11[a]	4^{-10}	$4{,}7 \times 10^{-6}$	38.40	0.59	1.53	100	4
S12	4^{-11}	$1{,}2 \times 10^{-6}$	37.83	0.85	2.26	78	1
S13	4^{-12}	$3{,}0 \times 10^{-7}$	38.29	0.03	22.22	22	0-1

[a] limit of detection.

The limit of detection (LOD) has more than one definition (Burns and Valdivia, 2008). In this study, the LOD is considered as the amount of analyte at which the qPCR method detects the presence of *A. astaci* DNA at least 95% of the time, which is in accordance with Vrålstad et al. (2009). We detected the presence of *A. astaci* DNA (a sample yielded a Ct value) in 7 out of the 9 replicates of dilution 4^{-11} and in all replicates of dilution 4^{-10} (Table S1), thus the LOD corresponds approximately to dilution 4^{-10} in the standard 4-fold serial dilution. The most sensitive LOD theoretically possible is 3 copies per PCR, assuming a Poisson distribution, a 95% chance of including at least 1 copy in the PCR, and single-copy detection (Bustin et al., 2009). Therefore, the number of PCR forming units (PFU) was roughly set at 1 for dilution 4^{-11} and the numbers of PFU in the other standards were calculated according to the 4-fold serial dilution.

The definition of the limit of quantification (LOQ) is highly dependent on the degree of measurement uncertainty accepted (Vrålstad et al., 2009). In congruence with Vrålstad et al. (2009) we roughly estimated LOQ to be 10-fold higher than the LOD, which corresponds to 40 PFU.

A standard curve was constructed from the data and the regression $y = –3.5482x + 39.47$ was obtained with $R^2 = 0.99562$ and amplification efficiency of 91.35 % (Fig. S1).

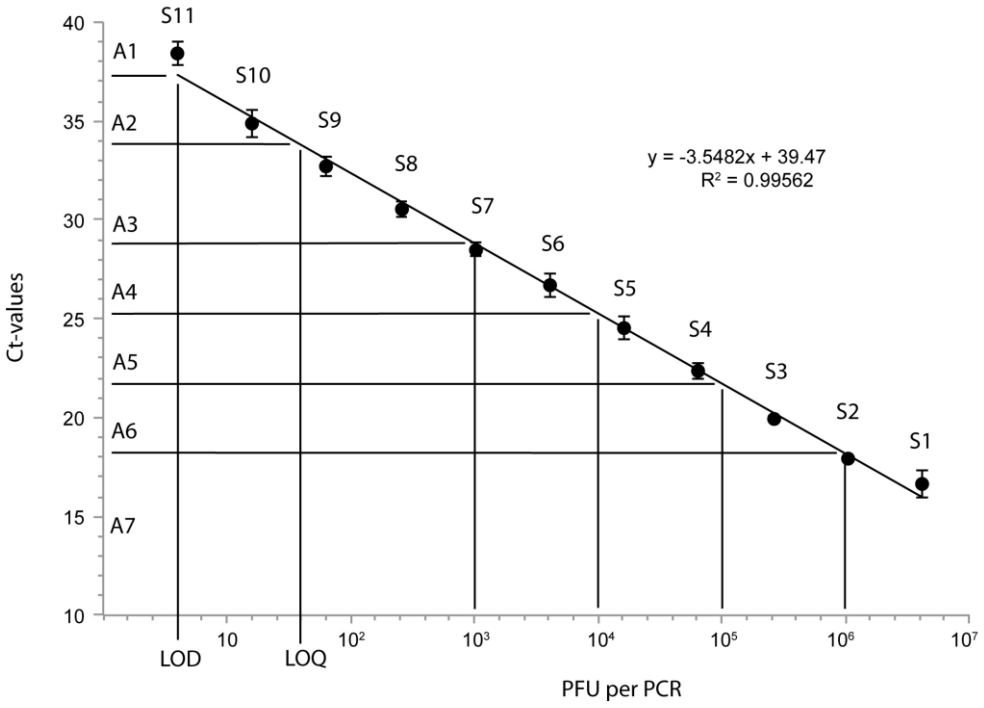

Figure S1. The standard curve based on the mean Ct-values from the 4-fold serial dilution shown in Table S1 plotted against the estimated number of PCR-forming units (PFU) per reaction volume. The limits of detection (LOD) and quantification (LOQ) are indicated. The translation of the qPCR results in semi-quantitative agent levels was adopted from Vrålstad et al. (2009).

Comparison of results obtained at the Wageningen University and Research Centre in Wageningen (CVI) and at the Charles University in Prague (CUNI)

To compare the quantitative results obtained at CVI and CUNI, several DNA isolates were analyzed in both laboratories. These included three isolates of agent level A0 (no agent), seven of A2 (very low agent level), six of A3 (low level), one A4 (moderate level), one A5 (high level) and one A7 (exceptionally high level). Although we observed some variation in quantified amount of *A. astaci* DNA (expressed in PCR-forming units, PFU), the results in generally corresponded between both laboratories if expressed in semi-quantitative agent levels (Table S2). Negative samples were tested as negative in both laboratories, and different agent levels were only detected if the PFU value obtained in one or both laboratories were below the level of quantification, and close to the threshold separating those agent levels.

Table S2. The comparison of results obtained for selected DNA isolates at CVI and at CUNI.

DNA isolate no.	Agent level according to CVI	Number of PFU in 5 µl of the DNA isolate according to CVI	Agent level according to CUNI	Number of PFU in 5 µl of the DNA isolate according to CUNI	Ratio of number of PFU obtained at CVI to the number obtained at CUNI
1	A0	0	A0	0	-
2	A0	0	A0	0	-
3	A0	0	A0	0	-
4	A2	6*	A1	5*	1.20
6	A2	6*	A2	8*	0.75
8	A2	9*	A2	17*	0.53
5	A2	10*	A2	7*	1.43
11	A2	24*	A2	26*	0.92
9	A2	29*	A2	19*	1.53
7	A2	30*	A2	17*	1.76
13	A3	41	A3	73	0.56
10	A3	52	A2	25*	2.08
15	A3	150	A3	256	0.59
14	A3	199	A3	167	1.19
12	A3	393	A2	49*	8.02
16	A3	699	A3	267	2.62
17	A4	3142	A4	4096	0.77
18	A5	55217	A5	65536	0.84
19	A7	1017562	A7	1048576	0.97
				Median	1.08

*asterisk indicates a number of PFU below the limit of quantification.

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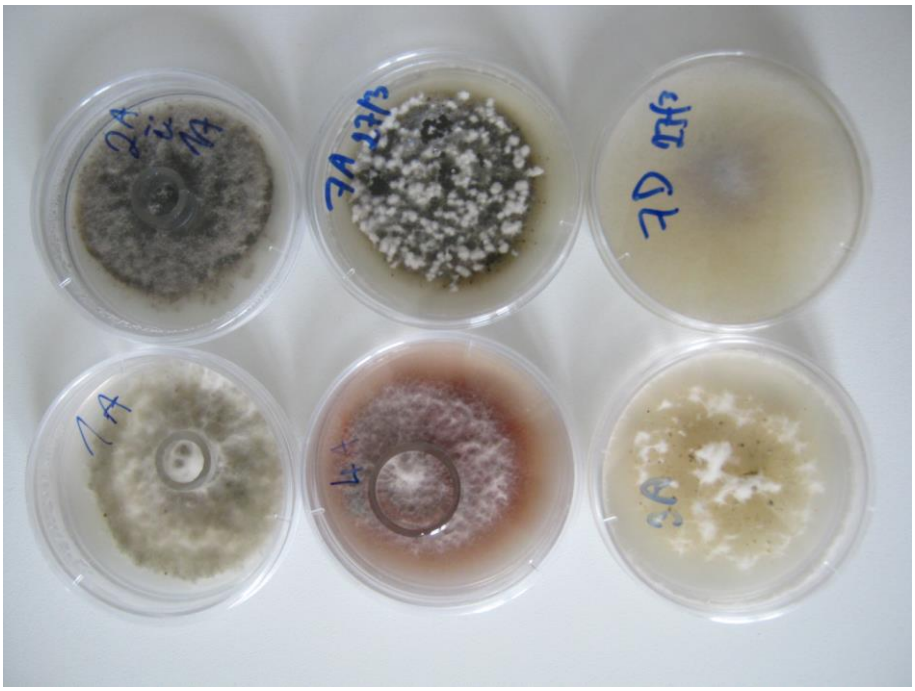
Chapter 7

Kozubíková-Balcarová, E., Koukol, O., Martín, M.P., Svoboda, J., Petrusek, A., 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.**



"Fungi-like organisms also complicate the isolation of *Aphanomyces astaci* from crayfish, as some species, particularly saprobiotic, grow faster in artificial media than animal-specific parasites, and often overgrow the target species."

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The diversity of oomycetes on crayfish: Morphological *vs.* molecular identification of cultures obtained while isolating the crayfish plague pathogen

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ABSTRACT

Numerous oomycetes colonise the crayfish cuticle, the best known being the crayfish plague pathogen *Aphanomyces astaci*. Although other oomycetes associated with crayfish complicate the isolation and molecular detection of *A. astaci*, their diversity is little known. To improve this knowledge, we analysed 95 oomycete isolates obtained during attempts to isolate A. astaci from crayfish presumably infected by this pathogen. We characterized the isolates morphologically and by sequencing of the nuclear internal transcribed spacer (ITS) region. We identified 13 taxa by molecular analysis. Ten of them were assigned to five genera; the remaining three were affiliated with the order *Saprolegniales* but could not be reliably assigned to any genus. Morphological identification to species level was only possible for 15 % of isolates; all corresponded to *Saprolegnia ferax*, which was confirmed by ITS sequencing. The most frequently isolated species were *S. ferax* and *Saprolegnia australis*. Only seven isolates of A. astaci were obtained, all from one disease outbreak. We show that oomycete cultures obtained as by-products of parasite isolation are valuable for oomycete diversity studies, but morphological identification may uncover only a fraction of their diversity. Further, we show that crayfish may be frequently associated with potentially serious parasites of other organisms.

Introduction

Aquatic oomycetes (*Heteroconta*, *Chromista*) are nearly ubiquitous in freshwater environments and have evolved a wide range of life strategies. Saprobiotic species as well as parasites ranging from opportunistic to highly specific can be found in this group (Johnson *et al.* 2002). While animal parasites causing severe losses in both aquacultures and in the wild have been studied intensively (van West 2006), much less scientific attention has been paid to other oomycetes.

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There is thus insufficient knowledge of the diversity of this group.

Aphanomyces astaci Schikora (*Saprolegniales*) is an example of an intensively studied oomycetous parasite. *Aphanomyces astaci* is highly adapted to living in freshwater crayfish and causes the crayfish plague, a devastating disease of European crayfish (Söderhäll & Cerenius 1999). On the contrary, it does not usually harm North American crayfish species, which are regarded as its original hosts, and some of these species have become widespread invaders in Europe (Souty-Grosset *et al.* 2006). Apart from *A. astaci*, crayfish may also host a variety of other filamentous microorganisms such as the parasitic oomycete *Saprolegnia parasitica* Coker (Söderhäll *et al.* 1991), saprobiotic oomycete *Aphanomyces frigidophilus* Kitanch. & Hatai (Ballesteros *et al.* 2006) or fungi (Dörr *et al.* 2012). Growth of these organisms into tissues of the host is usually limited by the host's immune reaction (Söderhäll & Cerenius 1992). Nevertheless, their hyphae may considerably expand when crayfish are wounded, stressed, or dying (e.g., Hirsch *et al.* 2008).

Research on *A. astaci* often encounters problems related to the presence of these organisms. The DNA of species most related to *A. astaci* may cross-react with molecular methods for detecting this parasite, resulting in false positive detections (Oidtmann *et al.* 2006; Kozubíková *et al.* 2009). Recent descriptions of several new *Aphanomyces* species from various hosts (O'Rourke *et al.* 2010; Takuma *et al.* 2010, 2011) confirm that our knowledge of the diversity of this genus is still insufficient, and that other taxa with potential influence on DNA-based pathogen detection methods may be found. Fungi-like organisms also complicate the isolation of *A. astaci* from crayfish, as some species, particularly saprobiotic, grow faster in artificial media than animal-specific parasites, and often overgrow the target species (Cerenius *et al.* 1988).

Isolates of oomycetes other than *A. astaci* obtained as by-products in attempts to isolate *A. astaci* are usually discarded. However, such material might be valuable for obtaining better insight into the diversity of species that use living crayfish as hosts or moribund crayfish as substrates, and which may influence *A. astaci* diagnostics. Understanding which taxa most often complicate the isolation of this pathogen may allow the development of more selective cultivation methods. Furthermore, material obtained in such cultivation attempts may contain unknown or little characterized lineages relevant for taxonomic and phylogenetic studies.

The taxonomy of oomycetes is still incompletely resolved. The traditional approach for identifying genera and species is usually based on the morphological characters of asexual (zoosporangia) and sexual (oogonia and antheridia) structures (Seymour 1970; Johnson *et al.* 2002). However, a high level of phenotypic plasticity occurs in this group (Johnson *et al.* 2002; Hulvey *et al.* 2007) and many isolates fail to produce any diagnostic features in laboratory cultures (Diéguez-Uribeondo *et al.* 2007). Moreover, recent phylogenetic studies based on molecular markers often do not support traditional taxonomy, especially on the generic and species level (e.g., Cooke *et al.* 2000; Diéguez-Uribeondo *et al.* 2007). Hulvey *et al.* (2007) suggested revising the system of oomycetes by performing phylogenetic analyses for available oomycete isolates and then redefining species on the basis of both molecular and morphological characteristics. Such an approach has been partly applied in phylogenetic studies on the genera *Saprolegnia* (Diéguez-Uribeondo *et al.* 2007) and *Aphanomyces* (Diéguez-Uribeondo *et al.* 2009). Phylogeny-based taxonomical revisions will probably result in the uncovering of many synonymous names which refer to the same or very closely related taxa. On the other hand, substantial genetic variation may be found among isolates seemingly belonging to the same taxon.

During attempts to isolate *A. astaci* from several crayfish species we have obtained an extensive collection of oomycetes. We decided to identify these oomycete isolates using traditional morphological methods as well as molecular tools. We aimed to (1) extend the knowledge of the diversity of oomycete microbiota on crayfish, and (2) compare the results of traditional morphological and molecular approaches for taxon identification.

Material and methods

Isolates of oomycetes

We obtained oomycete isolates from three species of crayfish from European waters certainly or presumably infected with *Aphanomyces astaci*. We included two native European species (23 individuals of *Astacus astacus* and four individuals of *Austropotamobius pallipes*), and one invasive North American species (eight individuals of *Orconectes limosus*) in the study. *Astacus astacus* that were dead or dying due to crayfish plague (confirmed by a PCR-based procedure after Oidtmann *et al.* 2006) originated from three streams in the Czech Republic: Žebrákovský brook near Světlá nad Sázavou (49°41′N, 15°22′E), a tributary of Pěnenský pond near Horní Pěna (49°06′N, 15°03′E; an outbreak described in Kozubíková *et al.* 2008), and the stream Litavka in Příbram (49°40′N, 13°58′E). Two additional specimens of *A. astacus* originating from the Světlohorská reservoir near Vimperk (49°00′N, 13°44′E) were used in an experiment on the transmission of crayfish plague pathogen from *O. limosus* from the locality Smečno (see below). *Orconectes limosus* for the isolation attempts came from two Czech localities, a stream Pšovka near Lhotka u Mělníka (50°23′N, 14°33′E) and a town pond in Smečno (50°11′N, 14°02′E). Both populations are known to host *A. astaci* (Kozubíková *et al.* 2009) but to a different extent (Matasová *et al.* 2011). Most *O. limosus* were sampled alive but were dead or dying at the time of oomycete isolation for unclear reasons (e.g., handling stress). *Austropotamobius pallipes* presumably dying from crayfish plague originated from three localities in Spain: the river Rio Frío near Granada (37°9′N, 4°12′W), the river Borosa near Jaén (38°1′N, 2°52′W), and a stream near Olot near Girona (42°11′N, 2°30′E). Some of the crayfish were kept in aquaria after capture, and moulted or died during that time. As we primarily intended to isolate *A. astaci*, we chose material suitable for that purpose, i.e., moribund or dead crayfish from crayfish plague outbreaks or *A. astaci* carriers and their exuviae. Dead individuals and exuviae were either fresh or up to 2 d old; only in a few cases were the dead crayfish bodies already in the early stage of decomposition. Dead or moribund European crayfish prevailed as starting material for oomycete isolation in this study (60 % of all obtained isolates came from such material). In one case, we

used a living and apparently healthy *O. limosus* individual with macroscopic melanised depositions for isolation, all remaining *O. limosus* were dead at the time of isolation or their exuviae were used. More detailed information on the state of crayfish used as starting material for the isolation of each oomycete strain is provided in Supplementary Table 1.

Crayfish were euthanized before dissecting the tissues if needed. Soft abdominal cuticle, limbs, telson, uropods, eye stalks, pieces of cuticle with melanin depositions, and pieces of crayfish exuviae were used as inocula. Samples of crayfish tissues were rinsed in penicillin G solution (at a concentration of 100 mg L^{-1}) and placed on sterilised river water-glucose-yeast (RGY) media (containing filtered pond water, glucose, yeast extract, agar, penicillin G, and oxolinic acid, as described in Alderman & Polglase 1986). Plates were incubated at room temperature and checked daily for the growth of filamentous organisms. Tips of growing hyphae were repeatedly transferred to fresh RGY media to eliminate possible bacterial contamination and obtain presumably single-strain cultures.

Morphological identification

Asexual structures

To induce the production of asexual structures (zoosporangia), a drop culture procedure after Cerenius *et al.* (1988) was followed. A peptone-glucose medium PG1 according to Unestam (1965) developed for cultivation of *Aphanomyces astaci* but suitable also for other groups of oomycetes and fungi was used. This medium contains peptone, glucose, a specific salt solution (for details see Unestam 1965), a phosphate buffer, and penicillin G. About 1 ml of the liquid medium was placed on Petri dishes and inoculated with a piece of the agar medium containing growing hyphae. After 3 d of incubation at room temperature, the liquid medium was washed out using autoclaved tap water, and the drop cultures were checked daily with a microscope for the presence of zoosporangia. The types of zoosporangia were evaluated to identify the genera of the isolates (Scott 1961; van der Plaats-Niterink 1981; Daugherty *et al.* 1998; Johnson *et al.* 2002).

Sexual structures

To obtain sexual structures (oogonia, antheridia), autoclaved hemp seeds were cultivated on PG1 agar cultures of the isolates as in Diéguez-Uribeondo *et al.* (2007). Seeds overgrown with hyphae were transferred to Petri dishes with autoclaved tap water, incubated at room temperature, and inspected using an inverted microscope every 3 d for 2–3 weeks. If sexual structures appeared, classical identification keys for oomycetes (Cejp 1959; Johnson *et al.* 2002) were used to identify species, and some of those structures were documented by microphotographs.

Molecular identification

DNA extraction, amplification, and sequencing

DNA was extracted from mycelia grown in drop cultures prepared as described above. About 10 mg of mycelium per isolate was frozen at −80 °C before use. Total genomic DNA was extracted using the E.Z.N.A. fungi DNA miniprep kit (Omega Biotek, Doraville, USA) or DNeasy Plant Mini kit (QIAGEN, Valencia, California, USA), according to the manufacturer's instructions for DNA extraction.

Universal primers ITS5 and ITS4 (White *et al.* 1990) were used to amplify a fragment of the nuclear ribosomal DNA including internal transcribed spacers (ITS1 and ITS2) and the gene for 5.8S rRNA. PCR was performed using Ready-To-Go™ PCR Beads (GE Healthcare, UK) in a final volume of 25 μl as described in Winka *et al.* (1998) or Reddy Mix PCR Mastermix (with 1.5 mM $MgCl_2$, ABgene, Epsom, UK) in a final volume of 25 μl, with final concentration of each primer 0.5 μM.

The cycling protocol followed Martín & Winka (2000). PCR products were visualised by agarose electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA). Both strands were sequenced separately at Secugen (Madrid, Spain) or Macrogen (Seoul, Korea).

Sequence analysis

Sequencher™ version 4.2 (Gene Codes Corporation, Ann Arbor, Michigan, USA) was used to assemble the consensus sequence from the two DNA strands of the ITS nrDNA of each isolate. The resulting sequences were deposited to GenBank under acc. nos. KF386637–KF386727 (see Supplementary Table 1 for details). A BLAST search was performed for all sequences to reveal the most closely related taxa, from which sequences are available in GenBank. As the taxonomy and nomenclature of oomycetes is likely to undergo substantial changes in the future (Hulvey *et al.* 2007), we primarily relied on publicly available reference sequences from recent phylogenetic studies on *Saprolegnia* and *Aphanomyces* (Diéguez-Uribeondo *et al.* 2007, 2009), *Phytophthora* (Burgess *et al.* 2009), and *Pythium* (Lévesque & de Cock 2004) or a barcoding study for oomycetes (Robideau *et al.* 2011). When possible, morphological evidence was also considered.

The sequence data were further processed in MEGA v. 5 (Tamura *et al.* 2011). Newly obtained sequences together with reference sequences acquired from GenBank were aligned using the ClustalW algorithm (Thompson *et al.* 1994), with gap opening and extension penalties values of 15 and 6.66, respectively. For detailed comparisons of sequence divergence among closely related isolates and taxa, separate alignments were created for each such group of similar sequences, and pairwise distances (expressed as p-distance, i.e., percentage of nucleotides differing between sequences) were calculated with pairwise deletion of gaps and sites with missing data. Furthermore, all obtained sequences were aligned together with selected reference sequences representing relevant closely related taxa, and we used this alignment to construct a neighbour-joining tree graphically depicting the patterns of sequence similarity.

Results

Isolates of oomycetes

We obtained a total of 95 oomycete isolates (35 from Czech *Orconectes limosus*, 51 from Czech *Astacus astacus*, and nine from Spanish *Austropotamobius pallipes*), one to 14 isolates per crayfish individual. A list of all isolates, including information on the presence of asexual and sexual structures and the

taxon identified on the basis of the ITS sequence, is provided in Supplementary Table 1.

Morphological identification

We treated 83 isolates in an attempt to induce reproductive structures. Asexual structures appeared in 49 isolates (59 %). In seven of them, we found the spore balls of primary cysts at the tips of zoosporangia (achlyoid sporangial type) typical for the genus *Aphanomyces* (Scott 1961). In three isolates we observed structures resembling sporangia of the genus *Pythium* (inflated filamentous or globose zoosporangial type; van der Plaats-Niterink 1981). In the remaining isolates producing zoosporangia, saprolegnioid release of zoospores (Daugherty *et al.* 1998) was observed, suggesting the presence of *Saprolegnia* spp.

Induction of sexual structures was successful in 13 isolates out of 83 tested (15 %). Oogonia had very similar appearance in all these isolates (Fig 1). We examined 60 oogonia from nine isolates in detail. They were spherical, in most cases with clearly pitted walls. The length of oogonial stalks usually substantially exceeded one half of the oogonium diameter. Mature oospores, usually 5–20 oospores per oogonium, were centric or rarely slightly subcentric with a diameter of about 22 μm. We did not observe many antheridia (12 per 60 oogonia in the nine isolates inspected in detail), and it was usually impossible to determine if they were monoclinous or diclinous (with the exception of one diclinous antheridium, Fig 1C). Based on the morphological characters described above, all isolates producing sexual structures were assigned to *Saprolegnia ferax* (Gruith.) Kütz. (Cejp 1959; Johnson *et al.* 2002).

Molecular identification

ITS sequences of all isolates obtained during our study clustered into 13 clearly distinct groups. The number of isolates of the identified taxa, together with information on the production of sexual or asexual structures and the origin (host) of the isolates, are provided in Table 1. By ITS sequencing, we identified oomycetes of five genera (*Aphanomyces*, *Saprolegnia*, *Phytophthora*, *Phytopythium*, and *Pythium*), and three more taxa for which generic assignment based on ITS sequences was not feasible (Table 1). The molecular analysis supported morphological identification of the genera *Pythium*, *Aphanomyces*, and *Saprolegnia*. One isolate (Li12) suggested to be *Pythium* sp. according to its morphology appeared to belong to the related genus *Phytopythium* on the basis of ITS sequencing. The patterns of ITS similarity, including one representative sequence from each group of our isolates, and sequences of the same and/or most related taxa retrieved from GenBank, are summarised in the neighbour-joining tree (Fig 2).

The only two isolates assigned to the genus *Phytophthora* (SAP716 and SAP717) had identical ITS sequences. They were identical to sequences labelled *Phytophthora inundata* Brasier, Sánch. Hern. & S.A. Kirk originating from the phylogenetic

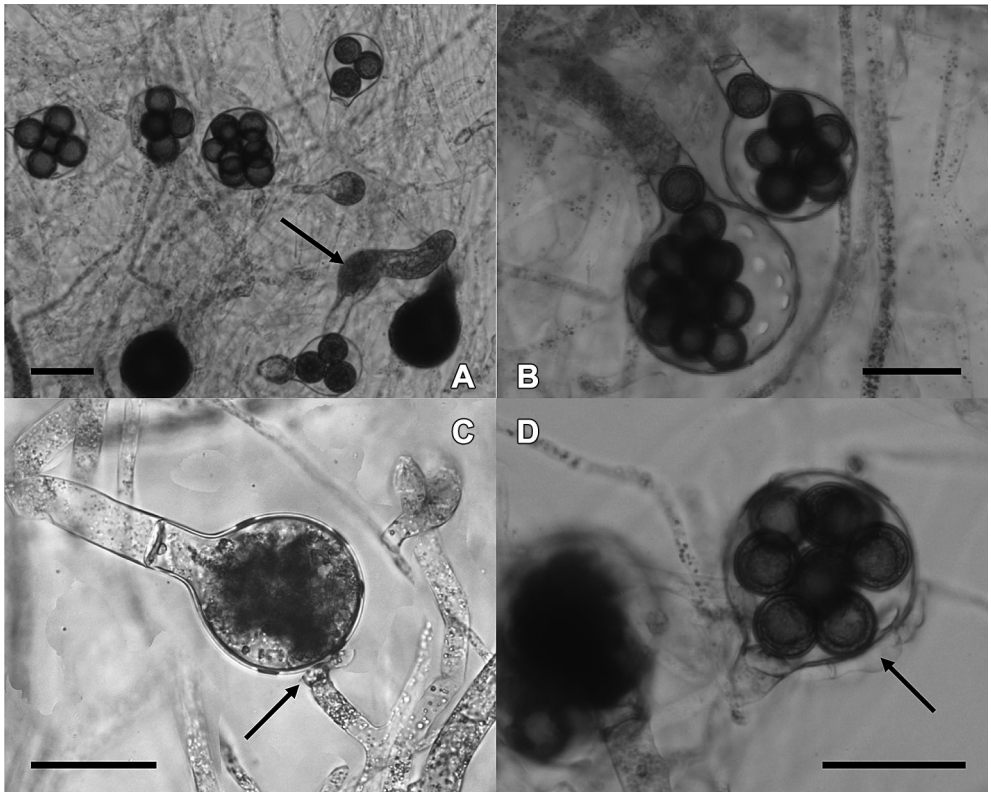

Fig 1 – Microphotographs of reproduction structures of the isolates identified as *Saprolegnia ferax*. (A) saprolegnious zoosporangium (marked with arrow) and oogonia with various numbers of oospores; (B) mature oogonia with clearly pitted walls containing centric oospores; (C) immature oogonium with antheridium (marked with arrow); (D) mature oogonium with antheridium (marked with arrow). Bars: 50 μm.

Table 1 – Summary of the isolated taxa. For each taxon we provide the number of obtained isolates, number of crayfish individuals from which they were isolated, and proportion of isolates which produced oogonia (sexual structures) and sporangia (asexual structures). Further, we provide examples of other hosts and material from which the taxa have also been isolated (only studies including molecular evidence were included).

Taxon	No. of isolates	Isolated from (no. of crayfish)			Asexual structures (%)	Sexual structures (%)	Previous records from crayfish	Previous records from other organisms and sources
		AA	OL	AP				
Peronosporales								
clade *Phytophthora inundata–P. humicola*[a]	**2**		1		0	0		Decaying leafs[o], plant roots[o,p,q], soil[o]
Pythiales								
Phytopythium sp.	**1**	1			100	0		
Pythium sp. I (subclade B2)[b]	**3**	3			0	0		Soil[o,q,r], plant roots[q,r], water[o]
Pythium sp. II (clade B)[b]	**2**	1			100	0		
Saprolegniales								
Aphanomyces astaci[c]	**7**	3			100	0	Numerous species[e,f,g]	No reliable
clade *Aphanomyces laevis–A. repetans*[c]	**6**		3		33	0	AP[h], PC[i] PL[i,e]	Water[e,s]
Saprolegnia australis (clade IV)[d]	**24**	4	2	2	68	0	AA[j,k], OL[l], OP[m]	Fish[n,t], fish eggs[q], soil[t]
Saprolegnia ferax (clade II)[d]	**32**	5	2	2	48	52	AA[n], OL[l]	Amphibian eggs[u,v], crab eggs[o], fish eggs[q], fish[n,q], planktonic crustacean[w], soil[q,t], water[n,s,t]
Saprolegnia hypogyna (clade Ia)[d]	**1**	1			100	0		Fish eggs[n,o], water[n]
Saprolegnia parasitica (clade I)[d]	**10**		1		90	0	AA[j], AL[n]	Fish[n,o], fish eggs[q], water[n,o]
Saprolegniales I	**2**	1			N/A	N/A		Amphibian eggs[v,x], fish[q], soil[t], water[t,o]
Saprolegniales II	**4**	2	1		0	0	AA[k]	Planktonic crustacean[w]
Saprolegniales III	**1**	1			100	0		Planktonic crustacean[q]
Summary	**95**				59	15		

Abbreviations of crayfish species: AA, *Astacus astacus*; AL, *Astacus leptodactylus*; AP, *Austropotamobius pallipes*; OL, *Orconectes limosus*; OP, *Orconectes propinquus*; PL, *Pacifastacus leniusculus*; PC, *Procambarus clarkii*.

a Subclade *P. inundata–P. humicola* of clade 6 according to Burgess *et al.* (2009).
b Clade B or its subclade B2 according to Lévesque & de Cock (2004).
c Clades according to Diéguez-Uribeondo *et al.* (2009).
d Clades according to Diéguez-Uribeondo *et al.* (2007).
e Diéguez-Uribeondo *et al.* (2009).
f Svoboda *et al.* (2012).
g Diéguez-Uribeondo *et al.* (1995).
h Cammà *et al.* (2010).
i Royo *et al.* (2004).
j Makkonen *et al.* (2010).
k Vrålstad *et al.* (2009).
l Hirsch *et al.* (2008).
m Krugner-Higby *et al.* (2010).
n Diéguez-Uribeondo *et al.* (2007).
o Strain database of the Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/collections/Biolomics.aspx?Table=CBS%20strain%20database).
p Brasier *et al.* (2003).
q GenBank (unpubl. data).
r Lévesque & de Cock (2004).
s E. Kozubíková-Balcarová (unpubl. data).
t Hulvey *et al.* (2007).
u Fernández-Benéitez *et al.* (2011).
v Ruthig (2009).
w Wolinska *et al.* (2008).
x Petrisko *et al.* (2008).

study on this genus by Burgess *et al.* (2009). However, sequences labelled as *Phytophthora humicola* W.H. Ko & Ann were also very similar to those obtained by us; those from Burgess *et al.* (2009) diverged by 0.6 %, and those obtained by other authors were even more similar (down to 0.12 % divergence). As it is not clear whether these two taxa represent one or two biological species, we label the isolates for the purpose of our study as the *P. inundata*–*P. humicola* clade, which is a subclade of the clade 6 previously defined by Burgess *et al.* (2009).

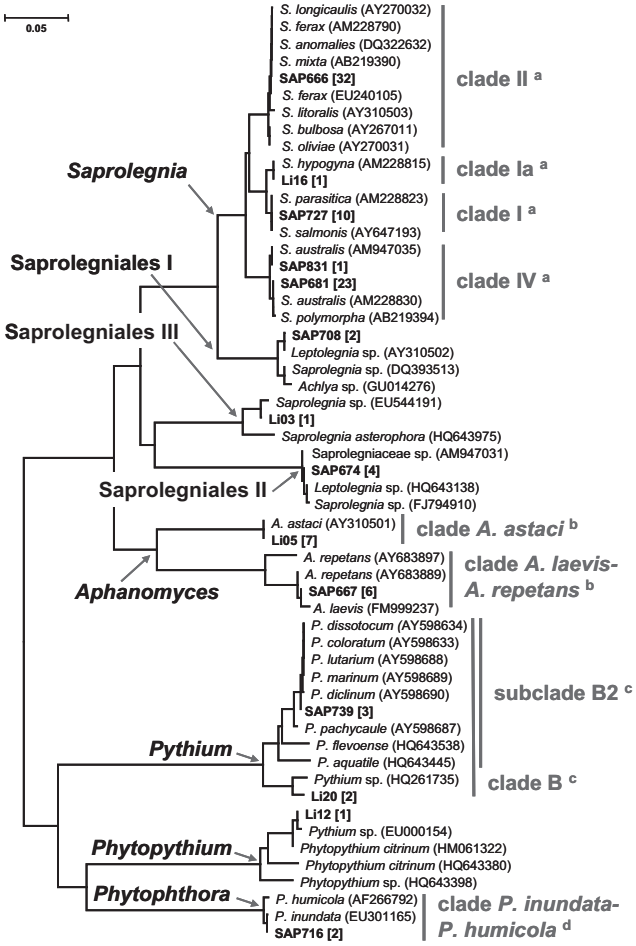

Fig 2 – Neighbour-joining tree demonstrating the ITS sequence similarity of representative oomycete isolates obtained from crayfish in our study, and their most similar ITS sequences obtained from GenBank. In each clade, one or two representative sequences of our isolates are presented in bold followed by the total number of isolates with identical or very similar sequences in brackets. Accession numbers are provided in parentheses for sequences retrieved from GenBank. We refer to the clades previously defined by phylogenetic studies: [a]Diéguez-Uribeondo *et al.* (2007), [b]Diéguez-Uribeondo *et al.* (2009), [c]Lévesque & de Cock (2004), [d]Burgess *et al.* (2009). Branch lengths corresponding to sequence divergence (p-distance) are relevant for closely related taxa. Genera representing different orders are phylogenetically so distinct that the ITS alignment can no longer be considered reliable, and thus the tree should not be considered as an attempt to reconstruct phylogenetic relationship among the groups.

Among five isolates assigned to the genus *Pythium* we found two distinct groups of ITS sequences. Three isolates included in the first group, each obtained from *Astacus astacus* from different Czech localities, were very similar to each other (two, SAP706 and SAP739, were identical and the third, Li02, differed from them at a single variable position). All matched completely to sequences labelled with several different species names (*Pythium coloratum* Vaatarja., *Pythium lutarium* Ali-Shtayeh, *Pythium marinum* Sparrow, *Pythium dissotocum* Drechsler, and *Pythium diclinum* Tokun.), originating either from a phylogenetic study on the genus *Pythium* (Lévesque & de Cock 2004) or the barcoding study of Robideau *et al.* (2011). As sequences with the above-mentioned names form a single cluster that might represent one species, and the nomenclature of such a complex is unclear, we refer to our isolates as *Pythium* sp. I subclade B2 (after Lévesque & de Cock 2004).

The two identical sequences from the second group of isolates (Li18 and Li20; both from a single locality and producing the same type of filamentous, inflated sporangia) clearly belonged to the genus *Pythium*. They differed from the closest available sequences, identified to the generic level only, by more than 1.8 %, and thus could not be assigned with certainty to any species. However, the closest sequence available in GenBank originated from the barcoding study of Robideau *et al.* (2011), and clearly belonged to clade B defined previously by Lévesque & de Cock (2004). Thus, we labelled the two isolates *Pythium* sp. II clade B.

The sequence of one of our isolates originating from Czech *A. astacus* (Li12) differed from two sequences labelled *Pythium* sp. only by ambiguous positions and a 1-bp indel (i.e., potential sequencing errors), and from one labelled *Pythium citrinum* by an additional 3-bp long indel. The closest sequences included in the barcoding study of Robideau *et al.* (2011), diverging by *ca* 4.6 %, belonged to the genus *Phytopythium*, recently established by Bala *et al.* (2010) from *Pythium* clade K after Lévesque & de Cock (2004). As most other available sequences of *P. citrinum* (the combination *Phytopythium citrinum* has not been formally proposed; André Lévesque, pers. comm.) were distinct from the sequence from our isolate (diverging by more than 4.7 %) and thus identification as *P. citrinum* does not seem to be well supported, we labelled our isolate *Phytopythium* sp.

Isolates assigned to the genus *Aphanomyces* represented two different taxa. Seven of them (Li05–Li11), obtained from one Czech mass mortality of *A. astacus*, belonged to the crayfish plague pathogen *Aphanomyces astaci*, which was confirmed by a complete match of the ITS sequence to reference sequences from this species. Two of these isolates were provided to the Reference Laboratory for Crayfish Plague, Finnish Food Safety Authority Evira, Kuopio, Finland.

The remaining six isolates of the genus *Aphanomyces* (e.g., SAP667) had similar ITS sequences, differing by no more than 0.7 %. They conformed to sequences of the *Aphanomyces laevis*–*Aphanomyces repetans* clade after Diéguez-Uribeondo *et al.* (2009), with the divergence from sequences of this clade retrieved from GenBank ranging between 0 and 8.5 %. As the taxonomy of this clade is apparently unresolved, we also use the name *A. laevis*–*A. repetans* clade in this study.

Within *Saprolegnia* we found three distinct groups, corresponding to clades I (including subclade Ia), II, and IV according to Diéguez-Uribeondo *et al.* (2007). Clade I (excluding subclade Ia) has been assigned to *Saprolegnia parasitica sensu stricto* by Diéguez-Uribeondo *et al.* (2007), so we use this name for all (e.g., SAP727) but one of our isolates that belong to this clade and have ITS sequences diverging from each other by no more than 0.6 % (four variable positions; the range of variation observed within clade I by Diéguez-Uribeondo *et al.* (2007)). Each of these ITS sequence variants had a complete match in GenBank labelled as *S. parasitica* or *Saprolegnia salmonis* Hussein & Hatai. The sequence of one isolate from a Czech noble crayfish (Li16) diverged from the rest of the obtained sequences of clade I by 0.7–1 %, falling into the subclade Ia of Diéguez-Uribeondo *et al.* (2007). As it completely matched two different sequences assigned to *Saprolegnia hypogyna* (Pringsh.) de Bary, we label the isolate with this name.

All isolates that produced oogonia (e.g., SAP666) had nearly identical ITS sequences (differing by no more than 0.3 %) and corresponded to clade II in Diéguez-Uribeondo *et al.* (2007). ITS sequences closely matching those from our isolates (divergence below 0.5 %) were labelled in GenBank with various species names: *Saprolegnia ferax*, *Saprolegnia longicaulis* Steciow, *Saprolegnia bulbosa* Steciow, *Saprolegnia mixta* de Bary, *Saprolegnia oliviae* Steciow, *Saprolegnia litoralis* Coker, *Saprolegnia anomalies* Gandhe & Kurne, and *Saprolegnia diclina* Humphrey. We adopted the name *S. ferax* suggested by the morphological identification for the purpose of this study. However, only 52 % of isolates sharing this ITS sequence (and treated to induce production of sexual structures) produced oogonia; the rest remained sterile.

All but one of 24 sequences corresponding to *Saprolegnia* clade IV, originating from all three host crayfish species from both Czechia and Spain, were identical. The remaining sequence (isolate SAP831) differed from the rest at three variable positions (0.4 %). As clade IV contained predominantly *Saprolegnia australis* R.F. Elliott in Diéguez-Uribeondo *et al.* (2007), and both sequence variants from our study had an exact match in GenBank, we label our isolates belonging to clade IV with this species name (*S. australis*).

Identification to the generic level was unclear for the remaining seven isolates, all apparently belonging to the order *Saprolegniales*. Two isolates with identical ITS sequences (SAP708, SAP709), isolated from one Czech *A. astacus*, matched completely to a GenBank sequence labelled *Leptolegnia* sp., and two other sequences assigned to this genus diverged by less than 1 %. However, other publicly available sequences with less than 1.5 % divergence have been assigned to genera *Saprolegnia* and *Achlya*, and most *Leptolegnia* sequences were much more divergent. Therefore, we labelled these isolates '*Saprolegniales* I'.

Another four identical sequences (e.g., SAP674), originating from three Czech localities and two crayfish host species, were also identified only to the order level, as the publicly available sequences with a complete match were labelled Saprolegniaceae sp., *Saprolegnia* sp., and *Leptolegnia* sp. For this group, we thus used the label '*Saprolegniales* II'.

The last taxon with unclear generic status was represented in our collection by only a single isolate from Czech *A. astacus* (Li03). The closest match in GenBank, differing at three variable positions and a 5-bp long indel was labelled *Saprolegnia* sp., and originated from an oomycete parasite of the planktonic crustacean *Daphnia* (Table 1). However, the next best matching sequences, diverging by 3.8 %, were assigned to species *Scoliolegnia asterophora* (de Bary) M.W. Dick (formerly *Saprolegnia asterophora*; Dick 1969). Thus we labelled our isolate '*Saprolegniales* III'.

Up to four oomycete taxa were obtained per one crayfish individual. *Saprolegnia ferax* (32 isolates from nine crayfish individuals) and *S. australis* (24 isolates from eight crayfish) were the most commonly isolated oomycetes, obtained from all three studied crayfish species. Both of these *Saprolegnia* species were found in Czechia as well as in Spain; the remaining 11 taxa were obtained only from crayfish collected in Czechia.

Discussion

Although some recent studies on oomycete diversity still rely on identification based on the morphology of reproduction structures observed directly on biological material (e.g., Czeczuga *et al.* 2002, 2004), more advanced methods, particularly molecular analyses, are more and more required for oomycete identification (Hulvey *et al.* 2007). In this study, we used both morphological and molecular approaches to identify a collection of oomycete isolates obtained from crayfish. Identification of three genera and one species made on the basis of morphology of asexual and sexual structures was supported with sequencing of ITS region of rDNA. However, molecular methods allowed us to uncover further diversity among isolates that did not produce any reproduction structures, and they were important for confirmation of the morphological identification. In conclusion, a combination of both approaches may certainly be useful when redefining oomycete taxa.

The low production of sexual structures in our isolate collection might have been caused by the narrow range of cultivation conditions. However, some isolates of *Saprolegnia parasitica* are not known to produce oogonia and antheridia at all (Diéguez-Uribeondo *et al.* 2007), similarly to *Aphanomyces repetans*, which has been suggested to be an asexual form of *Aphanomyces laevis* de Bary (Diéguez-Uribeondo *et al.* 2009). Thus, the varying results of induction of sexual reproduction may suggest that the isolates, although identified as the same taxa, differed in their physiological characteristics.

The only taxon reliably identified to the species level by both morphological and molecular approaches was *Saprolegnia ferax* (clade II after Diéguez-Uribeondo *et al.* 2007). In our isolates, this species was morphologically recognisable (in isolates producing sexual structures). However, substantial phenotypic plasticity may occur in this taxon, as demonstrated by the presence of identical or very similar sequences with several species names in the GenBank. For example, *Saprolegnia longicaulis* and *Saprolegnia bulbosa* were described on the basis of morphology as new species distinct from *S. ferax* in Argentina (Steciow 2001; Steciow *et al.* 2007). However, all ITS sequences of isolates identified as *S. longicaulis* in GenBank and the sequence of the isolate described as *S. bulbosa* by Steciow *et al.* (2007) belong to clade II after Diéguez-Uribeondo *et al.* (2007) together with our isolates. This calls

for a critical evaluation of recently described species, to check whether they are not only extreme phenotypic forms of already known species. A taxon diagnosis system for the genus *Saprolegnia* based on ITS nrDNA has just been described by Sandoval-Sierra *et al.* (2013), and is thus recommended for further use in accurate species delineation. A similar situation as in *Saprolegnia* may have arisen, with various names referring to the very close sequences of the genus *Pythium* of subclade B2 according to Lévesque & de Cock (2004), and in pairs *Phytophthora inundata* and *Phytophthora humicola*, and *S. parasitica* and *Saprolegnia salmonis*, respectively. It is apparent that identification characteristics for many oomycete taxa require reassessment, and the taxonomy of many groups should undergo major revisions using different molecular markers besides the ITS region.

Our study confirms previous findings that crayfish, especially dead or dying, may host various oomycete species (Hirsch *et al.* 2008; Krugner-Higby *et al.* 2010; Makkonen *et al.* 2010), some of them still not well defined. Apart from *Aphanomyces astaci*, the oomycetes obtained in this study do not seem to be host-specific for crayfish. A number of them have been already isolated from other hosts or substrates (see examples in Table 1). The plant parasite *P. inundata*, previously found to be associated with roots of trees growing in wet soils (Brasier *et al.* 2003), was also isolated from crayfish in this study. This can be explained by the crayfish origin. Specimens from which this oomycete was isolated came from a muddy pond where roots of surrounding vegetation penetrated into the littoral zone. Crayfish in such a pond are in frequent contact with vegetation using it as a shelter as well as for feeding. However, it is not clear whether *P. inundata* is able to grow directly in crayfish tissues and/or whether the crayfish may act as vectors.

Similarly, the relationships of other isolated taxa to crayfish are unclear. With the exception of *A. astaci*, we cannot rule out that instead of growing within the crayfish, the isolated oomycetes were simply associated with the crayfish surfaces. Apart from being parasites growing in the host body (which might be partly restricted by the immune reaction of the host), the taxa isolated may have other life strategies. It is likely that some of them are saprobionts that colonise only moribund or dead crayfish, or facultative parasites that succeed in colonising weak crayfish but are unable to grow in healthy host individuals. Furthermore, the strains isolated could have originated from the biofilm on the crayfish body surface, or even from spores or cysts accidentally attached to it (despite our efforts to eliminate such cases by the preinoculation treatment of crayfish tissues).

From the species isolated in this study, only *S. parasitica* was directly confirmed to be pathogenic to crayfish. It is able to kill even healthy crayfish specimens, although wounded animals are more susceptible to death from this species (Diéguez-Uribeondo *et al.* 1994). *Saprolegnia australis* was also suggested to be associated with mortality of crayfish (Krugner-Higby *et al.* 2010) but infection experiments that would directly confirm its pathogenicity to crayfish have not been performed. In our study, it is most likely that the main negative impact on the analysed specimens of *Astacus astacus* and *Austropotamobius pallipes* was due to infection by *A. astaci*; however, the oomycetes isolated may have caused secondary infections.

Although we do not know the exact relationships of the isolated oomycetes to crayfish, it is apparent that they were associated with these animals. In the case of crayfish translocations, such microbiota may be transferred to new localities along with their hosts. Most of the studied taxa were isolated from both European and North American crayfish, or from other hosts from various regions (Table 1). Therefore, none of them seems to be primarily associated with any of these geographical regions and its indigenous crayfish (as is the case for *A. astaci* transferred from North American crayfish to European ones; Vey *et al.* 1983). The accidental spread of some oomycete strains might nevertheless have negative impacts. *Saprolegnia parasitica* and *S. ferax*, which we isolated from crayfish in Czechia as well as Spain, are known parasites of fish and amphibians (van West 2006; Ruthig 2009; Fernández-Benéitez *et al.* 2011), and little is known about differences in pathogenicity among strains. It is possible that anthropogenic dispersal of some strains along with crayfish or other aquatic animals (van West 2006), particularly over long ranges, may result in serious infections of nonadapted host populations.

The two most commonly isolated taxa, *S. ferax* and *S. australis*, were obtained from all three crayfish species, in both Czechia and Spain. Several factors, which are not mutually exclusive, may explain their high prevalence among the isolated strains: (1) these species may prefer growth on animal hosts including crayfish, (2) they belong to most common species in aquatic environments, or (3) they are easily cultured, and thus overgrow other taxa. Fast growth might not be limited to laboratory conditions. We usually isolated only one or two oomycete taxa from each crayfish individual, and these may represent the most successful species that dominate this substrate at the time of isolation (particularly a moribund individual or cadaver). Then, slowly growing species such as *A. astaci* might be suppressed, and their isolation may become increasingly difficult. This is also apparent from the results of our study – despite the fact that the original aim was to obtain cultures of the crayfish plague pathogen, and we used material from several confirmed or presumed crayfish plague outbreaks, we were successful in only one case and only seven out of 95 obtained isolates belonged to *A. astaci*.

The oomycete collection studied by us was mostly obtained from moribund or dead crayfish. Therefore, it was probably enriched by microbiota preferably colonising dying crayfish. As such taxa are also those most likely influencing the results of *A. astaci* diagnostics, it is important to further improve knowledge of their diversity. In general, cultivation of microorganisms from crayfish is motivated by attempts to obtain causative agents of diseases or atypical symptoms. Reasons for such studies include crayfish mass mortalities or declines (Krugner-Higby *et al.* 2010), and the presence of melanised spots (Söderhäll *et al.* 1991), lesions (Makkonen *et al.* 2010), or substantial growths of epibiotic microbiota ('Aufwuchs'; Hirsch *et al.* 2008) on living crayfish. Laboratory cultures obtained as by-product of parasite cultivation are therefore valuable material for further analyses. However, healthy-looking disease carriers may also be tested for the presence of pathogens, as is more and more often the case for *A. astaci* (e.g., Kozubíková *et al.* 2009; Skov *et al.* 2011; Schrimpf *et al.* 2012). Thus, the diversity of microbiota

colonising healthy crayfish should be also more intensively studied, to uncover which taxa may cross-react with molecular tools for pathogen detections.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2013.07.005.

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Supplementary table 1. List of oomycete isolates included in the study.
Abbreviations: AA – *Astacus astacus*, AP – *Austropotamobius pallipes*, OL – *Orconectes limosus*, CZ – Czech Republic.
The isolates are sorted according to the 1) taxon, 2) crayfish species, 3) locality, 4) source, and 5) isolate code. Note that isolates of the same taxon from the same source might represent the same oomycete strain.

Isolate	GenBank acc. no.	Taxon	Crayfish species	Locality	Source [a]	Zoosporangia	Oogonia/ antheridia
SAP716	KF386637	clade *Phytophthora inundata-P. humicola*	OL	Smečno town pond, CZ	healthy-looking crayfish with melanisation	no	no
SAP717	KF386638	clade *Phytophthora inundata-P. humicola*	OL	Smečno town pond, CZ	healthy-looking crayfish with melanisation	no	no
Li12	KF386639	*Phytopythium* sp.	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	globose	no
Li02	KF386640	*Pythium* sp. I	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	zoospores [b]	no
SAP706	KF386641	*Pythium* sp. I	AA	tributary of Pěnenský pond, CZ	moribund crayfish from plague outbreak	N/A	N/A
SAP739	KF386642	*Pythium* sp. I	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
Li18	KF386643	*Pythium* sp. II	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	filamentous, inflated	no
Li20	KF386644	*Pythium* sp. II	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	filamentous, inflated	no
Li05	n.s.	*Aphanomyces astaci*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	achlyoid	no
Li06	n.s.	*Aphanomyces astaci*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	achlyoid	no
Li07	n.s.	*Aphanomyces astaci*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	achlyoid	no
Li08	KF386645	*Aphanomyces astaci*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	achlyoid	no
Li09	KF386646	*Aphanomyces astaci*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	achlyoid	no
Li10	n.s.	*Aphanomyces astaci*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	achlyoid	no
Li11	KF386647	*Aphanomyces astaci*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	achlyoid	no
SAP665	KF386648	clade *Aphanomyces laevis-A. repetans*	OL	Smečno town pond, CZ	dead crayfish	no	no
SAP678	KF386649	clade *Aphanomyces laevis-A. repetans*	OL	Smečno town pond, CZ	dead crayfish	no	no
SAP659	KF386650	clade *Aphanomyces laevis-A. repetans*	OL	Smečno town pond, CZ	exuvia with hyphae and spore balls	no	no
SAP760	KF386651	clade *Aphanomyces laevis-A. repetans*	OL	Smečno town pond, CZ	exuvia with hyphae and spore balls	achlyoid	no
SAP761	KF386652	clade *Aphanomyces laevis-A. repetans*	OL	Smečno town pond, CZ	exuvia with hyphae and spore balls	achlyoid	no
SAP667	KF386653	clade *Aphanomyces laevis-A. repetans*	OL	Smečno town pond, CZ	exuvia with melanised spots	no	no
SAP657	KF386654	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP671	KF386655	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	no	no

SAP681	KF386656	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP682	KF386657	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP683	KF386658	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP684	KF386659	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP685	KF386660	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP721	KF386661	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP722	KF386662	*Saprolegnia australis*	AA	Světlohorská reservoir, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP703	KF386663	*Saprolegnia australis*	AA	Žebrákovský brook, CZ	dead crayfish from plague outbreak	saprolegnioid	no
SAP704	KF386664	*Saprolegnia australis*	AA	Žebrákovský brook, CZ	dead crayfish from plague outbreak	saprolegnioid	no
SAP747	KF386665	*Saprolegnia australis*	AA	Žebrákovský brook, CZ	dead crayfish from plague outbreak	no	no
SAP734	KF386666	*Saprolegnia australis*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
SAP737	KF386667	*Saprolegnia australis*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
SAP746	KF386668	*Saprolegnia australis*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	saprolegnioid	no
SAP748	KF386669	*Saprolegnia australis*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
SAP413	KF386670	*Saprolegnia australis*	AP	a brook near Olot, Spain	dead crayfish	N/A	N/A
SAP831	KF386671	*Saprolegnia australis*	AP	river Borosa, Spain	dead crayfish	N/A	N/A
SAP658	KF386672	*Saprolegnia australis*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP672	KF386673	*Saprolegnia australis*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP699	KF386674	*Saprolegnia australis*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP759	KF386675	*Saprolegnia australis*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	no	no
SAP675	KF386676	*Saprolegnia australis*	OL	Smečno town pond, CZ	freshly dead crayfish	no	no
SAP730	KF386677	*Saprolegnia australis*	OL	Smečno town pond, CZ	freshly dead crayfish	saprolegnioid	no
Li19	KF386678	*Saprolegnia ferax*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	saprolegnioid	yes
SAP660	KF386679	*Saprolegnia ferax*	AA	Světlohorská reservoir, CZ	dead crayfish	saprolegnioid	no
SAP664	KF386680	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	dead crayfish from plague outbreak	saprolegnioid	yes
SAP677	KF386681	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	dead crayfish from plague outbreak	saprolegnioid	yes
SAP732	KF386682	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	dead crayfish from plague outbreak	saprolegnioid	no
SAP758	KF386683	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	dead crayfish from plague outbreak	saprolegnioid	yes
SAP666	KF386684	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	yes
SAP668	KF386685	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	yes
SAP679	KF386686	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	yes
SAP735	KF386687	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	yes
SAP736	KF386688	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	yes
SAP738	KF386689	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
SAP740	KF386690	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	yes

SAP745	KF386691	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	yes
SAP749	KF386692	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
SAP750	KF386693	*Saprolegnia ferax*	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
SAP411	KF386694	*Saprolegnia ferax*	AP	a brook near Olot, Spain	dead crayfish	N/A	N/A
SAP414	KF386695	*Saprolegnia ferax*	AP	a brook near Olot, Spain	dead crayfish	N/A	N/A
SAP416	KF386696	*Saprolegnia ferax*	AP	a brook near Olot, Spain	dead crayfish	N/A	N/A
SAP408	KF386697	*Saprolegnia ferax*	AP	river Rio frío, Spain	dead crayfish	N/A	N/A
SAP409	KF386698	*Saprolegnia ferax*	AP	river Rio frío, Spain	dead crayfish	N/A	N/A
SAP410	KF386699	*Saprolegnia ferax*	AP	river Rio frío, Spain	dead crayfish	N/A	N/A
SAP412	KF386700	*Saprolegnia ferax*	AP	river Rio frío, Spain	dead crayfish	N/A	N/A
SAP662	KF386701	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly dead crayfish	no	no
SAP691	KF386702	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly dead crayfish	no	no
SAP663	KF386703	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly moulted dead crayfish and its exuvia	saprolegnioid	no
SAP676	KF386704	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly moulted dead crayfish and its exuvia	saprolegnioid	no
SAP692	KF386705	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly moulted dead crayfish and its exuvia	saprolegnioid	yes
SAP693	KF386706	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly moulted dead crayfish and its exuvia	saprolegnioid	yes
SAP731	KF386707	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly moulted dead crayfish and its exuvia	saprolegnioid	no
SAP743	KF386708	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly moulted dead crayfish and its exuvia	no	no
SAP744	KF386709	*Saprolegnia ferax*	OL	Smečno town pond, CZ	freshly moulted dead crayfish and its exuvia	saprolegnioid	no
Li16	KF386710	*Saprolegnia hypogyna*	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	saprolegnioid	no
SAP694	KF386711	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP695	KF386712	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP696	KF386713	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP697	KF386714	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP698	KF386715	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP700	KF386716	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP719	KF386717	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP723	KF386718	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP726	KF386719	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	saprolegnioid	no
SAP727	KF386720	*Saprolegnia parasitica*	OL	Smečno town pond, CZ	dead crayfish overgrown with hyphae	no	no
SAP708	KF386721	Saprolegniales I	AA	tributary of Pěnenský pond, CZ	moribund crayfish from plague outbreak	N/A	N/A
SAP709	KF386722	Saprolegniales I	AA	tributary of Pěnenský pond, CZ	moribund crayfish from plague outbreak	N/A	N/A
Li01	KF386723	Saprolegniales II	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	zoospores [b]	no
SAP669	KF386724	Saprolegniales II	AA	Žebrákovský brook, CZ	freshly dead crayfish from plague outbreak	no	no
SAP661	KF386725	Saprolegniales II	OL	stream Pšovka, CZ	freshly dead crayfish from plague outbreak	no	no

SAP674	KF386726	Saprolegniales II	OL	stream Pšovka, CZ	freshly dead crayfish from plague outbreak	no	no
Li03	KF386727	Saprolegniales III	AA	stream Litavka, CZ	freshly dead crayfish from plague outbreak	saprolegnioid	no

[a] bodies of crayfish described as “freshly dead” did not show any signs of decay, those described as “dead” could have already been in the early stage of decomposition but none of those used for oomycete isolation were dead for longer than two days

[b] zoospores present but no zoosporangia found

n.s. - not sequenced