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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 **Svoboda, J.**, Fischer, D., Kozubíková-Balcarová, E., Šťástková, A., Brůčková, M., Kouba, A., Petrusek, A. (*unpublished manuscript*).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).

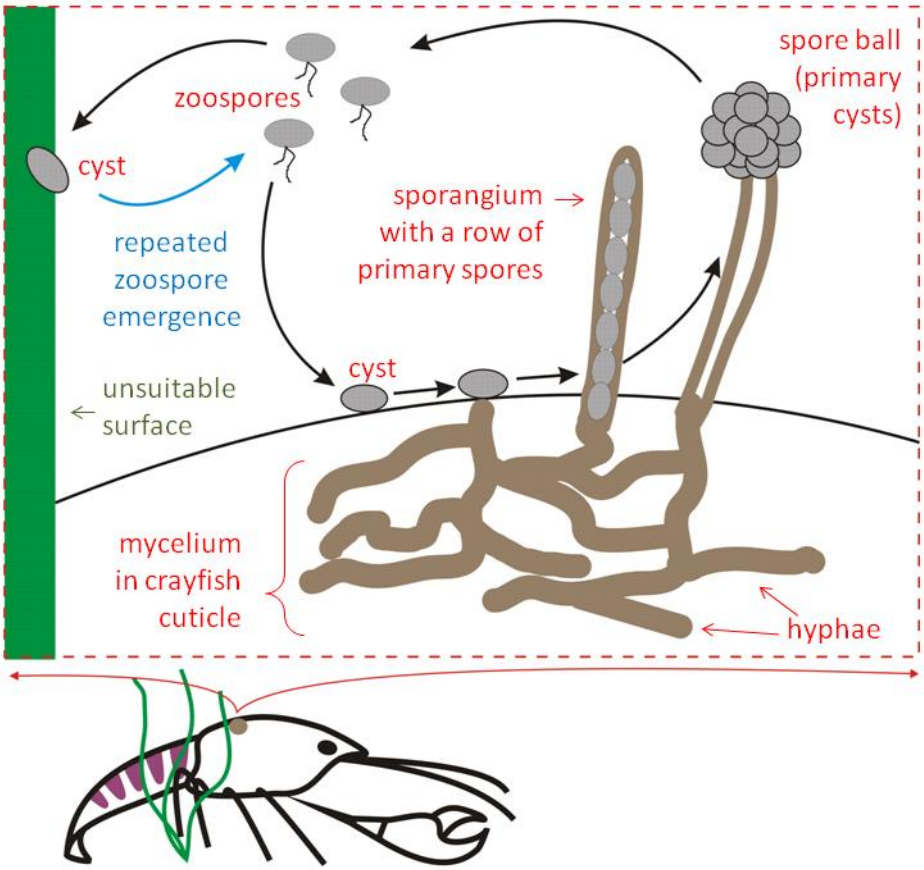

Figure 1: Summary of *A. astaci* life cycle, with a crayfish host and unsuitable substrate represented by an aquatic macrophyte. The figure was inspired by Cerenius *et al.* (1988) and Diéguez-Uribeondo *et al.* (2006).

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.

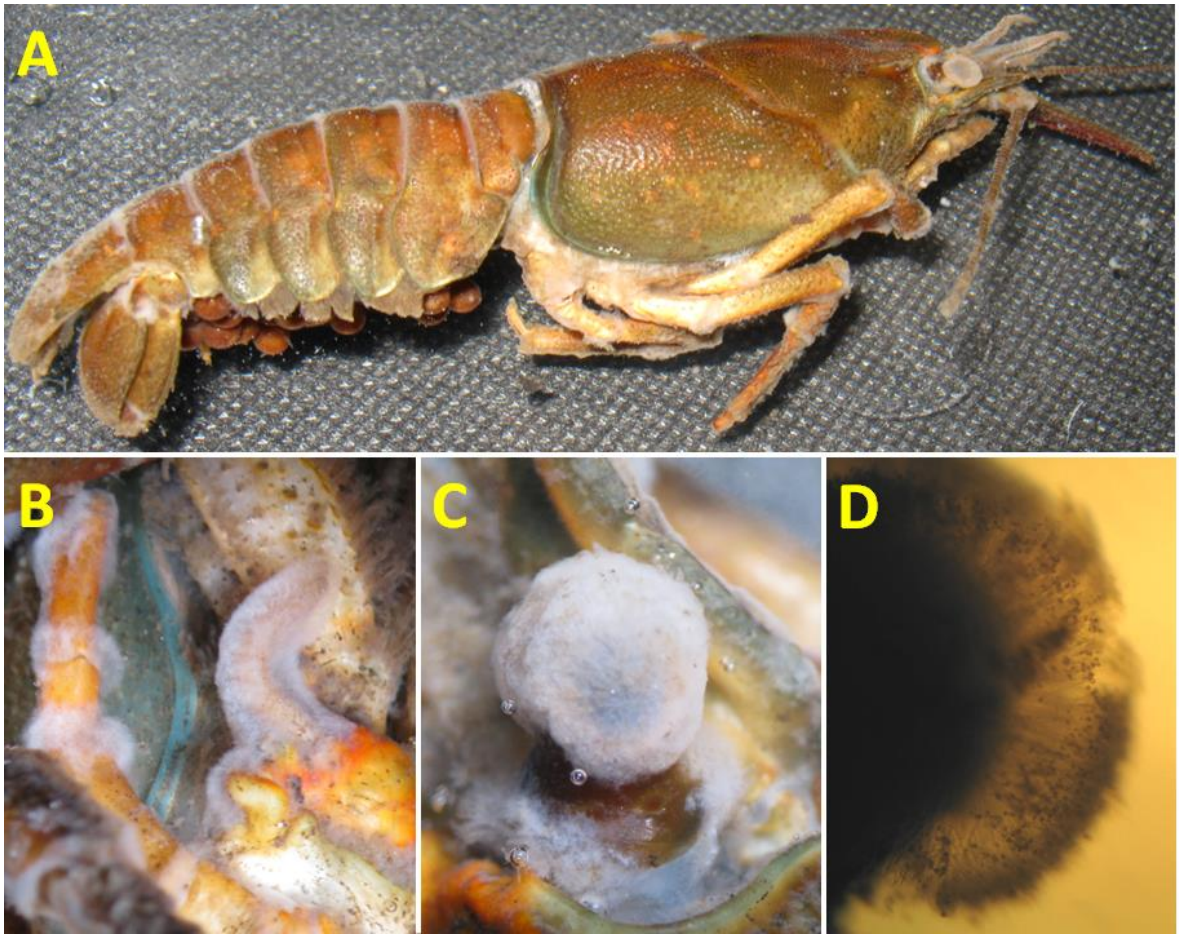

Figure 2: A dead individual of the noble crayfish *Astacus astacus* collected during a crayfish plague outbreak in the brook Černý near the village Pec (Czech Republic). Cotton-like mycelium of *A. astaci* can be seen on the soft cuticle between the segments of abdomen, legs and antennae (A). Detailed 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 *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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