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Study programme: Ecology

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The crayfish plague pathogen *Aphanomyces astaci* in its introduced ranges:
vectors, introduction pathways, genetic variation and host-pathogen interactions

Vektory, šíření a genetická variabilita patogenu račího moru v oblastech, kam byl zavlečen

Ph.D. Thesis

Supervisor: Adam Petrusek

Prague, 2016

I declare that this thesis has not been submitted for the purpose of obtaining 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.
All literature sources I used when writing this thesis have been properly cited.

Prague, July 2016

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The research presented in this thesis was financially supported by the STARS scholarship of the Faculty of Science, the Czech Science Foundation, the Czech Ministry of Education, the Mobility Fund of the Charles University, and the Erasmus+ programme.

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– PUBLICATIONS –

chapter 1

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(shared first authorship)*

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– APPENDICES –

appendix 1

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appendix 2

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appendix 3

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– PREFACE & ACKNOWLEDGEMENTS –

The time has passed so fast! These were, without doubt, exciting and scientifically enriching four years. When I decided to come to Prague for my PhD studies I did not know where it may lead me but I can say now with all my confidence that it was a good decision. Going through this PhD thesis you will notice that it resulted from a joint collaborative effort of many great astacologists with whom I had the pleasure to work during my studies. I have also spent a great time on various conferences and meetings that enabled me not only to travel but most of all to meet many excellent scientists from all over the world. All these encounters were very inspiring to me. In short, I am very glad for this experience and I would like to thank all the people that contributed to this PhD thesis and supported me during these four years.

My biggest thanks go to my supervisor Adam Petrusek whose enthusiasm, invaluable advice and encouragement made my PhD studies an exciting journey through the intricacies of *Aphanomyces astaci* research. Eva Kozubíková-Balcarová and Jiří Svoboda, thank you both not only for guidance and support during all these years but also for being great friends. I could always count on you and for that I am very grateful! Děkuju!

Many thanks to Jasna Vukić for her help in the molecular lab, discussions on various topics and sharing the love for Asian cuisine :) and to Jana Vokurková for technical support in the lab as well as for her big heart and patience during our little talks in Czech. Děkuji moc!

I thank also Ondřej Koukol from the Department of Botany for the permission to work with *A. astaci* cultures in the mycological lab.

As I have already mentioned, my research on *A. astaci* benefited greatly from fruitful collaborations with many scientists involved in astacological research. Special thanks go to Christoph Chucholl (from Fisheries Research Station Baden-Württemberg, Germany), Tadashi Kawai (Wakkanai Fisheries Institute, Hokkaido, Japan), Birgit Oidtmann (CEFAS, the United Kingdom), Ivo Roessink and Maurice Tilmans (Alterra, Wageningen University and Research Centre, the Netherlands), Menno Soes (Bureau Waardenburg, the Netherlands) and Satu Viljamaa-Dirks (EVIRA, Kuopio, Finland). Danke schön! どうもありがとうございました！ Dankuwel! Kittos paljon!

Furthermore, I have had an opportunity to spend part of my PhD studies in France at the University of Poitiers where I could study immunological aspects of *A. astaci* infection. I thank Frédéric Grandjean, Christine Braquart-Varnier, Thomas Becking, Carine Delaunay and Maryline Raymond for their help, fruitful discussions and warm hospitality. I would also like to thank all the students and postdocs for a very nice time outside the lab. Merci beaucoup!

My special thanks go also to colleagues from the Research Institute of Fish Culture and Hydrobiology in Vodňany. I especially thank Antonín Kouba for his enthusiasm and head full of ideas as well as Lukáš Veselý and Boris Lipták. Many thanks to the whole team of Pavel Kozák for a nice time on astacological conferences and their hospitality during my multiple stays in Vodňany.

I would also like to thank Sabrina Kumschick and Petr Pyšek for a possibility to participate in the sDIV workshop on impacts of invasive alien species and all workshop participants for a very enriching experience and inspiring time in Leipzig.

Many thanks to whole Department of Ecology, especially to former and recent students and postdocs. Thank you for a great time I could share with you (not only during our bowling sessions ;))!

Last but not least, my sincere thanks go to my family and friends who supported me during all these years! Especially, I would like to thank my parents for their love and encouragement when I needed it the most. Dziękuję Wam bardzo Kochani!

The PhD thesis was artistically enriched with watercolour prepared by my sister Kinga Mrugała. Dziękuję! :)

– ABSTRACT –

The crayfish plague pathogen, *Aphanomyces astaci,* is responsible for substantial declines and local extinctions of native European crayfish populations. As a consequence, the pathogen is now listed among 100 world's worst invasive alien species. The spread of *A. astaci* is greatly facilitated by its natural hosts, North American crayfish, that thanks to a long co-evolutionary history with the crayfish plague pathogen evolved efficient defence mechanisms. In contrast, European, Australian and Asian crayfish species are highly susceptible to this disease agent. However, progress of *A. astaci* infection in native European crayfish was observed to differ between distinct pathogen strains, indicating variability in their virulence. Indeed, we demonstrated a relationship between patterns in crayfish immune response and *A. astaci* virulence in an experimental infection involving the European noble crayfish and three differently virulent crayfish plague strains.

The European continent is currently inhabited by at least eight North American crayfish species. The carrier status was confirmed in six of them, including also *Orconectes* cf. *virilis* occurring in the Netherlands and the UK. In this country, we detected *Aphanomyces astaci* presence in some populations of the non-indigenous crayfish species as well as in individuals of the introduced catadromous crab, with the observed variation in pathogen prevalence among hosts linked to their introduction history and coexistence. Moreover, owing to aquaculture and stocking to open waters, several North American crayfish species established populations also on other continents as, e.g., *Procambarus clarkii* and *Pacifastacus leniusculus* in Japan. Therefore, as happened in Europe, they may pose a threat to endemic crayfish diversity. As the first such case, we confirmed the crayfish plague infection in Japanese populations of both crayfish species, indicating that *A. astaci* may have contributed to declines of the Japanese endemic crayfish *Cambaroides japonicus*.

The trade in ornamental crayfish species is nowadays a very popular hobby. Unfortunately, its rapid growth coincides in Europe with exotic crayfish releases to open waters. Our screening for *A. astaci* presence in various non-European crayfish species available for sale in Germany and the Czech Republic confirmed that aquarium trade may represent a source of crayfish plague pathogen (as well as other crustacean diseases), and hence may contribute to *A. astaci* spread to the natural environment with crayfish released from home aquaria. Furthermore, the crayfish plague pathogen may be also transmitted horizontally within shop facilities to presumably uninfected crayfish species, such as the parthenogenetically reproducing marbled crayfish. This widely traded crayfish taxon has been introduced to open waters in several European countries, including Slovakia where its recent expansion has been documented. Although I have not detected *A. astaci* in any of the three studied Slovak populations, the marbled crayfish might acquire the infection from the North American crayfish species encountered during its expansion.

Even ornamental crayfish of non-American origin may contribute to crayfish plague spread, if popular and widely available species exhibit elevated resistance. Experimental infection of the Australian *Cherax destructor* with *A. astaci* indeed indicated its decreased susceptibility, in comparison to European noble crayfish. Thus, *C. destructor* releases may result in formation of new pathogen reservoirs. Moreover, recently reported *A. astaci* infection in two crab species raised concerns that freshwater shrimps may also facilitate crayfish plague transmission to susceptible hosts. Although laboratory experiments with two ornamental Asian shrimp species revealed their resistance to *A. astaci*, pathogen growth was observed in some individuals and exuviae. Therefore, their potential to act as *A. astaci* vectors warrants further evaluation.

– ABSTRAKT (in Czech) –

Patogen račího moru, *Aphanomyces astaci*, je zodpovědný za významný úbytek a lokální vymizení populací původních evropských druhů raků, v důsledku čehož byl zařazen mezi 100 nejhorších invazních druhů světa. Při šíření *A. astaci* mají významnou roli jeho původní hostitelé, severoamerické druhy raků, jež si během dlouhé koevoluce vybudovaly efektivní obranné mechanismy proti tomuto patogenu. Naproti tomu evropské, asijské či australské druhy raků jsou vůči nákaze velmi citlivé. Průběh infekce evropských populací různými kmenu patogenu se ale často liší, což naznačuje, že existuje variabilita ve virulenci různých genotypů *A. astaci*. To se ukázalo i v experimentech, při kterých jsme evropské raky říční vystavili infekci třemi různými kmeny *A. astaci*: sledované parametry račí imunity skutečně závisely na virulenci příslušného patogenu.

V evropských vodách se v současnosti vyskytuje minimálně osm amerických druhů raků. U šesti z nich, včetně *Orconectes* cf. *virilis* v Nizozemí a Anglii, bylo prokázáno, že ve volné přírodě patogen račího moru přenášejí. V nizozemských vodách jsme potvrdili přítomnost *A. astaci* jak u několika amerických druhů raků, tak u migrujícího kraba čínského. Rozdíly v prevalenci patogenu v populacích jednotlivých potenciálních hostitelů souvisely zejména s jejich introdukční historií a lokální koexistencí druhů. Za účelem lovu nebo akvakultury byly americké druhy raků introdukovány i na další kontinenty, mimo jiné se tak dostal rak signální a rak červený do Japonska. V invadovaných oblastech proto tyto druhy mohou ohrožovat místní raky přenosem račího moru podobně, jako se to stalo v Evropě. Prvním dobře doloženým případem nákazy introdukovaných populací amerických raků mimo evropský kontinent je naše studie potvrzující přítomnost *A. astaci* v populacích obou výše zmíněných druhů v Japonsku. To naznačuje, že račí mor mohl mít významný podíl na úbytku japonského endemického druhu *Cambaroides japonicus*.

Chov akvarijních raků se v poslední době stal rozšířeným fenoménem. Bohužel s nárůstem jeho popularity v Evropě přibylo i vysazování exotických račích druhů do volných vod. Naše studie zaměřená na potenciální výskyt *A. astaci* v různých druzích raků dostupných v online i kamenných obchodech v Německu a ČR potvrdila, že obchod s akvarijními druhy může být zdrojem šíření patogenu račího moru i jiných významných nemocí korýšů. V důsledku toho se může z domácích akvárií dostat račí mor i do volné přírody. Patogen se může u akvaristů šířit mezi raky horizontálně a tak nakazit i hostitelské druhy, u nichž by se nákaza neočekávala – např. partenogenetického raka pruhovaného. Tento mezi akvaristy velmi rozšířený taxon byl nalezen ve vodách několika evropských zemí včetně Slovenska, kde jsme nedávno zdokumentovali jeho šíření do nových lokalit. Ačkoli nebyl patogen račího moru potvrzen v žádné ze třech studovaných slovenských populací, v případě kontaktu šířícího se raka mramorovaného s jinými nakaženými hostitelskými druhy může k jeho nakažení v budoucnosti dojít.

I okrasné druhy raků pocházející z jiných oblastí než Severní Ameriky mohou potenciálně přispívat k šíření račího moru, zejména pokud se ukáže, že některé populární druhy mají vůči *A. astaci* zvýšenou odolnost. Experimentální infekce australského raka *Cherax destructor* skutečně prokázaly, že je méně citlivý na nákazu patogenem račího moru než rak říční. Vysazování *C. destructor* by proto mohlo vést ke vzniku nových rezervoárů této nemoci. Nedávno potvrzená schopnost sladkovodních krabů hostit její patogen navíc naznačila, že i další desetinožci včetně sladkovodních krevet by mohly přispívat k šíření patogenu na citlivé hostitele. Ačkoli naše laboratorní experimenty se dvěma druhy akvarijních krevet prokázaly jejich rezistenci vůči *A. astaci*, v některých jedincích a jejich svlečkách jsme dokumentovali růst patogenu. Studium této skupiny jako potenciálních hostitelů nebo vektorů patogenu račího moru proto vyžaduje další pozornost.

– OUTLINE OF PUBLICATIONS –

My thesis consists of eight chapters focused on four interconnected topics: 1) distribution and prevalence of *A. astaci* infection in populations of its natural hosts introduced to new regions, 2) novel introduction pathways as well as vectors of *A. astaci*, 3) genetic variation of the crayfish plague pathogen, 4) *A. astaci* pathogenicity and crayfish immune defence dynamics. These include three first-author peer-reviewed papers in international journals, two papers where I share the first-author position, and three second-author papers (**chapters 1-8**). My contributions to the three second-author studies include laboratory work, i.e., detection of *A. astaci* in crayfish tissues, participation in infection experiments as well as in analyses and discussions of obtained results. I have also provided comments and suggestions during manuscript preparations and approved their final versions. Furthermore, three additional studies are included as appendices (**appendix 1-3**). These publications are an outcome of a workshop focusing on the impacts of invasive alien species that I have attended. I have actively participated in discussions and writing of these manuscripts.

Natural spread and/or human-aided translocations of the North American crayfish species are responsible for the widespread presence of their established populations throughout Europe. Despite the threat of *A. astaci* transmission to native European crayfish species, neither *A. astaci* prevalence in these populations nor an actual carrier status of new crayfish invaders were evaluated in many European countries. **Chapter 1** investigates *A. astaci* presence in the Netherlands that harbours one of the greatest number of potential plague carriers in Europe. *A. astaci* prevalence was evaluated in Dutch populations of five North American crayfish species including three confirmed crayfish plague carriers (*Orconectes limosus*, *Pacifastacus leniusculus* and *Procambarus clarkii*) and two recently introduced and yet unstudied taxa (*Orconectes* cf. *virilis* and *Procambarus* cf. *acutus*) as well as a catadromous crab *Eriocheir sinensis.* Moderate *A. astaci* prevalence was observed in populations of *O. limosus*, *P. leniusculus* and *E. sinensis,* whereas the pathogen was only sporadically detected in populations of *P. clarkii.* For the first time, *A. astaci* infection was reported in the populations of *Orconectes* cf. *virilis* confirming this taxon as a new crayfish plague carrier in European freshwaters. Contrastingly, despite an extensive sampling, no pathogen was detected in *P.* cf. *acutus*. The study confirms *A. astaci* presence in the Netherlands and demonstrates substantial variation in pathogen prevalence in local host populations.

Parallel to introductions of North American crayfish species to Europe, *P. clarkii* and *P. leniusculus* started to be used for food production all over the world. Both of these North American crayfish species were also introduced to Japan where they have successfully expanded their ranges. **Chapter 2** explores the presence of *A. astaci* infection in Japanese populations of these introduced crayfish species. The crayfish plague pathogen was detected in every sampled location with moderate to very high prevalence in *P. leniusculus* and *P. clarkii* populations, respectively. The endemic Japanese crayfish, *Cambaroides japonicus*, is according to laboratory experiments highly susceptible to the crayfish plague pathogen. Therefore, the presence of *A. astaci* carriers pose a threat of pathogen transmission and might have contributed to declines in *C. japonicus* populations.

Another emerging crayfish invader with a potential to act as a successful *A. astaci* vector is the parthenogenetically reproducing marbled crayfish, *Procambarus fallax* f. *virginalis*. Discovered in the German aquarium trade in the 1990s, it is now one of the most popular ornamental crayfish species. Its popularity coupled with the parthenogenetic reproductive mode are responsible for a series of marbled crayfish introductions into open waters throughout Europe. In Slovakia, this crayfish species was first discovered in 2010. **Chapter 3** reports the presence of three newly established marbled crayfish populations in this country. Due to their location close to major rivers in the Danube basin, these populations may serve as a source of further expansion of this parthenogenetic crayfish. Although the crayfish plague pathogen was not detected in Slovak populations of the marbled crayfish, if these

crayfish manage to successfully spread to the River Danube, they may encounter established populations of crayfish plague carriers and thus further contribute to *A. astaci* transmission to susceptible European crayfish.

Besides the marbled crayfish, more than 120 non-European crayfish species have been available for sale in the German aquarium trade. The majority of these crayfish species originate from North America and are, therefore, suspected to be carriers of the crayfish plague pathogen. The recent rapid growth in ornamental trade coincides in Europe with crayfish releases into open waters. Hence, exotic pathogens may be transmitted with infected crayfish and contaminated water from aquaria to susceptible populations of European crayfish species. **Chapter 4** investigates the presence of two important agents of crustacean diseases, the crayfish plague pathogen and the white spot syndrome virus (WSSV), in the aquarium trade in Germany and the Czech Republic. Both pathogenic agents have been observed in ornamental crayfish available for sale. While WSSV was only confirmed in three individuals of Australian *Cherax quadricarinatus*, crayfish plague pathogen was detected in eight American and one Australian crayfish species, comprising in total 27 % of screened crayfish batches. Furthermore, the study revealed *A. astaci* horizontal transmission between crayfish species within shop facilities, indicating that also presumably uninfected crayfish, as the parthenogenetically reproducing marbled crayfish, might acquire the crayfish plague infection. Finally, the study highlights that 1) trade in ornamental crayfish may act as a potential introduction pathway of new pathogen strains differing in virulence or climate requirements and 2) incorrect labelling of ornamental crayfish species may contribute to crayfish and pathogen introductions.

Due to the lack of a long co-evolutionary history, after which pathogens often show low virulence and host populations high level of resistance, the introduced pathogenic agents may be highly virulent and cause population crashes in naïve hosts. The introduction of the crayfish plague pathogen and subsequent declines in populations of European crayfish species well exemplifies such a scenario. Recent studies, however, demonstrated that even European crayfish may occasionally carry *A. astaci* without a development of an acute infection, phenomenon likely explained by variation in pathogen virulence. Four different *A. astaci* genotype groups (A, B, D & E), at least some of them differing in virulence, are known at present in Europe. **Chapter 5** investigates the variation in immune defence parameters (haemocyte density, phenoloxidase activity, and production of reactive oxygen species) of the European noble crayfish *Astacus astacus*. The experimental crayfish were exposed to two dosages of *A. astaci* zoospores (1 and 10 spores ml^{-1}) of three *A. astaci* strains responsible for the crayfish plague outbreaks in Central Europe and belonging to two highly virulent (B & E) and one of lower virulence (A) genotype groups. The intensity and timing of the immune response differed between the strains as well as between the spore concentrations. Moreover, the study revealed a stronger and faster change in immune parameters after an infection with two more virulent strains, indicating a relationship between crayfish immune response and *A. astaci* virulence. In addition, the virulence of *A. astaci* strain belonging to the genotype group E (isolated from *Orconectes limosus*) was experimentally tested for the first time, revealing equally high and rapid mortality as caused by the genotype group B (from *Pacifastacus leniusculus*).

The Australian crayfish, similarly to European and Japanese crayfish species, were assessed as highly susceptible to crayfish plague infection. However, moderate resistance to *A. astaci* has been suggested for the widely traded Australian yabby, *Cherax destructor*. This ornamental crayfish species has not only been assessed as a high-risk species in regard to its invasiveness but exhibits also a high potential to establish in Central Europe. Therefore, if *C. destructor* is indeed less susceptible to *A. astaci* than European noble crayfish, its releases may result in formation of new pathogen reservoirs. **Chapter 6** investigates *C. destructor* susceptibility to three *A. astaci* strains, the same as used in the experiment described in **chapter 5.** The individuals of *C. destructor* were exposed to two doses of *A. astaci* zoospores (10 and 100 spores ml^{-1}), and their mortality was further compared with

that of European noble crayfish. In contrast to *A. astacus,* some survival among *C. destructor* individuals was observed after exposure to the least virulent *A. astaci* strain (from the genotype group A). Moreover, despite no survival of Australian crayfish after infection with the two more virulent strains, their mortality was significantly delayed, compared with *A. astacus* mortality. Therefore, we suggest that under favourable conditions *C. destructor* may contribute to crayfish plague spread in Central Europe.

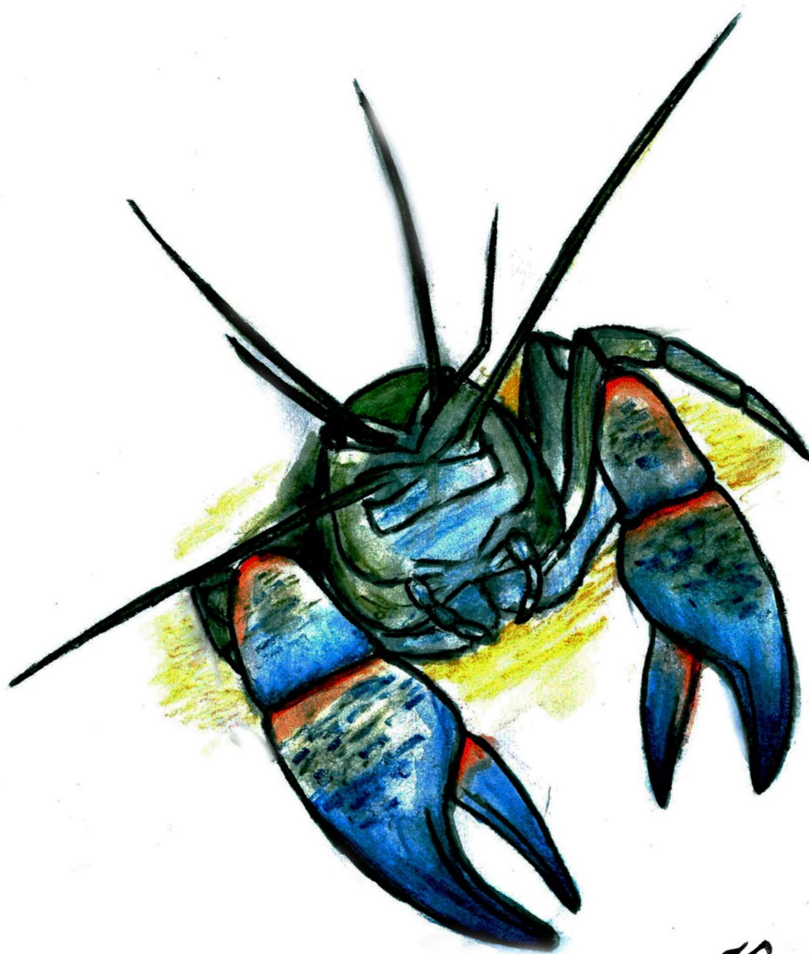
Although *A. astaci* host range has long been considered to be limited to freshwater crayfish, it was recently suggested that other freshwater decapod crustaceans, i.e., crabs and shrimps, may also acquire *A. astaci* infection, and thus contribute to crayfish plague transmission to European crayfish species. For example, the growth of *A. astaci* was already confirmed in tissues of the freshwater crabs coexisting with infected crayfish species. **Chapter 7** investigates the resistance of two Asian shrimps species, *Macrobrachium dayanum* and *Neocaridina davidi,* to crayfish plague infection. *A. astaci* strain belonging to genotype group D, i.e., its original host was *Procambarus clarkii*, a known crayfish plague carrier that may get into contact with shrimps, was used in the experimental trial. In addition, individuals of the highly susceptible European crayfish, *A. astacus* were infected to serve as a control for pathogen virulence. In contrast to *A. astacus*, no mortality was observed among infected shrimp individuals. In *N. davidi*, frequent moulting was suggested as a factor contributing to decreasing levels of *A. astaci* DNA. In contrast, high pathogen DNA levels were detected in some non-moulting individuals of *M. dayanum*, indicating that *A. astaci* growth may have occurred in tissues of this shrimp species. Further experiments, however, are crucial to evaluate its potential contribution to *A. astaci* spread. Finally, these and other recent advances in *A. astaci* biology with respect to its host range and transmission are summarized in **chapter 8.** The review highlights several aspects as, e.g., newly confirmed or suspected *A. astaci* hosts, latent crayfish plague infections in populations of European crayfish species, and the relationship between *A. astaci* genotype groups and host taxa.

The non-native species cause broad range of changes in the recipient ecosystems. Although some of these changes (usually termed impacts) are potentially damaging to ecosystems and biodiversity, they are still poorly understood. The progress toward understanding of these impacts is largely hindered by a lack of 1) consolidated terminology for impacts, 2) unified strategies for detecting and quantifying impacts, and 3) standardized framework for interpreting impacts. These issues were addressed in the **Appendices 1-3**. The explicit definition of impacts will not only help discriminate between disparate definitions and scientific discord but also improve communication between scientists, managers and policy makers. **Appendix 1** presents seven key questions that fall into four categories: directionality, classification and measurement, ecological or socio-economic changes, and scale. These questions should help in formulating clear and practical definitions of impact to suit specific scientific, stakeholder, or legislative contexts.

Moreover, several other factors hinder accurate predictions of alien species impact, and hence limit management decisions aiming to effectively allocate scarce resources. These include context-dependent nature of ecological impacts, research projects limited to certain taxa and habitat types as well as lack of standardized methodology for detecting and quantifying impacts. **Appendix 2** summarizes different strategies, including specific experimental and observational approaches, for detecting and quantifying ecological impacts of alien species. Furthermore, the review identifies approaches for 1) maximizing the insights into the nature of the impacts, and 2) recognizing high-impact species. Finally, it provides recommendations for the development of systematic quantitative measurements allowing temporal and spatial comparisons of alien species impacts across taxa.

The impacts of alien species vary greatly across species and their recipient ecosystems. Therefore, standardised methods to evaluate, compare and eventually predict the magnitudes of these different impacts are critically needed. **Appendix 3** proposes a straightforward system for

classification of alien species according to the magnitude of their environmental impacts. The classification system is based on the mechanisms of impact used to code species in the International Union for Conservation of Nature (IUCN) Global Invasive Species Database (GISD). It uses five semi-quantitative scenarios that describe impacts under each mechanism to assign species to different levels of impacts (ranging from Minimal to Massive), with the assignment corresponding to the highest level of deleterious impact associated with any of the mechanisms. Additionally, the scheme includes categories for species that are Not Evaluated, have No Alien Population, or are Data Deficient. As such, this classification system is applicable at different levels of ecological complexity, spatial and temporal scales, and embraces existing impact metrics.



– INTRODUCTION –

Freshwater ecosystems are threatened by a variety of factors that include overexploitation, habitat degradation and modification, climate change, water pollution and invasion of non-native species (Dudgeon *et al*. 2006). In fact, the successful biological invasions of aquatic animals, plants and pathogens, along with extensive land use, are leading causes of freshwater biodiversity declines (Sala *et al*. 2000), estimated to happen at a much higher rate than in the terrestrial ecosystems (Ricciardi & Rasmussen 1999). The particular susceptibility of aquatic ecosystems to the spread of non-native invasive species is to a great extent caused by intensive human use, connectivity between water bodies and hence great dispersal opportunities for aquatic organisms (Beisel 2001). These introduced organisms may impose a wide range of negative impacts on ecosystem and community functioning through predation, competition for shelter and resources, hybridization or habitat modification (**appendix 3**). Furthermore, they may threaten native competitors also through transmission of pathogens (Daszak *et al*. 2000; Peeler *et al*. 2011), either facilitating the spread of the ones already occurring in their new ranges or introducing novel disease agents (Strauss *et al*. 2012).

In Europe, releases and escapes from aquaculture and aquarium trade were assessed as the most important pathways of non-native species introductions, especially in Central and Western European countries (Nunes *et al*. 2015). Both pathways contribute also to introductions of exotic pathogens (Peeler *et al*. 2011; Rodgers *et al*. 2011). Majority of the known records of emerging aquatic diseases are connected with aquaculture (Peeler *et al*. 2011); releases of such parasites as *Bonamia ostreae, Gyrodactylus salaris*, and *Anguillicoloides crassus* caused in Europe dramatic populations declines of wild oysters, salmonids and eels, respectively. The open design of many aquaculture systems allows parasite exchange between farmed and wild populations, even in the absence of host escapees (Peeler *et al.* 2011). Furthermore, although to a lesser extent than aquaculture, the ornamental trade also contributes to the spread of non-native disease agents. This entry pathway is responsible for transmission of, e.g., ranaviruses to common toads in UK, and iridoviruses to native fish species in Australia (Hyatt *et al*. 2000; Whittington & Chong 2007).

Aphanomyces astaci Schikora, the causative agent of the crayfish plague, is another example of pathogen introduced via aquaculture, stocking and aquarium trade of its host species. Since its introduction in the mid-19th century, the pathogen caused so far irreversible declines in populations of native European crayfish species (Diéguez-Uribeondo *et al*. 2006; Holdich *et al*. 2009). Consequently, *A. astaci* was listed among 100 world's worst invasive species (Lowe *et al*. 2004) and belongs to the best studied pathogens of aquatic invertebrates (Diéguez-Uribeondo *et al*. 2006). Studies on this deadly pathogen constitute also the core of this PhD thesis, which focuses on *A. astaci* vectors, introduction pathways, genetic variation and host-pathogen interactions.

Biology of Aphanomyces astaci *& pathogen diagnostic methods*

The biology of *A. astaci* has been well described in several reviews and book chapters (e.g., Cerenius *et al*. 1988; Söderhäll & Cerenius 1999; Diéguez-Uribeondo *et al*. 2006; Rezinciuc *et al*. 2015; **chapter 8**). Briefly, *Aphanomyces astaci* is a fungal-like organism belonging to Oomycetes (family Saprolegniaceae). The genus *Aphanomyces* comprises saprophytes as well as plant and animal parasites, such as *A. cochlioides* parasitizing roots of sugar beets or *A. invadans* infecting a wide range of freshwater and estuarine fish species (Diéguez-Uribeondo *et al*. 2009). In contrast to plant pathogens, sexual reproduction is rare or absent in *Aphanomyces* spp. parasitizing animals (Diéguez-Uribeondo *et al*. 2009), and the presence of sexual apparatus was never confirmed in *Aphanomyces astaci* (Söderhäll & Cerenius 1999).

The asexual life cycle, allowing a rapid dispersal of the pathogen, is one of *A. astaci*

adaptations to the parasitic mode of life. It is characterized by a formation of motile, biflagellate zoospores, the only infective units of crayfish plague pathogen. The production of zoospores (i.e., sporulation) takes place when vegetative hyphae growing in the tissues of infected hosts protrude to the surrounding water. During this process, a row of primary spores is formed from the subdivided hyphal cytoplasm within sporangia. These spores are subsequently extruded through the hyphal tip, where they encyst and attach to each other forming clusters called "spore balls". From each primary cyst a secondary spore (i.e., zoospore) equipped with two flagellae is released and swims freely in the water column in order to find a new host. The completion of *A. astaci* life cycle occurs when a zoospore encysts on a host cuticle, germinates and penetrates host tissues with an emerging hypha. However, the zoospore may also undergo a process called a repeated zoospore emergence (RZE) if accidentally encysted on an unsuitable substrate or due to a wide range of stimuli, such as temperature changes, presence of salts, or physical agitation (Cerenius & Söderhäll 1984, 1985). If this happens, instead of germination, a new zoospore is released from the secondary cyst, which allows *A. astaci* to retry a search for a suitable host up to three times (Cerenius & Söderhäll 1984, 1985).

The lack of sexual structures as well as high morphological similarity during asexual stages within the genus *Aphanomyces* hinders *A. astaci* reliable identification solely based on microscopic examination (Cerenius *et al*. 1988; Oidtmann *et al*. 1999b). Early diagnostics of crayfish plague outbreaks involved *A. astaci* isolation to axenic cultures followed by experimental infection of susceptible crayfish (Oidtmann *et al*. 1999b). However, this approach was not only time-consuming and required experienced personnel but was often unsuccessful (Oidtmann *et al*. 1999b). *Aphanomyces astaci* cultured on artificial media is especially prone to bacterial infections that may inhibit its growth and/or it may be easily overgrown by saprotrophic oomycetes and fungi (Oidtmann *et al*. 1999b, 2004; Diéguez-Uribeondo *et al*. 2009).

The use of PCR and restriction fragment length polymorphism (RFLP)-based assay allowed *A. astaci* determination without the need of performing infection experiments, thereby shortening the time span of the analysis and omitting the use of experimental animals (Oidtmann *et al*. 2002). Nevertheless, the procedure still involved isolation of *A. astaci* to axenic cultures and was, therefore, unsuitable for fast *A. astaci* detection in tissues of infected crayfish individuals (Oidtmann *et al*. 2002). Direct verification of pathogen growth in host tissues was enabled by the development of three assays based on polymerase chain reaction (PCR) technique; the conventional PCR assay targeting the internal transcribed spacer (ITS) regions of the ribosomal DNA (Oidtmann *et al*. 2004, 2006), as well as two quantitative real-time PCR assays targeting either ITS1 region (Vrålstad *et al*. 2009) or chitinase encoding genes (Hochwimmer *et al*. 2009; Makkonen *et al*. 2012a). In particular, the ITS qPCR is not only useful in fast and reliable pathogen detection but also allows assigning the relative levels of *A. astaci* infection to semi-quantitative categories, based on the strength of the PCR signal (Vrålstad *et al*. 2009). This simple and comprehensible classification is suitable for interpretation of analytical results by any stakeholder group interested in *A. astaci* presence.

Tuffs & Oidtmann (2011) compared the sensitivity and specificity of these three assays and reported that the qPCR targeting the ITS region is most sensitive; this was also confirmed by Kozubíková *et al*. (2011b). A lower detectability was observed for the conventional PCR, whereas the chitinase-based assay turned out to be the least sensitive. All three assays were observed to be *A. astaci*-specific with a lack of cross-reaction with other tested oomycetes, crayfish pathogens and bacteria. However, Hochwimmer *et al*. (2009) observed an amplification of two oomycete species in the chitinase-based assay and Kozubíková *et al*. (2009) noted a cross-reaction in the conventional PCR with an unknown *Aphanomyces* spp. closely related to *A. astaci*. Given that several new *Aphanomyces* spp. present on American crayfish species have been described in the recent years (Diéguez-Uribeondo *et al*. 2009), the potential interference of so far undetected *Aphanomyces* spp. with the detection methods should not be ignored. Therefore, to ensure reliable pathogen detection, it was

recommended to support *A. astaci*-positive detections obtained with the ITS qPCR assay with sequencing of the ITS region amplified with the conventional PCR assay (Kozubíková *et al*. 2011b; OIE 2015).

Both of the PCR assays targeting the ITS region proved to produce reliable conclusions on *A. astaci* presence in North American crayfish species acting as reservoirs of the pathogen as well as in European crayfish species suffering from the disease outbreaks (e.g., Oidtmann *et al*. 2006; Vrålstad *et al*. 2009, 2011; Kozubíková *et al*. 2011b; **chapter 1**). While the North American crayfish species can effectively limit pathogen development in their cuticles through melanisation, in European crayfish the unlimited growth of *A. astaci* mycelium leads to host death within few days (Söderhäll & Cerenius 1999; Cerenius *et al*. 2003); a difference reflected also in *A. astaci* detectability in crayfish tissues. *Aphanomyces astaci* zoospores preferentially attach to soft parts of the crayfish integument, including the soft abdominal cuticle, tailfan (consisting of uropods and telson) and joints (OIE 2015) as well as in the vicinity of fresh wounds (Nyhlén & Unestam 1980). Vrålstad *et al*. (2011) and Oidtmann *et al*. (2006) evaluated presence of *A. astaci* infection on different types of crayfish tissues. The use of soft abdominal cuticle and parts of the tailfan resulted in the highest number of positive *A. astaci* detections. It is, therefore, recommended to combine both of these tissues, with the addition of any melanised part of the crayfish body. However, it should be stressed that melanisation is a general immune defense mechanism in invertebrates (Cerenius *et al.* 2008), and hence its presence is not always associated with a positive *A. astaci* carrier status.

Parallel to the improvement of crayfish plague diagnostic methods, molecular approaches focusing on *A. astaci* genetic diversity were developed. They allow unique genetic fingerprinting of particular crayfish plague strains and hence also tracking of *A. astaci* dissemination. First PCR-based tool, the randomly amplified polymorphic DNA (RAPD; Huang *et al*. 1994) enabled identification of distinct *A. astaci* genotypes and initiated further research on *A. astaci* genetic diversity. The results obtained with RAPD-PCR were later supported with another PCR-based approach, the amplified fragment length polymorphism (AFLP; Rezinciuc *et al*. 2014). Nevertheless, both methods are limited to *A. astaci* axenic cultures that are difficult to obtain and maintain, and also unavailable for large-scale and retrospective studies. Recently, a panel of nine co-dominant microsatellite markers was developed (Grandjean *et al*. 2014) that allows *A. astaci* genotyping of DNA isolates from laboratory axenic cultures as well as from mixed genome isolates from pathogen hosts. These microsatellite loci enable not only unambiguous separation of all known RAPD-defined *A. astaci* genotype groups but are also suitable for revealing genotypic variation within these groups (e.g., Grandjean *et al*. 2014; Maguire *et al*. 2016; **chapter 2**). Therefore, they are especially useful for identification of novel *A. astaci* genotypes (Grandjean *et al*. 2014) as well as evaluation of potential infection sources or horizontal transmission between *A. astaci* hosts (Svoboda *et al*. 2014). However, an unambiguous identification of mixed patterns, as e.g., the one observed from the narrow clawed crayfish *Astacus leptodactylus* in Jagodno Lake, Croatia (Maguire *et al*. 2016), will still require an isolation of *A. astaci* to axenic cultures.

Spread of Aphanomyces astaci *in Europe*

The first mass mortalities of native European crayfish species are presumed to be a result of an accidental introduction of *A. astaci* to Lombardy, northern Italy in 1859 (Alderman 1996). The pathogen may have been transported to this region with infected crayfish contaminating a batch of fishes from North America (Cornalia 1860 in Gherardi *et al*. 2011a). The second wave of crayfish mortalities started 15 years later in Plateau de Langres in France, from where the subsequent expansion of the crayfish plague pathogen affected almost an entire continent by the end of the century (Alderman 1996).

To compensate for extensive losses of European crayfish stocks three North American crayfish were introduced for stocking to open waters and aquaculture: the spiny-cheek crayfish *Orconectes limosus* (first introduced in 1890), the signal crayfish *Pacifastacus leniusculus* (1959), and the red swamp crayfish *Procambarus clarkii* (1973; Holdich *et al*. 2009). In the literature, these crayfish species are referred as Old NICS (Non-Indigenous Crayfish Species) to distinguish from additional non-European crayfish (New NICS) introduced after 1980 that were mainly released from home aquaria and as bait specimens or escaped from ponds (Holdich *et al*. 2009; Chucholl 2013; Kouba *et al*. 2014). Up to now, seven New NICS have established populations in European freshwaters. These include five species of North American origin (the calico crayfish *Orconectes immunis*, the virile crayfish *Orconectes* cf. *virilis*, the Kentucky River crayfish *Orconectes juvenilis*, the white river crayfish *Procambarus* cf. *acutus*), one crayfish taxon originating from aquarium trade (the marbled crayfish *Procambarus fallax* f. *virginalis*), and two Australian crayfish species (the common yabby *Cherax destructor* and the redclaw *Cherax quadricarinatus*; Kouba *et al*. 2014).

As discovered decades later, the North American crayfish species, including the three Old NICS, are natural carriers of *A. astaci* (Unestam 1972; Vey *et al*. 1983; Diéguez-Uribeondo & Söderhäll 1993). Recently, the crayfish plague infection was also reported in wild populations of *O. immunis* in France and Germany, *O.* cf. *virilis* in the Netherlands and the United Kingdom as well as the marbled crayfish in Germany (Filipová *et al*. 2013; Schrimpf *et al*. 2013a; Keller *et al*. 2014; **chapter 1**).

The introduction of *A. astaci* natural carriers to European freshwaters accelerated the spread of the crayfish plague agent, thus contributing further to mortalities in the native crayfish populations (Vennerström *et al*. 1998; Souty-Grosset *et al*. 2006; Kozubíková *et al*. 2008; Holdich *et al*. 2009). For instance, in Sweden the introduction of *P. leniusculus* resulted in an increased rate of crayfish plague outbreaks in populations of the native noble crayfish *Astacus astacus* (Bohman *et al*. 2006). It was estimated that as a consequence of crayfish plague spread in this country only 3% of *A. astacus* populations remain, compared to the number prior to *A. astaci* introduction (Bohman *et al*. 2006). The presence of *P. leniusculus* was also linked to population declines in the white-clawed crayfish *Austropotamobius pallipes* in France (Bramard *et al*. 2006; Collas *et al*. 2007) and in Spain (Diéguez-Uribeondo 2006) as well as *A. astacus* in Finland (Vennerström *et al*. 1998) and in Germany (Oidtmann *et al*. 1999a). Consequently, these two native European crayfish are listed in the IUCN (International Union for Conservation of Nature) Red List as vulnerable (*A. astacus*) and endangered (*A. pallipes*). Furthermore, both species as well as the stone crayfish *Austropotamobius torrentium* are included in Appendix III of the Bern Convention stating that their exploitation and harvest are subject to control and in Annex V of the European Habitat Directive requiring the designation of special areas of conservation for their protection.

Due to the substantial impact of crayfish plague pathogen on native European crayfish species, it is important to gather reliable information about *A. astaci* reservoirs in European freshwaters, especially about distribution and prevalence of *A. astaci* in the populations of North American crayfish species. Identification of localities inhabited by *A. astaci*-infected non-native crayfish will aid selection of native crayfish populations under a severe threat of *A. astaci* transmission, which may benefit from translocations to isolated “Ark Sites” (Peay & Guthrie 2008; Peay & Füreder 2011). Furthermore, it will enable determination of unsuitable areas in the close vicinity of crayfish plague carriers and may support successful reintroduction of native crayfish (Souty-Grosset & Reynolds 2009). Moreover, as an eradication of the non-native crayfish species populations is extremely difficult and/or not feasible after their establishment (Genovesi 2005; Gherardi *et al*. 2011a), it is first and foremost pivotal to prevent new crayfish introductions and if already present restrict their spread. The monitoring of *A. astaci* prevalence may facilitate identification of populations with the highest risk of pathogen transmission that should be included in control measures aiming to eradicate or limit their

expansion. Furthermore, waterbodies with highly infected crayfish populations should be also targeted in information campaigns to prevent the spread of infective zoospores to other localities with, e.g., contaminated fishing or diving equipment.

The studies on the distribution and prevalence of the crayfish plague infection in populations of North American crayfish species have been performed all over Europe (e.g., Kozubíková *et al*. 2009, 2010; Skov *et al*. 2011; Vrålstad *et al*. 2011; Filipová *et al*. 2013; **chapter 1**). Although it was long assumed that, e.g., the signal crayfish is "a permanent carrier of the parasite and there are no *A. astaci*-free *P. leniusculus"* (Cerenius *et al.* 2003), very low prevalence or an apparent absence of *A. astaci* infection were reported not only in populations of *P. leniusculus* in Denmark, France, the Czech Republic (Kozubíková *et al*. 2009; Skov *et al*. 2011; Filipová *et al*. 2013) but also *O. limosus* in Germany and the Czech Republic (Kozubíková *et al*. 2009; Schrimpf *et al*. 2013b), and *P.* cf. *acutus* and *P. clarkii* in the Netherlands (**chapter 1**).

These results indicate that *A. astaci* detectability varies in populations of North American crayfish, and it may be affected by several interconnected factors, such as density of crayfish population, temporal fluctuations in pathogen presence, type of waterbody inhabited by crayfish and/or age and sex of sampled crayfish individuals (Kozubíková *et al*. 2009, 2010; Matasová *et al*. 2011; Vrålstad *et al*. 2011). Moreover, another factor affecting *A. astaci* prevalence is the introduction history of non-native species that may greatly reduce the probability of pathogen introduction as source populations often consist of a small subset of native populations and/or uninfected life-history stages (Torchin *et al*. 2003). Therefore, the results obtained during monitoring of *A. astaci* prevalence should be interpreted with caution. In particular, the absence or low prevalence of crayfish plague infection should be carefully evaluated as only long-term extensive sampling over several seasons may provide sufficient results representative for the real *A. astaci* status in pathogen's natural hosts. Therefore, all populations of North American crayfish species should be considered as potential reservoirs of the crayfish plague pathogen.

Detection of the crayfish plague pathogen outside Europe

Owing to anthropogenic introductions for commercial purposes, some crayfish species have been translocated outside their native ranges (Lodge *et al*. 2012). Among them, *Procambarus clarkii*, native to north-eastern Mexico and south-eastern states of USA, is nowadays the globally most widespread crayfish species (Hobbs *et al*. 1989; Lodge *et al*. 2012; Loureiro *et al*. 2015). Apart from Europe, *P. clarkii* was introduced to tropical and subtropical regions of Asia, Africa, Central and South America (including, e.g., Brazil, China, Japan, Kenya, Egypt, Israel; Suko 1958; Hobbs *et al*. 1989; Xingyong 1995; Ibrahim *et al*. 1997; Magalhães *et al*. 2005; Wizen *et al*. 2008; Gherardi *et al*. 2011b) as well as western and eastern states of USA (ISSG). Although *P. clarkii* translocations were mainly aimed for aquaculture and natural fisheries (as in USA and China, respectively; FAO 2007), it was also introduced as a food for fish, e.g., in Kenya (Gherardi *et al*. 2011b) and other edible species as American bullfrog, *Rana catesbeiana* in Japan and Hawaii (Suko 1958; Hobbs *et al*. 1989). Furthermore, as suspected in Brazil and Venezuela, the trade in ornamental crayfish and subsequent releases of crayfish specimens from home aquaria, may have been responsible for *P. clarkii* entry to the natural environment (Rodríguez & Suárez 2001; Magalhães *et al*. 2005).

Another North American crayfish species used commercially for food production is *Pacifastacus leniusculus*, native to North American regions westwards from the Rocky Mountains (Taylor *et al*. 2007). Its introduced range includes western states of the USA (California, Nevada, Utah), and temperate regions of Europe and Japan (Kawai *et al.* 2002; Taylor *et al*. 2007; Kouba *et al*. 2014). In contrast to Europe, where *P. leniusculus* imports were aimed to boost wild crayfish stocks, in Japan the signal crayfish was introduced as aquaculture species to farm ponds across the country

(Kawai *et al*. 2002; Holdich *et al*. 2009). Nevertheless, in both cases its commercial use initiated subsequent human-aided spread into the natural environment (Kawai *et al*. 2002; Kouba *et al*. 2014).

Both of these North American crayfish species are known *A. astaci* carriers in Europe (Holdich *et al*. 2009). We have recently reported the presence of *A. astaci* in *P. clarkii* and *P. leniusculus* populations in Japan, being thus the first report of *A. astaci* infection in these two commercially important crayfish species outside Europe and its native range in North America (**chapter 2**). Nakata & Goshima (2003) suggested that *A. astaci* transmission to the endemic Japanese crayfish *Cambaroides japonicus* might have contributed, along with habitat modification and competition with alien species, to drastic declines in its populations in the past decades. This had not been evaluated before our work, which provided some indirect support for this hypothesis. Given that *C. japonicus* was assessed as highly susceptible to crayfish plague pathogen (Unestam 1969), widespread presence of *A. astaci* infection in populations of North American crayfish species may further endanger this endemic crayfish species.

The presence of *Aphanomyces astaci* outside Europe and its native range in North America has been also reported from crayfish farms in Israel and Taiwan. Both incidents were confirmed by DNA analyses and notified to World Organization for Animal Health (OIE). In Israel, *A. astaci* was detected in extensively farmed North American crayfish *Procambarus alleni.* In Taiwan, five *A. astaci* outbreaks were recorded in aquaculture of the Australian *Cherax quadricarinatus* (Hsieh *et al*. 2016).

Trade in ornamental crayfish as a new Aphanomyces astaci *introduction pathway*

In Europe, the introductions of new non-indigenous crayfish species are recently a result of illegal stocking activities, bait introductions, garden pond escapes and aquarium releases (Chucholl 2013 and references therein). Even though import, trade and transport of ornamental non-European crayfish species are forbidden or restricted in many European regions (Edsman 2004; Holdich & Pöckl 2005; Hefti & Stucki 2006; Peay 2009; Svobodová *et al*. 2010), the market for ornamental crayfish has grown rapidly in some European countries in the last two decades and keeping crayfish in home aquaria gained in popularity (Chucholl 2013; Patoka *et al*. 2014). In consequence, the aquarium trade is nowadays considered the main introduction pathway of non-indigenous crayfish species into European freshwaters (Peay 2009; Chucholl 2013).

The boom for keeping crayfish as ornamental pet species started in Germany in early 1990s (for details see Chucholl 2015). At present, the German aquarium market is regarded as the main importer of non-indigenous crayfish to Europe, with an impressive number of more than 120 non-indigenous crayfish species (mainly of North and Central American origin) available for sale (data from mid-2009; Chucholl 2013). The Czech Republic is the second leading importer as well as the main European exporter of ornamental crayfish (Patoka *et al*. 2014, 2015); with the domestic production of American crayfish from the family Cambaridae greatly exceeding the number of crayfish individuals imported from Asia (mainly *Cherax* spp. from Indonesia; Patoka *et al*. 2015). In total, 26 non-European crayfish species were recorded in the Czech aquarium trade (Patoka *et al*. 2014) that similarly to other European ornamental markets belong mainly to three genera: *Cambarellus*, *Procambarus* and *Cherax.*

Risk assessments of ornamental crayfish species were undertaken in four of these European countries: Germany, the Czech Republic, Italy and Greece, with the use of the Freshwater Invertebrate Invasiveness Scoring Kit (FI-ISK; Tricarico *et al*. 2010; Chucholl 2013; Papavlasopoulou *et al*. 2014; Patoka *et al*. 2014). This screening tool provides a risk score indicating crayfish potential invasiveness in a given country and is based on their size, biological characteristics and availability in the aquarium trade (Tricarico *et al*. 2010). Four crayfish species: *P. clarkii, P. fallax* f. *virginalis*, *P. alleni* as well as *C. destructor* consistently attained the highest risk rating (Tricarico *et al*. 2010; Chucholl 2013;

Papavlasopoulou *et al*. 2014; Patoka *et al*. 2014). Releases of these four ornamental crayfish species were already recorded in Europe (e.g., Souty-Grosset *et al*. 2006; Holdich *et al*. 2009; Chucholl 2013; Gross 2013; Kouba *et al*. 2014 and references therein), and are considered responsible for *P. clarkii* and *P. fallax* f. *virginalis* established populations in Central Europe (**chapter 3**; Chucholl 2013; Kouba *et al*. 2014; Chucholl 2015 and references therein).

The presence of *A. astaci*-infected crayfish was also recorded in the ornamental trade markets in Germany and the Czech Republic (**chapter 4**). *Aphanomyces astaci* DNA was detected in 8 out of 19 tested crayfish taxa, including the three high-risk species from the genus *Procambarus* (**chapter 4**). Moreover, presence of *A. astaci* infection in Australian *Cherax quadricarinatus* indicated that the crayfish plague pathogen may be horizontally transmitted between crayfish individuals within shop facilities, likely due to flow-through aquaria installations, contaminated equipment, or during handling and packing. This finding highlights that releases of any non-European crayfish species may be followed by the spread of crayfish plague pathogen. On the whole, the ornamental trade may act as *A. astaci* reservoir and facilitate pathogen transmission to the natural environment in a twofold manner 1) with discarded water, or 2) with infected crayfish individuals that were either released from home aquaria or escaped from garden ponds (**chapter 4**).

Horizontal transmission is also considered responsible for detection of *A. astaci*-infected individuals of the marbled crayfish in the German aquarium trade (Viljamaa-Dirks *et al*. 2014; **chapter 4**) as well as in its wild population in Lake Moosweiher where this crayfish coexists with infected *O. limosus* (Keller *et al*. 2014). The marbled crayfish was first discovered in German aquarium trade in mid-1990s (Scholtz *et al*. 2003) and up to now the ornamental trade is its only known "natural habitat" (Chucholl 2015). Interestingly, this crayfish taxon reproduces solely through parthenogenesis, and hence consists entirely of genetically identical females (Vogt *et al*. 2015). Attractive coloration as well as an undemanding nature result in its high popularity among aquarists (Chucholl 2015). However, the marbled crayfish can easily overpopulate home aquarium in a short time that often results in crayfish sale or disposal (Patoka *et al*. 2014). Such activities are especially undesirable as even a single specimen is in principle sufficient to start a viable population (Scholtz *et al*. 2003).

In fact, established populations of this crayfish invader have been already reported from Germany and Slovakia (Chucholl *et al.* 2012; **chapter 3**), and the number of its encounters in the wild still increases (Kouba *et al.* 2014; Samardžić *et al*. 2014; Lőkkös *et al*. 2016; Novitsky & Son 2016). Moreover, a single specimen of the marbled crayfish, believed to have an aquarium origin, was found near Sapporo in Japan (Faulkes *et al*. 2012), and this crayfish taxon is well established in Madagascar (Jones *et al*. 2009; Kawai *et al*. 2009). As suggested by Faulkes *et al.* (2012) considerable lag phase may separate discovery of a single specimen and the establishment of a viable population. Therefore, discovery of new established populations of the marbled crayfish should be expected in the near future. Considering its potential to act as a crayfish plague vector, the presence of marbled crayfish in the wild may also facilitate *A. astaci* transmission to native European crayfish species. Therefore, it is of utmost importance to prevent its further releases via aquarium trade as well as limit crayfish further expansion in the natural environment (for details see **chapter 3**).

Aphanomyces astaci *genetic variation & its implications in host-pathogen interactions*

The knowledge of *A. astaci* genetic variation is still scarce, with almost no information about its diversity in the native range in North America. Up to now, five distinct *A. astaci* genotype groups (A-E) have been identified with RAPD profiling, four of which are present in Europe. Group A is considered to be involved in the first mass mortalities of European crayfish species in the 19th century and its original host remains unknown (Huang *et al*. 1994). It was solely isolated from native

European crayfish species, *A. astacus* and *A. leptodactylus* (Huang *et al*. 1994). Three other *A. astaci* genotype groups present in Europe are associated with certain North American crayfish species: *P. leniusculus* (group B; Huang *et al*. 1994), *P. clarkii* (group D; Diéguez-Uribeondo *et al*. 1995) and *O. limosus* (group E; Kozubíková *et al*. 2011a). *A. astaci* isolates belonging to these groups were obtained either from North American crayfish inhabiting European freshwaters or European crayfish species dying in crayfish plague outbreaks (reviewed in Rezinciuc *et al*. 2015 and **chapter 8**). The fifth genotype group (C) is known from Canadian *P. leniusculus*, and so far has not officially reported in Europe (Huang *et al*. 1994; Rezinciuc *et al*. 2015).

Recent development of microsatellite markers allows discovery of new genotypes within the RAPD-defined groups. Some variation from the reference axenic culture of the genotype group D was reported in the ornamental crayfish (Viljamaa-Dirks *et al*. 2014). Several *A. astaci* isolates were obtained from three ornamental crayfish species (*P. clarkii*, *P. alleni* and *P. fallax* f. *virginalis*; **chapter 4**). While the RAPD-PCR analysis indicated that all isolates belong to the genotype group D, the microsatellite genotyping revealed a presence of two similar but distinct genotypes that were not host-specific and differed at one locus (Viljamaa-Dirks *et al*. 2014). One of these *A. astaci* genotypes (with allele pattern different from typical D-group strains) was also recorded in *P. clarkii* populations present in Japan (**chapter 2**). Similarly, differences at some loci (from reference genotype groups A & B) were observed in field samples of European and North American crayfish species in Croatia (Maguire *et al.* 2016). Moreover, *A. astaci* genotype, not matching known RAPD-defined genotype groups, was involved in crayfish plague outbreak in the Czech Republic (*A. astaci* isolate from Úpořský brook; Grandjean *et al*. 2014), indicating much higher amount of genetic variation in the crayfish plague pathogen.

Aphanomyces astaci strains belonging to different genotype groups may differ in such ecological properties as, e.g., climate requirements (Diéguez-Uribeondo *et al*. 1995; Rezinciuc *et al*. 2014) and virulence (Makkonen *et al*. 2012b, 2014; Viljamaa-Dirks *et al*. 2013, 2016; **chapter 5**). Studies examining physiological properties of various *A. astaci* strains revealed that D-group strains are, similarly to their original crayfish host *P. clarkii*, adapted to higher temperatures (Diéguez-Uribeondo *et al*. 1995; Rezinciuc *et al*. 2014). Also virulence was recently demonstrated to vary between *A. astaci* strains belonging to different genotype groups (Makkonen *et al*. 2012b, 2014; Viljamaa-Dirks *et al*. 2013, 2016; **chapter 5**). Substantial differences were observed between pathogen strains of genotype groups A, B & E (Makkonen *et al*. 2012b, 2014; **chapter 5**). Total mortality was repeatedly observed in *A. astacus* exposed to *A. astaci* strains from the genotype groups B & E, confirming their very high virulence towards European crayfish species (Makkonen *et al*. 2012b, 2014; **chapter 5**). In contrast, the crayfish plague infection developed more slowly and only sporadically caused mortality of *A. astacus* challenged with *A. astaci* strains from genotype group A (Makkonen *et al*. 2014; **chapter 5**).

All of the *A. astaci* genotype groups have been documented in numerous crayfish plague outbreaks in Europe (e.g., Filipová *et al*. 2013; Kozubíková-Balcarová *et al*. 2014; Rezinciuc *et al*. 2014), however *A. astaci* strains from group A have been also implicated in latent crayfish plague infections carried by various European crayfish species (reviewed in **chapter 8**). It was hypothesised that decreased virulence of *A. astaci* strains from this genotype group may be a result of more than century-long coexistence of *A. astaci* and the European crayfish species (Jussila *et al*. 2014). In fact, individuals of the European noble crayfish survived exposure to *A. astaci* zoospores of A-group strains from geographically distinct sources (originating either from Fennoscandia: Makkonen *et al*. 2014; or from Armenia: **chapter 5**). Moreover, Makkonen *et al*. (2012b) suggested that some tested populations of noble crayfish exhibited higher resistance to *A. astaci*, indicating that variable host resistance may be another factor involved in chronic crayfish plague infections. In agreement with this suggestion, latent *A. astaci* infections in Turkish populations of *A. leptodactylus* caused by pathogen

strain from genotype group B seem to be a result of host increased resistance (Svoboda *et al*. 2014).

The balanced host-pathogen relationship between North American crayfish species and the crayfish plague pathogen (Cerenius *et al*. 2003) is considered to be an outcome of a long co-evolutionary history that results in lowered virulence of pathogens and higher resistance of hosts (May & Anderson 1990). In contrast to highly susceptible European, Australian and Asian crayfish species, the North American crayfish evolved efficient immune defence mechanisms to prevent development of *A. astaci* infection. Thanks to a continuous production of high levels of prophenoloxidase (proPO) transcripts, unless stressed, the North American crayfish are able to immediately limit pathogen growth through melanisation of the penetrating hyphae (Unestam & Weiss 1970; Cerenius *et al*. 2003). This immune defence mechanism was not observed in highly susceptible European *A. astacus* (Cerenius *et al*. 2003; **chapter 5**). Nevertheless, at least two non-American crayfish species *A. leptodactylus* and *C. destructor* seem less susceptible to *A. astaci* than noble crayfish, based on laboratory experiments, and in case of *A. leptodactylus* also on field observations.

Early study of Unestam (1969) demonstrated *A. leptodactylus* partial mortality after exposure to *A. astaci* zoospores. This European crayfish species was also reported to carry chronic crayfish plague infections in Romania and Turkey (Kokko *et al*. 2012; Pârvulescu *et al*. 2012; Schrimpf *et al*. 2012; Svoboda *et al*. 2012); with the Turkish populations confirmed to carry B-group *A. astaci* strain (Svoboda *et al*. 2014). Similarly, some individuals of *C. destructor* survived an experimental exposure to zoospores of B-group *A. astaci* strain (Unestam 1975). An elevated resistance of this Australian crayfish species was recently suggested after an experimental exposure to three *A. astaci* strains differing in virulence (groups A, B & E; **chapter 6**). While *A. astaci* infection with A-group strain resulted in total mortality of *A. astacus*, some *C. destructor* individuals survived the experimental trial. Furthermore, although all yabby died after infection with the two more virulent pathogen strains, in contrast to *A. astacus*, their mortality was significantly delayed (**chapter 6**). Nevertheless, it does not imply a general elevated resistance of the species; *A. astaci* strain from the genotype group D was used in a successful eradication of established populations of *C. destructor* in Spain (Souty-Grosset *et al*. 2006). Rather, it seems that the Australian yabby (and similarly other non-American crayfish species) may exhibit substantial variation in susceptibility to various *A. astaci* genotypes.

Based on experimental results with *C. destructor,* we nevertheless suggest that under favourable conditions established populations of this Australian crayfish species may act as pathogen reservoirs in European freshwaters (**chapter 6**). Therefore, the potential contribution of non-American crayfish species to *A. astaci* spread should not be ignored. Other freshwater decapods could also be involved in *A. astaci* transmission to European crayfish species. The host range as well as *A. astaci* transmission are comprehensibly described in **chapter 8**. Briefly, at least two freshwater-inhabiting crab species *Eriocheir sinensis* and *Potamon potamios* were suggested as *A. astaci* non-symptomatic hosts. Especially worrying is *A. astaci* detection in tissues of the former species, which is a migratory crab capable of covering long distances (Herborg *et al*. 2003). Infected *E. sinensis* were collected from multiple localities where the crab likely acquired *A. astaci* infection from coexisting North American crayfish species (Schrimpf *et al*. 2014; Svoboda *et al*. 2014; **chapter 1**). Furthermore, two Asian shrimp species, *Neocaridina davidi* and *Macrobrachium dayanum,* were tested for their potential resistance to *A. astaci* (**chapter 7**). The experimental infection with *A. astaci* zoospores was not followed shrimp mortality, indicating their resistance to the crayfish plague pathogen. However, based on high pathogen DNA levels detected in some individuals and exuviae of *M. dayanum*, it was suggested that *A. astaci* may grow in tissues of this shrimps species and this possibility should be further evaluated (**chapter 7**). Several other invertebrate species inhabiting European freshwaters were either exposed to *A. astaci* zoospores or collected from waterbodies inhabited by infected crayfish populations and subsequently tested for *A. astaci* infection. Neither *A. astaci* growth nor presence of pathogen DNA were detected in tissues of these organisms (reviewed in **chapter 8**).

– CONCLUSIONS –

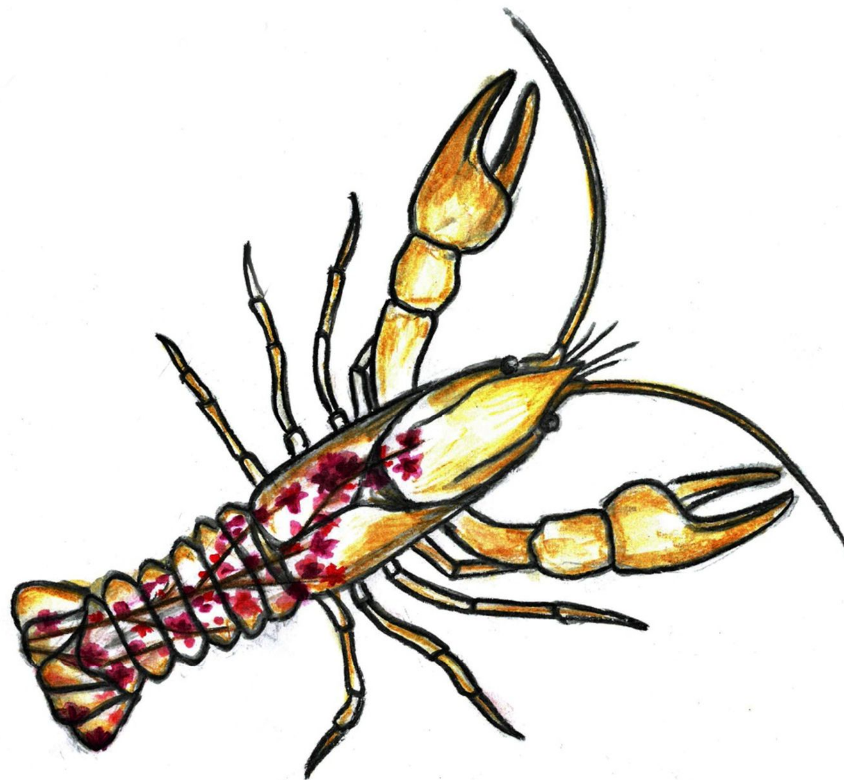
Introduction of infectious pathogens along with their natural hosts may have devastating impacts not only on naïve wildlife populations but also on whole ecosystems, as exemplified by severe declines and losses of native European crayfish populations. Therefore, the non-indigenous hosts and their associated pathogens should be subjected to management efforts aiming to mitigate consequences of their introduction. However, as in the case of crayfish plague, any management actions will only produce significant outcomes if supported by research on pathogen introduction pathways, vectors and reservoirs as well as its genetic variation and interactions with host species. I hope that studies included in my PhD thesis contributed to our understanding of this deadly crayfish pathogen and will eventually prove useful also in management efforts.

The prevalence of *A. astaci* infections in populations of North American crayfish species may substantially vary depending on multiple factors, including their introduction history and coexistence. The monitoring of *A. astaci* distribution is, therefore, crucial if such management actions, as e.g., reintroduction of European crayfish species in Dutch freshwaters, are foreseen. However, it should be taken into account that "plague-free" status of North American crayfish populations may change with time and hence all populations of these non-indigenous crayfish species should be considered as *A. astaci* reservoirs. In addition, spreading individuals of non-indigenous crayfish, including the parthenogenetically reproducing marbled crayfish, may acquire *A. astaci* infection from other hosts. Furthermore, the North American crayfish species have been successfully introduced to many other regions of the world. Similarly as observed in Japan, *A. astaci* may be present in populations of these crayfish, and therefore contribute to mortalities of susceptible to *A. astaci* astacofauna present not only in Europe but also in Asia and Australia. Therefore, prevention of new introductions and further spread of North American crayfish species, and thus also *A. astaci* transmission and emergence of novel pathogen strains, is especially recommended.

The trade in ornamental crayfish species has gained in importance in recent decades and is nowadays considered as the main introduction pathway of non-indigenous crayfish species in Europe. Detection of the crayfish plague pathogen in the ornamental non-European crayfish indicates that the aquarium trade may also act as *A. astaci* reservoir and introduction pathway to the natural environment. As the aquarium trade is a highly unregulated industry subjected to limited regulations, education of crayfish hobby keepers is critically needed. It should focus on mechanisms of *A. astaci* introduction to the aquatic environments and be especially implemented in regions characterized by high popularity of ornamental crayfish. Moreover, the observed decreased susceptibility to *A. astaci* infection of the Australian yabby *C. destructor* as well as the apparent resistance of ornamental shrimp species highlights that other hosts may potentially facilitate crayfish plague transmission and spread.

Our knowledge of *A. astaci* genetic variation has just started to improve together with the development of molecular methods for pathogen fingerprinting. Based on the findings in the German aquarium trade and wild *P. clarkii* populations in Japan, it should be assumed that many more pathogen genotypes are yet to be discovered, some of which might have been already introduced with recently established North American crayfish species. The novel pathogen strains may vary in virulence and pathogenicity towards the European crayfish species as well as *A. astaci* natural hosts, North American crayfish species. Consequently, research on host-pathogen interactions, especially focused on immune response of host crayfish species to *A. astaci* infection, should be conducted along with the studies on *A. astaci* genetic variation. The outcomes may, among others, explain mechanisms behind local host adaptations and thus elucidate future prospects of native European crayfish populations.

I hope that studies included in my PhD thesis will inspire future research on *A. astaci* that may focus on such aspects as 1) *A. astaci* diversity in Europe and in its native range in North America, 2) decreased susceptibility of non-American crayfish species, including *A. leptodactylus* and adult *C. destructor* as well as exposure to differently virulent *A. astaci* strains, 2) resistance of other freshwater shrimps, e.g., less frequently moulting, as well as their potential to become long-term *A. astaci* carriers capable of pathogen transmission to other host species, 3) immune response of native European crayfish suffering from latent *A. astaci* infections, 4) mechanisms behind *C. destructor* elevated resistance from an immunological perspective, 5) and many others contributing to protection of endemic crayfish diversity. Moreover, I believe that effective conservation of indigenous crayfish species will be only possible if other crayfish pathogens are also thoroughly studied.



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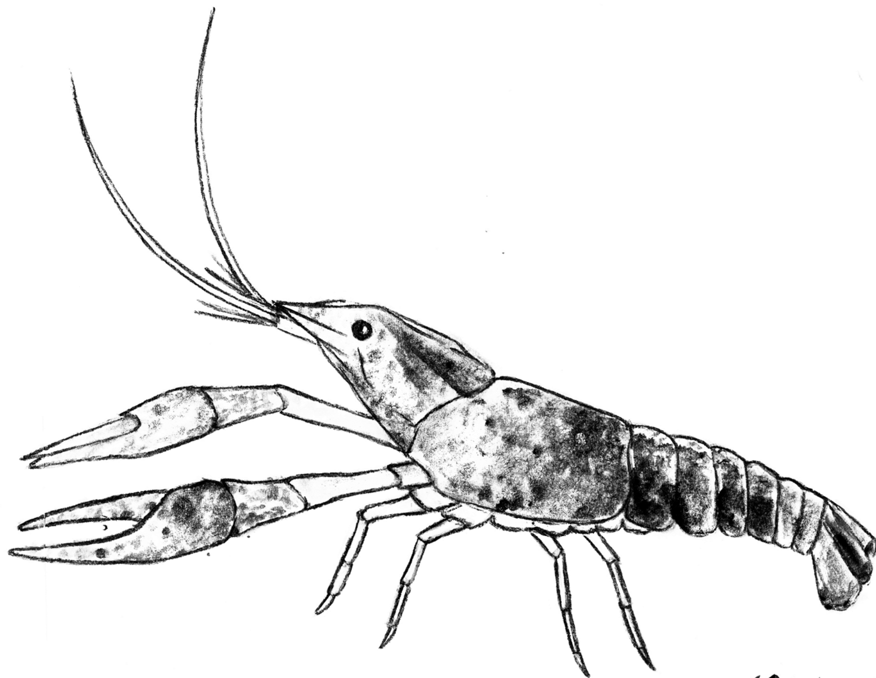

– chapter 1 –

Tilmans M*, Mrugała A*, Svoboda J, Engelsma MY, Petie M, Soes DM, 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 *(* shared first authorship)*

Journal of Invertebrate Pathology 120 (2014) 74–79

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

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

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ARTICLE INFO

Article history:
Received 14 January 2014
Accepted 5 June 2014
Available online 13 June 2014

Keywords:
Crayfish plague
Aphanomyces astaci
Host range
Orconectes virilis
Eriocheir sinensis
Real-time PCR

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.

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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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http://dx.doi.org/10.1016/j.jip.2014.06.002
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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.

Acknowledgments

The study has been partially funded by the Czech Science Foundation (Project no. P505/12/0545). AM was supported by the Charles University in Prague (Project SVV 267204). Michal Voorbergen-Laarman is greatly acknowledged for his assistance in the analysis of the crayfish samples at the Central Veterinary Institute and André Blokland for providing part of the crayfish samples. We thank two anonymous referees for comments on the previous version of the manuscript.

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).

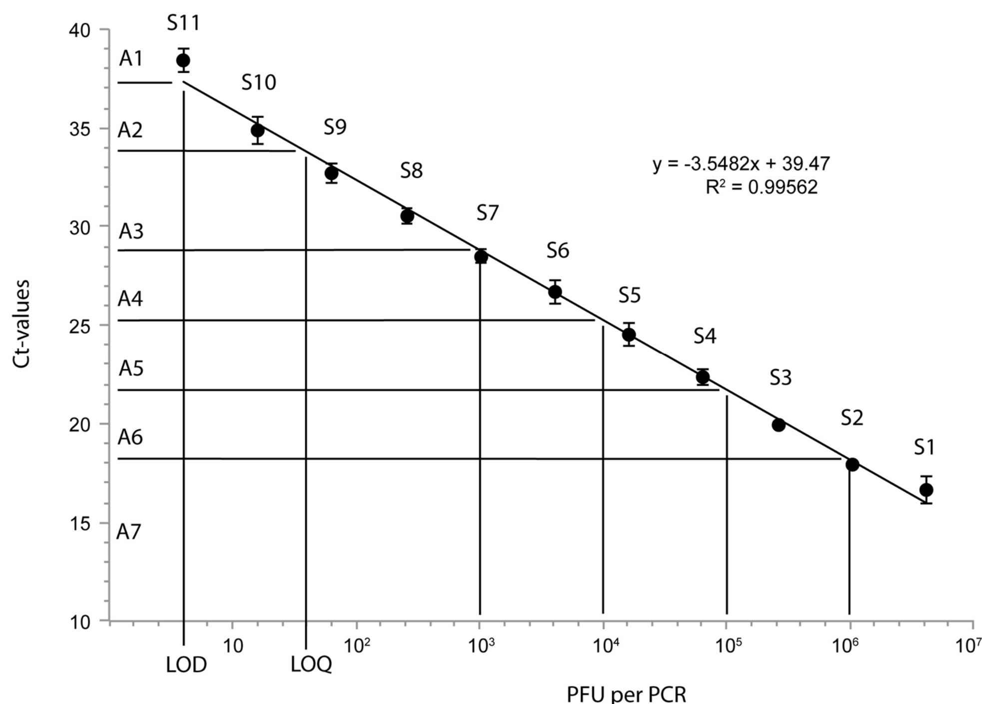

Figure S1. The standard curve based on the mean Ct-values from the 4-fold serial dilution shown in Table S1 plotted against the estimated number of PCR-forming units (PFU) per reaction volume. The limits of detection (LOD) and quantification (LOQ) are indicated. The translation of the qPCR results in 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 2 –

Mrugała A, Kawai T, Kozubíková-Balcarová E, Petrusek A (2016)
Aphanomyces astaci **presence in Japan: a threat to the endemic and endangered crayfish species** ***Cambaroides japonicus*****?** ***Aquatic Conservation: Marine and Freshwater Ecosystems*****,**
doi: 10.1002/aqc.2674

Aphanomyces astaci presence in Japan: a threat to the endemic and endangered crayfish species *Cambaroides japonicus*?

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Abstract

1. Spread of the crayfish plague pathogen, *Aphanomyces astaci,* by North American crayfish species is considered one of the main reasons for substantial declines and local extinctions of native European crayfish populations. Owing to anthropogenic introductions, several American crayfish species got established throughout the world, and thus pose a potential threat to indigenous crayfish populations susceptible to crayfish plague.
2. In Japan, two such widespread alien species, *Procambarus clarkii* and *Pacifastacus leniusculus*, were introduced for aquaculture purposes in the late 1920s and since then successfully expanded their ranges.
3. Aggressive interactions with alien crayfish along with habitat modifications have been primarily considered responsible for drastic declines of populations of the Japanese endemic crayfish, *Cambaroides japonicus,* observed in the last few decades. However, the presence of the crayfish plague pathogen, to which Japanese crayfish are susceptible, may be expected, and could contribute to these declines.
4. Only recently, *A. astaci* has been reported from Taiwan, and to our knowledge no study focusing on its presence outside of the Western Palearctic has been conducted.
5. To fill this gap, we screened 54 *P. clarkii* and 47 *P. leniusculus* individuals from five different Japanese locations using molecular methods recommended by the World Organization for Animal Health. *A. astaci* DNA was detected in all studied populations, altogether in 61% and 21% of examined individuals of *P. clarkii* and *P. leniusculus*, respectively.
6. Our results provide the first evidence of *A. astaci* presence in Japan and highlight the threat of pathogen transmission to *Cambaroides japonicus* populations.

Keywords: crayfish plague, *Procambarus clarkii*, *Pacifastacus leniusculus*, Asia, real-time PCR, aquaculture

Introduction

The Japanese crayfish, *Cambaroides japonicus,* is the only endemic crayfish in Japan, inhabiting cold, clear brooks in Hokkaido and the Aomori, Akita and Iwate Prefectures in northern Honshu (Kawai and Fitzpatrick, 2004). Drastic declines in natural populations of *C. japonicus* owing to invasion of alien species (i.e., crayfish, fish and mammals), deforestation, habitat fragmentation and water quality deterioration, resulted in its designation as an endangered species by the Ministry of Environment of Japan (Category II: vulnerable; Usio, 2007). Several studies implied competition for shelter as well as predation by the North American signal crayfish, *Pacifastacus leniusculus* to be directly involved in these declines (Nakata and Goshima, 2003, 2006). Moreover, crayfish plague, a disease transmitted by North American crayfish, has been suggested as a potential contributory factor (Nakata and Goshima, 2003).

Aphanomyces astaci, the infective agent of crayfish plague, is listed among 100 'world's worst' invasive species (Lowe *et al.*, 2004) and causes one of the most studied invertebrate diseases of conservation relevance in Europe (Rezinciuc *et al.*, 2015). Its accidental introduction to European freshwaters is an example of devastating consequences that may be imposed on susceptible native fauna after pathogen emergence in formerly uninvaded regions. The first mass mortalities of European crayfish populations caused by *A. astaci* date back to the second half of the 19th century, expanding from outbreaks in Italy and France to almost an entire continent by the end of the century (Alderman, 1996). Extensive losses of crayfish stocks resulted in introductions for restocking and aquaculture of three North American crayfish species: spiny-cheek crayfish, *Orconectes limosus* (first introduced in 1890), signal crayfish, *Pacifastacus leniusculus* (1959), and red swamp crayfish, *Procambarus clarkii* (1973; Holdich *et al.*, 2009). As discovered decades later, these three species (as well as other North American crayfish) are natural carriers of *A. astaci*, a pathogen native to their original range (Unestam, 1972; Vey *et al.*, 1983; Diéguez-Uribeondo and Söderhäll, 1993). Their subsequent spread, both natural and aided by humans, led to huge losses of native European crayfish populations (Alderman, 1996).

Parallel to introductions of North American crayfish to Europe, two *A. astaci* carriers, *P. clarkii* and *P. leniusculus*, started to be used intensively for commercial purposes across the world (Hobbs *et al.*, 1989). As a result, the red swamp crayfish became globally the most widespread crayfish species. Apart from Europe, this warm-water crayfish native to north-eastern Mexico and south-eastern states of USA had successfully spread and established in tropical and subtropical regions of Asia, Africa, and Central and South America (including, e.g., China, Japan, Egypt, Israel, Kenya, Brazil; Lowery and Mendes, 1977; Hobbs *et al.*, 1989; Xingyong, 1995; Ibrahim *et al.*, 1997; Magalhães *et al.*, 2005; Wizen *et al.*, 2008). The introduced range of the second crayfish invader, *P. leniusculus*, native to North American regions westwards from the Rocky Mountains, is limited to western states of the USA (California, Nevada, Utah), and temperate regions of Europe and Japan (Kawai *et al.*, 2002; Taylor *et al.*, 2007; Kouba *et al.*, 2014). Owing to its climatic requirements and environmental plasticity, the signal crayfish is now the most widespread crayfish in Europe (Kouba *et al.*, 2014). The presence of these two invasive crayfish species imposes a wide range of negative impacts not only on native crayfish through competition and disease transmission, but also on whole aquatic communities and habitat structure (e.g., Rodríguez *et al.*, 2005; Gherardi and Acquistapace, 2007; Matsuzaki *et al.*, 2009; Reynolds, 2011).

Both of these North American crayfish species were introduced to Japan, where they successfully expanded their ranges (Figure 1). *Procambarus clarkii* was imported from the USA in 1927

as a food for the bullfrog *Rana catesbeiana* used in aquaculture in Kanagawa and Saitama Prefectures, central Honshu (Suko, 1958). At present, the distribution range of this crayfish includes entire Honshu, Shikoku, Kyushu, and a few locations in thermal waters of Hokkaido (Saito and Hiruta, 1995; Nakata *et al.*, 2001, 2005; Kawai and Kobayashi, 2006). *Pacifastacus leniusculus* was introduced as an aquaculture species to farm ponds across Japan on five separate occasions between 1926 and 1930 (Kawai *et al*., 2002). Although most of these populations died out shortly after introduction, two of them in the Tankai Reservoir in central Honshu (Shiga Prefecture) and in Lake Mashu in eastern Hokkaido survived and are considered source populations for the human-aided spread of this crayfish into the natural environments (Kawai *et al*., 2002). Unlike *P. clarkii*, *P. leniusculus* is widely distributed in Hokkaido and several populations have been found in Honshu (Figure 1; Nakatani and Yokoyama, 2003; Usio *et al.*, 2007; Nakata *et al.*, 2010). Furthermore, both species coexist in a small river in Hokkaido where a hot spring flows in and warms the river water to temperatures exceeding 25 °C (Nakata *et al.*, 2005). The Ministry of Environment of Japan designated both crayfish as Invasive Alien Species, formally prohibiting their raising, import, transport and releases into the natural environment (Mito and Uesugi, 2004). However, *P. clarkii* is still used for educational and research purposes, as well as an ornamental pet in household aquaria (Goto and Kawai, 2012; Sunagawa *et al*., 2015), whereas *P. leniusculus* is sometimes illegally used as bait in the recreational fishing (Kawai T., unpublished).

Until now, the presence of *Aphanomyces astaci* outside Europe and its native range in North America has been confirmed by DNA analyses in farmed crayfish species in Israel and Taiwan. Both incidents have been notified to World Organization for Animal Health (OIE). In Israel, *A. astaci* was detected in extensively farmed North American crayfish *Procambarus alleni.* In Taiwan, five crayfish plague outbreaks were recorded in aquacultures of the Australian redclaw crayfish *Cherax quadricarinatus;* four of them resulted in a total mortality of the stocks (Hsieh *et al*., 2016). In those regions, no native crayfish species are known; however, the European experience of widespread crayfish mass mortalities may become a reality for any susceptible crayfish species inhabiting regions invaded by *A. astaci* carriers.

To prevent such a scenario, conservation actions aiming to preserve global crayfish diversity should include the assessment of *A. astaci* presence and threat. The Japanese crayfish, similarly to European and Australasian crayfish species, has been assessed as highly susceptible to crayfish plague infection (Unestam, 1969). However, despite the fact that decline of the Japanese endemic crayfish had already been attributed to crayfish plague more than a decade ago (Nakata and Goshima, 2003), no studies on *A. astaci* presence have been undertaken in Japan.

The main aim of this study was to evaluate the presence and prevalence of *Aphanomyces astaci* in selected North American crayfish populations introduced to non-European locations. The hypothesis that the alien crayfish in Japan indeed carry the pathogen, and thus may act as reservoirs of the crayfish plague agent in this country, was tested. For this purpose, several populations of both *A. astaci* carriers were screened using molecular methods recommended by the World Organisation for Animal Health (OIE, 2012). Based on the outcome, the potential contribution of crayfish plague to *C. japonicus* declines was assessed, together with a likely scenario of further spread of *A. astaci* in Japan. In addition, *P. clarkii* individuals originating from two other regions (Lake Naivasha in Kenya and an aquaculture installation in Indonesia) were screened for the presence of *A. astaci*.

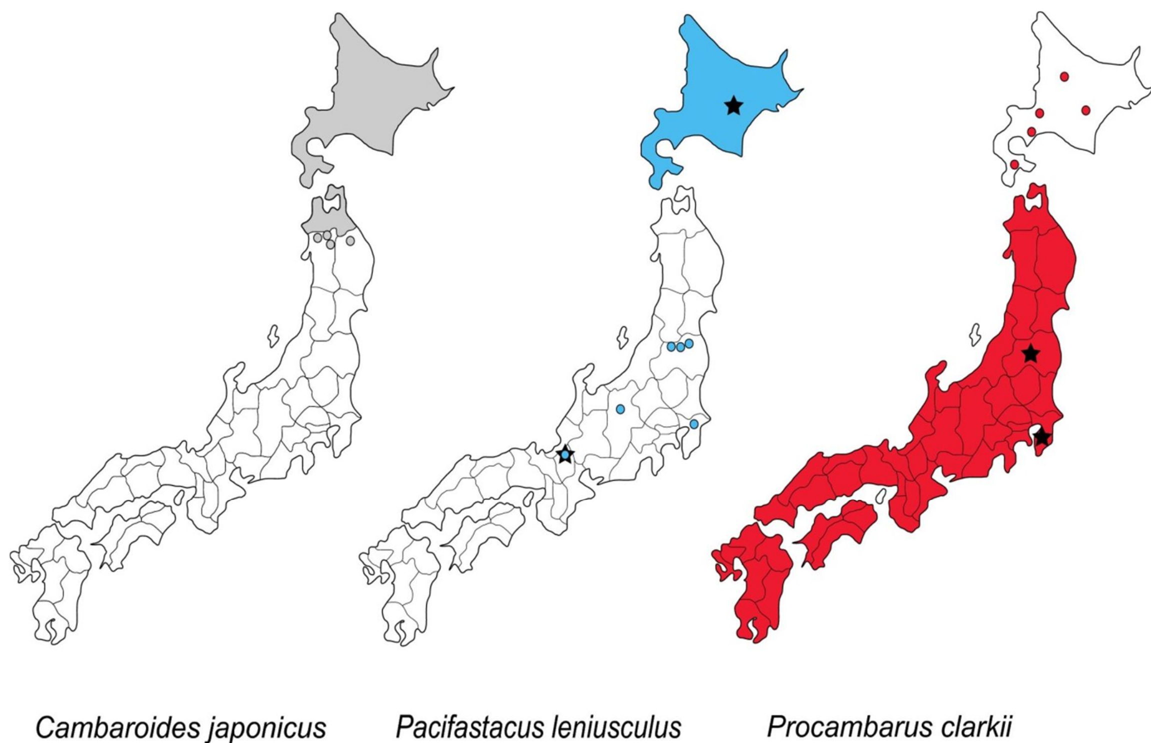

Figure 1. Maps of Japan with administrative division to prefectures, showing simplified distributions of all three crayfish species populations (prefectures marked in colour indicate crayfish wide-spread distributions therein, coloured circles indicate single known populations). Approximate locations of analysed populations of *P. clarkii* and *P. leniusculus* are marked with black stars on the respective maps.

Distribution of *C. japonicus* populations is based on Kawai and Fitzpatrick (2004), Kawai and Labay (2011), *P. leniusculus* on Nakatani and Yokoyama (2003), Usio *et al.* (2007), Nakata *et al.* (2010), and *P. clarkii* on Nakata *et al.* (2005), Kawai and Kobayashi (2011).

Methods

For evaluating the presence of *A. astaci* in Japanese freshwaters, individuals of two North American crayfish species were obtained from locations in Honshu and Hokkaido between 2012 and 2013. The crayfish were captured using baited traps on two separate occasions. During the first sampling, 20 specimens of each species were collected: *P. clarkii* from one location in the Chiba Prefecture (Honshu Island) and *P. leniusculus* from Lake Shikaribetsu in Hokkaido. Positive detection of the crayfish plague pathogen resulted in a second sampling event. Thirty-four individuals of *P. clarkii* were collected from two locations in the Chiba Prefecture (including the previously sampled one) and from one location in the Fukushima Prefecture (Honshu Island). Twenty-seven specimens of *P. leniusculus* were caught at one location in the Shiba Prefecture (Honshu Island) and again from Lake Shikaribetsu in Hokkaido. The approximate locations are marked on maps (Figure 1) that present a simplified distribution of all three crayfish species occurring in Japan.

In addition, individuals of *P. clarkii* from two non-European locations were screened for the presence of *A. astaci* infection. Twelve specimens were obtained from Lake Naivasha in Kenya (0°46'33"S, 36°25'06"E), and 20 specimens from a large batch imported for Czech ornamental trade from aquaculture in Indonesia.

Upon sampling, crayfish were usually stored separately in plastic bottles filled with ethanol; Kenyan individuals were dissected after sampling, and only uropods were preserved in ethanol. The subsequent sample processing and molecular analyses were performed at the Department of Ecology, Charles University in Prague, following the protocols described in detail in Mrugała *et al*. (2015). The soft abdominal cuticle, two pieces of uropods, and any melanized tissues visible by naked eye (for the specimens from Kenya only two parts of uropods) were dissected from each crayfish. Before DNA extraction, these mixed tissue samples were mechanically homogenized by grinding with liquid nitrogen. The extraction was performed from up to 40 mg of the homogenized tissues with the DNeasy tissue kit (Qiagen) following the manufacturer's protocol.

The detection of *A. astaci* was performed with TaqMan MGB quantitative PCR (qPCR) on the iQ5 BioRad thermal cycler as described in Vrålstad *et al.* (2009), with minor modifications of the original protocol to reduce likelihood of false positive results (as in Svoboda *et al.*, 2014). The standard curve for quantification was calibrated with a four-fold dilution series of genomic *A. astaci* DNA (Vrålstad *et al*., 2009). The relative levels of infection were assigned to semi-quantitative agent levels, based on the strength of the PCR signal (according to Vrålstad *et al*., 2009; Kozubíková *et al.*, 2011). Samples yielding agent levels A2 or higher were considered *A. astaci*-positive. Negative controls were included in every step of the process to check for contamination; these remained negative in all cases.

To confirm the presence of *A. astaci* DNA in samples that yielded positive qPCR results, 569 bp long amplicons including parts of internal transcribed spacers (ITS) 1 and 2 and 5.8S rDNA were sequenced according to Oidtmann *et al.* (2006). Owing to a lower sensitivity of conventional PCR compared with the qPCR approach (Kozubíková *et al*., 2011; Tuffs and Oidtmann, 2011), the ITS amplicons were obtained only from infected individuals with agent levels A3. The PCR products were purified with ethanol precipitation and sequenced in one direction on the ABI 3130xl Genetic Analyzer (Applied Biosystems). Resulting sequences were compared with publicly available sequences of *A. astaci*.

For an identification of the *A. astaci* genotype group present in Japan, genotyping of the *A. astaci*-positive samples was performed with microsatellite markers as described in Grandjean *et al.* (2014). As the amplification success depends on the amount of *A. astaci* DNA, consistent amplifications of microsatellite loci were reported for samples with agent level A4 or higher, i.e., exceeding 1000 PCR-forming units (PFU) in the reaction, and no amplifications were obtained for samples with agent level A3 below 250 PFU (Grandjean *et al.*, 2014). For that reason, genotyping was only attempted for samples with agent level A3 in our study. In the case of an initial lack of amplification, the DNA isolates were concentrated on Concentrator Plus 5305 (Eppendorf).

For estimating the prevalence of *A. astaci* in studied populations, 95% confidence intervals were calculated as in Filipová *et al.* (2013). This was performed for the prevalence values obtained from the number of *A. astaci*-positive and total number of tested individuals per population using the function 'epi.conf' from the library epiR (Stevenson *et al*., 2013) for R v. 3.0 (R Development Core Team, 2013).

Results

The presence of *Aphanomyces astaci* DNA was detected in all sampled populations of North American crayfish species in Japan. Thirty-three of 54 *P. clarkii* and 13 of 47 *P. leniusculus* individuals tested positive for the pathogen. While *P. leniusculus* populations were moderately infected (24 to 40% of analysed individuals testing positive), the pathogen prevalence in *P. clarkii* populations was very high (75 to 100%) in three out of four samples (Table 1). Moreover, in contrast to a stable *A. astaci* prevalence in the resampled populations of *P. leniusculus* in Lake Shikaribetsu, a substantial increase in pathogen detectability was noted in samples from a *P. clarkii* population in a reservoir in the Chiba Prefecture. The *A. astaci*-positive isolates reached very low (A2) and low (A3) agent levels (Table 1), according to Vrålstad *et al.* (2009).

For confirmation of *A. astaci* detection, ITS sequences were obtained from two *P. clarkii* populations sampled in the Chiba Prefecture. They matched completely the *A. astaci* sequences available in the GenBank (e.g., KF944439). No ITS sequences were obtained from infected *P. leniusculus* owing to a very low level of pathogen infection.

The *A. astaci* genotyping was only possible for three DNA isolates obtained from infected *P. clarkii*. These three crayfish specimens were caught in the Chiba Prefecture. Owing to low amount of *A. astaci* DNA in all three isolates, reliable amplification and scoring of the microsatellites was not possible for three out of the nine loci. However, the multilocus genotype corresponded to the reference axenic culture of the genotype group D that was originally obtained from this host species at five out of the six loci (allele sizes: Aast4: 131 bp, Aast6: 148 bp, Aast9: 180 bp, Aast13: 194 bp, Aast14: 250 bp). A consistent difference was observed at the Aast10 locus, where a homozygote with allele sizes of 156 bp (instead of 142 bp as reported for the genotype group D) was scored. The amplification of this allele was successful for two out of three genotyped samples.

No *A. astaci* DNA was detected in *P. clarkii* individuals from Lake Naivasha in Kenya, and from the aquaculture samples from Indonesia. However, as indicated by wide confidence intervals, the lack of the pathogen infection in analysed material cannot be considered as confirmation of *A. astaci* absence in those sources.

Table 1. Results of *Aphanomyces astaci* detection in populations of two North American crayfish occurring in Japan. Semi-quantitative agent levels are provided for both positive and negative (below the level of detection) values. Asterisks indicate locations from where crayfish were collected during the first sampling occasion.

Sp.	Sampling site	Type of water body	Individuals tested	*A. astaci* infected	Prevalence (95% CI)	Agent level		
						A1	A2	A3
Procambarus clarkii								
	Chiba *	Reservoir	20	3	15% (3-38%)	7	3	
	Chiba I	Reservoir	15	15	100% (70-100%)		7	8
	Chiba II	Reservoir	16	12	75% (48-93%)	3	7	5
	Fukushima	Lake	3	3	100% (19-100%)		1	2
Pacifastacus leniusculus								
	Shikaribetsu *	Lake	20	5	25% (9-49%)	1	4	1
	Shikaribetsu	Lake	17	4	24% (7-50%)	3	4	
	Shiga	Reservoir	10	4	40% (12-74%)		4	
Procambarus clarkii								
	Kenya, Naivasha	Lake	12	0	0% (0-36%)			
	Indonesia	Aquaculture	20	0	0% (0-24%)			

Discussion

Owing to its devastating and irreversible impacts on native European crayfish populations, *Aphanomyces astaci* is considered one of the worst pathogens affecting freshwater invertebrates. All crayfish species that lack long co-evolutionary history with the pathogen should be regarded as potentially susceptible (Unestam, 1972). Therefore, this disease agent is a threat to native crayfish diversity worldwide. Evaluating its presence should be extremely important in every region where introduced North American crayfish may come into contact with non-resistant native crayfish species. The screening of populations of North American crayfish species in this study unambiguously confirmed the widespread presence of the crayfish plague pathogen in Japan, and provided the first evidence of *A. astaci* infection in its natural hosts in Asia-Pacific region.

The detection of *Aphanomyces astaci* in Japan is especially worrying as the pathogen may spread to the native Japanese crayfish, if *A. astaci* carriers occur in the same water catchment. The distribution of *C. japonicus* is limited to the northern part of Honshu and Hokkaido, overlapping greatly with the range invaded by *P. leniusculus*. Experimental investigations demonstrated *P. leniusculus* superiority in shelter competition as well as its predation on Japanese crayfish, and those factors certainly contributed to drastic population declines and *C. japonicus* displacements (Nakata and Goshima, 2003, 2006). At present, no shared locality between *P. leniusculus* and *C. japonicus* is known in Japan (Nakata and Goshima, 2006). In Europe, expansion of the invasive signal crayfish is usually correlated with declines in populations of the native European crayfish species (e.g., Almeida *et al*., 2014); with the primary reason being the transmission of crayfish plague, and the superior competitive ability of the invading crayfish possibly further contributing to these declines (Alderman *et al*., 1990; Bohman *et al*., 2006; Diéguez-Uribeondo, 2006; Chucholl and Schrimpf, 2015). The results described here suggest that crayfish plague might also have played an important role in *C. japonicus* declines, and poses a threat to the remaining Japanese crayfish populations.

The presence of crayfish plague infection was revealed in every studied population of *P. clarkii* and *P. leniusculus* in Japan. The pathogen prevalence in these populations ranged from very high to moderate, in line with the *A. astaci* prevalence observed in North American crayfish populations in Europe (Kozubíková *et al*., 2009, 2010; Vrålstad *et al*., 2011; Filipová *et al*., 2013; Tilmans *et al*., 2014). The detectability of *A. astaci* may depend on temporal fluctuations in pathogen presence, type of water body inhabited by crayfish, density of crayfish population, and the age of sampled crayfish individuals (Kozubíková *et al*., 2009; Matasová *et al*., 2011; Vrålstad *et al*., 2011). The direction of temporal changes in pathogen presence may also vary. In contrast to Matasová *et al.* (2011), an increase in *A. astaci* prevalence was observed in *P. clarkii* populations sampled in the Chiba Prefecture. Therefore, results from a single sampling event should be interpreted with caution, as the proportion of infected individuals may not adequately represent the long-term pathogen prevalence.

The low levels of *A. astaci* infections detected in *P. clarkii* and *P. leniusculus* in Japan are typical for carriers of latent pathogen infections (Kozubíková *et al*., 2011; Filipová *et al*., 2013; Tilmans *et al*., 2014). Long co-evolutionary history of North American host crayfish with *A. astaci* equipped them with effective defence mechanisms limiting pathogen growth in their cuticles (Cerenius *et al*., 2003). Consequently, even if weakly infected, these crayfish may act as reservoirs of the disease, repeatedly releasing spores to the water (Strand *et al*., 2012; Svoboda *et al*., 2013).

In populations of non-indigenous species, the pathogen presence and prevalence may also be strongly shaped by their introduction history. As the source populations often consist of relatively small subsets of native populations or uninfected life-history stages, the probability of pathogen introduction may be greatly reduced (Torchin *et al*., 2003). Therefore, even the lack of crayfish plague detection in the red swamp crayfish populations from Kenya and Indonesia is not unexpected. Very low prevalence or an apparent absence of *A. astaci* infection were reported in, for example, in some populations of *Procambarus* cf. *acutus* and *P. clarkii* in the Netherlands (Tilmans *et al*., 2014), *P. leniusculus* in Denmark and France (Skov *et al*., 2011; Filipová *et al*., 2013), and *O. limosus* in Germany (Schrimpf *et al*., 2013) but only long-term and/or very extensive sampling over several seasons may provide sufficiently robust results to allow conclusions about the real *A. astaci* absence in these populations. Especially in Indonesia inhabited by many endemic crayfish species (Furse *et al*., 2015), the releases of farmed *P. clarkii* individuals to open waters should be prevented not only because of the risk of disease transmission but also because of their high competitive abilities. The use of cultivation enclosures, e.g. with closed recirculating water systems as well as preventing illegal releases of crayfish should substantially reduce the number of crayfish escapees (Liu and Li, 2009; Conde and Domínguez, 2015).

Transmission experiments, in which susceptible *C. japonicus* were placed in tanks with presumably infected *P. leniusculus*, were used to assess the threat this North American invader may pose to Japanese endemic crayfish (Nakata and Goshima, 2003; Ichijyo *et al*., 2011). This method of choice may be useful in determining crayfish susceptibility to *A. astaci*, but it will not provide reliable evidence of its presence or absence in its natural carriers. Therefore, the lack of *C. japonicus* mortality in the experiments described here should not be considered as a confirmation that the signal crayfish populations in Japan do not harbour crayfish plague infections. In infection trials performed by Svoboda *et al.* (2013), the transmission rate from confirmed *A. astaci*-positive donors to susceptible noble crayfish *Astacus astacus* was less than 50%.

Moreover, many different factors may also influence success of infection. For example, water temperature can influence *A. astaci* growth and spore release (Diéguez-Uribeondo *et al.*, 1995; Rezinciuc *et al*., 2014). In addition, the increased resistance of susceptible crayfish species, as observed in Australian yabby, *Cherax destructor* (Unestam, 1969; Mrugała *et al*., in press) may hinder the detection of crayfish mortality if the experimental trial is terminated too early. As documented by Makkonen *et al.* (2012), certain noble crayfish populations exhibited elevated resistance towards *A. astaci*, most likely linked to their previous experience with the pathogen. The moderate prevalence of *A. astaci* and the very low levels of pathogen infections in tested populations of *P. leniusculus,* together with the high *C. japonicus* susceptibility assessed by Unestam (1969), suggest that uninfected or weakly infected *P. leniusculus* individuals were used in the above-mentioned experiments (Nakata and Goshima, 2003; Ichijyo *et al.*, 2011). Thus, it is advisable to support such experiments with the use of very specific molecular methods (also recommended by OIE; OIE, 2012) when evaluating the presence of *A. astaci* in populations of North American crayfish species.

Although it can be argued that direct evidence is lacking for crayfish plague contribution to *C. japonicus* declines, the disease outbreaks may happen unnoticed (especially if crayfish populations got infected with a highly virulent *A. astaci* strain), or are not reported to relevant authorities (particularly if the public and shareholders are unaware of the pathogen threat; see Kozubíková *et al.*, 2008). The pathogen genotype identified in this study apparently belongs to the genotype group D,

isolated in Europe from *P. clarkii* and assessed as highly virulent towards the native European crayfish species (Diéguez-Uribeondo *et al*., 1995); the same allele at the Aast10 locus has already been observed in isolates from aquarium *P. clarkii* (Viljamaa-Dirks *et al*., 2014). Even though Diéguez-Uribeondo *et al*. (1995) observed that the *A. astaci* strains belonging to this group may, like their original host, be adapted to subtropical waters, the observed genetic variation among strains may affect the adaptability and survival potential of *A. astaci* in temperate regions of Japan. Moreover, it is likely that more *A. astaci* strains have been introduced to Japan. *Pacifastacus leniusculus* may be a carrier of other *A. astaci* strains (see Huang *et al*., 1994; Grandjean *et al*., 2014) that are, similarly as its host, more adapted to colder climates.

At present, northern Japan is mainly inhabited by *P. leniusculus*; however, this situation may change with the recent northward expansion of *P. clarkii.* The present distribution of this warm-water crayfish in Hokkaido is restricted to thermal waters (Saito and Hiruta, 1995; Nakata *et al*., 2001, 2005; Kawai and Kobayashi, 2006); however the red swamp crayfish introduced to temperate Central and Western Europe is apparently thriving (Kouba *et al*., 2014). Chucholl (2011) reported that *P. clarkii* is able to cope well with cold habitats in Germany by modulating its life history. Furthermore, even without the red swamp crayfish spreading beyond the thermal waters, the pathogen can still be transmitted to *P. leniusculus* if both species come into contact. A syntopic occurrence of both invaders has indeed been observed in a river in Hokkaido (Nakata *et al*., 2005).

Introduction of new North American crayfish species that may act as *A. astaci* carriers should also be considered. A single specimen of the marbled crayfish, *Procambarus fallax* f. *virginalis* was discovered in 2006 in a river near Sapporo in Hokkaido (Faulkes *et al.,* 2012). Infected marbled crayfish have already been observed in the German aquarium trade and laboratory cultures, as well as in the wild (Keller *et al*., 2014; Mrugała *et al*., 2015). Thus, this crayfish species may pose a threat to the Japanese crayfish not only through a direct competition but also as a vector of the pathogen. Although no further records of this crayfish are known from Japan, Faulkes *et al.* (2012) suggested that considerable lag phase may separate discovery of a single specimen from the establishment of a marbled crayfish population, as observed in Europe. Moreover, marbled crayfish reproduces parthenogenetically and even a single specimen may be a founder of a new population (Scholtz *et al*., 2003). Therefore, it may only be a matter of time until established populations of *P. fallax* f. *virginalis* are found in Japan. Faulkes *et al.* (2012) predicted with the niche-based species distribution models that the most suitable habitats for marbled crayfish in Japan include eastern Honshu, Shikoku and Kyushu, and to a lesser extent Hokkaido. However, the presence of established marbled crayfish populations in temperate Europe in Germany and Slovakia (Chucholl *et al*., 2012; Lipták *et al*., 2016) may indicate its potential to establish successfully in northern Japan.

The trade in ornamental crayfish is considered nowadays to be the main introduction pathway of these freshwater crustaceans in Europe (Peay, 2009), mainly as a result of its rapid growth in popularity during the past decades (Chucholl, 2013). It is therefore likely that this introduction pathway may become relevant in any region of the world where crayfish are sold as pets for home aquaria. In Japan, despite a national ban of alien species imports, several North American crayfish species from the family Cambaridae circulate in the aquarium trade (Sunagawa *et al*., 2015), and the single marbled crayfish specimen found near Sapporo is believed to have an aquarium origin (Faulkes *et al*., 2012). As the *A. astaci* infections have been already detected in aquarium shops in Central Europe (Mrugała *et al*.,

2015), it should be emphasised that any crayfish species released to the natural environment may carry the crayfish plague pathogen. We believe that education of the general public and crayfish retailers may substantially reduce such risks (for details see Mrugała *et al.*, 2015).

The lesson learnt from European crayfish experience with the highly destructive crayfish plague pathogen should prompt conservation actions to protect the remaining populations of Japanese endemic crayfish. Prevention of further spread and new introductions of North American crayfish species, and thus also transmission of *A. astaci* and emergence of novel pathogen strains, is especially recommended. A wide variety of control methods limiting the spread of invasive non-native crayfish species has been tested already in Europe (Freeman *et al.*, 2010; Gherardi *et al.*, 2011). Although eradication of the alien invasive species may often be very difficult or not feasible after their establishment (Genovesi, 2005; Vilà *et al.*, 2010), several approaches may restrict or slow the spread of invasive crayfish species to localities inhabited by *C. japonicus*. These may include: physical constraints to species dispersal through natural and man-made barriers (Gherardi *et al.*, 2011; Frings *et al.*, 2013), intensive trapping during periods with the highest crayfish catches (Sousa *et al.*, 2013), or use of an electric shock treatment (Peay *et al.*, 2015) as well as a biological control (Freeman *et al.*, 2010). In addition, if restoration of *C. japonicus* populations is foreseen, much wider screening as well as extensive monitoring of crayfish plague presence in *P. clarkii* and *P. leniusculus* populations in northern Japan will be crucial to identify sites that are less likely to get the pathogen. However, all such activities will only produce successful, long-term outcomes if supported with the appropriate educational initiatives.

Acknowledgements

Agata Mrugała was supported by the Charles University in Prague (project SVV 260312). We thank M. Sunagawa for the collection of crayfish specimens in Japan, and I. Přikryl for samples from Lake Naivasha. Philip J. Boon and two anonymous referees provided valuable comments on the previous version of the manuscript.

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– chapter 3 –

Lipták B, Mrugała A, Pekárik L, Mutkovič A, Gruľa D, Petrusek A, Kouba A (2016) Expansion of the marbled crayfish in Slovakia: beginning of an invasion in the Danube catchment? *Journal of Limnology* 75: 305-312

J. Limnol., 2016; 75(2): 305-312
DOI: 10.4081/jlimnol.2016.1313

ORIGINAL ARTICLE

Expansion of the marbled crayfish in Slovakia: beginning of an invasion in the Danube catchment?

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ABSTRACT

The marbled crayfish, Procambarus fallax *f.* virginalis, *is a taxon widely available in the aquarium pet trade, which has been introduced to open waters in several European countries and in Madagascar. Recent studies confirmed this parthenogenetically reproducing crayfish as a high-risk invasive species, and vector of the crayfish plague pathogen,* Aphanomyces astaci. *It has been first discovered in Slovakia in 2010, but the status of the local population was not studied since then. Due to enlarged sampling area around the first report and one locality, where we presupposed the crayfish occurrence, we identified three new marbled crayfish populations in Slovakia. Two populations are located critically close to the Váh River, a major tributary of the Danube River; one of them being directly connected to the Váh River* via *a side channel during occasional floods. The third established marbled crayfish population was found at the mouth of a thermal stream flowing into the Nitra River, a tributary of the Váh River. In this stream, crayfish coexist with exotic fish and gastropod species of aquarium origin. We presume that the reported localities may serve as a source for further expansion of the marbled crayfish in the mid-part of the Danube catchment. Floods, active dispersal (including overland), passive dispersal by zoochory or anthropogenic translocations are among the major drivers facilitating the marbled crayfish colonization. We have not detected the crayfish plague pathogen in any of the studied populations. However, if spreading further, the marbled crayfish will encounter established populations of crayfish plague carriers in the Danube River, in which case they may acquire the pathogen by horizontal transmission and contribute to spread of this disease to indigenous European crayfish species.*

Key words: Aquarium pet trade; crayfish plague; freshwater crayfish; Procambarus fallax *f.* virginalis*; species introductions.*

Received: August 2015. Accepted: January 2016.

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INTRODUCTION

Biological invasions and human impacts (*e.g.*, habitat destruction and pollution) are the major factors negatively influencing global biodiversity (Sala *et al*., 2000; Dudgeon *et al*., 2006). One of the important introduction pathways of potential invaders is the global pet trade, of which aquatic organisms represent a great portion (Padilla and Williams, 2004). The problem of releases of aquarium fish in both freshwater and marine environments has been recognized for a long time (*e.g.*, Courtenay and Stauffer, 1990; Semmens *et al.*, 2004), but scantly addressed. More recently, establishment of ornamental crayfish populations received attention, particularly in Europe. Rising density of human population and increasing socio-economic conditions favor the chances of crayfish releases (Perdikaris *et al*., 2012; Chucholl, 2014) and some of the species kept in aquaria become established in the wild (Holdich *et al*., 2009; Kouba *et al*., 2014). Native European crayfish species are challenged by the ever increasing number of newly introduced alien crayfish and the risks associated with them (particularly disease transmission), which substantially complicates their population recovery and conservation (Peay and Füreder, 2011; Capinha *et al*., 2013).

One of the emerging crayfish invaders in European freshwaters is the marbled crayfish, also known as Mamorkrebs, a parthenogenetically reproducing form of *Procambarus fallax* (Hagen, 1870) (Martin *et al.*, 2010a) discovered originally in the aquarium trade (Scholtz *et al.*, 2003). Marbled crayfish are widely available undemanding pets, frequently sold both in brick and mortar shops and online (Chucholl, 2013, 2015; Faulkes, 2013; Mrugała *et al.*, 2015; Lipták and Vitázková, 2015). Due to its asexual mode of reproduction, marbled crayfish can overpopulate a home aquarium in a short time. Such situation often leads to sale or disposal of redundant individuals by aquarium holders (Patoka *et al*., 2014a).

In the wild, marbled crayfish were first recorded in

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Germany in 2003 (Marten *et al*., 2004). Since then, their presence was reported from various European countries, including the Netherlands, Italy, Slovakia, Croatia, and even Sweden (summarized in Chucholl *et al*., 2012; Kouba *et al*., 2014; Samardžić, 2014), and the crayfish got apparently well established in Madagascar (Jones *et al*., 2009; Kawai *et al*., 2009). The first reliable record of an established population in Central Europe had been reported in 2010 from southwestern Germany (Chucholl and Pfeiffer, 2010), and by 2012 at least six established marbled crayfish populations were known in Europe (Chucholl *et al*., 2012). Moreover, the marbled crayfish has been recently confirmed as a vector of the crayfish plague pathogen, *Aphanomyces astaci* (Keller *et al.*, 2014; Mrugała *et al.*, 2015), which is responsible for substantial population declines and local extinctions of native European crayfish species (for review, see Holdich *et al*., 2009). The presence of the marbled crayfish in natural ecosystems may, therefore, facilitate the spread of this disease and thus affect native European crayfish species if they get into contact with an infected carrier.

The marbled crayfish had been first detected in Slovak surface waters in 2010, when more than 150 individuals were collected from a small gravel pit near the village Koplotovce (Janský and Mutkovič, 2010). The aim of our study was to evaluate the present status of the marbled crayfish in Slovakia, and its potential for further spread and impact. We report additional sites with the established marbled crayfish populations, in which we assessed the population structure. Furthermore, we tested the collected animals for the potential presence of the crayfish plague pathogen, *Aphanomyces astaci.*

METHODS

Study sites

The Slovak Republic is located in the heart of Europe, and its lowland regions are characterized by a continental climate with warm summers and cold winters. All three studied sites with marbled crayfish populations are located in the southwestern part of the country at relatively low elevations (Fig. 1; Tab. 1).

Koplotovce site

This is the first site from which the marbled crayfish was first reported in Slovakia (Janský and Mutkovič, 2010), it comprises seven adjacent groundwater-fed gravel pits (Fig. 1) ranging in area from 1600 m^2 to 21,600 m^2. The gravel pits freeze over in winter (with the bottom temperatures not exceeding 4°C), while the epilimnion warms up to 23-25°C in summer. Two of these pits (one being the site of the first marbled crayfish record for Slovakia) are privately owned. The area of the gravel pits is separated from the adjacent Váh River by an embankment that provides protection from the occasional floods. The pits have fluctuating water level and varying depth (up to

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Fig. 1. The marbled crayfish (*Procambarus fallax* f. *virginalis*) occurrence in Slovakia. Black lines in the central map indicate country borders, blue lines indicate the river network, and the orange rectangle represents privately owned and thus inaccessible sites. Red stars represent newly discovered marbled crayfish populations, while the yellow star represents the original site of the first record in the country.

2-3 m), and are partially overgrown with macrophytes. Although isolated under standard hydrological and meteorological conditions, all gravel pits get periodically interconnected following excessive rainfall (last such events occurred in 2006 and 2010). The pits are frequently visited fishing grounds, seasonally restocked with fish.

Leopoldov site

It is represented by a single large gravel pit (surface area ca. 130,600 m^2), connected during floods with a side channel (Drahovský kanál) of the Váh River (last such event occurred in 2010). Depth of the gravel pit varies, reaching 5-7 m at its southern and 4 m in its northern section (Fig. 1). The water temperature regime of this gravel pit is similar to those in Koplotovce. The site is a frequently visited fishing ground, seasonally restocked with fish.

Opatovce site

It is a thermal stream flowing through Opatovce nad Nitrou, a small village next to the popular thermal spa town Bojnice. The water temperature in the stream varies little during the year, ranging from 29 to 31°C; pH values increase from 7.15 in the middle section to 8.30 at the stream mouth (Májsky 2007). The stream (ca. 1 m wide) empties into the Nitra River (ca. 8 m wide), a tributary of the Váh River. The stream bed is formed by concrete blocks, and the stream banks are continuously lined with dense vegetation.

Field work

We failed to get an access to the privately owned gravel pit in Koplotovce to inspect the original site of the marbled crayfish record. Therefore, the adjoining gravel pits were surveyed. A pilot study at the Koplotovce site was carried out in three gravel pits and one adjoining periodical pool on 15 May 2014. The survey was performed manually with small hand-held net and with 30 fishmeat-baited crayfish traps. The traps were left overnight and collected in the morning. Subsequently, the Koplotovce site was visited on 6 September 2014. Two gravel pits (previously sampled on 15 May 2014) were investigated with electrofishing equipment. In the larger gravel pit (ca. 17,000 m^2), the sampling was conducted along ca. 40 m of the shore; the smaller adjoining pit (ca. 1600 m^2) was surveyed for 10 min in a 10 m long shore area. Finally, an additional survey with a standardized sampling effort was carried out on 17 October 2014 in one of the pits where crayfish had been recorded during a previous visit. The animals were collected with a small hand-held net for 45 min in a shore area approximately 30 m long.

The Leopoldov site was inspected for three days on 16, 17 and 18 September 2014. On this occasion, crayfish were mainly observed and photographed. The collection of crayfish took place on 18 October 2014, with the same effort as during the last-mentioned samping at the Koplotovce site (*i.e.*, by manual search with a hand-held net for 45 min along an approx. 30 m long shore area).

The thermal stream in Opatovce was visited for the first time on 19 September 2013, when the site was inspected for crayfish by electrofishing. Subsequently, on 17 October 2014, crayfish individuals were sampled as in the gravel pits by a small hand-held net for 45 min along an approx. 30 m long stream section.

Upon capture, the carapace length of crayfish individuals was measured. The numbers of juveniles carried by females obtained at the Koplotovce site were counted; brood sizes of females from the Leopoldov site were roughly estimated from available photographs. Subsequently, all individuals were stored in 96% ethanol for further analyses.

Molecular analyses

From each locality, up to 16 specimens (as given in Tab. 1) were used for screening for the presence of the crayfish plague pathogen. From each crayfish, we dissected the whole soft abdominal cuticle, the tail fan, and two joints of walking legs (in individuals smaller than 4.5 cm all basal joints with legs). Furthermore, we inspected the crayfish for the presence of melanized spots, potentially indicating an immune response to pathogens; if these were observed, the respective body part was included in the analysis.

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Tab. 1. Details on the sampled localities with the marbled crayfish (*Procambarus fallax* f. *virginalis*) populations in Slovakia, and on collected crayfish individuals.

Sampling site	River basin	Type of water body	Coordinates		Elevation (m)	Sampling date	Collected crayfish	CL (mm)	Crayfish tested for *A. astaci*
			Latitude (N)	Longitude (E)					
Koplotovce	Váh	Gravel pit	48°28’11”	17°48’15”	141	6 Sep 2014	11	13.3-44.8	6
						17 Oct 2014	10	5.6-24.3	10
Leopoldov	Váh	Gravel pit	48°27’02”	17°47’11”	140	18 Oct 2014	21	5.4-30.9	12
Opatovce	Nitra	Thermal stream	48°46’01”	18°34’39”	254	17 Oct 2014	38	7.4-35.7	12

CL, carapace length.

The genomic DNA was extracted with the DNeasy tissue kit (Qiagen) from up to 50 mg of the mix-tissue samples ground beforehand in liquid nitrogen (as in Kozubíková *et al*., 2009). The molecular detection of *A. astaci* was performed with the TaqMan minor groove binder quantitative polymerase chain reaction (qPCR; after Vrålstad *et al.*, 2009) as described in Svoboda *et al*. (2014). Additionally, the identity of the crayfish species was investigated by sequencing of a 648 bp long fragment of the mitochondrial gene for the cytochrome c oxidase subunit I (COI) from one crayfish individual per population. We used the universal primer pair LCO1490/ HCO2198 (Folmer *et al*., 1994), following the protocols described in Mrugała *et al*. (2015).

RESULTS

Three new established populations of the marbled crayfish have been confirmed in Slovakia. As expected for this parthenogenetically reproducing taxon, all captured individuals were females. At the Koplotovce site, only exuviae of a single crayfish individual was found on 15 May 2014, despite the overnight use of crayfish traps and manual sampling effort. Five adult and six medium-sized (carapace lenght (CL) 10-20 mm) individuals were caught at this site on 6 September 2014 (Fig. 2A). Three mature females, captured on 6 September 2014, carried 372, 412 and 455 juveniles, respectively. The fourth female lost some of the offspring during manipulation, and thus carried only 81 juveniles at the time of counting. Ten more marbled crayfish were collected on 17 October 2014 (Fig. 2B), in the survey with a standardized sampling effort. Two mature marbled crayfish females with eggs were photo-documented at the Leopoldov site on 16 September 2014. Although the egg numbers were not counted, the assessment of photographs suggests that both brood sizes reached at least 300 eggs. Furthermore, young individuals were observed on 17 and 18 September 2014. During the survey on 18 October 2014, 21 medium-sized (CL 5-15 mm) individuals were collected (Fig. 2C). The crayfish were found mainly in the leaf litter accumulated at the banks of the gravel pit.

At the Opatovce site, four crayfish individuals were collected on 19 September 2013 and further 38 crayfish were caught during a standardized sampling on 18 October 2014 (Fig. 2D). Three ornamental fish species, the guppy (*Poecilia reticulata*), the Mozambique tilapia (*Oreochromis mossambicus*) and the convict cichlid (*Amatitlania nigrofasciata*), were observed at the site as well. Moreover, the stream bottom substrate was dominated by a dense population of the red-rimmed melania (*Melanoides tuberculata*), an alien gastropod frequently

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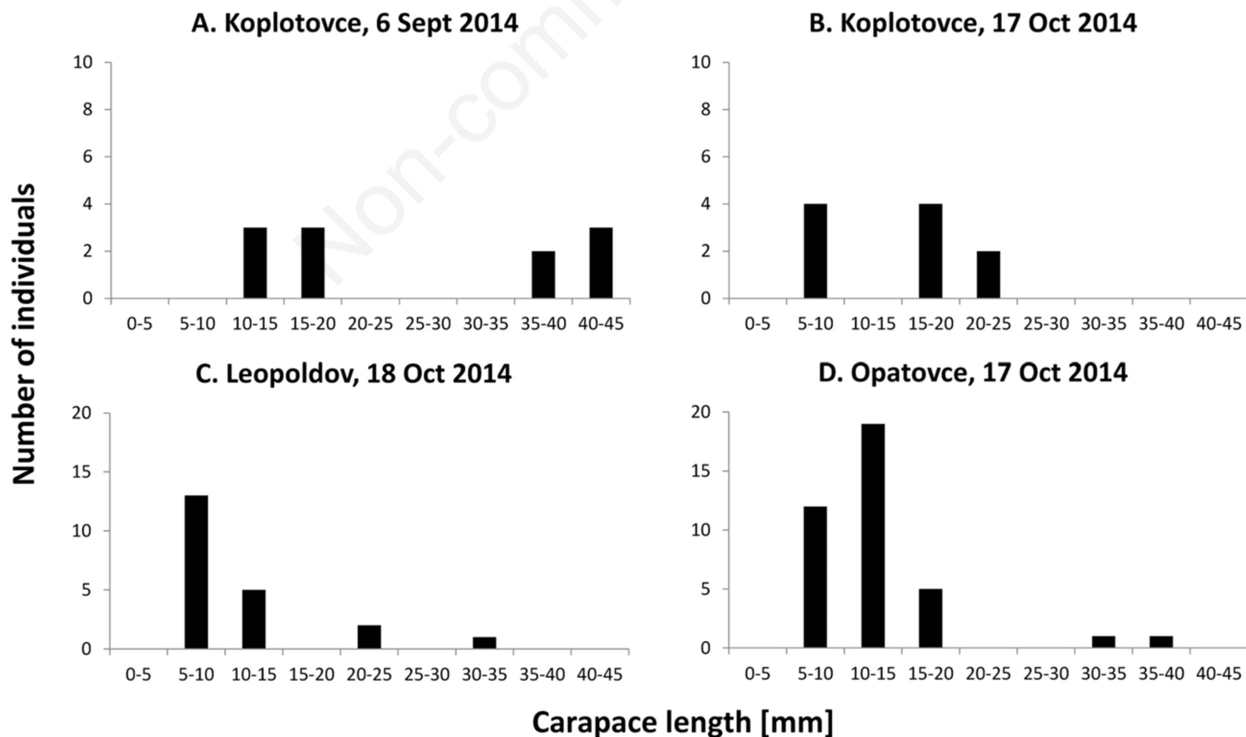

Fig. 2. Body size distribution (expressed as carapace length) of the marbled crayfish (*Procambarus fallax* f. *virginalis*) from the inspected Slovak crayfish populations.

kept in aquaria. All four crayfish individuals sampled on 19 September 2013 were transferred alive to laboratory for breeding; two individuals died soon but the two others were still alive in January 2015. By that time, both surviving females had reproduced five times, approximately every three months. First reproduction of their offspring was observed at the age of 6 months, in synchrony with the maternal generation.

The DNA barcoding confirmed the morphological identification of captured crayfish as *P. fallax* f. *virginalis*. All obtained COI fragments matched completely the publicly available reference sequences of the marbled crayfish from GenBank (acc. nos. KC107813, HM358011, JF438007; Martin *et al.*, 2010a; Filipová *et al.*, 2011; Shen *et al.*, 2013). No traces of *Aphanomyses astaci* DNA were detected in any of the analyzed marbled crayfish.

DISCUSSION

The marbled crayfish presence in Central Europe is an excellent example of successful introductions of an ornamental species. Although original prognoses questioned its survival in the wild, especially in temperate climate (Martin *et al.*, 2010b), it is now recognized as an established invader both in Europe and in Madagascar (Jones *et al*., 2009; Kawai *et al*., 2009; Chucholl *et al*., 2012; Kouba *et al*., 2014). Due to its parthenogenetic reproduction strategy, theoretically no more than one individual is needed to establish a viable population (Scholtz *et al*., 2003). Based on our data and on findings from Germany (Chucholl and Pfeiffer, 2010; Chucholl *et al*., 2012), it is evident that the species survives and successfully reproduces in Central European climatic conditions. Its overwinterintg ability, with successful survival at 2 to 3°C for three months, was also confirmed experimentally (Veselý *et al*., 2015). As the marbled crayfish is widely available in the aquarium pet trade in Europe, this raises concerns of its further introductions (Chucholl, 2013, 2014; Patoka *et al*., 2014b).

However, even if no new marbled crayfish populations become established in near future in Slovakia or adjoining countries, the already known Slovak populations are an obvious threat, as they may serve as the foothold for the spread of this species in the Danube basin. Several North American invasive crayfish are known for their considerable capacity for active migration and colonization. For example, survival potential of a desiccation for up to several hours has been documented for the congeneric red swamp crayfish *Procambarus clarkii* as well as for the signal crayfish *Pacifastacus leniusculus* (Banha and Anastácio, 2014). This may promote crayfish passive dispersal over long distances, but also allows crossing of terrestrial barriers to new suitable habitats. Active overland dispersal has been recently documented for the spiny-cheek crayfish *Orconectes limosus* (Puky, 2014), and living marbled crayfish have been also observed out of water, over 100 m from a lake (Chucholl *et al*., 2012). Moreover, water birds may possibly serve as the translocation vectors for crayfish. Small juveniles of the red swamp crayfish were reported to climb to mallard feet, remain there for several minutes and survive on air for up to three hours (Águas *et al*., 2014).

If the marbled crayfish manages to successfully colonize rivers in the Danube basin (which seems likely as the population in the thermal stream in Opatovce is not separated from the Nitra River by any barrier, and the other populations are in a close vicinity of the Váh River and its side channel), the species' relatively fast dispersal can be expected unless restricted by environmental factors. The colonization potential of invasive crayfish can be dramatic, spreading downstream and even upstream in a considerable speed (Bubb *et al*., 2004). A good example is the colonization of the Danube River by the spiny-cheek crayfish in Hungary and adjoining countries (Puky and Shád, 2006; Pârvulescu *et al*., 2012; Lipták and Vitázková, 2014). The expansion of marbled crayfish may be further enhanced by passive dispersal along the rivers, in particular downstream by currents and floods. Single individuals of sexually reproducing crayfish invaders, when dispersing over long distances, are highly unlikely to establish a population unless a mated mature female or female with a clutch survives the translocation (note, however, that it remains unclear under which conditions facultative asexual reproduction, reported for spiny-cheek crayfish, takes place; Buřič *et al*., 2011). However, due to the obligate asexual reproduction of the marbled crayfish, this taxon is not limited by the Allee effect at very low population densities, and even dispersal of juvenile individuals may allow their subsequent reproduction in newly colonized sites. Floods (such as those occurring in the Váh basin in 2006 and 2010) may thus not only allow the crayfish to spread from the gravel pits to the river system, but also facilitate their rapid downstream dispersal.

Thermal streams, both fed by natural warm springs and those thermally polluted by human activities (*i.e.*, cooling water from industry), represent a specific category of habitats that may support introductions of ornamental aquatic species in temperate regions (Emde *et al*., 2016). Many of such species are elsewhere limited by the low water temperatures and are unable to proliferate outside the thermal streams; however, some of them tolerate a wide range of temperatures and may disperse successfully out of these sites of introduction. Numerous cases of establishment of aquarium crustaceans, in particular crayfish, have been documented in such habitats across Europe. The red swamp crayfish in a thermal stream in Austria (Petutschnig *et al.*, 2008) and the tropical redclaw crayfish, *Cherax quadricarinatus*, in an oxbow lake in Slovenia (Jaklič and Vrezec, 2011) seem so far restricted to thermal waters. In Germany, establishment of two

aquarium shrimp species, one of which may tolerate also lower temperatures, has been documented in a stream fed by cooling water from a coal power plant (Klotz *et al*., 2013). In case of the marbled crayfish in a thermal stream in Slovakia, the temperature does not seem a limiting factor (as apparent from the other established marbled crayfish populations in Central Europe; Chucholl *et al*., 2012). Furthermore, the relatively fast current of the thermal stream can facilitate crayfish movement into the Nitra River.

Juveniles observed in autumn, and the presence of medium-sized individuals in our samples (Fig. 2), indicate at least two seasonal clutches of the marbled crayfish in studied sites. It is estimated that under the laboratory conditions, the marbled crayfish can complete up to seven reproduction cycles during its lifespan of 2 to 3 years, and the generation time is about 6-7 months (Vogt, 2010). The amount of juveniles increases with each cycle in relation to size increase of the maternal individuals (Vogt, 2011), and may reach very high values for large females. Under laboratory conditions, Vogt (2011) reported the maximum number of 427 juveniles in one clutch. Some field-collected individuals were nevertheless even more fecund: one female from Madagascar studied by Jones *et al.* (2009) carried approximately 530 eggs (see Fig. 2 in Jones *et al*., 2009), and Chucholl and Pfeiffer (2000) reported as many as 724 eggs in a single marbled crayfish clutch from a German population. Thus, 455 juveniles carried by one marbled crayfish from the Koplotovce site do not seem to be exceptional, even under Central European conditions, and this number confirms a substantial reproduction potential of this invasive species.

The ability of the marbled crayfish to act as an *A. astaci* vector deserves considerable attention as well. Although no *A. astaci* infection was detected in our study, a complete absence of the pathogen cannot be ascertained. Infected marbled crayfish have been already confirmed in the aquarium pet trade, laboratory cultures, as well as in the wild (Keller *et al*., 2014; Mrugała *et al*., 2015), and genotyping of the pathogen suggested that the species got infected by horizontal transmission from another species (Mrugała *et al*., 2015). If the marbled crayfish successfully colonizes the Danube, it is thus likely that it will acquire the infection from the spiny-cheek crayfish, confirmed to carry the crayfish plague pathogen in this river (Kozubíková *et al*., 2010; Pârvulescu *et al*., 2012). Due to the marbled crayfish potential to rapidly expand its range, it is possible that it might spread the infection also into habitats that the other American species has not reached yet.

CONCLUSIONS

The presence and potential spread of the marbled crayfish in Slovak freshwaters represents a threat not only to the native astacofauna but potentially also to other aquatic biota. Fast growth, early maturation, high fecundity and parthenogenetic reproduction strategy combined with a capacity for competition with other crayfish species (Jimenez and Faulkes, 2010) and an ability to spread crayfish plague pathogen (Keller *et al*., 2014; Mrugała *et al*., 2015), characterize a very successful invader. Given the fact that the species is widely available in the aquarium trade and already introduced to several locations in Europe, a management aiming to prevent further expansion is crucial.

This situation increases the pressure on local public and environmental agencies to promote adequate preventive actions, as the lack of proper education may promote translocations and introduction of the crayfish to new waterbodies, and thus contribute substantially to the marbled crayfish further colonization of the Danube catchment. The socioeconomic drivers increase the likelihood of species introductions, particularly in areas with high gross domestic product and high human population density (Perdikaris *et al*., 2012; Chucholl, 2014), such as the Vienna-Bratislava region and nearby Budapest metropolitan area in Hungary. Thus, our findings of established marbled crayfish might not be the last from this region. We believe that public education focusing on the mechanisms and consequences of crayfish spread, along with the development of more intensive regulation of ornamental trade, should constitute a basis of any management action. Furthermore, it should be supported by a further research evaluating marbled crayfish impacts on the native communities and habitats, and eventually, a development of the effective elimination means of alien crayfish from the natural environments.

ACKNOWLEDGMENTS

The Czech Science Foundation supported this work through project P505/12/0545. Partial funding was provided by the Ministry of Education, Youth, and Sports of the Czech Republic (projects CENAKVA – CZ.1.05/2.1.00/ 01.0024 and CENAKVA II – LO1205 under the NPU I program) and the Grant Agency of the University of South Bohemia (012/2016/Z). A.M. was supported by the Charles University in Prague (project SVV 260312). We thank local fishermen and Samuel Baranovič for the help in collecting the crayfish. Two anonymous referees provided comments on the previous version of the manuscript.

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– chapter 4 –

Mrugała A, Kozubíková-Balcarová E, Chucholl C, Cabanillas Resino S, Viljamaa-Dirks S, Vukić J, Petrusek A (2015) Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: crayfish plague and white spot syndrome. *Biological Invasions* 17: 1313-1326

Biol Invasions (2015) 17:1313–1326
DOI 10.1007/s10530-014-0795-x

ORIGINAL PAPER

Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: crayfish plague and white spot syndrome

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Received: 16 April 2014 / Accepted: 13 October 2014 / Published online: 19 October 2014
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Abstract Rapidly growing trade of ornamental animals may represent an entry pathway for emerging pathogens; this may concern freshwater crayfish that are increasingly popular pets. Infected crayfish and contaminated water from aquaria may be released to open waters, thus endangering native crustacean fauna. We tested whether various non-European crayfish species available in the pet trade in Germany and the Czech Republic are carriers of two significant crustacean pathogens, the crayfish plague agent *Aphanomyces astaci* and the white spot syndrome virus (WSSV). The former infects primarily freshwater crayfish (causing substantial losses in native European species), the latter is particularly known for economic losses in shrimp aquacultures. We screened 242 individuals of 19 North American and Australasian crayfish taxa (the identity of which was validated by DNA barcoding) for these pathogens, using molecular methods recommended by the World Organisation for Animal Health. *A. astaci* DNA was detected in eight American and one Australian crayfish species, comprising in total 27 % of screened batches. Furthermore, viability of *A. astaci* was confirmed by its isolation to axenic cultures from three host taxa, including the parthenogenetic invader Marmorkrebs (*Procambarus fallax* f. *virginalis*). In contrast, WSSV was only confirmed in three individuals of Australian *Cherax quadricarinatus*. Despite modest prevalence of detected infections, our results demonstrate the potential of disease entry and spread through this pathway, and should be considered if any trade regulations are imposed. Our study highlights the need for screening for pathogens in the ornamental trade as one of the steps to prevent the transmission of emerging diseases to wildlife.

Keywords Aquarium trade · Exotic pathogens · *Aphanomyces astaci* · White spot syndrome virus · Marmorkrebs · DNA barcoding

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Introduction

The impacts of infectious diseases on wildlife and ecosystem health have become a critical conservation issue. In particular, newly emerging diseases may lead to elimination or substantial reduction of host populations, which may result in local species extinctions

(Smith et al. 2006) and unpredictable secondary impacts (Smith et al. 2009). The emergence of novel pathogens is associated with three main factors: increase in pathogenicity via genetic change, natural range expansions of infected species, and human-mediated movements of organisms (Cunningham 2002; Gozlan et al. 2006). Anthropogenic movements of biota often lead to the establishment of non-native species carrying exotic pathogens in new areas. As observed worldwide, including Europe, global trade is one of the major vectors of new species introductions (DAISIE 2009; Hulme 2009), and hence a potential entry pathway of exotic diseases.

Aquatic environments are particularly vulnerable to the spread of non-native species and exotic diseases. From the multitude of pathways connected with global trade, commercial trade appears to be responsible for the majority of non-native species introductions into aquatic environments (Bartley and Subasinghe 1996; Hulme et al. 2008; Tricarico 2012). Although there are many reports available on emerging diseases connected with aquaculture activities (Gozlan et al. 2006; Peeler et al. 2011; Rodgers et al. 2011), only a few examples are known of pathogen spread through the trade of live organisms for direct consumption, ornamental purposes, and research (e.g., Kim et al. 2002; McColl et al. 2004). One of the most striking cases is undoubtedly the introduction of the chytrid fungus *Batrachochytrium dendrobatidis*, a pathogenic agent responsible for the global decline of amphibians and species extinctions, which seems to have spread, among other pathways, through the trade of experimental and ornamental amphibians (Fisher and Garner 2007).

Freshwater crayfish (Crustacea, Decapoda, Astacidea) are another group of aquatic animals endangered in some regions of the world by related invasive species and diseases spread by them (Holdich et al. 2009). Some of the non-native crayfish established at present in European waters have apparently been introduced via the aquarium trade (Ahern et al. 2008; Chucholl et al. 2012; Kouba et al. 2014), which seems nowadays the most likely entry pathway of new crayfish introductions in Europe (Peay 2009). Furthermore, many other crayfish species that have not yet been found to be established in Europe are widely available for sale (Chucholl 2013). Yet, it remains unclear to what extent the crayfish available in the aquarium trade may contribute to the spread of important crustacean diseases. In our study, we focused on the presence of pathogens responsible for crayfish plague (a disease of high conservational relevance in Europe), and the white spot syndrome (a disease of substantial economic importance). Both of these are listed diseases by the World Organisation of Animal Health (OIE 2012).

The crayfish plague pathogen, *Aphanomyces astaci* Schikora (Oomycetes), has seriously affected native European crayfish since the second half of the nineteenth century, causing dramatic declines or losses of entire populations (Alderman 1996; Holdich et al. 2009). Unlike in European crayfish, the infection does not normally cause death in North American species (Söderhäll and Cerenius 1999), which act as its vectors. It has been assumed that North American crayfish in general are potential vectors of this disease agent (Oidtmann 2012) but direct evidence for this statement is still meagre, as only a few species were tested for the presence of this pathogen. Five North American crayfish species (out of eight established in Europe) have been confirmed as *A. astaci* carriers in European open waters so far (Holdich et al. 2009; Filipová et al. 2013; Schrimpf et al. 2013; Tilmans et al. 2014): *Pacifastacus leniusculus* (Dana 1852), *Orconectes limosus* (Rafinesque 1817), *Procambarus clarkii* (Girard 1852), *O. immunis* (Hagen 1870) and *O.* cf. *virilis*. Furthermore, Culas (2003, p. 16) reported detection of *A. astaci* in two individuals of marbled crayfish, *Procambarus fallax* (Hagen 1870) f. *virginalis*, unfortunately without any details of their origin. A substantial variation in *A. astaci* prevalence has been documented among taxa and populations of alien crayfish in various European countries (e.g., Kozubíková et al. 2011; Filipová et al. 2013), and no data exist on *A. astaci* infection status or prevalence from the native range of American crayfish.

In contrast to the crayfish plague pathogen with narrow host specificity (but see Schrimpf et al. 2014; Svoboda et al. 2014a, b), white spot syndrome virus has been reported from a wide range of crustacean hosts (Stentiford et al. 2009). It is particularly important due to significant economic losses in shrimp aquacultures worldwide (Flegel 2009; Lightner 2011). Experimental transmission also demonstrated its ability to infect and kill crayfish species (Edgerton 2004; Jiravanichpaisal et al. 2004), and hence crayfish may become vectors of this virus. As only scarce records of

its presence in wild crayfish populations are available (Baumgartner et al. 2009), it is not clear whether it may cause disease outbreaks in native European crayfish. Nevertheless, virus detection in *P. clarkii* illegally imported to the UK for sale from Singapore (Longshaw et al. 2012) indicates that crayfish trade may be one pathway of its spread.

In this study, we screened for potential carriers of the crayfish plague pathogen and white spot syndrome virus in a large collection of crayfish obtained from pet shops and breeders, using the molecular methods recommended by the World Organisation for Animal Health (OIE 2012). Furthermore, we also tested the viability of detected *A. astaci* by isolation to laboratory cultures. As we focused on the European ornamental trade, the chosen sources are considered the most representative for this particular region. Screening of crayfish species from other potential associated sources, such as airport quarantines, direct imports, and bait releases was omitted due to their low relevance and/or hampered access to such sources.

Our study focused on two Central European countries, Germany and the Czech Republic. Germany represents a large aquarium trade market with around 120 non-European crayfish available for sale, and is most likely the main importer of non-native crayfish to Europe (Chucholl 2013). Furthermore, German online shops readily deliver purchased crayfish to other countries within the European Union. Therefore, the results from this country should reflect the general state of crayfish infestations in the European pet trade. Moreover, the availability of numerous species in the German pet trade allows screening of a broad range of crayfish circulating in Europe. The Czech crayfish pet trade, though with a relatively short tradition, is nowadays on the increase and the Czech Republic may be regarded as a second leading European country in the crayfish ornamental trade (Patoka et al. 2014); thus it represents a potential future situation in other European countries.

Information on whether crayfish available in the pet trade act as vectors of important crustacean diseases is essential for risk assessment and should be considered if any trade regulations are imposed. In particular, the trade of ornamental crustaceans (specifically crayfish) should be maintained without posing unnecessary risks of pathogen transmission to endangered native European species.

Materials and methods

Crayfish samples

The crayfish were obtained from various sources in Germany and the Czech Republic within the period 2010–2013. The sources included four private on-line aquarium shops in Germany, one German hobby breeder, two private aquarium store shops in the Czech Republic, one Czech breeder, and one direct import from Singapore (Table 1). During the study, two German shops (shop DE 2 and shop DE 3) merged and are labelled thereafter as shop DE 3. Crayfish stocks in those two shops were, to our knowledge, of the same origin. In total, 242 crayfish individuals were purchased in 41 batches (one batch being a group of individuals of one taxon from one retailer obtained at the same time) covering 19 taxa (three Australasian and 16 North American). If available, *Cherax* spp. from Asia and Australia, two regions where *A. astaci* is not known to occur naturally (Unestam 1975), were purchased together with North American species from the same shops. We expected them not to be infected, as species of this genus are considered sensitive to crayfish plague (Unestam 1975) and individuals with signs of the disease should be removed by the sellers. After delivery, crayfish were stored separately in plastic bags at −80 °C.

DNA extraction

Prior to dissection, each crayfish was examined for external conditions; any presence of melanized spots on the body that may indicate immune reaction to pathogens was noted. The total body length (from the tip of the rostrum to the end of the telson) of each crayfish was measured and the sex determined. We dissected whole soft abdominal cuticle, halves of two uropods and telson, two proximal joints of walking legs (in individuals smaller than 2 cm all basal joints and the whole tail fan), and melanized spots (if present). Such mixed-tissue samples were stored in 96 % ethanol. We extracted the DNA using the DNeasy tissue kit (Qiagen) from up to 50 mg of the tissue ground in liquid nitrogen.

DNA barcoding of crayfish

As the purchased individuals were often juveniles or females, and thus lacked characteristics necessary for

Table 1 List of analysed batches of crayfish obtained from the aquarium trade, results of their identification by DNA barcoding, and molecular detection of *Aphanomyces astaci* and white spot syndrome virus

Shop	Assigned species name	Original label (if different)	Average size (mm)	Size range (mm)	Indiv. tested	*A. astaci* detected	Agent levels				WSSV detected
							A2	A3	A4	A5	
Shop CZ 1	*Cambarellus* sp.	*Cambarellus texanus*	19.7	17–27	10	–					–
	Cambarellus patzcuarensis		19.7	17–22	10	**1**		1			–
	Procambarus clarkii		52.4	49–57	9	–					–
	Procambarus fallax–Marmorkrebs		60.7	51–80	10	–					–
Shop CZ 2	*Cambarellus shufeldtii*	*Cambarellus patzcuarensis*	19.3	16–24	10	–					–
Breeder CZ 3	*Cambarellus* sp.	*Cambarellus texanus*	21.9	16–27	10	–					–
	Cambarellus shufeldtii		10.9	9–12	15	–					–
	Procambarus alleni		32.4	28–35	5	–					–
	Procambarus fallax–Marmorkrebs		22.2	20–24	6	–					–
Import CZ 4	*Procambarus alleni*		60.8	55–68	9	–					–
Shop DE 1	*Cherax quadricarinatus*		50.6	48–52	5	–					–
	Cambarellus patzcuarensis		15.0	15	5	–					–
	Orconectes limosus		62.0	62	1	**1**	1				–
	Procambarus alleni		38.6	31–44	5	**3**		3			–
	Procambarus fallax–Marmorkrebs		40.0	33–45	5	–					–
Shop DE 2	***Cherax quadricarinatus***		82.7	80–84	3	–					**1**
	Cambarellus patzcuarensis	Marmorkrebs	37.6	29–48	5	–					–
	Cambarellus patzcuarensis		17.7	16–20	6	–					–
	Cambarellus shufeldtii		15.3	14–16	4	–					–
	Procambarus clarkii		69.4	62–77	5	–					–
	Procambarus alleni	*Procambarus fallax*	29.5	26–33	6	–					–
Shop DE 3	*Cherax lorentzi*		41.6	40–43	5	–					–
	Cherax quadricarinatus		70.7	59–80	10	**1**		1			2
	Procambarus alleni[a]		56.1	39–70	11	**5**		3	2		–
	Procambarus clarkii[a]		46.0	41–49	5	**5**	3	2			–
	"*Procambarus enoplosternum*"[b]		49.0	37–60	5	**1**	1				–
	"*Procambarus enoplosternum*"[c]		43.5	33–47	6	**6**	1	5			–
	***Procambarus* fallax–Marmorkrebs**[a]		29.4	21–40	14	**2**	1	1			–
	Procambarus* cf. *llamasi	*Procambarus llamasi*	55.0	48–65	3	**3**		2	1		–
	"*Procambarus vazquezae*"		29.0	25–33	5	**5**	1	2	1	1	–

Table 1 continued

Shop	Assigned species name	Original label (if different)	Average size (mm)	Size range (mm)	Indiv. tested	*A. astaci* detected	Agent levels				WSSV detected
							A2	A3	A4	A5	
Shop DE 4	*Cherax holthuisi*		38.8	30–47	5	–					–
	Procambarus alleni		67.0	67	2	–					–
	Procambarus fallax–Marmorkrebs		32.0	32	1	–					–
	"*Procambarus vazquezae*"		33.0	30–36	3	–					–
Breeder DE 5	*Orconectes harrisoni*		21.5	21–22	2	–					–
	"*Orconectes leptogonopodus*"		36.0	35–37	2	–					–
	Orconectes tricuspis	*Orconectes margorectus*	27.0	27	2	–					–
	Orconectes palmeri–longimanus		38.0	35–41	2	–					–
	Orconectes cf. *virilis*	*Orconectes punctimanus*	34.5	34–35	2	–					–
	Orconectes rusticus	*Orconectes* cf. *rusticus*	44.0	43–45	2	–					–
	Procambarus cf. *llamasi*	*Procambarus llamasi*	26.9	24–32	10	–					–

For detections of *A. astaci*, semi-quantitative agent levels are provided. Crayfish names enclosed in quotation marks indicate taxa of which identification could not be verified by DNA barcoding; "cf." indicates presumed members of species complexes (see "Materials and methods"). Batches in which *A. astaci* or WSSV were detected are marked in bold

[a] These species from the shop DE 3 were used also for cultivation of *A. astaci*

[b] First batch of *P. enoplosternum*

[c] Second batch of *P. enoplosternum*

reliable species identification (often present on sexually mature males only), we relied on molecular tools to check for the correct labelling of examined crayfish species. From most batches of American crayfish, we amplified from one individual a 658 bp long fragment of the mitochondrial gene for the cytochrome c oxidase subunit I (COI), as is recommended for DNA barcoding of animals (Hebert et al. 2003). Polymerase chain reactions (PCR) were performed in a total volume of 25 µl containing: 2 µl of DNA template, 0.4 µM primers HCO 2198 and LCO 1490 (Folmer et al. 1994), 1× PCR Buffer (Promega), 2.2 mM $MgCl_2$, 0.15 mM dNTPs, 1 U of DNA Taq polymerase (Promega). The reaction PCR thermal protocol included: an initial denaturation at 94 °C for 2:30 min, 35 cycles of denaturing at 94 °C for 45 s, annealing at 48 °C for 1 min and an extension at 72 °C for 1 min, followed by the final extension at 72 °C for 10 min. The PCR products were sequenced using the forward primer, and compared with publicly available COI sequences.

The COI amplification repeatedly failed for two crayfish batches (*Orconectes rusticus* from breeder DE 5 and *Procambarus alleni* from shop DE 1); for these, we sequenced another widely studied mitochondrial marker, the gene for the 16S rRNA, for the purpose of DNA-based identification. The reactions were performed in a total volume of 25 µl containing: 6 µl of DNA template, 0.2 µM primers AR and BR (Palumbi et al. 1991), 1× PCR Buffer (Promega), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 U of DNA Taq polymerase (Promega). The PCR protocol included: an initial denaturation at 90 °C for 2:30 min, 10 cycles of 92 °C for 50 s, 48 °C for 30 s, 72 °C for 40 s, and 30 cycles of 92 °C for 30 s, 48 °C for 40 s, 72 °C for 40 s, followed by the final extension at 72 °C for 3 min.

We compared the obtained sequences with those available for relevant crayfish species. To check identification, two complementary strategies were applied. We downloaded already available sequences representing the taxa presumably sold by the retailers, and we also used the BLAST search to find the most similar sequences to those obtained from studied crayfish. If sequences unambiguously corresponding to the respective species (i.e., matching completely or diverging by no more than 1 %) were obtained, we considered the identification confirmed. If obtained sequences corresponded to another species than originally labelled, we used a corrected name based on GenBank records originating from taxonomically reliable studies (Taylor and Knouft 2006; Filipová et al. 2010; Pedraza-Lara et al. 2012). In such cases, the original as well as the corrected names are provided in Table 1. Taxa apparently belonging to species complexes that require systematic reassessment are indicated by "cf.", and for species where DNA-based identification was ambiguous, we use only the genus name. Finally, to indicate that the correct identification could not be confirmed, we enclose in quotation marks names of taxa for which reference sequences were unavailable but for which the above-mentioned approach did not clearly suggest misidentification (as no closely matching sequence was found in GenBank). The results of the COI-based identification were visually summarized in a Neighbour-Joining tree constructed in Mega 5.2 (Tamura et al. 2011), based on an alignment including sequences obtained in our study, and reference sequences representing all relevant taxa and most closely matching sequences for taxa that could not be identified reliably. Sequences representing newly sequenced taxa or substantially diverging from those publicly available were deposited to GenBank (KF944431–KF944435).

Molecular detection of *Aphanomyces astaci*

The assessment of *A. astaci* presence was performed with the TaqMan minor groove binder quantitative PCR (qPCR) according to Vrålstad et al. (2009), with minor changes from the original protocol to reduce likelihood of false positive results. These included an increase of the annealing temperature (from 58 to 62 °C), and decrease of the annealing time (from 60 to 30 s) (as in Svoboda et al. 2014b). The relative levels of infection were calculated based on the strength of the PCR signal and assigned to semi-quantitative agent levels (according to Vrålstad et al. 2009; Kozubíková et al. 2011). Individuals with agent levels A2 and higher were considered infected with *A. astaci*. We included negative controls in every step of the process to check for contamination; these remained negative in all cases.

The presence of *A. astaci* DNA in representative samples that yielded positive qPCR results was confirmed by the sequencing of 569 bp long amplicons including parts of internal transcribed spacers

(ITS) 1 and 2 and the 5.8S rDNA according to Oidtmann et al. (2006), as recommended by the World Organisation for Animal Health (OIE 2012). Purified PCR products of DNA isolates were sequenced in both directions using BigDye v. 3.1 Terminator kit on the ABi 3130xl Genetic Analyser (Applied Biosystems). Sequences were compared to publicly available sequences of *A. astaci*, and the representative ones were deposited to GenBank (KF944436–KF944439).

Isolation of *Aphanomyces astaci* to axenic cultures

Furthermore, additional individuals of three crayfish species (*P. alleni*, *P. clarkii* and Marmorkrebs), which were found to be infected with *A. astaci* in the shop DE 3, were purchased from this shop (14, 12, and 18 specimens of the mentioned species, respectively) and delivered alive to the OIE reference laboratory in Finland for isolation of the crayfish plague agent. Crayfish were kept in the laboratory in separate tanks containing about 10 l mixture of tap water and UV-treated lake water, plastic pipes for shelter, and an aeration system to prevent oxygen depletion. They were fed daily with peas or carrot, and the water in the tanks was changed regularly. Once found dead, the crayfish were examined for melanized areas in the exoskeleton appropriate for inoculation. The isolation of the crayfish plague agent was performed as described by Viljamaa-Dirks and Heinikainen (2006). In case of moulting, exuviae were also used for isolation. Inoculated plates were inspected daily and unseptate oomycete hyphae with branches 5–10 μm wide were collected for further study. Isolates were confirmed as *A. astaci* by amplification of a 569 bp long fragment in the ITS region with primers 42 and 640 as described in Oidtmann et al. (2006).

White spot syndrome virus detection

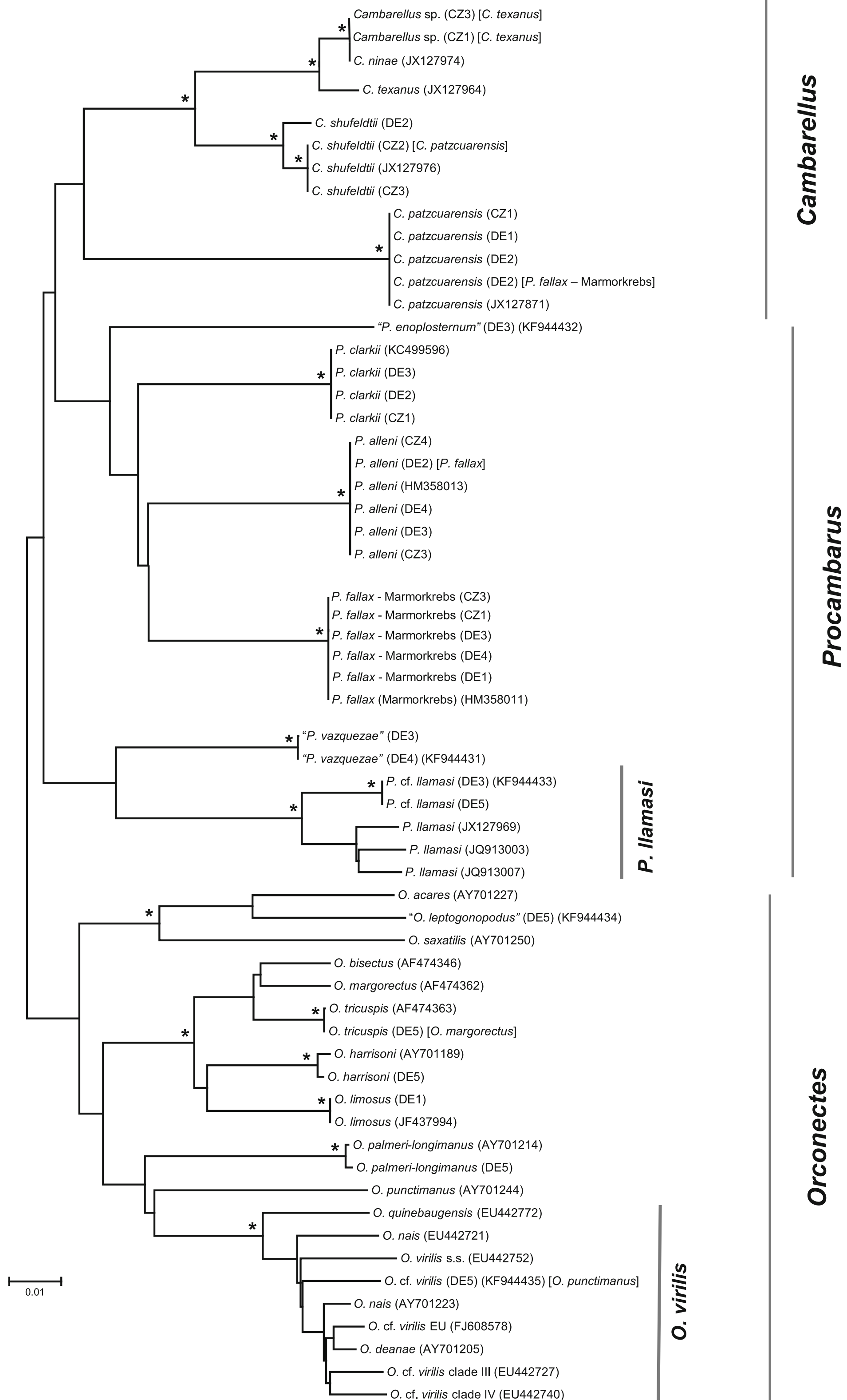
The same DNA isolates used for the DNA barcoding and *A. astaci* detection were used for detection of the white spot syndrome virus, as the virus infects, among other tissues, also the cells of cuticular epidermis (Wang et al. 1997; OIE 2012). We applied a nested PCR by Lo et al. (1996) to detect the viral DNA. Both reactions were performed in a total volume of 25 μl containing in both cases: 12.5 μl ReddyMix Master-Mix (Thermo Scientific) and primers for amplification of the viral DNA (final concentration 100 μM). In the first reaction, we used primers for the viral DNA 146F1 and 146R1 amplifying a 1,447 bp long DNA fragment and in the second (nested) PCR primers 146F2 and 146R2 producing a 941 bp DNA fragment (Lo et al. 1996). We added 5 μl of the DNA isolate to the first reaction and 1 μl of the product of the first PCR to the second reaction. The PCR protocol included: an initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and the final extension step at 72 °C for 2 min. PCR products were visualized in 50 ml 2 % agarose gels with 1.0 μl of GelRed stain (Biotium, USA). As a positive control we used DNA isolated from shrimp pleopods infected with WSSV, obtained from the Weymouth laboratory of the Centre for Environment, Fisheries and Aquaculture Science (Cefas), UK. Bands of the required length were considered as positive results of the reaction. The amplification was subsequently followed by the DNA sequencing to confirm the presence of the specific viral DNA. The product of the second PCR was purified and sequenced in the forward direction (see above). The obtained sequences were compared to the publicly available sequences of the WSSV DNA. A representative sequence was afterwards deposited to GenBank (Acc. No. KF981443).

Results

DNA barcoding of crayfish

The DNA barcoding revealed several inconsistencies between shop labelling and species identity (Table 1; Fig. 1). Apparently incorrect names were mostly associated either with juvenile individuals or species with relatively small body sizes and rare in the aquarium trade. In particular, dwarf crayfish of the genus *Cambarellus* were apparently misidentified (e.g., *C. shufeldtii* sold as *C. patzcuarensis*) or could not be reliably assigned to species based on COI sequences. In one case, crayfish species sold under different names (*C. patzcuarensis* and Marmorkrebs) by shop DE 2 belonged in fact to the same taxon (*C. patzcuarensis*).

The sequence from a specimen sold under the name *Orconectes punctimanus* did not match a GenBank record of that species from Taylor and Knouft (2006); rather, it was close to various members of the *O. virilis* complex (Filipová et al. 2010). Identical sequences


Cambarellus sp. (CZ3) [C. texanus]
Cambarellus sp. (CZ1) [C. texanus]
C. ninae (JX127974)
C. texanus (JX127964)
C. shufeldtii (DE2)
C. shufeldtii (CZ2) [C. patzcuarensis]
C. shufeldtii (JX127976)
C. shufeldtii (CZ3)
C. patzcuarensis (CZ1)
C. patzcuarensis (DE1)
C. patzcuarensis (DE2)
C. patzcuarensis (DE2) [P. fallax – Marmorkrebs]
C. patzcuarensis (JX127871)
"P. enoplosternum" (DE3) (KF944432)
P. clarkii (KC499596)
P. clarkii (DE3)
P. clarkii (DE2)
P. clarkii (CZ1)
P. alleni (CZ4)
P. alleni (DE2) [P. fallax]
P. alleni (HM358013)
P. alleni (DE4)
P. alleni (DE3)
P. alleni (CZ3)
P. fallax - Marmorkrebs (CZ3)
P. fallax - Marmorkrebs (CZ1)
P. fallax - Marmorkrebs (DE3)
P. fallax - Marmorkrebs (DE4)
P. fallax - Marmorkrebs (DE1)
P. fallax (Marmorkrebs) (HM358011)
"P. vazquezae" (DE3)
"P. vazquezae" (DE4) (KF944431)
P. cf. llamasi (DE3) (KF944433)
P. cf. llamasi (DE5)
P. llamasi (JX127969)
P. llamasi (JQ913003)
P. llamasi (JQ913007)
O. acares (AY701227)
"O. leptogonopodus" (DE5) (KF944434)
O. saxatilis (AY701250)
O. bisectus (AF474346)
O. margorectus (AF474362)
O. tricuspis (AF474363)
O. tricuspis (DE5) [O. margorectus]
O. harrisoni (AY701189)
O. harrisoni (DE5)
O. limosus (DE1)
O. limosus (JF437994)
O. palmeri-longimanus (AY701214)
O. palmeri-longimanus (DE5)
O. punctimanus (AY701244)
O. quinebaugensis (EU442772)
O. nais (EU442721)
O. virilis s.s. (EU442752)
O. cf. virilis (DE5) (KF944435) [O. punctimanus]
O. nais (AY701223)
O. cf. virilis EU (FJ608578)
O. deanae (AY701205)
O. cf. virilis clade III (EU442727)
O. cf. virilis clade IV (EU442740)
0.01
Cambarellus
Procambarus
P. llamasi
Orconectes
O. virilis

◀**Fig. 1** DNA barcoding of studied aquarium crayfish. A neighbour-joining tree was created from a 605 bp long alignment of a part of the mitochondrial gene for the cytochrome c oxidase subunit I (COI). COI sequences were obtained from one individual representing each analysed crayfish batch, and compared with publicly available reference sequences (see "Materials and methods" for details). Codes of sources from where the crayfish were purchased and GenBank accession numbers of reference sequences are listed in parentheses. Apparently incorrect or ambiguous original labels are presented in *square brackets*. Species names in quotation marks highlight these species for which COI sequences were absent in GenBank. *Vertical lines* indicate genera and species complexes; taxa of the *O. virilis* complex were chosen and labelled according to Filipová et al. (2010). Asterisks indicate nodes with 99–100 % bootstrap support from 1,000 replications

from two batches delivered as *Procambarus llamasi* were both distinct from those labelled as *P. llamasi* in GenBank (diverging by 3 % and more at COI). As these sequences formed three distinct (though related) clades, it is possible that *P. llamasi* is actually a cryptic species complex, and we consequently labelled the specimens from the aquarium trade as *P.* cf. *llamasi*.

Detection of *Aphanomyces astaci*

Eight North American and one Australian crayfish species tested positive for *A. astaci*, comprising in total 27 % of the screened batches, originating from three different shops (Table 1). The isolates positive for *A. astaci* DNA mostly reached low (A2) to moderate (A4) agent levels, with one individual (*P. vazquezae*) revealing an even higher infection level (A5). *Cherax* spp. tested negative for *A. astaci* presence in all but one individual of *C. quadricarinatus* (A3), which originated from the same shop as most of the infected North American species (Table 1). All but two of the infected North American crayfish species (*C. patzcuarensis* and *O. limosus*) belonged to the genus *Procambarus*.

In two of the screened shops (DE 1 and CZ 1), *A. astaci* infections were low and seldom detected, in comparison to the very high prevalence of *A. astaci* in the shop DE 3. From this source, *A. astaci*-positive individuals were detected in all of the tested North American crayfish species and one of two *Cherax* spp., although the agent levels and number of infected crayfish varied.

We obtained ITS sequences from 6 out of 9 *A. astaci*-positive batches. These were all identical to the *A. astaci* reference sequences available in GenBank. From the three remaining batches (*Cambarellus patzcuarensis*, *C. quadricarinatus* and Marmorkrebs) with low levels of *A. astaci* infections, no ITS sequences were obtained due to a lower sensitivity of the standard PCR approach in comparison to the qPCR detection method (Kozubíková et al. 2011). Forty-seven individuals examined for *A. astaci* presence showed signs of melanisation but only in six of these specimens did we detect the pathogen DNA.

Laboratory cultures of *A. astaci* were obtained from all of the three crayfish species (*P. alleni*, *P. clarkii* and Marmorkrebs) ordered for this purpose from the shop DE 3. Culture identification was confirmed for all three host species by the PCR approach, corroborating that these crayfish batches were all infected with a viable crayfish plague pathogen.

White spot syndrome virus detection

The WSSV was only detected in three individuals of *Cherax quadricarinatus* from shops DE 2 and DE 3 (Table 1), which likely supplied animals of the same origin (see "Materials and methods"). Analysis of the first purchase of three individuals of *C. quadricarinatus* from shop DE 2 revealed the presence of the virus in one crayfish. Out of ten *C. quadricarinatus* purchased afterwards from the same source (shop DE 3 after the merger), the infection was detected in two more individuals. Sequencing of the amplified virus DNA fragments confirmed the WSSV detection.

Discussion

Our study detecting the presence of two ecologically and economically significant crustacean pathogens in the European ornamental crayfish trade confirms that the aquarium trade may contribute to the transmission of these (and likely other) important infectious diseases (see also Longshaw et al. 2012), and is the first study to demonstrate this entry pathway for the crayfish plague pathogen. As aquarium crayfish seem to be frequently released into the natural environment in Germany (the main importer of non-indigenous crayfish to Europe; Chucholl 2013) as well as in other Central European countries (e.g., Janský and Mutkovič 2010; Chucholl et al. 2012; Gross 2013), this pathway is relevant from the conservation point of view, particularly with respect to the potential

transmission of crayfish plague to native European crayfish species.

North American crayfish have evolved defence mechanisms against the growth of *A. astaci* mycelium in their cuticles (Cerenius et al. 2003), thus becoming carriers of latent infections. The detected agent levels ranging from low to moderate are typical for these carriers. With suppressed infections, North American crayfish act as reservoirs of the disease, repeatedly releasing spores to the water (Strand et al. 2012; Svoboda et al. 2013). Out of eight North American crayfish species found infected with *A. astaci* in our study, only two, *P. clarkii* and *O. limosus,* had been previously recognized as vectors of crayfish plague (Holdich et al. 2009). The remaining six species had not been documented to carry the pathogen before.

Particularly important is the confirmation of the carrier status of the Marmorkrebs, *P. fallax* f. *virginalis*, which has become extremely widespread in the aquarium trade and which seems the most frequently released aquarium crayfish species in Europe (Chucholl et al. 2012). Recently, the number of Marmokrebs records has increased dramatically: whereas in 2010 there was only one established population known, by 2012 fifteen new findings with at least six established populations were recorded (Chucholl et al. 2012), and additional ones are being reported from various European countries (see Kouba et al. 2014). Furthermore, niche-based species distribution models developed by Feria and Faulkes (2011) predict considerable parts of Europe as suitable for this species. Our results show that even this parthenogenetic crayfish that spreads through captive breeding only (it is not known from the original range of *P. fallax*) is a potential vector of an important disease.

Infections detected in aquarium crayfish may be of different origins. The detection of *A. astaci* in all but one crayfish species in batches from the shop DE 3 (including the infected Marmorkrebs and even one specimen of Australian *C. quadricarinatus*) suggests that most of these species were not the original hosts of the pathogen. In fact, the RAPD genotype identification of *A. astaci* isolated to axenic cultures (Viljamaa-Dirks, unpubl. results) revealed that all three species were infected with the same genotype group (indicated D or Pc, originally isolated from *Procambarus clarkii*; Diéguez-Uribeondo et al. 1995). This points at the possibility that aquarium crayfish might acquire an infection through horizontal transmission of the disease within shop facilities, for instance due to flow-through aquaria installations, contaminated equipment, or during handling and packing. In particular, the detection of *A. astaci* infection in one *Cherax* individual suggests that the pathogen might have been acquired in the shop facilities. However, it should not be excluded that some of the infected aquarium taxa might also be the original carriers of the infection. It will be of particular concern if aquarium crayfish carry novel strains of the crayfish plague pathogen, which may differ in properties such as increased virulence (Viljamaa-Dirks et al. 2013; Makkonen et al. 2014) or climate requirements (Diéquez-Uribeondo et al. 1995; Rezinciuc et al. 2014). Nonetheless, under both scenarios the ornamental trade poses a danger not only to stocks of non-American crayfish species within pet shops or home aquaria but also to the native crustacean fauna in the wild.

Various decapod crustacean species and amongst them also freshwater crayfish were observed to be susceptible to the WSSV (Bateman et al. 2012). Moreover, it was reported that at low water temperature, crayfish species might act as carriers of the virus, permitting further spread of the disease (Jiravanichpaisal et al. 2004). Both factors, the species-specific vulnerability to the virus and the temperature-related variation in virulence, may explain the observed low prevalence of WSSV infection in the pet trade. Only three crayfish individuals of *C. quadricarinatus* tested positive for WSSV in our study. This is in concordance with the low prevalence of this disease reported by Longshaw et al. (2012), who only revealed an infection in tested individuals of *P. clarkii* directly imported from Singapore. Nevertheless, despite its low prevalence, this finding is of particular importance, as the white spot syndrome virus is listed not only by the World Organisation for Animal Health (OIE) but also as a notifiable disease in the European Aquatic Animal Health Directive (2006/88).

Pathogens of aquatic animals may be transmitted from ornamental cultures to open waters in a twofold manner: (1) with discarded water, or (2) with infected animals either released from aquaria or escaping from garden ponds. The latter mechanism deserves particular consideration as the transmission may occur even in the absence of the successful establishment of a new host population; infected crayfish may transmit the pathogen to other suitable hosts present in the aquatic

environment. The likelihood of disease transmission may therefore depend not only on the potential invasiveness of the released host species but also on the frequency of release events. With increasing popularity of various animals (including crayfish) as pets, the likelihood of release and, thus, disease transmission into open waters, also increases (Peay 2009; Chucholl 2013). For instance, the propagation of crayfish as pets in Germany since the late 1990s has been followed by many independent release events, which ultimately led to establishment of Marmorkrebs and *P. clarkii* populations in the wild (Dümpelmann et al. 2009; Chucholl et al. 2012). A specimen of *P. alleni*, recently found in the river Rhine (Gross 2013), was also most likely of aquarium origin. All of these species tested positive for *A. astaci* presence in our study, and were assessed as high-risk species regarding their potential invasiveness, based on their size, biological characteristics, and availability in the pet trade (Chucholl 2013; Patoka et al. 2014).

Orconectes limosus, despite its rare availability in the aquarium trade, is another species posing substantial risk. It is widely established in Europe (Holdich et al. 2009) and is frequently infected by *A. astaci* in Central European countries (Kozubíková et al. 2011). Most likely captured for the pet trade from the natural environment, this species may be sold already infected. Moreover, it was reported as being sold for stocking garden ponds (Chucholl 2013), from where the crayfish escape and disease spread is just a matter of time.

Not every released crayfish species is likely to establish a population and become invasive. For instance, *C. patzcuarensis, P. llamasi, P. vazquezae* and *P. enoplosternum* have been assessed as medium-risk species for Germany (Chucholl 2013), indicating only a limited potential for invasiveness in Central Europe (Pekny and Lukhaup 2005). However, we have detected *A. astaci* in at least some specimens assigned to these taxa, and thus their role as potential disease vectors should not be overlooked. This holds especially true for *C. patzcuarensis*, which is the most common crayfish species in the German aquarium trade and which is occasionally kept in garden ponds (Chucholl 2013).

The introduction of new non-indigenous pathogens may have a substantial effect on the energy flow, function and structure of whole ecosystems (Daszak et al. 2000), especially in regard to keystone species such as crayfish (Reynolds et al. 2013). In the current study, we strengthen evidence of a so far neglected potential entry pathway of important crustacean diseases: the trade of ornamental crayfish. The aquarium trade may not only act as a pathway for new non-native species introductions, but also as a reservoir of pathogens with the potential of their wider spread. Trade with ornamental aquatic animals is rapidly growing at present, which significantly increases numbers of potential disease carriers available for sale. In this situation, prevention of pathogen release and spread will require education, outreach and enforcement efforts directed at crayfish aquarists and the aquarium industry.

Public education focused on mechanisms of pathogen introduction into aquatic environments, their consequences and threats is critical and should serve as a basis for further actions. In our opinion, public education should be especially implemented in regions characterized by either a high popularity of ornamental crayfish linked to high release rates, or climatic and habitat conditions suitable for crayfish species common in the aquarium trade. In both cases a relatively high risk of pathogen introduction should be assumed.

Furthermore, direct actions aiming to reduce the potential release and spread risks should be applied. Since crayfish species' availability in the trade has been shown to be a major determinant of the likelihood of release, the availability of disease carrying species and species with a high potential invasiveness should be drastically reduced (Chucholl 2013; Patoka 2014). In this context, Whittington and Chong (2007) suggested a limitation of not only the number of aquarium species which are traded but also their countries of origin. With regard to crayfish plague, our results clearly support the general notion that most, if not all, crayfish species of North and Central American origin can be *A. astaci* carriers and should therefore be subjected to such limitation, unless proven to be disease-free. Moreover, disease risk assessment according to the existing OIE recommendations should be accomplished prior to the import of any new non-native crayfish species into Europe. A quarantine of non-native species with native species may also be an option in specific cases (Peeler et al. 2011). Regarding the maintenance of the stock, preventive measures may include for example proper water disposal from aquarium facilities, a controlled

water flow within shop facilities, and the usage of different tanks and other equipment for different species to avoid horizontal disease transmission. We strongly recommend that shop owners, breeders and aquarium hobbyists substantially reduce the risks of disease transmission by choosing only sources previously screened for infections. Thus, the implementation of regular screening for crayfish suppliers along with prohibition of disease carrying species in the ornamental trade should be considered. Finally however, all of these disease risk mitigation actions would be feasible only in the light of more intensive regulations of the ornamental crayfish trade (see Stentiford et al. 2010 for context).

Acknowledgments The study has been partially funded by the Czech Science Foundation (Project No. P505/12/0545). A.M. was supported by the Charles University in Prague (Project SVV 267204). E.K.-B. was supported by the Project No. CZ.1.07/2.3.00/30.0022 of the Education for Competitiveness Operational Programme (ECOP) co-financed by the European Social Fund and the State Budget of the Czech Republic. We thank Michelle Pond and Kelly Bateman for their support with the WSSV molecular detection, Jiří Patoka and Petr Difko for providing crayfish from some Czech sources, and Jiří Svoboda for technical support. Anonymous referees provided useful comments to previous versions of the manuscript.

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Becking T*, Mrugała A*, Delaunay C, Svoboda J, Raimond M, Viljamaa-Dirks S, Petrusek A, Grandjean F, Braquart-Varnier C (2015) Effect of experimental exposure to differently virulent *Aphanomyces astaci* strains on the immune response of the noble crayfish *Astacus astacus*. *Journal of Invertebrate Pathology* 132: 115-124 *(* shared first authorship)*

Journal of Invertebrate Pathology 132 (2015) 115–124

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Effect of experimental exposure to differently virulent *Aphanomyces astaci* strains on the immune response of the noble crayfish *Astacus astacus*

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ARTICLE INFO

Article history:
Received 17 April 2015
Revised 14 August 2015
Accepted 17 August 2015
Available online 26 September 2015

Keywords:
Crayfish plague
Astacus astacus
Virulence
Immune parameters
Mortality tests

ABSTRACT

European crayfish are sensitive to the crayfish plague pathogen, *Aphanomyces astaci*, carried by North American crayfish species due to their less effective immune defence mechanisms against this disease. During a controlled infection experiment with a susceptible crayfish species *Astacus astacus* using three *A. astaci* strains (representing genotype groups A, B, and E), we investigated variation in their virulence and in crayfish immune defence indicators (haemocyte density, phenoloxidase activity, and production of reactive oxygen species). Experimental crayfish were exposed to two dosages of *A. astaci* spores (1 and 10 spores mL^{-1}). The intensity and timing of the immune response differed between the strains as well as between the spore concentrations. Stronger and faster change in each immune parameter was observed in crayfish infected with two more virulent strains, indicating a relationship between crayfish immune response and *A. astaci* virulence. Similarly, the immune response was stronger and was observed earlier for the higher spore concentration. For the first time, the virulence of a strain of the genotype group E (isolated from *Orconectes limosus*) was experimentally tested. Total mortality was reached after 10 days for the two higher spore dosages (10 and 100 spores mL^{-1}), and after 16 days for the lowest (1 spore mL^{-1}), revealing equally high and rapid mortality as caused by the genotype group B (from *Pacifastacus leniusculus*). No mortality occurred after infection with genotype group A during 60 days of the experimental trial.

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1. Introduction

The introduction and spread of non-indigenous species is one of the most important problems in conservation biology worldwide, being after habitat loss the second most frequent cause of species extinction in freshwater ecosystems (Sala et al., 2000). Diseases carried by non-native species represent important mechanisms underpinning the invasion process (Prenter et al., 2004), and are in some cases the key factors behind the impact of invasions. Infectious pathogens introduced along with their original hosts often cause devastating impacts in newly colonized regions as a result of host switching (Desprez-Loustau et al., 2007). Due to the lack of a long co-evolutionary history, after which pathogens often show low virulence and host populations high level of resistance (Schmid-Hempel and Ebert, 2003), such non-native parasites may be highly virulent and cause population crashes in naïve hosts, as observed in amphibians, crayfish and fish (Bakke and Harris, 1998; Edgerton et al., 2004; Rachowicz et al., 2005).

The introduction of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes) to Europe exemplifies a situation where naïve hosts that lack a long co-evolutionary history with the pathogen are threatened by its presence. As responsible for substantial declines in native European crayfish populations (Alderman, 1996; Holdich et al., 2009), *A. astaci* is considered one of the 100 world's worst invasive species (Lowe et al., 2000). This parasite is spread by its natural hosts, North American crayfish species (for review, see Holdich et al., 2009), which are substantially more resistant than European crayfish (Cerenius et al., 2003).

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http://dx.doi.org/10.1016/j.jip.2015.08.007
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Invertebrate immunity is based on a complex innate immune system (Vazquez et al., 2009). Upon penetration by the pathogen into the crayfish circulatory system, the infectious agent meets a wide range of defence mechanisms launched mainly by and in haemocytes (Vazquez et al., 2009). The intruder can be phagocytized, encapsulated, trapped by the products of the coagulation system, killed by reactive oxygen species (ROS) or by products of melanization (i.e. products released by the phenoloxidase (PO) system) or can succumb to the action of antimicrobial peptides (Cerenius et al., 2010). North American crayfish have evolved efficient immune defence mechanisms to prevent a lethal *A. astaci* infection. The development of pathogen hyphae in the cuticle is firstly retained by encapsulation (Unestam and Nylund, 1972), and subsequently inhibited by the capsule melanization (Unestam and Weiss, 1970). In these crayfish species, continuous production of high levels of prophenoloxidase (proPO) transcripts keeps the host immune system on alert. Due to this mechanism, the *A. astaci* growth is usually immediately inhibited and the infection development prevented (Cerenius et al., 2003). Among European crayfish, such resistance phenomenon is not generally observed. Therefore, their immune defence mechanisms are usually insufficient to efficiently prevent infection by the crayfish plague pathogen.

Recent studies have shown, however, that even the native European crayfish species may at least occasionally carry the crayfish plague pathogen without developing visible symptoms and/or being affected by a massive mortality (Jussila et al., 2011; Viljamaa-Dirks et al., 2011; Svoboda et al., 2012; Kušar et al., 2013). Such differences in host reaction to *A. astaci* infection could be explained by variable virulence of *A. astaci* strains (Makkonen et al., 2014), and may exemplify a situation when parasites become less virulent, thus reducing their impact on the host and enhancing their propagule spread (Bull, 1994). Until now, four distinct *A. astaci* genotype groups have been isolated in Europe: group A was obtained from infected individuals of European crayfish species (*Astacus astacus* and *Astacus leptodactylus*), and groups B, D and E from different North American crayfish (*Pacifastacus leniusculus, Procambarus clarkii* and *Orconectes limosus*, respectively) (Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Kozubíková et al., 2011). It was recently demonstrated both in the field and experimental settings that virulence may substantially vary between strains belonging to different *A. astaci* genotype groups (Makkonen et al., 2012b, 2014; Viljamaa-Dirks et al., 2013). In particular, substantial differences were observed in a controlled infection experiment between genotypes from the groups A and B isolated in Fennoscandia (Makkonen et al., 2014). In crayfish challenged with the former, infection developed much more slowly and did not always result in crayfish death. In contrast, the latter killed all the infected crayfish within a few days (Makkonen et al., 2014). This supports the view that Finnish noble crayfish *A. astacus* populations carrying latent *A. astaci* infections are infected with genotype group A (Viljamaa-Dirks et al., 2013), associated with the first crayfish plague arrival to Europe (Huang et al., 1994). Such results seem to be an evidence of the pathogen's loss of virulence during more than a century of interactions with European crayfish (Jussila et al., 2014). Moreover, microsatellite genotyping of infected individuals indicated that at least one Turkish population of narrow-clawed crayfish carries a latent infection by the *A. astaci* genotype group B (Svoboda et al., 2014). Although this is yet to be confirmed by isolation of the pathogen to axenic culture, the results suggest that the virulence patterns may differ among regions or hosts.

Several studies have been performed on the specific crayfish immune response towards the *A. astaci* strains present in European waters. A recent study focused on the trade-offs between energy-demanding behaviour (such as exploration) and a strong immune reaction in the light of local crayfish adaptation to *A. astaci* (Gruber et al., 2014). It was hypothesized that noble crayfish subpopulations with a disease history would have been selected for stronger resistance towards the pathogen. Nevertheless, insufficient knowledge on temporal changes in crayfish immune response limits our understanding of the *A. astaci* pathogenicity.

Our primary aim was to evaluate whether there is a correlation between the *A. astaci* virulence and crayfish immune response after an experimental infection. Individuals of the noble crayfish were exposed to three crayfish plague strains presumably differing in virulence (belonging to genotype groups A, B, and E) in two spore dosages. Different parameters of immune response were quantified over time (total haemocytes count, PO activity, and ROS production). We expected a stronger and faster response in crayfish exposed to more virulent *A. astaci* strains and to higher spore concentrations. As a secondary aim, for the first time we assessed the virulence of a strain belonging to the genotype group E (originating from *O. limosus*) in a controlled infection experiment.

2. Materials and methods

2.1. Studied crayfish

In total, 380 individuals of the noble crayfish, *A. astacus*, were purchased from an aquaculture facility located in Thonnance-les-Joinville (Northeastern France). The weights and lengths of all crayfish ranged from 10 to 13 g and 7 to 8 cm, respectively. Upon arrival, the crayfish were placed in tanks containing 20 L of spring water (Quinçay, France), plastic pipes for shelter, and an aeration system. They were left to acclimate for at least one week prior to the beginning of the experiments.

2.2. *A. astaci* strains and zoospore production

Cultures of three *A. astaci* strains representing genotype groups A, B and E were used in the experiments. These included, respectively: strain Al7 isolated from *A. leptodactylus* carrying latent *A. astaci* infection, imported to the Czech Republic from Russia but most likely of Armenian origin, Pec14 isolated in January 2014 from dead *A. astacus* from a crayfish plague outbreak in the Černý Brook, Czech Republic (Kozubíková-Balcarová et al., 2014) and Evira4805a/10 isolated from infected *O. limosus* from a pond near Smečno, Czech Republic (Kozubíková et al., 2011). These strains were maintained in Petri dish cultures with RGY agar (Alderman, 1996). The genotyping of the strains was performed with microsatellite markers as described in Grandjean et al. (2014).

A. astaci zoospores were produced separately for each strain according to a protocol modified after Cerenius et al. (1988). A few small pieces (approximately 2 mm^2) of RGY-agar containing *A. astaci* hyphae were cut and transferred to glass flasks with 150 mL of liquid RG-medium. In order to obtain necessary zoospore numbers, six replicate cultures were prepared each time. The cultures were later placed on a shaker in a room at 17 °C (the same temperature as during the experiment) and left for three days to enable mycelium growth. On the third day, the mycelium was washed with autoclaved spring water to induce sporulation. The washing was repeated five times at hourly intervals. The cultures were afterwards incubated on a shaker for 16–20 h (depending on the strain used) and the spores were counted with a KOVA haemocytometer. Appropriate volumes of the spore suspension were added to tanks with crayfish.

2.3. Experimental design

The experiments took place at the laboratory of Ecology, Evolution and Symbiosis, University of Poitiers, in March and April 2014. Crayfish were kept at a controlled temperature of 17 °C in tanks containing 20 L of spring water, plastic pipes for shelter, and an aeration system. They were fed twice a week with carrots. Crayfish were monitored daily and excess food and dead individuals were removed. Each time prior to spore addition, shelters were removed and the aeration stopped for the first 5 h of the experiment. This was done in order to limit potential zoospore loss by attachment to additional substrates, and thus maximize the chances of infection.

2.3.1. Mortality experiment (genotype groups A and E)

Altogether 70 crayfish individuals were exposed to zoospores of two *A. astaci* strains, representing genotype groups A and E. Different aliquots of spore suspension were added directly to the tanks to reach concentrations of 1, 10, 100 and 380 spores mL^{-1} and 1, 10 and 100 spores mL^{-1}, respectively. Ten crayfish per group were used. In addition, eight individuals were treated as a negative control group. Unless the crayfish died earlier, the experiment was terminated after 60 days, and the remaining crayfish were euthanized by freezing at −20 °C.

2.3.2. Experimental monitoring of immune parameters (genotype groups A, B and E)

270 crayfish individuals were infected with three *A. astaci* strains with two different spore concentrations of 1 and 10 spores mL^{-1}. 45 individuals per strain and concentration were used, divided into three tanks (15 individuals each). In addition, 24 crayfish kept in two separate tanks (2 × 12 ind.) were used as a negative control group.

2.4. Immunological analyses

Depending on the strain virulence, the analyses were performed at different time intervals. For the least virulent strain (Al7, group A), we analysed the crayfish immunological response after 4, 7, 14, 47 and 60 days post-infection. Individuals exposed to more virulent strains Pec14 and Evira4805a/10 (groups B and E) were analysed more frequently, after 2, 4, 7 and 10 days post-infection. The control group was analysed at the beginning of the experiment (prior to spore addition to experimental groups; $n = 6$) and on days 14 ($n = 9$) and 61 ($n = 9$) to cover the entire period of the experiment.

2.4.1. Haemolymph collection

From each individual, we collected 600 μL of haemolymph using a 2.5–5 mL syringe (needle diameter 0.8 mm) containing an equal volume of cold anticoagulant (140 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6). The puncture was performed at the pericardial cavity. To reduce the bias caused by possible variation between individuals, haemolymph from three crayfish was pooled for each single measurement. As each experimental treatment (spore concentration/*A. astaci* strain combination) was replicated in three separate tanks, we obtained three pooled haemolymph samples (from three crayfish per tank) for every collection date and treatment. After the haemolymph collection, the crayfish were euthanized by a scalpel cut in the head region and stored in 95% ethanol. The haemolymph-anticoagulant mixture was used for the subsequent analyses of the immune parameters.

2.4.2. Haemocyte density

10 μL of anticoagulant and 10 μL of Trypan Blue solution (Invitrogen) were added to 10 μL of the haemolymph-anticoagulant mixture. 10 μL of this mixture were used to count the haemocyte density with an automatic cell counter (Countess® Cell Counting Chamberslide, Invitrogen). The total haemocyte density (THC) was expressed in number of cells mL^{-1}. For each pooled haemolymph sample, the measurement was repeated 3 times.

2.4.3. Phenoloxidase activity assay

Phenoloxidase (PO) activity was measured from the haemocyte lysate supernatant (HLS), as described in Söderhäll and Häll (1984). For each pooled haemolymph sample, the haemolymph–anticoagulant mixture (about 3.6 mL) was centrifuged for 10 min at 1300*g* (4 °C). Subsequently, the supernatant was removed and the pellet was resuspended in 1 mL of washing buffer (10 mM sodium cacodylate, 250 mM sucrose, 20 mM $CaCl_2$, pH 7) before being centrifuged for additional 10 min at 1300*g* (4 °C). After the supernatant removal, the pellet was resuspended in 200 μL of homogenization buffer (HB; 10 mM sodium cacodylate, 20 mM $CaCl_2$, pH 7), and then sonicated following a cycle of 3 × 30 s (30 mA). Finally, the homogenization buffer was added to the mixture (in order to obtain a final volume equal to the total volume of haemolymph collected initially from three crayfish) and centrifuged for 20 min at 16,000*g* (4 °C). The obtained supernatant (i.e. HLS) was kept on ice for the subsequent analyses.

Phenoloxidase activity was estimated spectrophotometrically using 20 mM L-DOPA as substrate and SDS as an elicitor in order to trigger the PO activity. Samples were distributed in 96-well microplate and 50 μL of HLS, 25 μL of 12 mM SDS and 25 μL of either a specific inhibitor (1 mg mL^{-1} of 1-phenyl-2-thiourea, i.e. PTU) or homogenization buffer were added. For negative controls, the elicitor was replaced with double-distilled water. The plate was finally incubated for 45 min at 37 °C and the substrates added accordingly. Both activities were measured at 490 nm using the TriStar Spectrophotometer (Berthold) at 15 min intervals. The final optical density (OD) values were recorded after 4 h.

Protein concentrations were obtained to create a baseline for PO activity calculations (Moreno-García et al., 2013). These concentrations were measured from the haemocyte lysate supernatant with the Micro BCA Protein Assay Kit (ThermoScientific) following the manufacturer's instructions. Five standards were used: 100, 50, 20, 10 and 5 μg protein mL^{-1} (prepared from bovine serum albumin). The absorbance was measured at 595 nm (Nanodrop 1000 Spectrophotometer, ThermoScientific) from 10 × diluted samples.

2.4.4. Reactive oxygen species quantification

The reduction of nitro blue tetrazolium (NBT) to blue formazan, following the protocol modified from Muñoz et al. (2000), was used as a probe for superoxide generation in the haemolymph. A determined volume of haemolymph (50 μL + 50 μL of anticoagulant solution) was deposited in triplicate in a 96-well microplate (100 μL of anticoagulant solution were used as negative controls). Next, 25 μL of L-15 Leibovitz and 25 μL of van Harreveld solution (270 mM NaCl, 25.74 mM $CaCl_2$, 4.63 mM KCl, 2.6 mM $MgCl_2$, 200 mM HEPES, pH 7.4; van Harreveld, 1936) were added to fix the haemocytes to the microplate. For haemocyte adherence, the microplate was incubated at room temperature for 30 min. Subsequently, after the supernatant removal, 50 μL of 0.3% NBT and 50 μL of L-15 Leibovitz/van Harreveld solution were added. After a 2-h incubation, the supernatant was again removed. The haemocytes were then fixed by a repeated washing; once with 200 μL of absolute methanol, and then twice with 200 μL of 70% methanol. This was followed by drying at room temperature. The obtained blue formazan was solubilised by addition of 120 μL of 2 M KOH and 140 μL of DMSO. The microplates were homogenized and the

extinction was read at 620 nm using the TriStar Spectrophotometer (Berthold).

2.5. Statistical analysis

2.5.1. Infection experiment

The Kaplan-Meier survival test was used to evaluate differences in mortality among experimental groups. The analyses were performed using the R software (version 3.0.2, R Development Core Team, 2013), with the packages "survival" (Therneau and Grambsch, 2000) and "KMsurv" (Klein and Moeschberger, 1997). The significance level was set at 0.05.

2.5.2. Experimental monitoring of immune parameters

All the values were tested for normality (Shapiro–Wilk test) and homoscedasticity of variances (Bartlett test). When the null hypothesis was rejected in ANOVA tests, the significance of differences was verified with a Tukey HSD test. For values not corresponding to the criteria of normality and/or homoscedasticity of variances, Kruskal–Wallis tests followed by Dunn's multiple comparison tests were performed. For all the analyses, we used the R3.0.2 with the packages "car" (Fox and Weisberg, 2011) and "pgirmess" (Giraudoux, 2013). The significance level was set at 0.05.

2.6. DNA extraction and detection of *A. astaci* DNA

All crayfish used in both experiments were tested for the presence of *A. astaci* infection. In addition, 8 crayfish individuals were analysed prior to the beginning of the experiment to evaluate the potential presence of this pathogen, acquired, e.g., in the aquaculture facilities.

After the immunological analyses, crayfish were stored in 95% ethanol. Prior to dissection, each crayfish was examined for any presence of melanized spots indicating immune reaction to pathogens. We dissected one half of the soft abdominal cuticle, one uropod and any melanized spots (if present). Dissected tissues from each individual were collected in a single 1.5 mL tube, dried and stored at −80 °C. The DNA was extracted with the DNeasy tissue kit (Qiagen) following the manufacturer's protocol with minor modifications. 360 μL of ATL buffer were added to the dissected material and the tissue was then crushed with one scoop (ca 50 μL) of stainless steel beads (diameter 1.6 mm) using BBX24B Bullet Blender (Next Advance) for 10 min at the maximum speed. Afterwards, the procedure followed the manufacturer's protocol with double volumes of chosen reagents (i.e., proteinase K solution, AL buffer, and ethanol).

The detection of *A. astaci* presence was performed with TaqMan MGB quantitative PCR (qPCR) with the LightCycler® 480 Instrument (Roche). The relative levels of infection were calculated based on the strength of the PCR signal and assigned to semi-quantitative agent levels (according to Vrålstad et al., 2009; Kozubíková et al., 2011). Individuals with agent levels A2 and higher were considered infected with *A. astaci*. We included negative controls in every step of the process to check for contamination; these remained negative in all cases.

3. Results

3.1. Mortality experiment (genotype groups A and E)

All crayfish infected with the Evira4805a/10 strain (genotype group E) died during the experiment (Fig. 1). The first dead crayfish were recorded 5 days post-infection in the treatment with the highest spore concentration (100 spores mL^{-1}), and 100% mortality was reached after 10 days for the two higher spore dosages (10 and 100 spores mL^{-1}), and after 16 days for the lowest (1 spore mL^{-1}). In contrast, no mortality was observed in all four treatments with the Al7 strain (genotype group A) during the whole period of the experiment (Table 1). No dead crayfish were recorded in the control group.

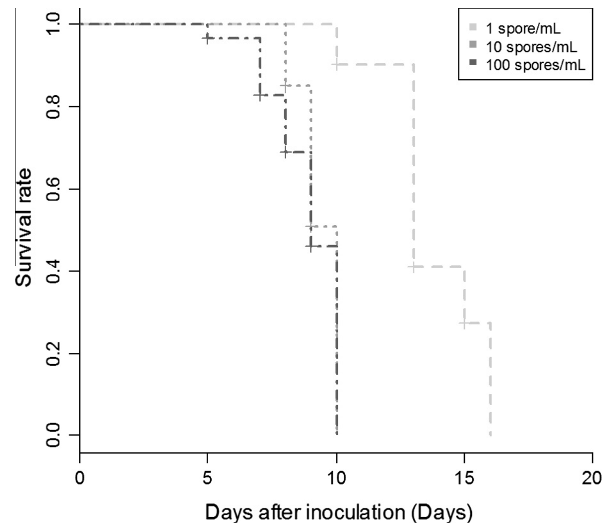

Fig. 1. Kaplan-Meier survival curves obtained after crayfish exposure to the strain of the genotype group E (Evira4805a/10).

The mortality rate was significantly faster in the treatments with the two higher spore concentrations than in the one with the lowest spore concentrations of the Evira4805a/10 strain (1 vs. 10 spores mL^{-1}: log-rank coeff = 3.32, df = 2, $p < 0.001$, 1 vs. 100 spores mL^{-1}: log-rank coeff = 3.65, df = 2, $p < 0.001$). No statistical difference was observed between the individuals exposed to 10 and 100 spores mL^{-1} (log-rank coeff = 0.43, df = 1, $p = 0.43$).

3.2. Monitoring of immune parameters (genotype groups A, B and E)

3.2.1. Haemocyte density

Infection with the crayfish plague pathogen resulted in a strong and significant ($p < 0.001$) decrease in total haemocyte number (THC) as compared to about $2.6 \pm 1.4 \times 10^6$ cells mL^{-1} in the control group (Fig. 2). This change was already noted on the second day post-infection in the crayfish infected with Pec14 and Evira4805a/10 strains, and on the seventh day in those exposed to Al7 strain. The haemocyte numbers remained below control value until the end of the experiment ($p < 0.001$). No significant difference between treatments was observed after day 7 post-infection ($p > 0.05$).

3.2.2. Phenoloxidase activity

No statistically significant variation in PO activity was observed in the negative control group before infection ($n = 6$), and on days 14 ($n = 9$) and 61 ($n = 9$) post-infection (Kruskal–Wallis: $\chi^2 = 0.0913$, df = 2, $p = 0.96$).

For the least virulent Al7 strain, infection with 1 spore mL^{-1} did not result in a statistically significant change in PO activity compared with the control for all but one measurement. A significant decrease in PO activity was noted on day 47 (Kruskal–Wallis: $\chi^2 = 8.53$, df = 1, $p < 0.001$), possibly due to an extensive crayfish moulting. In contrast, exposure to 10 spores mL^{-1} resulted in a significant increase in PO activity measured on days 7 and 14 post-infection (ANOVA: *F*-value = 36.24, $p < 0.001$, 7th day/control: $p < 0.001$, 14th day/control: $p < 0.001$).

Table 1
Details of experimental infection evaluating the virulence of strains of the genotype groups A and E: numbers of crayfish individuals (n), inoculation spore dosages (spore mL^{-1}), mortality estimates. Mortality rate describes a total mortality during the post-infection period and the mean day of death refers to a certain day after inoculation when 50% mortality was reached.

Strain (genotype group)	*n*	Spore mL^{-1}	Mortality	
			Mean day of death ± SD	Mortality rate (%)
Al7 (A)	10	1	–[a]	–
	10	10	–[a]	–
	10	100	–[a]	–
	10	380	–[a]	–
Evira4805a/10 (E)	10	1	12.9 ± 1.9	100
	10	10	9 ± 0.8	100
	10	100	8 ± 1.6	100
Control	8	–	–[a]	–

[a] No crayfish died in the group.

Infection of *A. astacus* with the Pec14 strain (genotype group B) caused a substantial increase in PO activity on day 2 of the experiment, followed in the next days by a gradual decrease. The change was more evident in response to the higher spore concentration. In the treatment with 1 spore mL^{-1}, a statistically stronger response was observed on days 2 and 4 post-infection (Kruskal–Wallis: $\chi^2 = 34.89$, df = 4, $p < 0.001$, 2nd day/control: $p = 0.018$, 4th day/control: $p = 0.043$), while in the treatment with 10 spores mL^{-1}, the differences were significant on days 2, 4 and 7 (Kruskal–Wallis: $\chi^2 = 42.70$, df = 4, $p < 0.001$, 2nd day/control: $p < 0.001$, 4th day/control: $p < 0.001$, 7th day/control: $p = 0.031$).

Although a similar pattern might have been expected after infection with Evira4805a/10 strain (genotype group E) due to its presumed comparable high virulence, no significant increase in PO activity was recorded in the treatment with 1 spore mL^{-1}. Moreover, in the second treatment with 10 spores mL^{-1}, a gradual increase in PO activity, rather than decrease, was noted. A significantly stronger response was recorded on days 4 and 7 post-infection (Kruskal–Wallis: $\chi^2 = 31.52$, df = 3, $p < 0.001$, 4th day/control: $p < 0.001$, 7th day/control: $p = 0.031$). On day 10 of the experiment, a significant decrease in PO activity compared to the control was noted for both spore concentrations (for 1 spore mL^{-1} Kruskal–Wallis: $\chi^2 = 25.40$, df = 4, $p < 0.001$, 10th day/control: $p = 0.026$; for 10 spores mL^{-1} Kruskal–Wallis: $\chi^2 = 31.52$, df = 3, $p < 0.001$, 10th day/control: $p = 0.045$), which was likely linked to the moribund state of the tested crayfish (see Fig. 3).

For each tested strain, the recorded PO activity was significantly stronger when crayfish were challenged with higher spore dosage (Kruskal–Wallis (genotype group A): $\chi^2 = 21.09$, df = 1, $p < 0.001$; Kruskal–Wallis (genotype group B): $\chi^2 = 25.86$, df = 1, $p < 0.001$; Kruskal–Wallis (genotype group E): $\chi^2 = 9.26$, df = 1, $p = 0.0023$).

3.3. ROS production

The temporal trend of reactive oxygen species ROS production was not visualized due to the lack of observable pattern between the different time intervals. Thus, the variability in ROS production between infected crayfish and uninfected control group is presented from the pooled data over the whole period of the experiment, i.e. the results correspond to all the data recorded between 0 and 10 days for genotype groups B and E and between 0 and 60 days for genotype group A.

No significant difference was observed between the different genotype groups. Crayfish exposure to 10 spores mL^{-1} of *A. astaci* resulted in a significant increase in ROS production compared to the negative control group (Kruskal–Wallis: $\chi^2 = 30.23$, df = 3, $p < 0.001$) (Fig. 4). In contrast, no such increase was observed for the individuals infected with 1 spore mL^{-1} (data not shown).

3.4. *A. astaci* detection in challenged crayfish

No *A. astaci* DNA was detected in the negative control group, confirming a lack of infection in crayfish purchased from the aquaculture.

For the two more virulent strains (Pec14 and Evira4805a/10), we observed a gradual increase in pathogen detection and infection intensity. In the treatment with the higher spore concentration (10 spore mL^{-1}) low levels of *A. astaci* DNA were already detected on the day 2 post-infection, reaching high levels (agent level A5) on the day 7 of treatment (Table 2). In contrast, after exposure to zoospores of the least virulent strain (Al7), only low levels of *A. astaci* DNA (agent level A2) were detected in some crayfish hosts throughout the whole experimental period.

4. Discussion

In resistant North American crayfish, *A. astaci* becomes encapsulated by a sheath of melanin as a result of PO activity produced by haemocytes (Söderhäll and Häll, 1984; Cerenius et al., 2003; Aquiloni et al., 2011). In contrast, in highly susceptible crayfish species, such as *A. astacus* used in our experiment, the pathogen is rarely melanised (Söderhäll and Cerenius, 1999). This leads to an unhampered mycelium growth in the cuticle and may result in crayfish death within a few days depending on the virulence of the *A. astaci* strain (Makkonen et al., 2012b, 2014). However, melanization may also be occasionally observed in chronically infected population of noble crayfish (Viljamaa-Dirks et al., 2011). In our study, we quantified the variation of three host defence parameters (total haemocyte count, PO activity, and ROS production) in the susceptible crayfish *A. astacus* during infection by three *A. astaci* strains belonging to different genotype groups (A, B, E). As has already been reported, strains from group A appear much less virulent than strains from group B (Makkonen et al., 2012b, 2014). These observations were confirmed in our study, suggesting that also other strains (not of Finnish origin) from this genotype group may exhibit decreased virulence.

The mortality dynamics evaluated in our study confirmed the lower virulence of the Al7 strain (group A), with no mortality observed within 60 days in any treatment, not even after exposure to 380 spores mL^{-1}. Makkonen et al. (2012b) reported similar results, demonstrating the lack of or incomplete mortality of *A. astacus* within 42 days after exposure to other strains from the genotype group A (the mortality levels depending on both host population and pathogen strain). As the *A. astaci* strain from the group A used in the experimental infection of Makkonen et al. (2012b) originated from Finland, our observations revealed that also geographically distant pathogen strains might have co-evolved with crayfish hosts, and as a result decreased their virulence. In contrast, the strain belonging to genotype group E exhibited very high virulence, with 100% mortality observed 8 days after exposure to the highest spore dosage (100 spores mL^{-1}). Hence, it is not surprising that this genotype group has been recently involved in a series of outbreaks in the Czech Republic (Kozubíková-Balcarová et al., 2014) and at least one in France (Filipová et al., 2013). The virulence of this strain seems similar to that reported for strains of the genotype group B, as prior works reported 100% mortality of *A. astacus* within 6 days after exposure to pathogen spore concentration of 100 spores mL^{-1} (Makkonen et al., 2012b, 2014). However, it seems that some variability in virulence among strains from

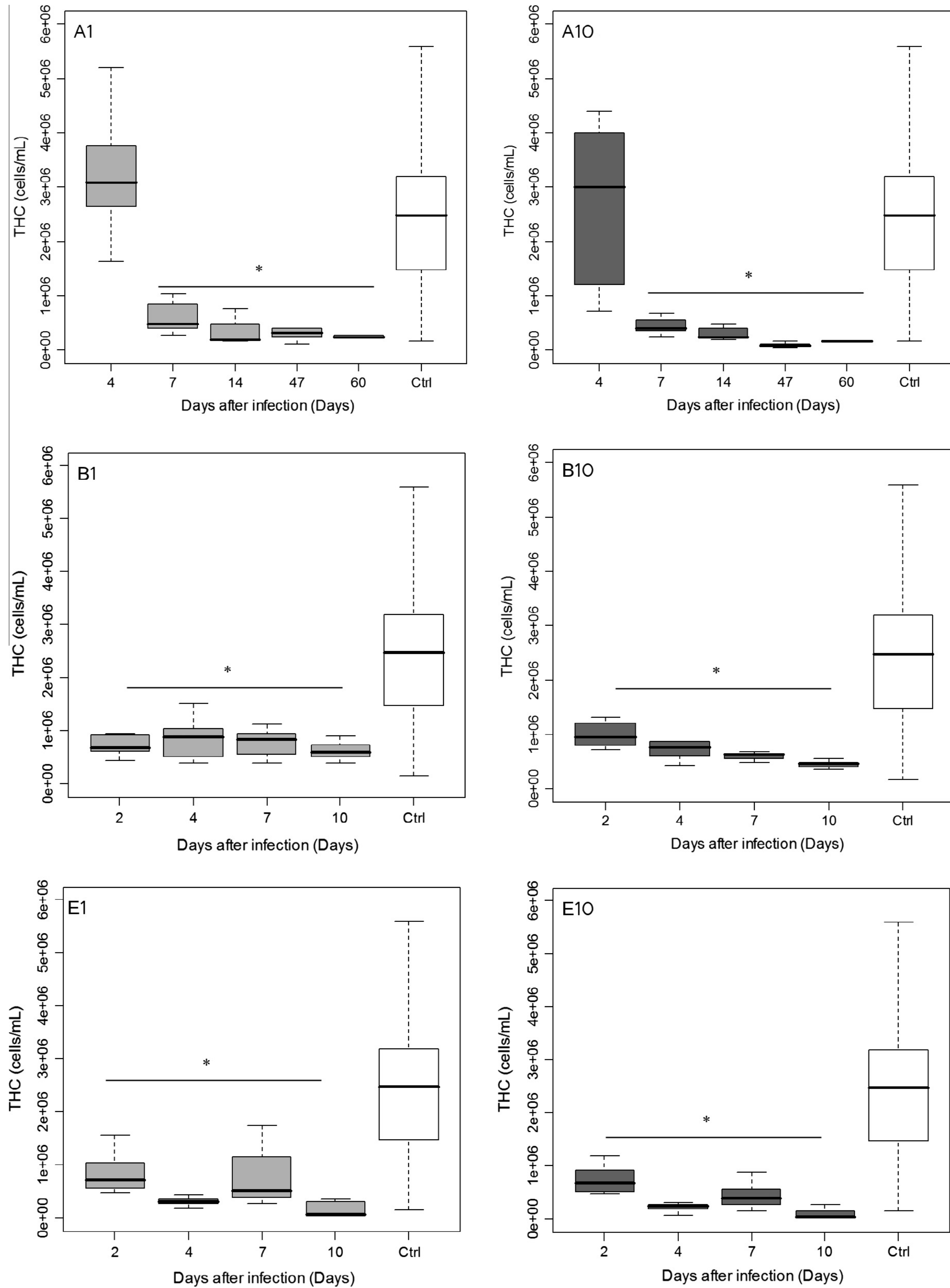

Fig. 2. Change in the total haemocyte counts (THC) presented for each *A. astaci* genotype group (A, B, E) and spore concentration (1 or 10 spores mL^{-1}). Graphs are marked accordingly (e.g. A1 standing for strain of the genotype group A and 1 spore mL^{-1}). Label "Ctrl" indicates values for THC measured in the non-infected control group. Asterisks indicate significant differences ($p < 0.001$) from the controls.

the B group also exists, and mortality rates may depend also on crayfish origin (Makkonen et al., 2012b; see also Svoboda et al., 2014).

The detected *A. astaci* agent levels in infected crayfish seem to be correlated with the different virulence of pathogen genotype groups. The infection proceeded faster and reached higher agent

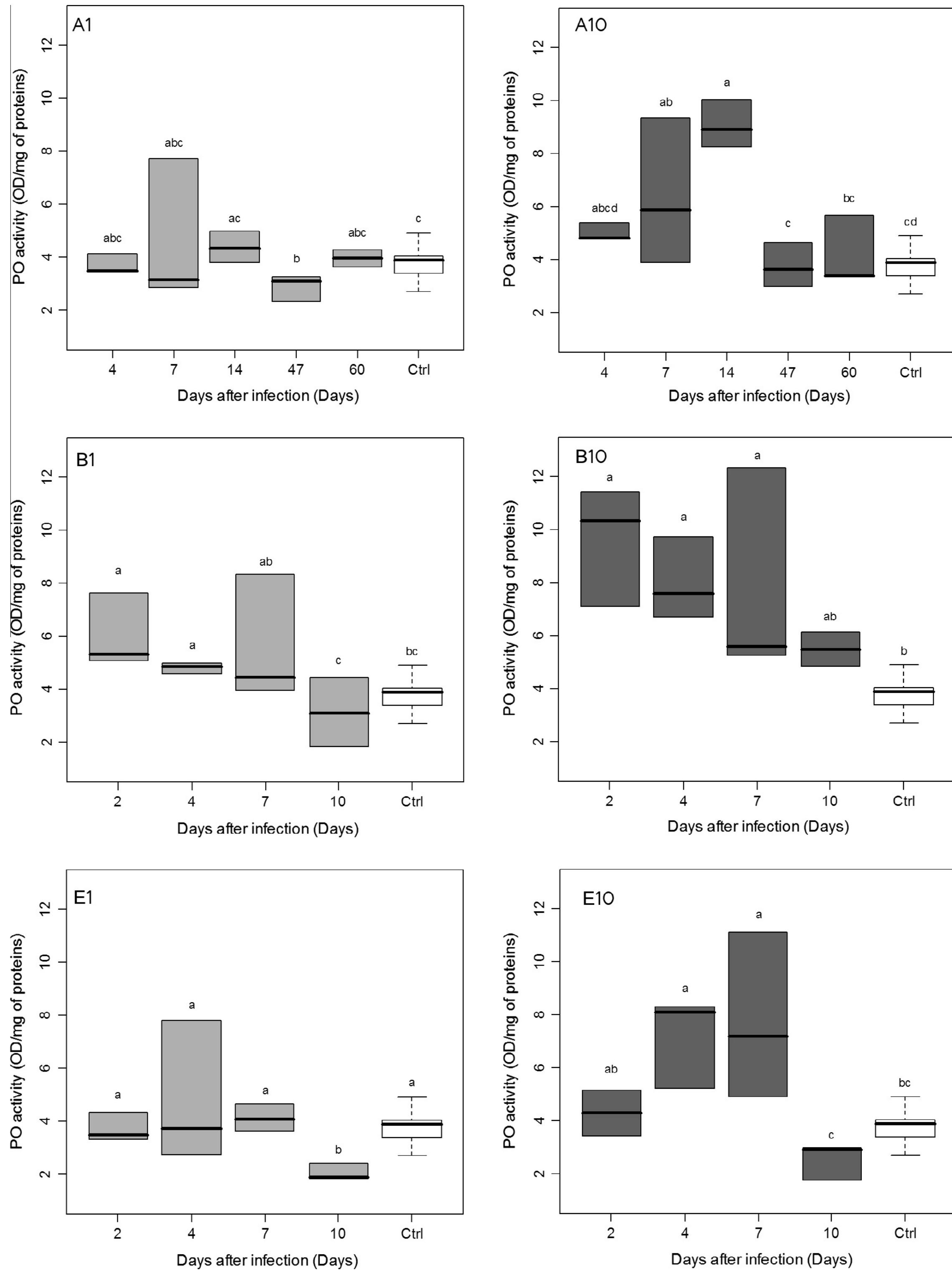

Fig. 3. Variation in PO activity (expressed as Optical Density (OD) mg of proteins^{-1}) presented for each *A. astaci* strain (A, B, E) and spore concentration (1 or 10 spores mL^{-1}). Graphs are marked accordingly (as in Fig. 2). "Ctrl" indicates values of PO activity measured in the non-infected control group. Plots marked with the same letter (a, b, c, d) within each panel are not statistically different from each other.

levels in the treatments with the more virulent groups B and E. In contrast, very low agent levels remained after exposure to the least virulent Al7 strain throughout the whole experimental period, indicating a slowly progressing (or even chronic) infection. It has been speculated that problems with attachment and germination of spores or an effective inhibition of weaker pathogen strains by crayfish immune defences may limit the pathogen's growth (Makkonen et al., 2012b). However, as no evidence for mechanisms behind *A. astaci* reduced virulence exists, further research is needed to evaluate these suggested explanations. Nonetheless, the rapid response of crayfish immune system implies that the pathogen was able to penetrate and colonize the noble crayfish.

Fig. 4. Variation in ROS production (expressed as Optical Density (OD)) presented for each *A. astaci* genotype group (A, B, E) with a spore density of 10 spores mL^{-1}. The plots correspond to values recorded during the entire experiment (60 days for the genotype group A and 10 days for the genotype groups B and E). Control values (Ctrl) for PO activity were measured in the non-infected control group. * indicates $p < 0.05$.

The drop in haemocyte numbers was faster in crayfish exposed to more virulent genotype groups (i.e. B and E) in contrast to the delayed response following the infection with a strain belonging to group A. A similar decrease was reported after laminarin injection (mimicking an infection) in *P. leniusculus*; however, in that case, the haemocyte decline was followed by a slow recovery to its normal levels within the next 24 h (Söderhäll et al., 2003). Such a renewal was not observed during our experiments, not even in crayfish infected by genotype group A, which survived the whole 60-day experimental trial. This observation may indicate either a pathogen's disturbance of the haemocyte recovery in native European crayfish (causing the crayfish inability to recruit new haemocytes), or a direct allocation of haemocytes in the defence mechanisms against *A. astaci* (such as nodule formation or phagocytosis) in the infected tissues. Nevertheless, low haemocyte numbers, observed in all infected crayfish regardless of the *A. astaci* strain virulence, do not exclusively explain crayfish high mortality, which is most likely caused by a synergistic effect of more factors disturbing crayfish homeostasis.

The PO activity in host crayfish showed similar patterns after infection by the three strains, although the maximum values were reached at different time intervals (for 10 spores mL^{-1}: at 2nd day for the group B, 4th day for E, and 14th day for A). This result may reflect varying degree of ability of pathogen strains to penetrate and grow in the cuticle, likely influenced by chitinases that play crucial role in cuticle penetration (Andersson and Cerenius, 2002). Recently, Makkonen et al. (2012a) have reported the existence of genetic variation of chitinase genes in strains belonging to genotype groups A and B, which may contribute to differences in the epidemiological properties of genotypes.

A clear link was observed between an increase in PO activity and a drop in haemocyte numbers. The haemocyte response to infection results in a rapid release of components of the PO cascade to the haemolymph, accompanied by a destruction of these immune cells (Vazquez et al., 2009). These processes likely contributed to lower THC in infected individuals in comparison to the control group. In crayfish exposed to the group B strain, the temporal trend of the PO activity corresponded to this scenario, increasing quickly after infection and then gradually declining to

Table 2
Results of *Aphanomyces astaci* detection in living crayfish from the monitoring of immune parameters. For negative and positive detections of *A. astaci*, semi-quantitative agent levels based on the estimated amount of PCR-forming units (PFU) in the reaction (according to Vrålstad et al., 2009) are provided: A0 (0 PFU), A1 ($1 \leqslant$ PFU < 5), A2 ($5 \leqslant$ PFU < 50), A3 ($50 \leqslant$ PFU < 10^3), A4 ($10^3 \leqslant$ PFU < 10^4), A5 ($10^4 \leqslant$ PFU < 10^5), A6 ($10^5 \leqslant$ PFU < 10^6). *n* – number of tested individuals.

Strain (genotype group)	Spore density (spores mL^{-1})	Days after infection (days)	*n*	Agent levels					
				A0	A1	A2	A3	A4	A5
Al7 (A)	1	4	9	7	2	–	–	–	–
		7	9	7	1	1	–	–	–
		14	9	6	2	1	–	–	–
		47	9	7	2	–	–	–	–
		60	4	3	1	–	–	–	–
	10	4	9	2	4	3	–	–	–
		7	9	–	8	1	–	–	–
		14	9	3	5	1	–	–	–
		47	9	1	7	1	–	–	–
		60	5	2	3	–	–	–	–
Pec14 (B)	1	2	9	1	6	2	–	–	–
		4	9	–	3	5	1	–	–
		7	9	–	1	4	3	1	–
		10	9	–	–	2	4	3	–
	10	2	9	–	5	4	–	–	–
		4	9	–	–	2	6	1	–
		7	9	–	–	2	2	3	2
		10	4	–	–	–	–	1	3
Evira4085a/10 (E)	1	2	9	4	5	–	–	–	–
		4	9	2	5	2	–	–	–
		7	9	2	3	2	2	–	–
		10	9	–	2	3	3	1	–
	10	2	9	–	1	5	3	–	–
		4	9	–	–	2	6	1	–
		7	9	–	–	–	5	3	1
		10	9	–	–	–	–	2	7
Control		0	6	6	–	–	–	–	–
		14	9	9	–	–	–	–	–
		61	9	9	–	–	–	–	–

a level similar to the one observed in control individuals. This result is in line with the measurement of proPO transcript levels reported by Cerenius et al. (2003). In treatments with the two other strains, however, the PO activity showed a more complex pattern.

The PO activity in decapod crustaceans is mainly produced by granular and semi-granular haemocytes (Vazquez et al., 2009). The maintenance of PO activity from a reduced number of haemocytes may be explained by two non-exclusive hypotheses (as suggested by Söderhäll et al., 2003): (1) the remaining haemocytes could be mainly granular ones, (2) the expression of proPO activity in semi-granular haemocytes could be elevated in response to *Aphanomyces* infection. To evaluate these hypotheses and answer the question of disturbed haemocyte recovery, it would be interesting to (1) identify which haemocyte types are more prevalent during *A. astaci* infection of susceptible and resistant crayfish species in comparison with uninfected individuals, (2) analyse the release of newly synthesized haemocytes from the hematopoietic tissue using *in vivo* proliferation experiments, and (3) quantify the proPO gene expression rates in granular and semi-granular haemocytes of these different individuals.

In crustaceans, reactive oxygen species act as protective defence mechanisms against pathogen infection, however their high levels may also lead to host cell damage (for review see Avery, 2011). An increase in ROS production by haemocytes, two times higher in infected individuals than in the control group (regardless of the pathogen's strain) was thus a predictable outcome, indicating host immune response towards *A. astaci* infection. As the role of *A. astaci* virulence in cell host cytotoxicity remains unknown, further study focusing on the quantification of the gene expression coding for detoxification enzymes such as the superoxide dismutase, is recommended.

Our study highlights the significant impact of the *A. astaci* infection on cellular and metabolic levels of the susceptible noble crayfish, *A. astacus*, and provides valuable insights into interactions of this host-pathogen pair from an immunological perspective. The more virulent is an *A. astaci* strain, the faster and stronger is the host immune response. However, the response is not sufficient to prevent the development of the infection by the virulent strains, which results in high mortality of infected hosts. Gruber et al. (2014) hypothesized that the decreased survival of the noble crayfish may be caused by high self-reactivity costs of a strong immune response. Our study provides further evidence that such a reaction may impair *A. astacus* survival of the crayfish plague infection. However, to fully understand this phenomenon, it is essential to extend this study by an estimation of immunological parameters in natural populations of e.g. *A. astacus* carrying chronic crayfish plague infections. We believe that such data may explain mechanisms behind local host adaptations, and thus contribute to our understanding of future prospects of native European crayfish populations that are coming into contact with this pathogen.

Acknowledgments

Agata Mrugała and Jiří Svoboda were supported by the Charles University in Prague (project SVV 260198). We are grateful Jean-Pierre Geeraert for providing crayfish *Astacus astacus* from the farm "Le moulin aux écrevisses" (52300 Thonnance-les-Joinvilles, France). We would like to thank Lage Cerenius and two anonymous referees for useful comments on the manuscript.

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– chapter 6 –

Mrugała A, Veselý L, Petrusek A, Viljamaa-Dirks S, Kouba A (in press) May *Cherax destructor* contribute to *Aphanomyces astaci* spread in Central Europe? *Aquatic Invasions*

(corrected proof version)

REABIC

Aquatic Invasions (2016) Volume 11, Issue x: xxx–xxx
doi:
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Research Article

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

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Received: 20 January 2016 / Accepted: 29 June 2016/ Published online: xx xxxxx 2016
Handling editor: Christoph Chucholl

Abstract

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

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

Introduction

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

Freshwater ecosystems are particularly vulnerable to biological invasions (Ricciardi and Rasmussen 1999; Shea and Chesson 2002), with the key drivers of non-native species introductions being aquaculture and the associated trade of live organisms for direct consumption, ornamental purposes, or even research (Copp et al. 2007; Gozlan 2008; Peeler et al. 2011). Consequently, all these pathways also contribute to the introduction of exotic pathogens (Peeler et al. 2011; Rodgers et al. 2011). The crayfish plague agent, an oomycete *Aphanomyces astaci* Schikora, is an example of such introduced exotic pathogens. It is undoubtedly one of the most devastating emerging diseases in European freshwaters, also listed among worst invasive species in Europe as well as globally (Lowe et al. 2004; DAISIE 2009). Its unintentional introduction from North America to Europe resulted in substantial declines and local extinctions of native

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

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

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

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

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

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

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

Methods

Studied crayfish and Aphanomyces astaci *strains*

The yabby, *Cherax destructor*, originated from an experimental culture and were kept at the Research Institute of Fish Culture and Hydrobiology in Vodňany, Czech Republic. The noble crayfish, *Astacus astacus*, were caught with permission of the nature conservancy authorities from the pond Pařez (Kaliště, Czech Republic; 49°36′N, 15°19′E). Before the experiment, *A. astacus* were adapted to the communal rearing conditions in the laboratory for 3 weeks. All crayfish were approximately 4 months old at the beginning of the experiment; their total length ranged from 20 to 40 mm.

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

Experimental design

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

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

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

DNA extraction and A. astaci *detection*

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

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

Statistical analyses

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

Results

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

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

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

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

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

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

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

Similarly, a total mortality was observed after exposure to the two more virulent *A. astaci* strains. Very high and exceptionally high agent levels (A6–A7) were detected. No crayfish had moulted during the experiment. The first dead *A. astacus* were recorded on the 12th and 6th day post-inoculation with the zoospores of the Pec14 strain, and 100% mortality was reached on the 27th and 9th day, in concentrations of 10 and 100 spores ml^{-1}, respectively (Figure 1B). Furthermore, exposure to the Evira4805a/10 strain resulted in the first crayfish deaths on 8th and 7th day; no *A. astacus* survived longer than 15th or 13th day of the experimental trial (Figure 1C). Whereas no difference in mortality rate was observed between the treatments with the two spore concentrations after infection with the Evira4805a/10 (χ^2=1.3, df=1, p=0.258), the difference in mortality rate after exposure to the Pec14 strain was highly significant (χ^2=20, df=1, p≤0.001).

Prior to death, individuals of both crayfish species tended to lose their limbs (claws and legs) after the challenge with the two more virulent *A. astaci* strains. Infection with the Al7 strain was followed by limb loss only in challenged *A. astacus* (regardless

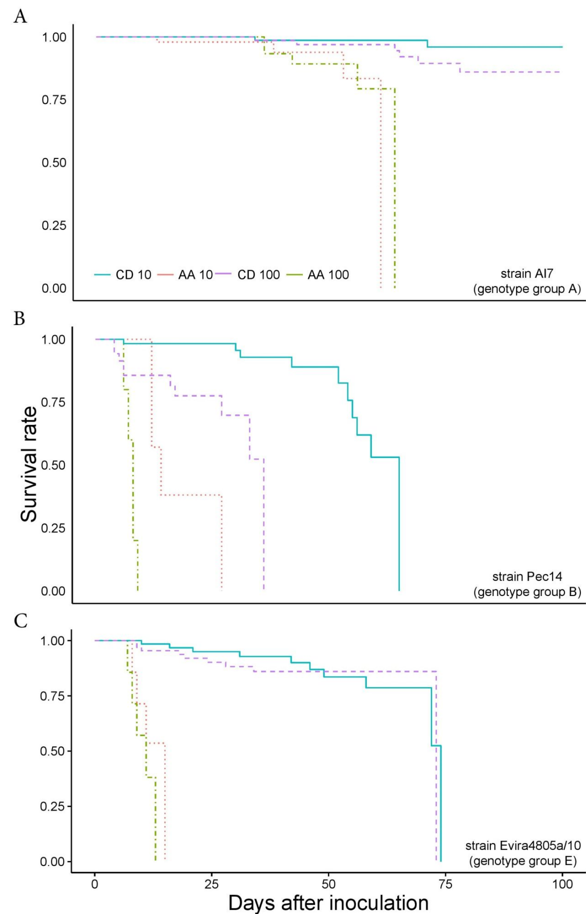

Figure 1. Kaplan-Meier survival analyses for both crayfish species: *Astacus astacus* (AA) and *Cherax destructor* (CD) after infection with three *A. astaci* strains (Al7, Pec14 and Evira4805a/10 representing genotype groups A, B and E, respectively) in two zoospore doses of 10 and 100 spores ml^{-1}.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

of the spore dose) and one *C. destructor* from the treatment with 100 spores ml^{-1}. Molecular analyses revealed exceptionally high pathogen load (more than 10^{6} PFU) in that crayfish individual.

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

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

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

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

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

Discussion

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

Long co-evolutionary history between pathogens and their hosts often results in lowered virulence of pathogens and higher resistance of hosts (May and Anderson 1990), a mechanism that explains balanced host-pathogen relationship between North American crayfish species and the crayfish plague pathogen (Cerenius et al. 2003). Recent field observations, however, provided evidence of latent *A. astaci* infections in most European native crayfish species, including *A. astacus* in Finland (Viljamaa-Dirks et al. 2011), *A. leptodactylus* in Turkey and Romania (Svoboda et al. 2012; Pârvulescu et al. 2012), the stone crayfish *Austropotamobius torrentium* in Slovenia (Schrank, 1803) (Kušar et al. 2013), the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) in Italy (Manfrin and Pretto 2014) as well as all four crayfish species in Croatia (Maguire et al. 2016). This confirms that even crayfish species generally considered highly susceptible may carry this pathogen without quickly progressing to acute infection. This phenomenon has been linked to a decreased virulence of some *A. astaci* strains belonging to genotype group A (Makkonen et al. 2012, 2014; Viljamaa-Dirks et al. 2013, 2016) but apparently other genotype groups may also be involved (see Svoboda et al. 2014a).

The variation in host resistance may contribute to chronic infections as well, as highlighted by considerably different survival rates of *C. destructor*

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

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

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

Although all *C. destructor* individuals exposed to *A. astaci* strains from the two more virulent genotype groups (B and E) died, the delayed mortality may be an indicator of its ability to slow down the progress of *A. astaci* infection. Unestam (1975) hypothesised that melanin deposition may be correlated with some degree of resistance to *A. astaci* infection in Australian yabby. In our study, the melanization on *C. destructor* individuals was sporadically observed and was mainly associated with broken limbs or injured uropods. This was most probably not directly associated with *A. astaci* infection, as melanization is a common invertebrate immune response towards any damage (Cerenius et al. 2008). Three non-exclusive reasons may explain the lack of observable *A. astaci*-associated cuticle melanization: 1) intensive moulting of juvenile crayfish, 2) micromelanization of areas of hyphal penetration (Aquiloni et al. 2011) that could be missed by the naked eye, and 3) less expressed and thus less competent immune systems of the young crayfish used in our experiment in comparison to adults. We presume that the immune response towards penetrating hyphae, i.e., encapsulation of hyphae by haemocytes and subsequent inhibition of its growth by capsule melanization (Unestam and Weiss 1970; Unestam and Nylund 1972), may be less effective in juvenile than in adult crayfish (as already observed in other groups of invertebrates; e.g., Dikkeboom et al. 1985; Dyrynda et al. 1995). If that is true, it may be expected that adult *C. destructor* may even more efficiently inhibit growth of *A. astaci* mycelium in their cuticles. Research focusing on differences in immunological responses between juvenile and adult crayfish is, however,

crucial to test this hypothesis. In any case, our results clearly demonstrate that a difference in resistance towards *A. astaci* exists between European *A. astacus* and Australian *C. destructor*, with the latter being able to slow down the infection progress even of the two more virulent *A. astaci* strains.

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

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

Acknowledgements

The Czech Science Foundation supported this work through project P505/12/0545. AM was supported by the Charles University in Prague (project SVV 260312). Partial funding was provided by the Ministry of Education, Youth, and Sports of the Czech Republic (projects CENAKVA – CZ.1.05/2.1.00/01.0024 and CENAKVA II – LO1205 under the NPU I program) and the Grant Agency of the University of South Bohemia (012/2016/Z). We thank C. Chucholl and two anonymous reviewers for valuable comments on the previous version of the manuscript.

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Journal of Invertebrate Pathology 121 (2014) 97–104

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

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

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ARTICLE INFO

Article history:
Received 7 March 2014
Accepted 16 July 2014
Available online 23 July 2014

Keywords:
Aphanomyces astaci
Crayfish plague
Freshwater shrimp
Host range
Pathogen transmission

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.

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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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http://dx.doi.org/10.1016/j.jip.2014.07.004
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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 ($N = 5$)

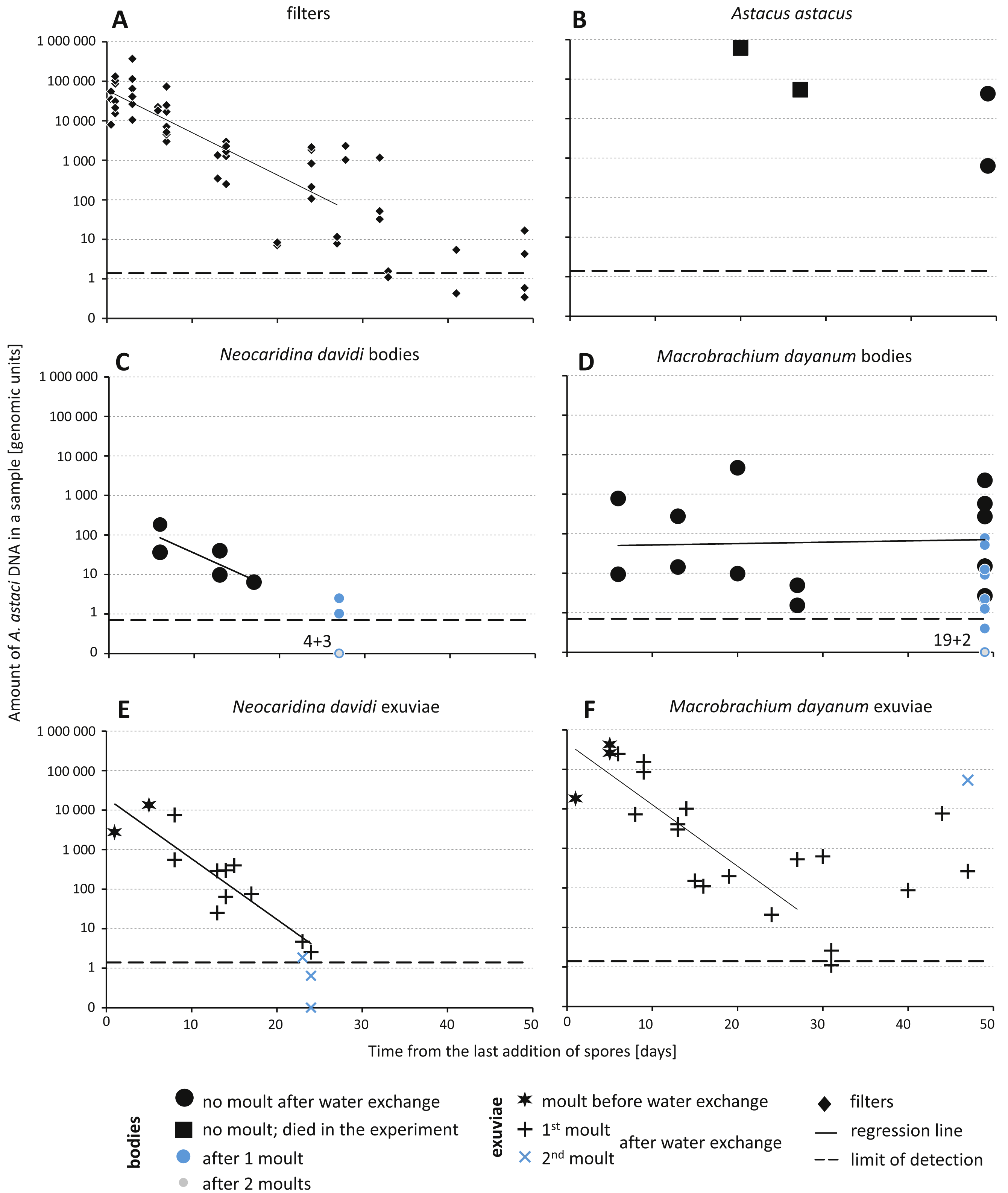

Fig. 1. Quantification of *A. astaci* DNA in samples exposed to *A. astaci* zoospores. The amount of *A. astaci* DNA isolated from filters (A), selected tissues of crayfish bodies (B), and whole bodies (C, D) and exuviae (E, F) of the shrimps *M. dayanum* and *N. davidi* is given in genomic units (C) 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.

Acknowledgments

We thank Jana Vokurková for the help with experiment maintenance, and Jiří Patoka and Pavel Šablatura, experienced breeders of ornamental crustaceans, for information on shrimp moulting frequency. J.S. and A.M. were partly funded by the Charles University in Prague (Project SVV 267204). E.K.-B. was supported by the Project No. CZ.1.07/2.3.00/30.0022 of the Education for Competitiveness Operational Programme (ECOP) co-financed by the European Social Fund and the State Budget of the Czech Republic. J.D.-U. was supported by Ministerio de Economía y Competitividad, Spain (CGL2012-39357). A.K. was funded by the projects CENAKVA (CZ.1.05/2.1.00/01.0024) and CENAKVA II (Project LO1205 with a financial support from the Ministry of Youth, Education, and Sports of the Czech Republic under the NPU I program). Three anonymous referees provided useful comments to a previous version of the manuscript.

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 8 –

Svoboda J, Mrugała A, Kozubíková-Balcarová E, Petrusek A (2016) Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *Journal of Fish Diseases*, doi: 10.1111/jfd.12472

Journal of **Fish Diseases**

Review

Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review

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Abstract

The crayfish plague pathogen, *Aphanomyces astaci* Schikora, has become one of the most well-studied pathogens of invertebrates. Since its introduction to Europe in the mid-19th century, it has caused mass crayfish mortalities, resulting in drastic declines of local populations. In contrast, North American crayfish usually serve as latent carriers, although they may also be negatively affected by *A. astaci* infections under some circumstances. Recent research benefiting from molecular tools has improved our knowledge about various aspects of *A. astaci* biology. In this review, we summarize these advances, particularly with respect to the host range and transmission. We highlight several aspects that have recently received particular attention, in particular newly confirmed or suspected *A. astaci* hosts, latent *A. astaci* infections in populations of European crayfish, and the relationship between *A. astaci* genotype groups and host taxa.

Keywords: crayfish plague, dispersal, genetic variation, host range.

The oomycete *Aphanomyces astaci* Schikora, the causative agent of crayfish plague, was probably introduced to Europe more than 150 years ago. Since then, it has decimated populations of native European crayfish (Alderman 1996) and still threatens the remaining populations of these ecologically, economically and conservationally important crustaceans (Füreder 2006; Holdich *et al.* 2009). Furthermore, experiments have indicated that Asian and Australian crayfish species will also be susceptible to crayfish plague if this pathogen is introduced to their areas of distribution (Unestam 1969b, 1975).

Thanks to decades of research, *A. astaci* is also considered one of the best-studied invertebrate pathogens (Diéguez-Uribeondo *et al.* 2006). Recently, research on the crayfish plague pathogen has received a substantial boost from newly developed tools allowing the sensitive detection and quantification of *A. astaci* DNA (including in non-symptomatic carriers and water samples; Oidtmann *et al.* 2006; Strand *et al.* 2011; Vrålstad *et al.* 2009) as well as genotyping of the pathogen in DNA isolates obtained from infected host tissues (Makkonen, Jussila & Kokko 2012a; Grandjean *et al.* 2014).

Many aspects of *A. astaci* biology have been well summarized in some recent publications. For general information on crayfish plague and some specific aspects such as the prevention of the disease, we recommend book chapters focusing on this topic (e.g. Kozubíková-Balcarová & Horká 2015; Rezinciuc *et al.* 2015). Overviews of the *A. astaci* life cycle have been provided in several reviews (e.g. Cerenius *et al.* 1988; Söderhäll & Cerenius 1999; Diéguez-Uribeondo *et al.* 2006), and the evolution of *A. astaci* virulence and resistance of its hosts has been recently discussed (Jussila *et al.* 2014a, 2016). In contrast, knowledge on the transmission of *A. astaci* was comprehensively reviewed by Oidtmann *et al.* (2002b) more

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than a decade ago. The aim of this review was thus to summarize and discuss recent advances in research on *A. astaci* with respect to its host range and transmission.

Hosts and the genetic variation of *Aphanomyces astaci*

Aphanomyces astaci has become well known as the species that causes mortalities in crayfish populations (Alderman 1996). Although other animals in localities with crayfish plague outbreaks do not seem harmed (Oidtmann 2012), the absence of harmful impacts does not mean that a particular species does not serve as a non-symptomatic host. Therefore, the host range of *A. astaci* might be wider than that currently recognized. Since the crayfish plague pathogen is only known to be transmitted to new hosts by zoospores that are restricted to freshwater environments (Unestam 1969a), all potential *A. astaci* hosts should inhabit freshwaters for at least a part of their life cycle. A vast number of crustacean groups fulfil this requirement, and their potential role as *A. astaci* hosts should be considered.

Crayfish non-indigenous to Europe

All North American crayfish species tested so far have shown a lower susceptibility to the crayfish plague pathogen than crayfish species from other regions (Table 1); they can become infected but restrict pathogen growth to their cuticle (Cerenius *et al.* 1988, 2003). This adaptation to *A. astaci* probably evolved in parallel in both North American crayfish lineages, the genus *Pacifastacus* and the species-rich family Cambaridae (Unestam 1972). It has therefore long been assumed that the crayfish plague pathogen originated in North America and that all North American crayfish

Table 1 Crayfish species experimentally tested for susceptibility to *Aphanomyces astaci*. High susceptibility – individuals frequently die after exposure to *A. astaci* spores; this class includes species classified as of low and moderate resistance by Unestam (1969b). Low susceptibility – individuals usually do not die after exposure to *A. astaci* spores; this class includes species classified as of higher resistance by Unestam (1969b). The regions of origin are characterized according to Holdich *et al.* (2006)

Species	Region of origin	Susceptibility to *A. astaci*	References
Cambarus acuminatus Faxon	North America	Low	5
Cambarus bartoni (Fabricius)	North America	Low	5
Cambarus latimanus (Le Conte)	North America	Low	5
Cambarus longulus Girard	North America	Low	5
Cambarus sp. (close to *C. extranius*)	North America	Low	5
Faxonella clypeata (Hay)	North America	Low	5
Orconectes erichsonianus Faxon	North America	Low	5
Orconectes limosus (Rafinesque)	North America	Low	5, 9
Orconectes propinquus (Girard)	North America	Low	5
Orconectes virilis (Hagen)	North America	Low	5
Pacifastacus leniusculus (Dana)	North America	Low	3, 5, 7, 8
Procambarus clarkii (Girard)	North America	Low	5
Procambarus hayi (Faxon)	North America	Low	5
Astacopsis fluviatilis Riek	Tasmania	High	8
Astacopsis gouldi Clark	Tasmania	High[a]	8
Astacus astacus (L.)	Europe	High	2, 5, 6, 7, 8, 9
Astacus leptodactylus Eschscholtz	Europe, Asia	High[a]	1, 5
Austropotamobius pallipes (Lereboullet)	Europe	High	1, 7
Austropotamobius torrentium (Schrank)	Europe	High	10
Cambaroides japonicus De Haan	Japan	High	5
Cherax destructor Clark	Australia	High[a]	8
Cherax papuanus Holthuis	Papua New Guinea	High	8
Cherax quadricarinatus (von Martens)	Australia	High	4
Cherax quinquicarinatus (Gray)	Australia	High[a]	8
Euastacus clydensis Riek	Australia	High	8
Euastacus crassus Riek	Australia	High	8
Euastacus kershawi Smith	Australia	High	7
Geocharax gracilis Clark	Australia	High[a]	8

References: 1: Alderman *et al.* (1987), 2: Diéguez-Uribeondo & Söderhäll (1993), 3: Persson & Söderhäll (1983), 4: Roy (1993) after Stephens *et al.* (2005), 5: Unestam (1969b), 6: Unestam (1969a), 7: Unestam (1972), 8: Unestam (1975), 9: Vey, Söderhäll & Ajaxon (1983), 10: Vorburger & Ribi (1999).

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

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species have elevated resistance to *A. astaci* (Unestam 1969b, 1972).

However, even North American crayfish, such as the signal crayfish *Pacifastacus leniusculus* (Dana), can succumb to *A. astaci* infection (Aydin *et al.* 2014; Edsman *et al.* 2015). Infected signal crayfish can suffer from extensive mortalities if their immune system is suppressed, which may happen during moulting, when attacked by other parasites, or in unfavourable environmental conditions (Cerenius *et al.* 2003). Aydin *et al.* (2014) suggested that *A. astaci* infections might be one of the main reasons behind recent losses in signal crayfish populations. However, neither the level of prevalence nor the crayfish plague pathogen load in infected specimens differed significantly between lakes with collapses of signal crayfish populations and lakes without those collapses (Sandström *et al.* 2014).

In European waters, eleven non-indigenous crayfish species have been found so far (Table 2) (Holdich *et al.* 2009; Kouba, Petrusek & Kozák 2014). *A. astaci* has been detected in natural populations of six of these and in captive individuals of two more species (Table 2). Some of them are not included in Table 1 (which summarizes only crayfish species experimentally exposed to *A. astaci* spores), however, as host susceptibility cannot be reliably assessed from the positive molecular detection of *A. astaci* DNA alone. Molecular detection of a low amount of pathogen DNA does not necessarily mean the respective individuals were actually infected (for example, *A. astaci* may be present in the form of attached spores). Similarly, without information on when a host individual was exposed to the pathogen spores, it is not possible to conclude that it is able to survive with the infection for long.

Thanks to effective defence mechanisms against *A. astaci* growth in their cuticles, North American crayfish can act as chronic carriers of the disease (Söderhäll & Cerenius 1999). It is likely that numerous North American crayfish species are *A. astaci* carriers in their native American range. The apparent absence of the pathogen in tested populations of some species might be due to the introduction of uninfected or weakly infected founder individuals (e.g. Schrimpf *et al.* 2013b; Tilmans *et al.* 2014). Presumably, *A. astaci*-free individuals may acquire the pathogen through contact with infected individuals, even of another host species. Such horizontal transmission between species has apparently occurred in aquarium facilities of the pet trade, with the red swamp crayfish *Procambarus clarkii* (Girard) being the likely source (Mrugała *et al.* 2015). The detection of *A. astaci* in the Australian redclaw crayfish *Cherax quadricarinatus* (von Martens) almost certainly resulted from such a horizontal transmission within pet shop facilities, or during handling and packing (Mrugała *et al.* 2015).

Genotype groups of *A. astaci*

Using random amplified polymorphic DNA (RAPD), five genotype groups of *A. astaci* have

Table 2 Non-indigenous crayfish taxa found in European waters and the detection of *Aphanomyces astaci* in these taxa

Species	Region of origin	*A. astaci* detection in wild populations; genotype group (references)	*A. astaci* detection in pet trade or aquaculture; genotype group (references)
Cherax destructor Clark	Australia		
Cherax quadricarinatus (von Martens)	Australia		Yes, unknown (4, 9)
Orconectes immunis (Hagen)	North America	Yes; unknown (6, 10)	
Orconectes juvenilis (Hagen)	North America		
Orconectes limosus (Rafinesque)	North America	Yes; E (3)	Yes, unknown (4)
Orconectes virilis (Hagen)	North America	Yes; unknown (5)	
Pacifastacus leniusculus (Dana)	North America	Yes; B, C (1)	
Procambarus cf. *acutus* (Girard)	North America		
Procambarus alleni (Faxon)	North America		Yes, D (4)
Procambarus clarkii (Girard)	North America	Yes, D (2, 8)	Yes, D (4)
Procambarus fallax f. *virginalis* Martin	North America	Yes, unknown (7)	Yes, D (4, 7)

References: 1: Huang *et al.* (1994), 2: Diéguez-Uribeondo *et al.* (1995), 3: Kozubíková *et al.* (2011a), 4: Mrugała *et al.* (2015), 5: Tilmans *et al.* (2014), 6: Schrimpf *et al.* (2013a), 7: Keller *et al.* (2014), 8: Rezinciuc *et al.* (2014), 9: Marino *et al.* (2014), 10: Filipová *et al.* (2013). Additional studies have reported *A. astaci* in some of these species (particularly *O. limosus*, *P. leniusculus*, *P. clarkii*); the table preferentially includes those reporting the *A. astaci* genotype group found in host tissues.

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been recognized so far (Huang, Cerenius & Söderhäll 1994; Diéguez-Uribeondo *et al.* 1995; Kozubíková *et al.* 2011a). Group A comprises the first genotype group that invaded Europe; it was isolated from infected crayfish of the genus *Astacus* and its original host remains unknown (Huang *et al.* 1994). Other genotype groups are linked to different North American host species that are widespread in Europe (Holdich *et al.* 2009; Kouba *et al.* 2014): the signal crayfish *P. leniusculus* (genotype groups B and C; Huang *et al.* 1994), the red swamp crayfish *P. clarkii* (group D; Diéguez-Uribeondo *et al.* 1995) and the spiny-cheek crayfish *Orconectes limosus* (Rafinesque) (group E; Kozubíková *et al.* 2011a). Strains of different genotype groups may differ in their virulence (Makkonen *et al.* 2012b, 2014; Viljamaa-Dirks *et al.* 2013, 2015; Becking *et al.* 2015) and climate requirements (Diéguez-Uribeondo *et al.* 1995; Rezinciuc *et al.* 2014).

Aphanomyces astaci strains of different genotype groups can also be differentiated by amplified fragment length polymorphism (AFLP) (Rezinciuc *et al.* 2014) and by the recently developed microsatellite genotyping (Grandjean *et al.* 2014). In contrast to RAPD or AFLP, microsatellite genotyping can be used also to analyse mixed-genome DNA isolates obtained 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 evaluating *A. astaci* horizontal transmission (Svoboda *et al.* 2014b). Additionally, the method can recognize new *A. astaci* genotypes, even those that would be characterized as belonging to the same genotype group by RAPD (Grandjean *et al.* 2014).

Strains from genotype groups A, B, D and E have been detected in crayfish plague outbreaks across Europe (e.g. Viljamaa-Dirks *et al.* 2013; Grandjean *et al.* 2014; Kozubíková-Balcarová *et al.* 2014; Rezinciuc *et al.* 2014). New data on the presence of *A. astaci* in the aquarium trade (Mrugała *et al.* 2015) suggest that *A. astaci* strains can be horizontally transmitted between various North American crayfish species, since, for example, the marbled crayfish *Procambarus fallax* f. *virginalis* Martin and *P. alleni* (Faxon) hosted a strain from genotype group D, that is the group originally isolated from *P. clarkii*. Although it cannot be entirely ruled out that all those species are natural hosts of this genotype group, at least for the parthenogenetically reproducing marbled crayfish that is widespread in the aquarium trade but unknown from natural waters in North America, horizontal pathogen transmission seems likely.

The genotype groups of *A. astaci* have also been referred to as strains belonging to genotypes 1, 2, 3 and 4 (Andersson & Cerenius 2002), or as the *Astacus* strain, *Pacifastacus* strain I, *Pacifastacus* strain II and *Procambarus* strain (Oidtmann *et al.* 2002a), or in 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 information about the species from which a strain belonging to the group was originally isolated (e.g. As stands for *Astacus*). However, as the pathogen can be transmitted horizontally among different hosts, 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 (although Huang *et al.* (1994) used the letters only when referring to clusters in a dendrogram). The abbreviations 'PsI' and 'PsII' do not appear in the study of Huang *et al.* (1994) at all, and 'Pc' was originally used as the name for a strain, not a genotype group (Diéguez-Uribeondo *et al.* 1995). Therefore, we believe that the system of *A. astaci* genotype groups being labelled alphabetically (A to E so far) is preferable for keeping the nomenclature consistent and simple. Regardless of the nomenclature used, however, it is important to differentiate between specific strains and genotype groups (which may comprise multiple genetically distinct strains, differing in some important characteristics such as virulence).

Latent infections of European crayfish species

In contrast to North American crayfish, the immune response to *A. astaci* in European, Asian and Australian crayfish species is so weak that the individuals usually die soon after infection (Cerenius *et al.* 2003; Table 1). However, some variation in susceptibility has been observed under laboratory conditions in two Western Palaearctic crayfish species. First, while some individuals of the narrow-clawed crayfish *Astacus leptodactylus* Eschscholtz survived exposure to *A. astaci* in some experiments (Unestam 1969b), all individuals exposed to *A. astaci* spores died in experiments by Alderman, Polglase & Frayling (1987). Second,

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the ability of the noble crayfish *Astacus astacus* (L.) to survive for several weeks with infections by some *A. astaci* strains has also been confirmed in laboratory conditions (Makkonen *et al.* 2012b, 2014; Becking *et al.* 2015).

In agreement with these experimental results, latent infections, that is individual crayfish being positive for *A. astaci* without the crayfish population suffering mass mortalities or showing gross signs of infection (Jussila *et al.* 2014a), have recently been reported in some populations of *A. leptodactylus* in Turkey (Kokko *et al.* 2012; Svoboda *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 have revealed the presence of at least two evolutionary lineages (Maguire *et al.* 2014), the results of infection with *A. astaci* might vary because individuals belonging to different lineages show different levels of resistance. However, latent infections have also been detected in some populations of *A. astacus* in Finland (Jussila *et al.* 2011b; Viljamaa-Dirks *et al.* 2011, 2013), as well as in populations of the stone crayfish *Austropotamobius torrentium* (Schrank) in Slovenia (Kušar *et al.* 2013) and the white-clawed crayfish *A. pallipes* (Lereboullet) in Italy (Manfrin & Pretto 2014).

Some populations of crayfish species originally classified as being of low and moderate resistance (Unestam 1969b) can even sustain commercial exploitation despite latent infections with the pathogen (Jussila *et al.* 2011b; Svoboda *et al.* 2012). We therefore believe that sorting hosts to the three categories of low, moderate and high resistance as suggested by Unestam (1969b) should be simplified, and only two levels of susceptibility to the crayfish plague pathogen, low and high, should be used. (Considering that even North American crayfish can succumb to infection by *A. astaci*, it is better to avoid the term 'resistant' in this context.) The species that usually do not die after exposure to *A. astaci* spores (i.e. the North American crayfish species) are of low susceptibility (Table 1), whereas those that frequently die (i.e. crayfish from Europe, Asia, Australia, Tasmania and New Guinea) are of high susceptibility. We are not aware of any study exposing parastacid crayfish from South America and Madagascar to *A. astaci* but considering their systematic position, they are likely to be susceptible as well.

The mechanism enabling latent infections of generally susceptible species can lie on either side of the host–parasite interactions between crayfish and *A. astaci*. The extent and timing of crayfish mortality may depend on the virulence of the particular *A. astaci* strain (Makkonen *et al.* 2012b; Jussila *et al.* 2013). In some cases of latent infections in susceptible crayfish species, the presence of genotype group A was identified (e.g., Jussila *et al.* 2011b; Viljamaa-Dirks *et al.* 2011, 2013; Manfrin & Pretto 2014), and in other cases, its involvement was assumed (Caprioli *et al.* 2013; Kušar *et al.* 2013). Jussila *et al.* (2014a) hypothesized that decreased virulence of *A. astaci* strains from this genotype group is a result of the more than a century-long coexistence of the crayfish plague pathogen and European crayfish species. The survival of noble crayfish after experimental exposure to zoospores from *A. astaci* strains of this genotype group from geographically distinct sources (originating either from Fennoscandia: Makkonen *et al.* 2014; or the Ponto–Caspian region: Becking *et al.* 2015) may be regarded as evidence for this hypothesis. However, latent infections in which genotype group B was involved have also been reported (Svoboda *et al.* 2012). Similarly, some noble crayfish individuals have apparently survived infection with a strain from genotype group B for weeks in laboratory experiments (Jussila, Makkonen & Kokko 2011a; Jussila *et al.* 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 (A, B or E), nor any differences in subsequent recovery of the affected crayfish populations.

The outcome of infection also depends 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, that is related to the stress and physiological condition of the host (Jussila *et al.* 2011b) and the presence of other pathogens (Jussila *et al.* 2013).

Crabs, shrimps and non-decapod crustaceans

Apart from crayfish, a few other taxa have been tested for resistance to *A. astaci* (Table 3). Over

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Table 3 Animals other than crayfish tested for resistance to *Aphanomyces astaci*

Species	Taxon	References
Eriocheir sinensis H. Milne-Edwards	Decapoda: Brachyura	1
Macrobrachium dayanum (Henderson)	Decapoda: Caridea	4
Neocaridina davidi Kubo	Decapoda: Caridea	4
Mysis relicta Lovén	Mysida	3
Daphnia longispina (O.F. Müller)	Branchiopoda: Cladocera	2
Leptodora kindtii (Focke)	Branchiopoda: Cladocera	2
Chydorus sphaericus (O.F. Müller)	Branchiopoda: Cladocera	2
Bythotrephes longimanus Leydig	Branchiopoda: Cladocera	2
Bosmina sp.	Branchiopoda: Cladocera	2
Cyclops strenuus Fischer	Maxillopoda: Cyclopoida	2
Mesocyclops leuckarti (Claus)	Maxillopoda: Cyclopoida	2
Eudiaptomus graciloides (Lilljeborg)	Maxillopoda: Calanoida	2
Asplanchna priodonta Gosse	Rotifera: Monogononta	2

References: 1: Benisch (1940); 2: Unestam (1969b); 3: Unestam (1972), 4: Svoboda *et al.* (2014a). Benisch (1940) suggested that Chinese mitten crabs *E. sinensis* may suffer higher mortality due to *A. astaci* infections; all other tested species seem resistant.

70 years ago, Chinese mitten crabs *Eriocheir sinensis* H. Milne-Edwards were reported to be infected and killed by the pathogen (Benisch 1940) and Unestam (1972) thus assumed that *A. astaci* may infect not only crayfish but freshwater decapods in general. Although the identification of the pathogen involved in the study of Benisch (1940) was not entirely convincing, infection with *A. astaci* has recently been confirmed in two freshwater-inhabiting crab species from multiple localities (Schrimpf, Schmidt & Schulz 2014; Svoboda *et al.* 2014b; Tilmans *et al.* 2014). The crayfish plague pathogen was detected by microscopic and molecular methods in *E. sinensis* coexisting with *A. astaci*-infected signal crayfish *P. leniusculus* in lake Vänern (Sweden) and in the semi-terrestrial crab *Potamon potamios* (Olivier) coexisting with infected narrow-clawed crayfish *A. leptodactylus* in the Turkish lake Eğirdir (Svoboda *et al.* 2014b). Moreover, *A. astaci* was detected by molecular methods in specimens of *E. sinensis* from three localities in the river Rhine in Germany where they coexist with spiny-cheek crayfish *O. limosus* and calico crayfish *O. immunis* (Hagen) (Schrimpf *et al.* 2014), confirmed to carry the crayfish plague pathogen (Schrimpf *et al.* 2013a), and in the Netherlands where these crabs coexist with *A. astaci*-infected *O. limosus* (Tilmans *et al.* 2014). It is likely that crabs become infected from local crayfish populations, as suggested by results of microsatellite genotyping of pathogen strains in samples from lakes Vänern and Eğirdir (Svoboda *et al.* 2014b). Although molecular detections alone cannot be considered an ultimate confirmation of infection, Schrimpf *et al.* (2014) demonstrated that *A. astaci* could be transmitted from infected *E. sinensis* to susceptible crayfish.

The results of laboratory exposure of the freshwater shrimps *Neocaridina davidi* Kubo and *Macrobrachium dayanum* (Henderson) to pathogen spores indicated that these shrimps are resistant to *A. astaci* (Table 3); however, the results also suggested that some growth of the pathogen may have occurred in at least some individuals and the exuviae of *M. dayanum* (Svoboda *et al.* 2014a). Further experiments are needed to evaluate this assumed pathogen growth and to test whether *A. astaci* can sporulate from shrimp hosts and thus further spread the infection.

Apart from the closest relatives of crayfish, that is crabs and shrimps, several other freshwater invertebrates have been exposed to *A. astaci* (Table 3), without any apparent mycelium growth in their bodies. Although some studies have reported that *A. astaci* was isolated from dead crustaceans other than decapods, for example amphipods and isopods (Czeczuga, Kozłowska & Godlewska 1999; Czeczuga, Kozłowska & Godlewska 2002), *A. astaci* was identified according to morphology only, even though it cannot be distinguished from its congeners by such traits (Oidtmann 2012). Therefore, it is likely that *Aphanomyces* strains isolated from crustaceans and reported as *A. astaci* by Czeczuga *et al.* (1999, 2002) were actually some saprophytic species (see, e.g., Diéguez-Uribeondo *et al.* 2009).

Molecular methods to screen for the presence of *A. astaci* in non-symptomatic hosts have been available for almost a decade (Oidtmann *et al.* 2006; Vrålstad *et al.* 2009). Nevertheless, no study has focused in detail on potential non-decapod crustacean hosts. Some pilot results were included in the study of Svoboda *et al.* (2014b): several individuals of the benthopelagic mysid *Mysis relicta* Lovén, the amphipod *Pallasea quadrispinosa* Sars and the benthic isopod *Asellus*

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aquaticus (L.) were not found to be infected with *A. astaci* despite the presence of the pathogen in coexisting crayfish populations. This corresponds with the fact that other aquatic animals coexisting with infected crayfish in natural localities do not seem affected by the pathogen (Oidtmann 2012). In addition, the crayfish plague pathogen usually does not survive long in the absence of a suitable host; any exceptions can be explained through other mechanisms such as latent infections or reintroductions of the pathogen (Oidtmann 2012). However, the possibility that some other crustaceans may become accidental hosts of the crayfish plague pathogen, for example when stressed, still cannot be rejected.

Transmission of *Aphanomyces astaci*

The only known infectious forms of *A. astaci* are spores, that is zoospores and cysts (Oidtmann *et al.* 2002b), which apparently only survive in freshwater (Unestam 1969a). Spores of *A. astaci* transmit the disease horizontally among distinct host individuals; this is undoubtedly the dominant mode of the pathogen dispersal.

Vertical transmission, in which the disease is spread from one generation to the next by infected eggs, was not supposed to be relevant for *A. astaci* (Stephens *et al.* 2005). Makkonen *et al.* (2010) nevertheless reported the detection of *A. astaci* DNA from the eggs of infected females and in one of the tested groups of artificially incubated newly hatched juveniles using molecular detection targeting the *A. astaci* chitinase gene. However, the positive detection was not supported by a quantitative PCR (qPCR) method according to Vrålstad *et al.* (2009) (Makkonen *et al.* 2010), although this method is very sensitive and able to detect a single *A. astaci* zoospore (Tuffs & Oidtmann 2011). Furthermore, as *A. astaci* spores and their DNA can persist for several weeks (Svoboda *et al.* 2014a), the detection of *A. astaci* DNA in eggs taken from infected females does not necessarily mean there was an actual infection, especially if the amount of detected *A. astaci* DNA is low. Thus, direct vertical transmission through eggs remains to be proven. Nevertheless, crayfish juveniles might get 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).

Even when we assume that transmission of *A. astaci* is limited only to spores in freshwater environments, there are still many possible pathways of pathogen dispersal (Oidtmann *et al.* 2002b). Generally, the crayfish plague pathogen may disperse from one locality to another either by the spores themselves, or in the tissues of infected hosts (from which the spores are released at a new locality).

Introductions and human-mediated transfer of live hosts

Human activities have played the most important role in the dispersal of the crayfish plague pathogen. The pathogen itself was most probably introduced to Europe due to transoceanic shipping (Alderman 1996). During the first decades of the pathogen spread, the wholesale trade of European crayfish and transport of contaminated crayfishing equipment substantially facilitated the dispersal of the disease (Alderman 1996). Moreover, people have intentionally introduced several North American crayfish species to European freshwaters. The first three American crayfish species introduced to Europe, *O. limosus*, *P. leniusculus* and *P. clarkii*, were released to boost crayfish stocks decimated by crayfish plague (Holdich *et al.* 2006). Although, as later shown, all three species frequently carry and transmit the crayfish plague pathogen (Holdich *et al.* 2009), they are still sometimes spread both legally and illegally (Holdich *et al.* 2006). In addition, *A. astaci* hosts might be transported unintentionally, for example during fish transport or shipping.

While the above-mentioned crayfish species were introduced to Europe for aquaculture purposes, recent species introductions have resulted from illegal stocking activities, such as aquarium releases, the use of live fishing bait or garden pond escapes (Chucholl 2013). Currently, two Central European countries, Germany and the Czech Republic, seem to be the leaders in crayfish imports (Chucholl 2013; Patoka, Kalous & Kopecký 2014). In total, 120 non-indigenous crayfish species have been available in the German ornamental crayfish trade, most of them of North or Central American origin and thus suspected to be crayfish plague vectors (Chucholl 2013). Indeed, a pilot screening of ornamental crayfish confirmed infections in some of them (Mrugała *et al.* 2015).

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Moreover, freshwater-inhabiting crabs have been confirmed as potential long-term hosts and vectors of *A. astaci* (Schrimpf *et al.* 2014; Svoboda *et al.* 2014b). Chinese mitten crabs are intensively harvested and produced in aquacultures in South-East Asia (FAO 2012). Although the aquaculture as well as intentional transport and stocking of *E. sinensis* is not common in Europe, these 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).

The movement of infected hosts and transmission through tissues of dead individuals

The crayfish plague pathogen can be dispersed by the movement of infected crayfish (Oidtmann *et al.* 2002b). Different types of migration barriers, both natural and man-made, might prevent the movement of crayfish, and at least occasionally stop the spread of the disease during crayfish plague outbreaks (e.g. Frings *et al.* 2013; Kozubíková-Balcarová *et al.* 2014; Benejam *et al.* 2015). Although man-made barriers are frequently removed to restore connectivity within aquatic habitats, intentional fragmentation may actually be an efficient strategy to prevent the spread of diseases including crayfish plague (Rahel 2013).

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

The crayfish plague may also be spread by dead hosts or their body parts, with dead crayfish bodies likely remaining a source of infection for at least 5 days at 21 °C, and probably longer at lower temperatures (Oidtmann *et al.* 2002b). Nearly 600 000 tonnes of the confirmed North American *A. astaci* carrier *P. clarkii* is produced and sold every year (FAO 2012) for culinary purposes. Fortunately, the pathogen can be eliminated by both low and high temperatures, for example either 1 week of freezing at −5 °C or 1 min at 100 °C is lethal for *A. astaci* (Alderman 2000; Oidtmann *et al.* 2002b).

Dead crayfish or their body parts can be also transported by other animals. The transmission of *A. astaci* through the digestive tract of fish has already been demonstrated (Oidtmann *et al.* 2002b). However, it seems unlikely that *A. astaci* can be transmitted through the digestive tract of warm-blooded predators. In a pilot exposure experiment, the pathogen was not transmitted through the excrements of two mammalian predators to susceptible stone crayfish (Svoboda 2015). In addition, experiments testing *A. astaci* survival at the body temperatures of mammals and birds have shown that the sole effect of temperature should prevent the pathogen spread through their digestive tracts (Oidtmann *et al.* 2002b, Svoboda 2015), unless the gut passage time of the crayfish body parts is extremely fast or they are regurgitated. Therefore, pathogen transmission through the digestive tract of warm-blooded predators is probably even less likely than the potential transmission of *A. astaci* spores on their surface.

The dispersal of *Aphanomyces astaci* spores

The success of *A. astaci* infection depends on the number of spores the host is exposed to (Unestam & Weiss 1970; Alderman *et al.* 1987; Diéguez-Uribeondo *et al.* 1995; Makkonen *et al.* 2014). However, the lethal dose probably varies with the virulence of the particular *A. astaci* strain and the susceptibility of the particular crayfish population (see, e.g., Makkonen *et al.* 2012b; Jussila *et al.* 2013). High *A. astaci* prevalence in a crayfish population and high pathogen loads in infected crayfish generally lead to a higher spore density in the water (Strand *et al.* 2014), and very high spore concentrations may be found in tanks where large numbers of crayfish are kept (Strand *et al.* 2011). Concentrations of *A. astaci* spores can be several

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hundred per litre in a river with a crayfish plague outbreak, while the results obtained in localities with North American crayfish varied from no detection of *A. astaci* to ca 100 spores L^{-1} (Strand *et al.* 2014). These results correspond to previous laboratory studies, which 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; Makkonen *et al.* 2013; Svoboda *et al.* 2013). The concentrations also vary among different microhabitats in a water body (Strand *et al.* 2012, 2014).

The lifespan of *A. astaci* spores has not yet been assessed in detail. Unestam (1969a) found that a spore suspension kept at 14 °C infected all crayfish placed in the spore water 6 days after addition of the spores, but not after 15 days. In sterile laboratory conditions, *A. astaci* zoospores remain motile for up to 5 days at 2 °C (Unestam 1966); however, even after two months, a spore suspension of *A. astaci* stored at 2 °C contained some viable spores (Unestam 1966). The half-life of *A. astaci* DNA isolated from inert substrates immersed in an *A. astaci* spore suspension was estimated at 3.1 days by qPCR-based quantification (Svoboda *et al.* 2014a). This suggests that the half-life of the spores themselves at 20 °C might be no more than three days (likely less, as the short fragments of DNA used for qPCR-based detection in that study were probably detectable even some time after spore death). However, the survival of spores in various natural conditions remains to be investigated.

Aphanomyces astaci apparently does not produce oospores, which in a typical oomycete life cycle serve to resist dry periods and extreme temperatures (Diéguez-Uribeondo *et al.* 2009). However, Unestam (1969a) reported that in a synthetic medium *A. astaci* may form thick-walled hyphal portions or structures similar to gemmae (i.e. segments of hyphae serving as asexual propagules in Saprolegniales). When we cultivated *A. astaci* in the same medium, we also observed a few round structures which morphologically resembled those described by Unestam (J. Svoboda unpublished data). However, to the best of our knowledge, no one has investigated whether these structures play any specific role in the life cycle of the species, for example if they are more resistant to stressful conditions.

Since *A. astaci* spores are sensitive to desiccation (Alderman & Polglase 1986; Smith & Söderhäll 1986), the dispersal of *A. astaci* spores in natural conditions seems to be facilitated mostly by the flow of water within a watershed. The transmission of *A. astaci* on the surface of fish seems improbable because of the continuous production and anti-infectious properties of fish mucus (Oidtmann *et al.* 2002b). The surfaces of animals not restricted to freshwaters (e.g. birds and mammals) may be suitable substrates for the occasional transport of *A. astaci* spores among watersheds. Nevertheless, lakes only several kilometres from crayfish plague outbreaks were not struck by the disease although water birds moved among these localities (Unestam 1973). Successful transmission of the disease on the surface of mammals and birds thus seems rather unlikely, although such low-probability events cannot be entirely ruled out.

Although some *Aphanomyces* species can withstand salinity of 20 ppt (Dykstra *et al.* 1986), *A. astaci* is more sensitive to higher salinities (Unestam 1969a). According to Unestam's experiments, it is improbable that *A. astaci* could survive in sea or brackish water; a mineral salt solution of lower salinity than sea water drastically reduced zoospore production and prevented spore release into the medium (Unestam 1969a). However, the concentrations of salts in the mixtures tested by Unestam (1969a) did not correspond to those found in brackish water. As the reaction of *A. astaci* to the same concentrations of different cations varies (Cerenius & Söderhäll 1984), and one cation might influence the effects of another (Söderhäll & Cerenius 1987), it would be prudent to further evaluate *A. astaci* survival in various salinities. In particular, it should be tested which salt concentrations effectively limit the pathogen spread, whether there is any variation in sensitivity to salinity among *A. astaci* strains and thus whether *A. astaci* can survive in brackish water long enough to infect a new host (in conditions relevant, e.g., in the Black and Caspian Seas, where native European crayfish are found; Kouba *et al.* 2014).

The potential to disperse *A. astaci* spores during many of the activities of humans is high, but dispersal can largely be prevented. Items that have been in contact with water from a locality with *A. astaci*-infected hosts (e.g. fishing, crayfishing, or diving gear) should be cleaned of organic matter first (Jussila *et al.* 2014b), preferentially with hot

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water. Subsequently, the disinfectants Proxitane® 5:14, Virkon® S (Jussila *et al.* 2014b), sodium hypochlorite (Alderman & Polglase 1985) or iodophors (Alderman & Polglase 1985; Lilley & Inglis 1997) may be applied, or the items should be at least thoroughly dried (Smith & Söderhäll 1986; Alderman *et al.* 1987). Water without fish can be decontaminated using peracetic acid at a concentration of 10 mg L^{-1} (Jussila *et al.* 2011a).

It has been shown that malachite green may prevent the transmission of *A. astaci* through water used in fish transport (Alderman *et al.* 1987; Lilley & Inglis 1997). However, the use of this dye has been banned in several countries because of its potential carcinogenicity, mutagenicity and teratogenicity; for example, the European Council imposed a strict ban on the use of malachite green for all age categories of fish intended for human consumption (Srivastava, Sinha & Roy 2004; Sudová *et al.* 2007). Furthermore, the effective concentration of peracetic acid that prevents *A. astaci* spore germination is considered unsuitable for fish intended for consumption (Jussila *et al.* 2011a). The potential of some other disinfectants for eliminating *A. astaci* has already been tested: formaldehyde and potassium permanganate (Häll & Unestam 1980), sodium chloride, hydrogen peroxide, sodium hypochlorite and FAM30®, acetic acid and povidone iodine (Lilley & Inglis 1997; Fuangsawat, Abking & Lawhavinit 2011). However, further studies should determine the most appropriate concentrations and immersion times, focus on the toxicity to transported fish and eventually deliver a protocol for the routine decontamination of water during the transport of fish intended for human consumption.

Future perspectives

There is no doubt that research on the crayfish plague will continue. Further studies will likely benefit from recently developed molecular tools, especially the sensitive detection and quantification of *A. astaci* DNA by species-specific quantitative PCR (Vrålstad *et al.* 2009), and direct genotyping of *A. astaci* from infected host tissues (Grandjean *et al.* 2014). Further techniques, such as fluorescence *in situ* hybridization, may also be developed for *A. astaci.* Any detection methods, however, must also be tested against the other oomycetes living in or on the crayfish cuticle (Kozubíková-Balcarová *et al.* 2013) to avoid potential cross-reactions with related taxa (see Kozubíková *et al.* 2009, 2011b). We hope that future research on *A. astaci* will contribute to the conservation of susceptible crayfish species as well as to the mitigation of negative effects caused by the crayfish plague pathogen.

Acknowledgements

We thank Ondřej Koukol for his long-time support of our experimental work with *A. astaci* cultures and feedback on the biology of fungus-like organisms, two anonymous referees for comments on the previous version of the manuscript and David Hardekopf for language corrections. Our research was partly funded by the Czech Science Foundation (project no. P505/12/0545). A.M. and J.S. were supported by Charles University in Prague (project SVV 260198).

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Received: 15 October 2015
Revision received: 25 January 2016
Accepted: 28 January 2016

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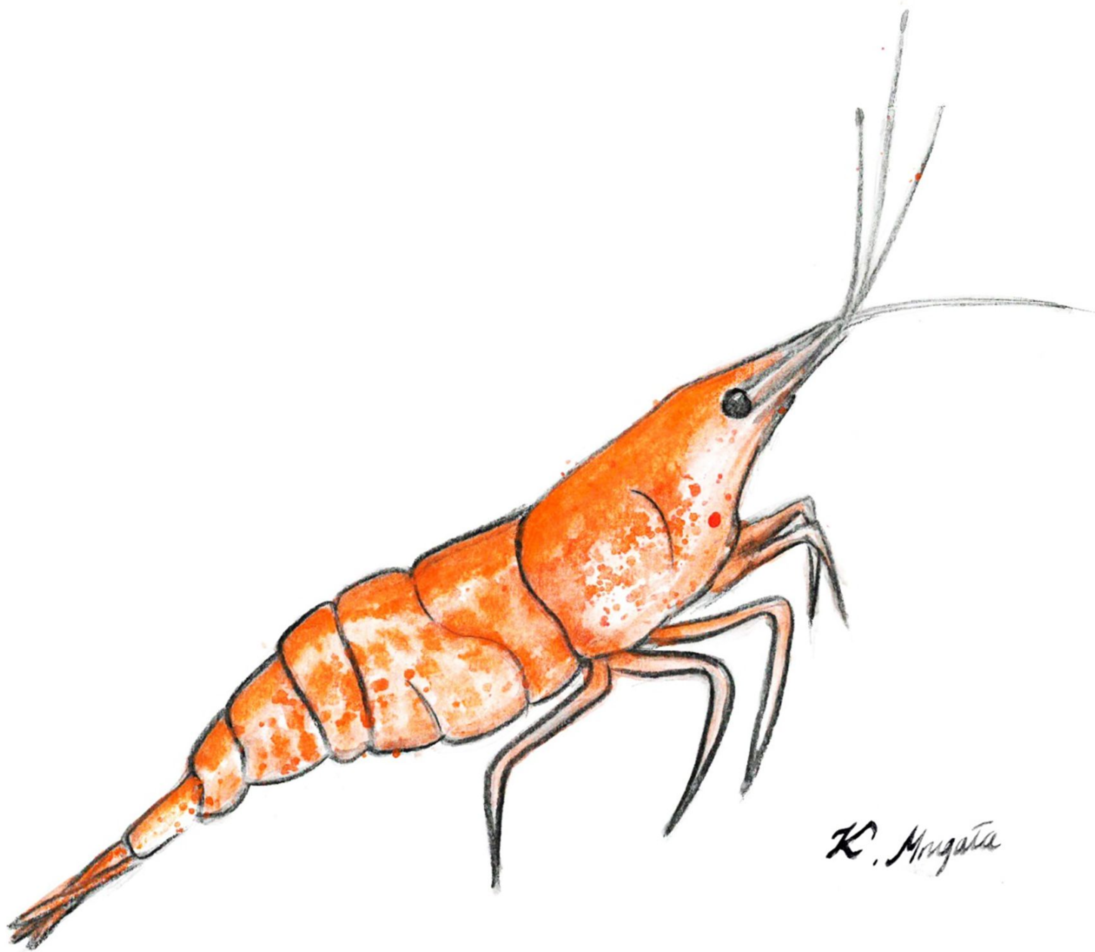
ATTACHED APPENDICES



– appendix 1 –

Jeschke JM, Bacher S, Blackburn TM, Dick JTA, Essl F, Evans T, Gaertner M, Hulme PE, Kühn I, Mrugała A, Pergl J, Pyšek P, Rabitsch W, Ricciardi A, Richardson DM, Sendek A, Vilà M, Winter M, Kumschick S (2014) Defining the Impact of Non-Native Species. *Conservation Biology* 28: 1188-1194

Conservation Biology

Essay

Defining the Impact of Non-Native Species

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Abstract: *Non-native species cause changes in the ecosystems to which they are introduced. These changes, or some of them, are usually termed impacts; they can be manifold and potentially damaging to ecosystems and biodiversity. However, the impacts of most non-native species are poorly understood, and a synthesis of available information is being hindered because authors often do not clearly define impact. We argue that explicitly defining the impact of non-native species will promote progress toward a better understanding of the implications of changes to biodiversity and ecosystems caused by non-native species; help disentangle which aspects of scientific debates about non-native species are due to disparate definitions and which represent true scientific discord; and improve communication between scientists from different research disciplines and between scientists, managers, and policy makers. For these reasons and based on examples from the literature, we devised seven key questions that fall into 4 categories: directionality, classification and measurement, ecological or socio-economic changes, and scale. These questions should help in formulating clear and practical definitions of impact to suit specific scientific, stakeholder, or legislative contexts.*

Keywords: biological invasions, definitions, ecological and socio-economic impacts, exotic species, human perception, invasion biology, invasive alien species

Paper submitted August 14, 2013; revised manuscript accepted January 14, 2014.

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DOI: 10.1111/cobi.12299

Definiendo el Impacto de las Especies No-Nativas

Resumen: *Las especies no-nativas pueden causar cambios en los ecosistemas donde son introducidas. Estos cambios, o algunos de ellos, usualmente se denominan como impactos; estos pueden ser variados y potencialmente dañinos para los ecosistemas y la biodiversidad. Sin embargo, los impactos de la mayoría de las especies no-nativas están pobremente entendidos y una síntesis de información disponible se ve obstaculizada porque los autores continuamente no definen claramente impacto. Discutimos que definir explícitamente el impacto de las especies no-nativas promoverá el progreso hacia un mejor entendimiento de las implicaciones de los cambios a la biodiversidad y los ecosistemas causados por especies no-nativas; ayudar a entender cuáles aspectos de los debates científicos sobre especies no-nativas son debidos a definiciones diversas y cuáles representan un verdadero desacuerdo científico; y mejorar la comunicación entre científicos de diferentes disciplinas y entre científicos, administradores y quienes hacen las políticas. Por estas razones y basándonos en ejemplos tomados de la literatura, concebimos siete preguntas clave que caen en cuatro categorías: direccionalidad, clasificación y medida, cambios ecológicos o socio-económicos, y escala. Estas preguntas deberían ayudar en la formulación de definiciones claras y prácticas del impacto para encajar mejor con contextos científicos, de las partes interesadas o legislativos específicos.*

Palabras Clave: biología de la invasión, especies exóticas, especies foráneas invasoras, definiciones, impactos ecológicos y socio-económicos, invasiones biológicas, percepción humana

A Call to Define the Impact of Non-Native Species

The introduction of species beyond their native range as a direct or indirect result of human action (termed *non-native species* here) causes changes in the ecosystems to which they are introduced. In some cases, these changes are dramatic and may result in the extinction of native species or radical changes in ecosystem functioning, but for the vast majority of non-native species no quantitative information is available on the consequences of such introductions (Kulhanek et al. 2011; Larson et al. 2013; Simberloff et al. 2013). We do know that the impacts of non-native species generally increase if the species establish themselves and spread in their new environment (i.e., if they become invasive sensu Blackburn et al. [2011]), but non-native species can have impacts even when they are not established or widespread (Ricciardi & Cohen 2007; Jeschke et al. 2013; Ricciardi et al. 2013). Indeed, non-native species can have impacts as soon as they are introduced; for example, pathogens can affect the health of animals, plants, or other organisms immediately after their arrival in the new environment. The breadth and potential severity of the impacts of non-native species means that a better understanding of them is of broad relevance, for example, for prioritizing management, conservation and restoration actions, and for appropriate policy responses to invasions.

Our collective experience is that progress toward this understanding is being hindered because authors often do not explicitly or clearly define the impacts of non-native species. The current literature on impacts is complicated by a plethora of different approaches to their quantification that are associated with a concomitant range of impact metrics (Hulme et al. 2013). We argue that if authors are routinely explicit about their definition of impacts of non-native species, it will be possible to synthesize the growing body of work on this topic more effectively. For instance, systematic reviews, comparative analyses, and meta-analyses (Koricheva et al. 2013) can be much more informative if the authors of studies included in such assessments clearly define impact and clearly explain how impact was measured. If authors do not, the synthesis of available data can become difficult or even impossible.

Explicit definitions of impact will also help disentangle which aspects of scientific debates about non-native species (see e.g., Gurevitch & Padilla 2004; Ricciardi 2004; Davis et al. 2011; Simberloff et al. 2011) are due to disparate definitions (including spatio-temporal scale, taxonomic focus, and consideration of human values) and which represent true scientific discord (i.e., a difference of opinion on a mutually understood argument, rather than on disjunct arguments). Distinguishing between these will help identify questions that should be research priorities.

A third reason for explicitly defining the impact of non-native species is that communication between scientists from different research disciplines and between scientists and stakeholders (e.g., managers, conservationists, and policy makers) will improve if clarity regarding the meaning of *impact* can be achieved. Decision science applies a clarity test to overcome the problem of different people assigning different meanings to the same term (see Howard [1988] for details). To pass a clarity test, *impact* must be explicitly and unambiguously defined. An area where clarity for improved communication is particularly relevant is the regulatory assessment of risks posed by non-native species (e.g., FAO 2004; EPPO 2007; EFSA 2011). In these assessments, experts from different sectors are typically involved, and they often have divergent views on how *impact* should be defined (Boonman-Berson et al. 2014).

For these 3 reasons—promoting progress toward a better understanding of impacts, discriminating between disparate definitions and scientific discord, and improving communication—we recommend that *impact* in the

context of non-native species be explicitly and clearly defined in scientific publications, stakeholder discussions, and other important contexts such as legislation. We formulated a set of questions to inform this exercise (see Heger et al. [2013] for questions that help define *alien* [i.e., *non-native*] and *invasive species*). Because different definitions can be suitable for different purposes, we do not call for a universal definition of impacts, but rather for explicit and clear definitions that reflect their particular context and audience.

Key Questions to Help Define the Impact of Non-Native Species

Questions that may serve as guidance to define the impact of non-native species fall into 4 categories: directionality, classification and measurement, ecological or socio-economic changes, and scale (Fig. 1). Many of the questions include the term *change*, reflecting the fact that the impacts of non-native species are due to changes caused by them. Such changes may occur proximally (i.e., within the regions or system in which they are introduced) or distally (e.g., downstream of the population of a non-native species that has changed water runoff or sedimentation rates [Zedler & Kercher 2004])—even over substantial distances (e.g., effects of allergenic pollen of non-native plants [Šikoparija et al. 2013]).

Directionality

Are only unidirectional changes considered (e.g., potential decrease in species diversity), or are bidirectional changes considered (e.g., potential increase or decrease in species diversity)? For example, Goodenough (2010), Schlaepfer et al. (2011), and Kumschick et al. (2012) looked at bidirectional changes caused by non-native species, whereas Olenin et al. (2007), Nentwig et al. (2010), and the international organizations FAO (2004), EPPO (2007), and EFSA (2011) considered only unidirectional changes. The latter makes sense for risk analyses, which typically focus on the potential for deleterious impacts of non-native species, whereas cost-benefit or multicriteria analyses (reviewed by Dana et al. [2014]) demand consideration of bidirectional changes (deleterious and beneficial impacts). Also, considering bidirectional changes may better capture the complexity of ecosystem dynamics. For instance, Pyšek et al. (2012) showed that plant species richness and measures of plant community structure tend to decrease following invasion, whereas the abundance and richness of the soil biota—as well as concentrations of soil nutrients and water—more often increase than decrease following invasion.

Classification and Measurement

Are impacts classified and quantified as neutrally as possible (e.g., solely based on the direction and magnitude of change), or are human values explicitly included? Daehler (2001), Rejmánek et al. (2002), Ricciardi et al. (2013), and Simberloff et al. (2013) define impacts neutrally. One challenge for a neutral definition is whether human values can (or indeed should) be excluded altogether. Larson et al. (2013) argue that the term *impact* is already value laden, and a more neutral term might be *change* or *effect*. Other authors define *impact* by explicitly including human values (e.g., Davis & Thompson 2000, 2001), and international regulatory bodies and organizations routinely do so (FAO 2004; EPPO 2007; EFSA 2011; IUCN 2013).

If definitions include human values, we suggest 2 components of an impact be discriminated: first, magnitude of change, which is neutrally quantifiable and, second, the value of the change assigned by humans (Kumschick et al. 2012). A challenge, however, is that the change may be perceived as valuable to one part of society but detrimental to another (e.g., Kumschick et al. 2012; Heger et al. 2013; Simberloff et al. 2013). For example, the presence of non-native fish and game species may be valued by anglers and hunters, but conservationists may perceive the same species as a threat to native biodiversity. Even economic stakeholders may have diametrically opposing views of non-native species, as in New Zealand, where non-native Scotch broom (*Cytisus scoparius*) is seen as valuable by beekeepers, while farmers and forestry industry people opt for releasing biocontrol agents against this species (Jarvis et al. 2006).

Keeney (1992) outlines how value-focused thinking can improve decision making. This approach may be useful in the context of non-native species. For example, the approach can be applied to classify changes caused by non-native species as either decision relevant or decision irrelevant. If stakeholders have different values, they will find different changes to be decision relevant and may differ in their views on the benefits of such changes (as illustrated by examples above). The application of decision science to this process can help managers and policy makers reach decisions despite conflicting viewpoints, although an exploration of this approach in practice is beyond the scope of this current article.

Is the term *impact* used only when the change caused by the non-native species exceeds a certain threshold, or is it used for any change? Ricciardi et al. (2013) define impact as a measurable change (recognizing detection thresholds), whereas Hulme et al. (2013) and Simberloff et al. (2013) define impact as a significant change (here, statistical significance should be discriminated against other types of significance, particularly biological significance). Thresholds of impact are potentially important

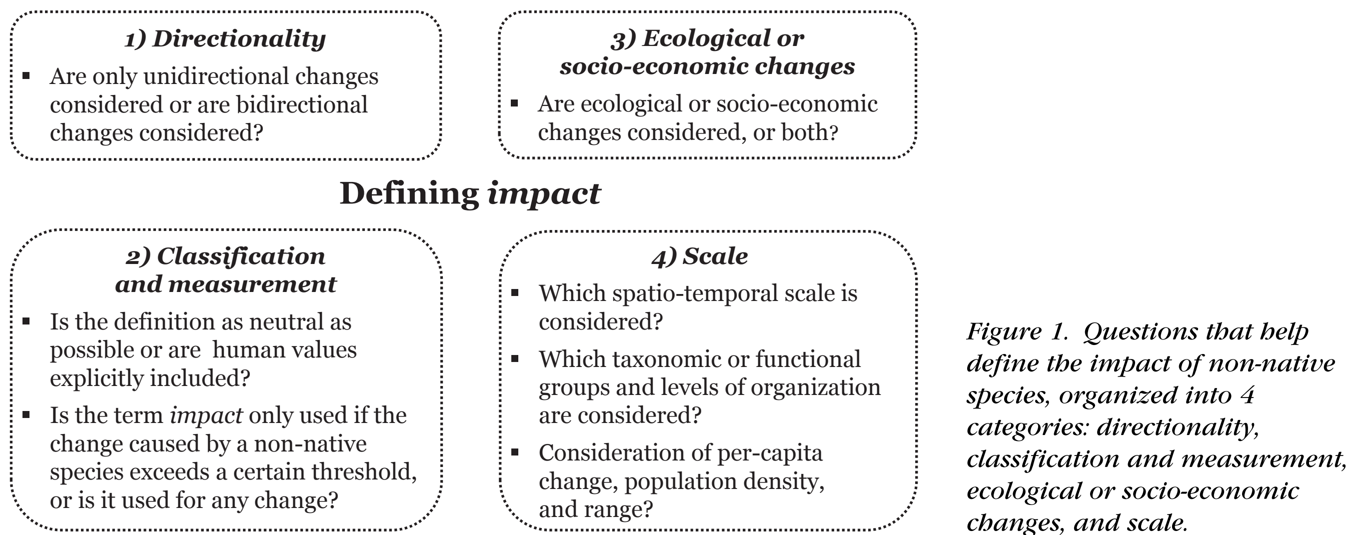

Figure 1. Questions that help define the impact of non-native species, organized into 4 categories: directionality, classification and measurement, ecological or socio-economic changes, and scale.

because they relate to the magnitude and potential reversibility of different changes. Some non-native species (i.e., *transformers* [Richardson et al. 2000]) can induce regime shifts and modify ecosystem functioning, enhancing their own abundance and persistence, and suppressing those of native species through modification of feedback processes (Nicholls et al. 2011; Seastedt & Pyšek 2011). Martin et al. (2009) outline a structured decision making framework for considering thresholds in the context of conservation and management that could be applied to impacts of non-native species.

Ecological or Socio-Economic Changes

Are ecological or socio-economic changes, or both, considered? Many studies (e.g., Pyšek et al. 2012; Ricciardi et al. 2013) have focused on ecological changes such as changes in population densities or ranges of native species, whereas other studies (e.g., Nentwig et al. 2010; Kumschick et al. 2012) also considered socio-economic changes such as those in agriculture, animal production, forestry, infrastructure, or human well-being. Although ecological and socio-economic impacts appear to be correlated (Vilà et al. 2010), the relationship between them needs to be better investigated: A strong ecological impact (e.g., extinction of a species) is not necessarily connected with a strong socio-economic impact.

Scale

Which spatial (e.g., local, regional, national, continental, global; or islands only) and temporal (e.g., intermittent, seasonal, transient, and permanent) scales are considered? The focal scale has a huge influence on almost any statement about impact. For instance, the introduction of non-native species can lead to net increases in species richness at small spatial scales (e.g., where fewer species, if any, become extirpated than are introduced) and cause a decline in global species richness through the extinction of endemic or locally rare native species (Sax & Gaines 2003; Clavero & García-Berthou 2005). Also, there can be large differences between the short- and long-term impacts of non-native species (Strayer et al. 2006). Results of a meta-analysis on declines of native species attributable to biological invasions in Mediterranean ecosystems showed that studies conducted at small scales or sampled over long periods reveal stronger impacts of non-native species than those conducted at large spatial scales and over short periods (Gaertner et al. 2009). The inclusion or exclusion of predicted future impacts of a non-native species should also be made explicit by authors (as is done by FAO 2004; EPPO 2007; and EFSA 2011). If the non-native species is still rare but rapidly spreading, currently documented impacts will typically be small, whereas anticipated future impacts (predicted from experiments or impacts caused in other regions invaded earlier or from species traits or high-impact congeners) may be much larger.

Which taxonomic or functional groups (e.g., animals, plants, fungi, parasites, parasitoids, viruses, or other pathogens) and levels of organization (e.g., genetic, population, species, community, ecosystem, and landscape) are considered? For example, Vilà et al. (2011) demonstrated that by the time changes in ecosystem processes (e.g., nutrient cycling) due to non-native species are detected, major impacts on plant species and communities are likely to have already occurred. In another study, Vilà et al. (2010) found taxonomic differences in the proportion of non-native species with known ecological and economic impacts in Europe. Sax (2002) provided a multitaxon analysis of invader impacts in Californian woodlands. In general, however, cross-taxonomic studies are rare in invasion ecology (Jeschke et al. 2012), and it would be useful for more studies to investigate impacts of non-native species across taxa and functional groups

(see also Sax et al. 2005). Such studies are vital for a general understanding of impacts.

Is change considered per capita or per biomass of the non-native species; locally for the non-native population (per capita change × population density); or over the full range of the non-native species (per capita change × population density × population range [cf. Parker et al. 1999])? For example, the impact scoring system of Kumschick and Nentwig (2010) and Nentwig et al. (2010) defines impact in 2 different ways: potential impact includes per capita impact and abundance, whereas actual impact additionally factors in the extent of the occupied range; species can rank high on potential but not actual impact or vice versa.

Resolving Disparity through Greater Clarity

These questions highlight considerations that may resolve substantial confusion about the impact of non-native species. They allow all—researchers, managers, policy makers, and others—who use the term *impact* to explicitly and clearly define it. In this way, progress toward a better understanding of impacts will be promoted, particularly because a synthesis of available information and data can be more informative. Aside from a suitable definition of *impact*, meta-analyses and other quantitative approaches for synthesis depend on studies that adequately measure impact. How impact should be measured depends on how it is defined. For example, if one is only interested in economic changes caused by non-native species (Are ecological or socio-economic changes, or both, considered?), impacts could be adequately measured in monetary terms. If ecological changes should be considered as well, a comprehensive impact score might be more adequate (see Kumschick & Nentwig [2010] and Nentwig et al. [2010] for examples of such scoring systems). Guidance on the choice of metrics is again provided by decision science (e.g., Keeney & Gregory 2005).

Aside from promoting progress toward a better understanding of impacts, explicit and clear definitions of *impact* will, as outlined above, also help one discriminate between disparate definitions and scientific discord and improve communication between scientists from different research disciplines and generally among scientists, managers, and policy makers. Regarding the latter, in our review of the literature on *impact* definitions, we found that many scientific studies quantify impact rather narrowly (e.g., restricted to unidirectional changes, ecological changes, and a limited scale [see above for references]). Yet, what is typically needed for appropriate management and policy actions is an understanding of *impact* in a broader sense. Indeed, regulatory bodies such as FAO (2004), EPPO (2007), or EFSA (2011) typically define the impact of non-native species rather broadly (although they are restricted to unidirectional changes if they follow a risk-assessment approach rather than a cost-benefit or multicriteria framework [Dana et al. 2014]). Explicit definitions of *impact* clearly expose this gap between what is needed by managers and policy makers and what scientists currently deliver. Scientists should be clear about the audience to which their assessment of impacts is directed and ensure their definition is appropriate for guiding subsequent action.

Acknowledgments

This paper is a joint effort of the working group sImpact that formed at a workshop supported by sDiv, the Synthesis Centre for Biodiversity Sciences within the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, funded by the German Research Foundation DFG (FZT 118). We thank the reviewers of previous versions of this paper for valuable comments. J.M.J. acknowledges support from the ERA-Net BiodivERsA (project FFII), with the national funder German Research Foundation DFG (JE 288/7-1); J.T.A.D. acknowledges support from NERC and The Leverhulme Trust; F.E. acknowledges support from the ERA-Net BiodivERsA (project WhoIsNext), with the national funder Austrian Science Foundation FWF; A.M. acknowledges support from the Charles University in Prague (project SVV 267204); and J.P. and P.P. acknowledge support from long-term research development project RVO 67985939 (Academy of Sciences of the Czech Republic), Centre of Excellence PLADIAS no. 14-36079G, grant P504/11/1028 (Czech Science Foundation), and institutional resources of Ministry of Education, Youth and Sports of the Czech Republic. P.P. also acknowledges support by the Praemium Academiae award from the Academy of Sciences of the Czech Republic. A.R. received support from the Canadian Aquatic Invasive Species Network; D.M.R. received support from the National Research Foundation (grant 85417); A.S. received support from the German Academic Exchange Service (DAAD); M.V. received support from projects Consolider-Ingenio MONTES (CSD2008-00040), FLORMAS (CGL2012-33801), and Severo Ochoa Program for Centres of Excellence in R+D+I (SEV-2012-0262); and S.K. received support from the Swiss National Science Foundation, the DST-NRF Centre of Excellence for Invasion Biology, and the Drakenstein Trust. This paper also contributes to COST Action TD1209.

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– appendix 2 –

Kumschick S, Gaertner M, Vilà M, Essl F, Jeschke JM, Pyšek P, Ricciardi A, Bacher S, Blackburn TM, Dick JTA, Evans T, Hulme PE, Kühn I, Mrugała A, Pergl J, Rabitsch W, Richardson DM, Sendek A, Winter M (2015) Ecological impacts of alien species: quantification, scope, caveats and recommendations. *BioScience* 65: 55-63

The supplemental material is available online at *http://bioscience.oxfordjournals.org/content/suppl/2014/12/13/biu193.DC1*

Ecological Impacts of Alien Species: Quantification, Scope, Caveats, and Recommendations

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Despite intensive research during the past decade on the effects of alien species, invasion science still lacks the capacity to accurately predict the impacts of those species and, therefore, to provide timely advice to managers on where limited resources should be allocated. This capacity has been limited partly by the context-dependent nature of ecological impacts, research highly skewed toward certain taxa and habitat types, and the lack of standardized methods for detecting and quantifying impacts. We review different strategies, including specific experimental and observational approaches, for detecting and quantifying the ecological impacts of alien species. These include a four-way experimental plot design for comparing impact studies of different organisms. Furthermore, we identify hypothesis-driven parameters that should be measured at invaded sites to maximize insights into the nature of the impact. We also present strategies for recognizing high-impact species. Our recommendations provide a foundation for developing systematic quantitative measurements to allow comparisons of impacts across alien species, sites, and time.

Keywords: biological invasions, context dependence, ecosystem functioning, management, prediction

The human-mediated translocation of species to regions outside their native ranges is one of the most distinguishing features of the Anthropocene (e.g., Ricciardi 2007). Although biological invasions are widely recognized as a key component of current global change, there is much debate among scientists and other stakeholders concerning, among other things, the scale of the changes caused by alien species and the extent to which management intervention is warranted (e.g., Richardson and Ricciardi 2013). This controversy is partly rooted in the lack of a widely accepted framework for interpreting impacts and a consolidated terminology for impacts to facilitate communication (Blackburn et al. 2014, Jeschke et al. 2014). One reason for this lack of consensus may be that such research has involved only a limited subset of alien species in a restricted number of regions and environments, which has hindered progress toward a predictive understanding of impacts in general (Hulme et al. 2013). There are, however, major gaps in our knowledge—in particular, how species traits and characteristics of the recipient environments interact to determine the level of impact (Drenovsky et al. 2012, Ricciardi et al. 2013), how spatial and temporal scales modulate the interpretation of impacts (Strayer et al. 2006, Powell et al. 2011), how the impacts of alien species can be distinguished from other concurrent and potentially synergistic stressors (e.g., climate change, landscape alteration; MacDougall and Turkington 2005, Didham et al. 2007), and how different types of impacts can be evaluated and compared using common metrics and currencies (Parker et al. 1999, Blackburn et al. 2014). Invasion science needs more-robust methods for reliably assessing the risks associated with alien species introductions (i.e., the likelihood of their establishment, spread, and impact), but there is ample research in which this has been attempted and on why it has been difficult (see, e.g., Leung et al. 2012, Kumschick and Richardson 2013).

The study of impacts is not a new phenomenon (see e.g., Lodge 1993, Mack and D'Antonio 1998). However, only recently have reviews of the magnitude, scope, and variation of the impacts of alien species, as well as their geographic and taxonomic distinctions and biases, substantially expanded our theoretical knowledge and provided useful conceptual frameworks (e.g., Vilà et al. 2010, Pyšek et al. 2012, Hulme et al. 2013, Ricciardi et al. 2013). Further progress hinges on a more-precise and -comparable quantification of impacts and on an elucidation of the mechanisms behind them, particularly in the context of local factors (coincident stressors,

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doi:10.1093/biosci/biu193 Advance Access publication 17 December 2014

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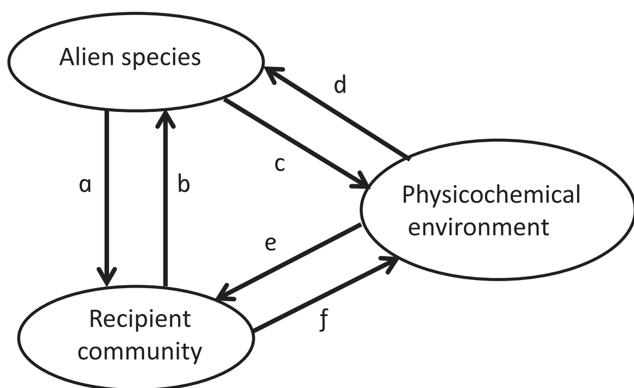

Moderating parameters and scales	Interactions affected
Composition of the recipient community	a, b, e, f
Abiotic changes	c, d, e, f
Abundance of the alien species	a, c
Time since introduction	a, b, c, d, e, f
Other stressors	a, b, c, d, e, f
Spatial scale (extent and grain)	a, b, c, d, e, f

Figure 1. The context dependence of alien species impacts. Knowledge of key interactions and moderating parameters is required to understand and properly quantify impacts. The details of these parameters are given in table 1 and appendix S1.

species interactions, and physicochemical conditions that vary over space and time)—all of which pose challenges for risk assessment and can misguide management decisions. Here, we assess approaches for quantifying and prioritizing impacts and provide recommendations for facilitating the risk assessment and management of alien species. Specifically, we propose guidelines on what information to collect on the invaded site to better understand the mechanisms of impact and to decide which alien species should be prioritized for management, on how to plan and conduct empirical studies to understand impacts, and on how to approach impact prediction. We follow Ricciardi and colleagues (2013) in defining an *impact* as a measurable change in the state of an invaded ecosystem that can be attributed to the alien species. This definition includes any change in ecological or ecosystem properties but excludes socioeconomic effects and human values (cf. Jeschke et al. 2014).

Quantifying ecological impacts in the field: What to measure

Quantitative assessments of alien species impacts are essential to ensure that resources spent on management are prioritized to target the most problematic species, threatened areas, and affected ecosystem processes (Hulme et al. 2013). However, in general, the selection of parameters used in quantitative studies of impact does not seem to have been sufficiently driven by hypotheses. The selection of appropriate parameters should account for impacts at different organizational levels, such as individuals, populations, communities, and ecosystem functions (Parker et al. 1999, Pyšek et al. 2012, Blackburn et al. 2014), and at different levels of diversity, such as genetic, functional, and taxonomic diversity. Quantifying several impact types at the same site allows for the determination of causal links among impacts and the identification of direct and indirect effects (figure 1; see also Hulme 2006).

Among the most important metrics is alien species abundance, which is correlated with the level of impact, although not necessarily linearly. The greater the number of individuals or biomass of the alien species is, the more resources they will use and the greater the extent and strength of their interactions with native species will be (e.g., Parker et al. 1999, Ricciardi 2003). Catford and colleagues (2012) provided a practical way of taking the abundance of alien species into account: identifying abundance thresholds and using categorical scores.

Time since invasion also influences the level of impact, through temporal changes in the abundance of the alien species, adaptation by the recipient community, postinvasion evolution, and variation in the physicochemical environment in the invaded range (Strayer et al. 2006, Dostál et al. 2013). The introduction or establishment date should therefore be noted. The magnitude, direction and type of impact also vary with the spatial extent and grain (resolution) of the study area (e.g., Gaertner et al. 2009). It is therefore important to indicate sampling plot size as well as the area over which plots were sampled, also in light of species–area curves. However, this measure might not always be straightforward—for example in the case of migrating animals.

The challenge of context dependence

The impacts of alien species vary both in their location and their duration or frequency, under the influence of local abiotic and biotic variables (Hulme 2006, Ricciardi et al. 2013). The abundance and performance (e.g., resource uptake, competitive success) of a species can vary predictably along physical environmental gradients (Ricciardi 2003, Jokela and Ricciardi 2008). In addition, the composition of the recipient community moderates impacts in several ways (e.g., through resistance or facilitation by resident species; Ricciardi et al. 2013). Interactions between native and alien species may also vary across physical gradients such that dominance patterns can even be reversed (Kestrup and Ricciardi 2009).

Finally, other anthropogenic stressors that simultaneously alter the physical and biological environment can affect many interactions and obscure the effects of alien species. Figure 1 illustrates this passenger–driver problem of impact attribution, which is a major challenge for management (MacDougall and Turkington 2005, Didham et al. 2007); impact attribution could be challenging if the passenger model dominated. In the driver model, interaction a (or c affecting e) is strong in both directions; in the passenger model, interaction d (or e affecting b) is strong, whereas a is weak. Also illustrated are additive (a and e are strong) and synergistic models (in which a, c, d, and e are strong).

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An increased understanding of context dependence is required in order to improve our ability to predict impacts. Resource managers can play a valuable role in their initial detection and by providing information on the shifting contexts of impacts, through their observation of environmental change. However, *quantifying* these changes requires considerable research and sufficient resources. Governments, landowners, and managers, as well as the general public, could profit from the outcomes of such studies. Moreover, funding should be allocated by all of these stakeholders to both research institutes and land management agencies. The outcomes can then feed into preventive measures—for example, to improve risk assessments and management plans.

The prioritization of management

It is beyond the scope of this study to discuss management prioritization if the passenger model dominates for a particular system. In the following section, we therefore only address impacts in situations in which the alien species is most likely to be a driver of the impact.

For efficient and cost-effective allocation of management resources, there is a strong need to flag those alien species with potentially high environmental impacts (Blackburn et al. 2014). It has been proposed that species with the potential to force ecosystems to cross biotic and abiotic thresholds—and, therefore, to change to alternative states (i.e., causing regime shifts)—should be considered as potentially the most disruptive and should be given top priority for intervention (Gaertner et al. 2014). Regime shifts are associated with a reorganization of the internal feedback mechanisms that structure an ecosystem, such as plant–soil feedbacks (Scheffer et al. 2012). However, at present, it is difficult to predict whether a given species can alter feedbacks in ways that could lead to a regime shift. The outcomes depend on the traits of the alien species, the characteristics of the invaded habitat and of the invaded community (figure 1; Pyšek et al. 2012, Kueffer et al. 2013), and interactions between these factors (Ricciardi et al. 2013). One way of tackling these challenges is to identify specific combinations of species traits, ecosystem characteristics, and impacts with a high probability of causing changes in ecosystem feedbacks (Gaertner et al. 2014). Such feedbacks are commonly associated with the impacts of ecosystem engineers (table 1 and supplemental appendix S1; Linder et al. 2012, Ricciardi et al. 2013).

If no quantitative or statistically comparable data are available, as is often the case, impact-scoring systems can be used to make very diverse data comparable. Furthermore, they allow comparisons between groups with different impact mechanisms (Kumschick et al. 2012, Blackburn et al. 2014). Scoring systems have been used to identify traits of alien mammals and birds associated with high levels of impact (Nentwig et al. 2010, Kumschick et al. 2013) and have shown that the diversity of habitats that an alien species can occupy could be a useful parameter in models predicting that species' impact (Evans et al. 2014).

Implications for prediction and prevention

We need to mitigate impacts not only when aliens are present but, ideally, also when they are expected to invade and likely to have an undesirable impact in the future. Preinvasion assessments with the purpose of predicting the risk of invasion and impact are used in many parts of the world (Kumschick and Richardson 2013), but the impact assessment is generally not convincingly incorporated, owing mainly to the same inherent difficulties and uncertainties that account for the lack of a robust predictive framework and a lack of data on impacts in general. A potential solution would be to identify predictable patterns via statistical synthesis of data from multiple sites for given species—ideally, those with a sufficiently documented impact history (figure 2; Kulhanek et al. 2011). Such studies can also contribute to the justification for labeling a species as a potential invasive or as causing a potential impact elsewhere as an often-suggested predictor of invasion success and impact, respectively, in the new range (Leung et al. 2012, Kumschick and Richardson 2013). Figure 2 outlines a logical series of empirical approaches for forecasting impacts, primarily on the basis of impact and invasion history. Vitousek (1990) posited that alien species that have large effects on ecosystem processes differ from the native species in their resource acquisition, resource efficiency, or capacity to alter disturbance regimes; examples of this include alien plants that change fire regimes following their introduction, such as many invasive grasses (D'Antonio and Vitousek 1992, Yelenik and D'Antonio 2013), and mammalian predators introduced to islands with no evolutionary history of such species or archetypes (e.g., Blackburn et al. 2004). The functional distinctiveness of the alien species may enhance its impact through novel resource use and exposure to ecologically naive residents or by introducing new ecosystem functions (e.g., nitrogen fixers in communities naturally without such a guild). Taxonomic or phylogenetic distinctiveness can serve as proxy parameters of functional distinctiveness (Ricciardi and Atkinson 2004, Strauss et al. 2006). In some cases, however, alien species may differ not in functional type but in performance and behavior. For example, alien and native predators may differ in their feeding behaviors toward a common prey, but these differences can be quantified and compared by testing their functional response (Dick et al. 2014).

Finally, one aspect of potentially high predictive value that has not been adequately explored is whether the impacts of alien species are similar to those of phylogenetically closely related or functionally similar alien species. This relationship is often assumed and used to assess the risk of species that have not been introduced elsewhere (e.g., Bomford 2008), but it has rarely been tested. A cursory examination of the freshwater literature indicates that taxonomic affiliation—whether a species is closely related to a proven invader—is not a consistent predictor of impact potential (Ricciardi 2003).

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Table 1. Suggested parameters important for quantifying, predicting, and prioritizing the management of the impact of alien species.

Purpose	Parameter	Rationale
Quantification	Changes to ecosystem function following invasion	Changes to ecosystem functions often affect ecosystem services.
	Per capita effects	The level of impact is a function of per capita effects (e.g., the rate of resource uptake), abundance, and interactions between organisms and their environment.
Context dependence	Composition and abundance of native species and traits in the recipient community	Recipient communities can be transformed rapidly by interacting with alien species. Native species may increase or decrease in abundance (or even become extirpated). Food webs may be altered because of the addition or deletion of energy pathways.
	Genetic composition of congeneric native species in the recipient community	Introgression may affect native gene pools.
	Abiotic changes following invasion	Altered physicochemical processes affect species interactions and ecosystem functions.
	Spatial scale	The overall spatial extent of impact depends on species distribution.
	Time since introduction	Impact varies over time, owing to changes to local abiotic conditions, the abundance of the invader, and the response of the recipient community.
	Other stressors during invasion	Identification of simultaneous biological (e.g., other invaders) and environmental stressors (e.g., climate change, nutrient pollution, land transformations) can have multiple additive or synergistic effects. It is necessary to disentangle these confounding effects to resolve whether the invasion is the cause or the symptom of any impact.
Prediction	Impact history of the invader	The impact history of a species, if well documented, is the most reliable predictor of its impact, although context-dependent influences can cause unexpected outcomes.
	Abundance of the invader	In many cases, the level of impact scales with abundance (at least initially). Elucidation of the relationship between abundance and impact will assist in developing species-specific predictive models and for determining thresholds for regime shifts.
	Functional or phylogenetic novelty (distinctiveness) of the invader respective to native community	Larger impacts are often caused by alien species that are functionally or phylogenetically distinct from the recipient community.
Management prioritization	Endemism	Native species that have been geographically isolated over evolutionary time scales are naive to the effects of a broad range of alien species.
	Ecosystem services	Identification of the affected ecosystem services can guide management prioritization and facilitate communication with various stakeholders.
	Rare and Red-listed species	Red-listed species are of priority conservation concern and should be protected against the threat of alien species.
	Conservation concern of the invaded ecosystem	Prioritization of alien species management depends on the nature of the ecosystem invaded (e.g., protected area, sanctuary).
	Native biodiversity	Diverse native assemblages are deemed to have more conservation value.
	Ecosystem engineers	Feedbacks, potentially leading to regime shifts, are commonly associated with the impacts of ecosystem engineers.

Note: The listed parameters do not cover every potential type of ecological impact (e.g., literature reviews of plant invasions have identified at least 15 broad types of impact that are repeatedly measured; see Pyšek et al. 2012, Hulme et al. 2013). Rather, the selection is driven by considerations for the provision of guidance for improving the consistency and comparability of the impacts of invasive species among studies (e.g., meta-analysis) and to elucidate context dependency, therefore increasing insights into species- and site-related variation and possibilities for predictions based on impacts previously recorded elsewhere. More detailed information on specific parameters and references appear in appendix S1.

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Understanding the mechanisms behind an impact is ultimately important to predicting the impacts of new alien species with no alien relatives. Trait-based models can give indications of such mechanisms, but, so far, it has not been explored to what degree traits correlated with impact have a predictive value for new invaders (Evans et al. 2014).

Experimental methods and approaches to investigate impacts

Various approaches have been taken to study the impacts of different taxa in different habitat types (supplemental appendix S2). Most of these studies have involved comparisons of invaded and uninvaded reference sites, primarily at the fine resolution of plots and their restricted extent (a in figure 3). This approach is commonly used to infer the impacts of alien species on particular native species, on community structure (i.e., species diversity), and on ecosystem processes such as nutrient pools and fluxes (Vilà et al. 2011). If suitable reference plots are available, it is the simplest observational approach, because it allows large amounts of data to be collected relatively easily and inexpensively. However, it does not demonstrate causality, because the

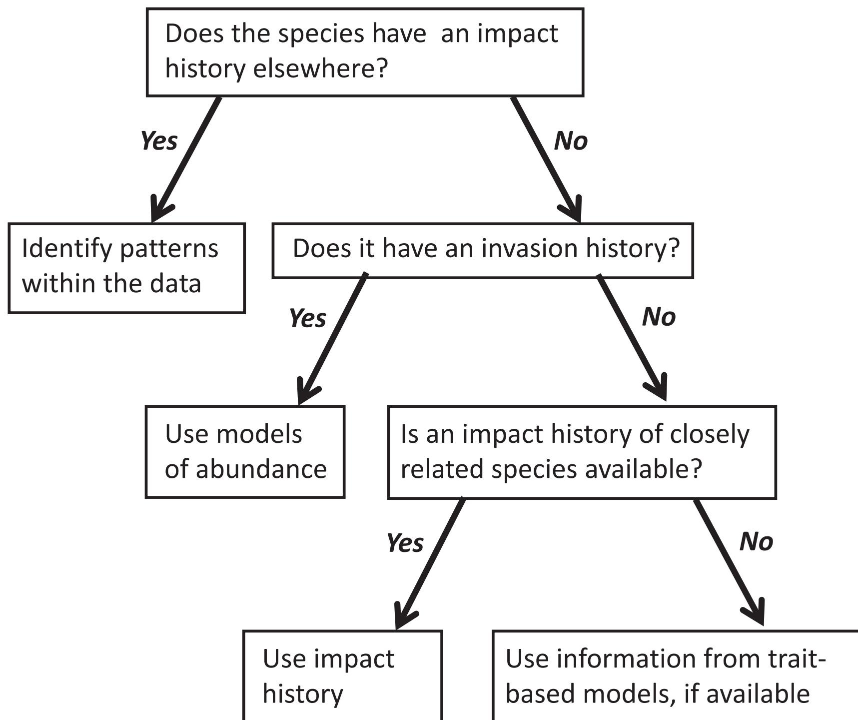

Figure 2. Empirical approaches for forecasting impacts of alien species, starting with the most desirable data. If an alien species has a sufficiently documented impact history in its invaded range, the patterns within the data could be analyzed statistically (e.g., using multivariate techniques or meta-analysis) to construct quantitative or qualitative models of its impact (e.g., Ricciardi 2003, Kulhanek et al. 2011). In cases in which no impact history is available, the invasion history of the species could be used to predict its abundance—a proxy for its level of impact—by relating variation in local abundance to limiting physicochemical variables (e.g., Jokela and Ricciardi 2008). Otherwise, predictive information might be obtained from the impact (invasion) history of functionally similar species or from trait-based models of high-impact invaders (e.g., Pyšek et al. 2012, Kumschick et al. 2013). Further information on the suggested parameters appears in appendix S1. Source: Adapted with permission from Ricciardi (2003).

observed outcome can be confounded with between-site differences not related to the introduced species. With this in mind, such studies should select plots that are as closely matched as possible for other abiotic and biotic features (Hejda et al. 2009). One approach is to correlate the magnitude of one or more impacts along a gradient of alien species abundance (b in figure 3). For instance, herbivore effects on plant fitness are often density dependent, such that their per capita effect is correlated with density (e.g., Trumble et al. 1993). However, the relationship between per capita impact and alien species abundance remains to be examined for a range of taxa, systems, and environmental conditions.

Unfortunately, it is often very difficult to find contemporaneous similar but uninvaded reference sites to contrast with invaded sites. Under such circumstances, it would be preferable to study genuine chronosequences that enable an analysis of the relationships in the time since invasion and the magnitude of impact, provided that there are good historical data to determine when the invasion began (c1 in figure 3). Of particular interest are comparisons of sites before and after invasion (c2 in figure 3). This is only feasible under certain circumstances, such as in locations in which there have been long-term monitoring programs (Magurran et al. 2010) or monitoring before an anticipated invasion took place (Roy et al. 2012). However, in such cases, the long-term temporal dynamics of the impacts of alien species are generally not sufficiently understood to give recommendations on the optimal time scale of impact studies (Yelenik and D'Antonio 2013). Moreover, time series studies might encounter the same confounding problems as comparisons between invaded and uninvaded sites, given that differences over time might be caused by other (confounding) stressors acting simultaneously during an invasion (figure 1, appendix S1).

If direct observations on the temporal dynamics of impacts are not feasible, changes in communities or ecosystem processes might not be attributable to the presence and activity of the alien species but, rather, to concurrent or preceding changes in the environment (e.g., grazing, eutrophication, changes in climate conditions). Whether alien species are passengers or drivers of change is difficult to resolve by observation alone (MacDougall and Turkington 2005). For example, the observed decline of native ladybird beetle species in arboreal habitats in the UK after invasion by the alien ladybird beetle *Harmonia axyridis* is also correlated with changes in maximum temperature and rainfall among years (Brown et al. 2011). However, path analysis and structural equation modeling can sometimes be applied to disentangle the relative importance of alien species and other stressors to native species declines (e.g., Light and Marchetti 2007, Hermoso et al. 2011).

Although, in any aspect of ecology, the manipulation of parameters is the best way to demonstrate causality, field removal experiments to identify the impacts of alien species (d in figure 3, appendix S2) have been reported in only a small number of studies. The most prominent examples concern the removal of alien plants, but field manipulation experiments represent less than 14% of the studies on the impacts of alien plants (Vilà et al. 2011). Comparing invaded plots with those from which alien species have been removed offers a straightforward method to demonstrate that ecological differences between these plots are linked

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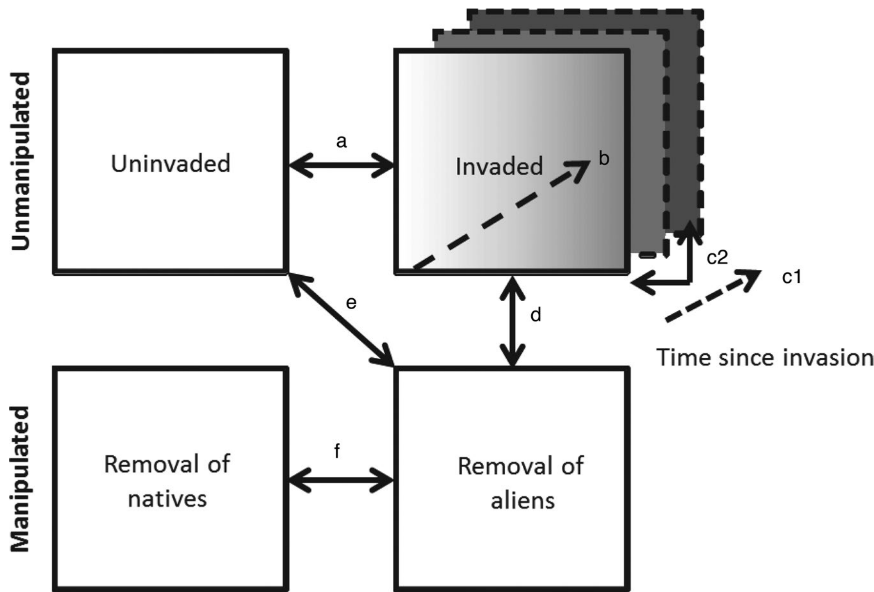

Figure 3. Empirical approaches for studying the impacts of invasive alien species using manipulated and unmanipulated plots: (a) Observational approach comparing invaded and uninvaded (reference) plots. (b) Observational approach along a gradient of alien species abundance (higher abundance is represented here by darker shading). (c1) Chronosequence of invasion (stages of different time since invasion shown as discontinuous squares). (c2) A special case of the previous approach: a before- and after-invasion approach comparing only two stages over time. (d) Experimental approach comparing invaded and removal plots. (e) Experimental approach comparing removal and uninvaded reference plots. (f) Experimental approach comparing plots from which the alien or the native species has been removed; these can be undertaken to account for the disturbance effect in the removal experiments (comparing panels (f), (e), and (d)) or to test whether functionally similar native and alien species have different effects.

to the effects of alien species. However, the outcomes of these experiments can be confounded with disturbance effects due to species removal. Disturbance can be minimized in various ways. For example, if the alien species is an annual plant, the invader can be removed at the seedling stage (Hulme and Bremner 2006). Disturbance is, however, often unavoidable if the invader is a perennial plant species. Consequently, removal plots are often set in an earlier successional stage than are intact invaded plots; even if they harbor high levels of species richness, their species composition can be different, and they are therefore not exactly comparable, because many species regenerating in the removal plots are early colonizers that can, themselves, be alien species (Truscott et al. 2008, Andreu et al. 2010). In such cases, it is advantageous to combine the experimental removal of alien species with the removal of native species, where that is deemed appropriate (f in figure 3), to distinguish the alien–native effect from the disturbance effect. For sessile species, comparing ecological differences between areas in which aliens and natives have been removed will elucidate whether the effect of the alien is due to species origin per se.

Removal experiments for mobile organisms are difficult to achieve in practice, and the results from such experiments are highly context dependent. There have now been many eradications of alien animal species worldwide (e.g., Pluess et al. 2012), with sometimes counterintuitive results on the dynamics of their prey (Rayner et al. 2007). Furthermore, compared with that of sessile species, the impact of mobile species with large home ranges (e.g., vertebrates) might be spatially diluted and difficult to quantify at the local scale. Eradications can be used for comparisons of invaded communities before and after the removal of the alien (e.g., Monks et al. 2014), but other approaches, such as comparisons with other invaded and uninvaded sites, might also be possible. For mobile species with large home ranges, the use of well-designed enclosures or fences to compare large invaded and uninvaded areas might be one of the most realistic options (Burns et al. 2012).

The removal of an alien species does not necessarily (or not immediately) lead to the restoration of preinvasion conditions, particularly for some ecosystem engineers that may have a legacy effect on habitat conditions (Magnoli et al. 2013). It is therefore crucial to compare removal plots with uninvaded and unmanipulated reference plots (e in figure 3). From a restoration perspective, a successful removal strategy would be one in which the ecosystem recovers along a trajectory leading to a state similar to that in a reference site, not only in terms of species richness but also in terms of species composition and ecosystem functioning. For example, following the removal of monkey flowers (*Mimulus guttatus*) from a riparian system, the resident plant community recovered and increased in species richness over time but toward a different community composition than that of uninvaded sites (Truscott et al. 2008). This demonstrates that different methodological approaches can lead to different conclusions based on extant impacts.

In some cases, removals of alien species could be compared with removals of closely related natives. For example, field removal experiments that have been conducted in the Bahamas to exclude the alien red lionfish (*Pterois volitans*) and test how the impact of this species compares with that of the coney grouper (*Cephalopholis fulva*, a native predator of similar size and diet) showed that the alien species reduced the abundance and richness of small coral-reef fishes more than of the native predator (Albins 2013). More studies

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of this kind are needed to discern whether alien species impacts represent the average effect or a magnified effect of a single species in the community when it is dominant (f in figure 3). However, such native-removal studies are only feasible and sensible if no negative conservation implications of removing those natives are expected.

Manipulative species-addition field experiments are technically feasible (appendix S2; Meffin et al. 2010) but highly challenging, because the prevention of the establishment and spread of the alien species outside experimental plots has to be a priority in the experimental setting. This is difficult to achieve and might jeopardize the value of an experiment intended to obtain observations of an interaction between the additional alien species individuals and the recipient community. An alternative is to perform species-addition experiments in restricted conditions mimicking field conditions as much as possible. Mesocosms have mainly been used to test the impacts of soil organisms and aquatic alien species (appendix S2). Such studies can be informative regarding particular impact mechanisms for species interactions but are problematic for inferring impacts at the community and ecosystem levels. Moreover, mesocosm and common-garden experiments are usually too short term or restricted in scale to predict long-term field conditions.

There are multiple ways to assess alien species impacts, but no single method appears to have a clear advantage. We advocate a four-way plot experimental design (uninvaded, invaded, removal of natives, removal of aliens; a, d, e, and f in figure 3), not only to reveal ecological impacts and to detect regime shifts but also to determine the potential success of restoration efforts. The use of large-scale removal programs as a source of experimental data can be highly valuable if they are carried out in such a way as to allow this recommended design. Spatial and temporal variation in impacts must also be taken into account by careful replication and monitoring of the sampled sites (Kueffer et al. 2013).

Conclusions

Not only is research on the impacts of alien species necessary to understand why some species are more disruptive than others and why some systems are more susceptible to being disturbed by alien species, but it is also of practical importance in determining how limited management resources should be allocated. The better our understanding of impacts, the better equipped we will be to implement effective management. Systematically gathering and synthesizing solid evidence of the impacts caused by alien species facilitates communication with the public and better informs policy- and decisionmakers. Disputes within the scientific community about the role of alien species increases the perception of them being innocuous or equally likely to have positive effects (but see Richardson and Ricciardi 2013). In fact, many alien species cause substantial and sometimes irreversible impacts, but we have not yet achieved a predictive understanding of when or where these impacts will occur or which species will cause them.

Furthermore, our synthesis points out that different experimental methodologies are appropriate for different taxa because of particular properties of the species and ecosystems involved, even though most methods are theoretically applicable for most organismal groups (appendix S2). It is known, however, that using different methodological approaches can lead to different conclusions (e.g., Truscott et al. 2008). Moreover, sessile organisms are more frequently studied than are mobile ones, which can potentially introduce bias. Further studies are required to determine the extent to which such issues influence our evaluation and knowledge of impacts and the perceived differences between organismal groups.

A more balanced view of impacts and a standardized protocol of how to quantify impacts—that is, which parameters to measure and which metrics to apply at invaded sites—are needed. Therefore, we have proposed a set of parameters on which to base the objective quantification of impacts. The collation of information on these parameters will contribute to a better understanding of context dependence and to a robust framework for prioritization.

Acknowledgements

This article is a joint effort of the working group sImpact that formed at a workshop supported by sDiv, the Synthesis Centre for Biodiversity Sciences within the German Centre for Integrative Biodiversity Research (iDiv) Halle–Jena–Leipzig, funded by the German Research Foundation (DFG; through grant no. FZT 118). We acknowledge support from the Swiss National Science Foundation and the Drakenstein Trust (to SK); the Spanish Ministry of Economy and Competitiveness and the Severo Ochoa Programme for Centres of Excellence (Projects Consolider-Ingenio MONTES no. CSD2008-00040, no. RIXFUTUR CGL2009-7515, and no. FLORMAS CGL2012-33801 to MV); the COST Action TD1209 Alien Challenge (to MV, FE, JMJ, PP, JP, SB, TMB, PEH, and WR); the Austrian Climate Research Program (project no. K10AC1K00061 "RAG-Clim" to FE); the ERA-Net BiodivERsA (project FFII), with national funding from the DFG (grant no. JE 288/7-1 to JMJ); the Academy of Sciences of the Czech Republic (grant no. RVO 67985939), the Czech Science Foundation (grant no. 14-36079G Centre of Excellence PLADIAS, and P504/11/1028 to PP and JP), and the Ministry of Education, Youth and Sports of the Czech Republic (to PP and AM); the Praemium Academiae award from the Academy of Sciences of the Czech Republic (to PP); the Canadian Aquatic Invasive Species Network and NSERC Canada (to AR); the UK Natural Environment Research Council and the Leverhulme Trust (to JTAD); the Department of Science and Technology and National Research Foundation's Centre of Excellence for Invasion Biology (to SK and DMR) and the National Research Foundation (grant no. 85417 to DMR); Charles University in Prague (project no. SVV 267204 to AM); and the German Academic Exchange Service DAAD (to AS).

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Supplemental material

The supplemental material is available online at *http://bioscience.oxfordjournals.org/lookup/suppl/doi:10.1093/biosci/biu193/-/DC1*.

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– appendix 3 –

Blackburn TM, Essl F, Evans T, Hulme PE, Jeschke JM, Kühn I, Kumschick S, Marková Z, Mrugała A, Nentwig W, Pergl J, Pyšek P, Rabitsch W, Ricciardi A, Richardson DM, Sendek A, Vilà M, Wilson JRU, Winter M, Genovesi P, Bacher S (2014) A Unified Classification of Alien Species Based on the Magnitude of their Environmental Impacts. *PLoS Biology* 12: e1001850

The supporting information is available online at *http://dx.doi.org/10.1371/journal.pbio.1001850*

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Essay

A Unified Classification of Alien Species Based on the Magnitude of their Environmental Impacts

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Abstract: Species moved by human activities beyond the limits of their native geographic ranges into areas in which they do not naturally occur (termed aliens) can cause a broad range of significant changes to recipient ecosystems; however, their impacts vary greatly across species and the ecosystems into which they are introduced. There is therefore a critical need for a standardised method to evaluate, compare, and eventually predict the magnitudes of these different impacts. Here, we propose a straightforward system for classifying alien species according to the magnitude of their environmental impacts, based on the mechanisms of impact used to code species in the International Union for Conservation of Nature (IUCN) Global Invasive Species Database, which are presented here for the first time. The classification system uses five semi-quantitative scenarios describing impacts under each mechanism to assign species to different levels of impact—ranging from Minimal to Massive—with assignment corresponding to the highest level of deleterious impact associated with any of the mechanisms. The scheme also includes categories for species that are Not Evaluated, have No Alien Population, or are Data Deficient, and a method for assigning uncertainty to all the classifications. We show how this classification system is applicable at different levels of ecological complexity and different spatial and temporal scales, and embraces existing impact metrics. In fact, the scheme is analogous to the already widely adopted and accepted Red List approach to categorising extinction risk, and so could conceivably be readily integrated with existing practices and policies in many regions.

Essays articulate a specific perspective on a topic of broad interest to scientists.

Introduction

Human activities are transforming natural environments by moving species beyond the limits of their native geographic ranges into areas in which they do not naturally occur. Many of these alien species (Box 1) have caused substantial changes to the recipient ecosystems. Such changes have been measured by a burgeoning number of studies that consider a

Citation: Blackburn TM, Essl F, Evans T, Hulme PE, Jeschke JM, et al. (2014) A Unified Classification of Alien Species Based on the Magnitude of their Environmental Impacts. PLoS Biol 12(5): e1001850. doi:10.1371/journal.pbio.1001850

Published May 6, 2014

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Funding: The sDiv workshop that led to the sImpact working group was funded by the German Research Foundation DFG (FZT 118). JP, PP, and ZM acknowledge the support from long-term research development project no. RVO 67985939 (Academy of Sciences of the Czech Republic), Centre of Excellence PLADIAS no. 14-36079G, and grant no. P504/11/1028 (Czech Science Foundation), and institutional resources of the Ministry of Education, Youth and Sports of the Czech Republic. PP acknowledges the support by the Praemium Academiae award from the Academy of Sciences of the Czech Republic. JMJ acknowledges support from the ERA-Net BiodivERsA (project FFII), with the national funder German Research Foundation DFG (JE 288/7-1). SK acknowledges financial support from the Swiss National Science Foundation, the DST-NRF Centre of Excellence for Invasion Biology, and the Drakenstein Trust. DMR and JRUW acknowledge support from the DST-NRF Centre of Excellence for Invasion Biology and the National Research Foundation (grants 85417 and 86894, respectively). AS acknowledges financial support of the German Academic Exchange Service (DAAD). MV acknowledges support from the Severo Ochoa Program for Centres of Excellence in R+D+I (SEV-2012-0262). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: CG, cryptogenic; DD, Data Deficient; GISD, Global Invasive Species Database; IUCN, International Union for Conservation of Nature; MA, Massive; MI, Minor; ML, Minimal; MO, Moderate; MR, Major; NA, No Alien Population; NE, Not Evaluated; SSC, Species Survival Commission.

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broad range of environmental impacts, defined here as measurable changes to the properties of an ecosystem by an alien species [1,2], at different levels of organisation (Box 1). For example, alien species have been shown to cause significant changes in native species extinction probabilities, genetic composition of native populations, behaviour patterns, species richness and abundance, phylogenetic and taxonomic diversity, trophic networks, ecosystem productivity, nutrient and contaminant cycling, hydrology, habitat structure, and various components of disturbance regimes [1–8]. Such changes are often indirect, and may involve subtle or poorly studied interactions that could yield substantial effects over time [9]. For these reasons, most scientists and conservation organisations consider alien species to be undesirable additions to ecosystems, and frequently devote considerable resources towards preventing or mitigating their impacts.

However, many alien species apparently have had little or no detectable effects on their new environment [1,10,11], and some effects may be considered to be positive [12–16]. It has been further claimed that alien species are no more likely to have undesirable impacts than natives, and therefore that management attention should be based on impacting species in general, rather than on the alien/native origin of species [17,18], although this view is controversial [19,20]. These commentators urge conservationists and land managers to organise priorities around whether species are producing net benefits or harm, so as to avoid wasting valuable conservation resources on the costs of excluding (e.g., through ballast-water treatment), eradicating, containing, or controlling alien species [21]. Recognising that impacts vary greatly among species and among recipient systems, and that many notable impacts only become obvious or significantly influential long after the onset of invasion, a critical need for invasion biology is the capacity to evaluate, compare, and predict the magnitudes of the impacts of different alien species, in order to determine and prioritise appropriate actions where necessary. The challenge is how to compare impacts attributable to diverse alien taxa on different levels of ecological complexity (individuals, populations, communities, ecosystems), at different spatial and temporal scales, assessed using a range of metrics and techniques [22].

In response to these issues, here we propose a simple standardised system for classifying alien species in terms of the magnitude of their impacts. Our aim is to produce a practical tool to report on the impacts caused by alien species, that can (i) be used to identify those species that have different levels of environmental impact, (ii) facilitate comparisons of the level of impact from alien species among regions and taxa, (iii) facilitate predictions of potential future impacts of the species in the target region and elsewhere, (iv) align with the mechanisms of impact identified in the International Union for Conservation of Nature (IUCN) Global Invasive Species Database ([GISD]; http://www.issg.org/database), and (v) prioritise management actions. The system we propose has the following properties, many of which also underlie the intentions of the IUCN Red List categories and criteria (our classification system can be viewed as broadly analogous to that approach) [23]:

1. The classification considers only environmental (as opposed to economic or societal) impacts (see Box 1 for definitions). Nevertheless, our scheme could be extended to social and economic impacts, as well as to environmental impacts on resident alien species that are perceived to be harmless or beneficial.
2. The classification identifies species that have deleterious abiotic or biotic impacts (Box 1). Its aim is not to weigh deleterious against beneficial impacts to determine the net value of an introduction, but rather to highlight potential consequences.
3. Species are classified on the basis of evidence of their most severe documented impacts in regions to which they have been introduced. The scheme is, therefore, not a predictive model of impact—however, by reporting on the worst observed case, it can be used to flag species with high potential impacts that need to be evaluated in detail in a particular introduction context.
4. The classification provides a consistent procedure for translating the broad range of impact types and measures into ranked levels of environmental impact. It therefore distinguishes between taxa with different magnitudes of impact.
5. The classification can be applied across taxa, so that different taxa can be compared using a common currency in terms of their environmental impact. It could also be applied at different taxonomic levels.
6. The classification considers consequences, not likelihoods; that is, it focuses on the consequences in terms of impact of an introduction, rather than on the likelihood of an invasion.
7. Classification is based on the best available evidence. Hence, species can move up and down impact categories as the quality of evidence improves, as conditions change, or as an invasion proceeds.
8. The scheme we propose here can be applied to impacts assessed at a range of spatial scales, from global to national or regional.

Classifying Impact

Our classification system is based on the Generic Impact Scoring System (GISS) to compare the impacts of alien animal species among members of large taxonomic groups, developed by Nentwig and colleagues [24] and subsequently extended by Kumschick and colleagues [25], modified to align it to the new impact scheme of the GISD implemented by the IUCN Species Survival Commission (SSC) Invasive Species Specialist Group (Figure 1).

The extended GISS [25] identified a set of six impact classes (herbivory; competition; predation; disease transmission; hybridisation; impact on ecosystem, other than those mentioned before, i.e., chemical, physical, or structural changes), which we here term impact mechanisms (Box 1). Each of these mechanisms was associated with one of a sequential series of six impact scenarios (ranked 0–5) describing increasing levels of impact by aliens by that mechanism. These semi-quantitative scenarios were designed such that each step change in category reflects an increase in the order of magnitude of the particular impact so that a new level of organisation is involved. Thus: (0) no discernible impact; (1) discernible impacts, but no effects on individual fitness; (2) effects on fitness, but not on populations; (3) changes to populations, but not to community composition; (4) community changes, which are reversible; and (5) irreversible community changes and extinctions. Species impacts are assessed and assigned to a scenario for each impact mechanism. The scenario ranks assigned for each impact mechanism can be summed to produce an overall impact score. Species can then be compared with respect to these scores, for example to identify traits associated with higher levels of impact [24,26].

Our classification scheme is based on the impact mechanisms and scenarios presented by Kumschick and colleagues [25], but modified in four ways. First, and most importantly, we added new scenarios for mechanisms of impact additionally identi-

fied in the IUCN GISD. The GISD scheme identifies 13 impact mechanisms (Figure 1), along with associated outcomes of those impacts in respect of changes to environmental or socio-economic parameters. Of these, numbers 1–4 and 8 correspond directly to scenarios in Kumschick and colleagues [25], while mechanisms 9–11 are captured under Kumschick and colleague's mechanism of impact on ecosystem (see above). We therefore expanded the Kumschick and colleagues scheme by adding explicit scenarios for four mechanisms of impact in the GISD schema: parasitism (impact 5 in Figure 1), poisoning/toxicity (impact 6), bio-fouling (impact 7), and interaction with other invasive species (impact 12). We ignored the thirteenth mechanism (other), as it is not possible to derive scenarios for unspecific impacts (although impacts not captured by the first 12 mechanisms can still be assigned on the basis of the general meanings identified in Table 1).

Second, Kumschick and colleagues [25] described scenarios of deleterious and beneficial environmental impacts by alien taxa, but here we consider only the deleterious impacts (see point 2 above). Third, we combined the two lowest ranking scenarios for each mechanism. The zero-ranked scenario in each case always refers to "No impact known or detectable," but as the presence of an alien individual in a new environment always produces a change to the properties of an ecosystem (e.g., by altering its genetic or species diversity), by definition it has a non-zero impact in some context. Note that there is a crucial distinction between species with no known impacts, and species for which there is insufficient evidence to assess impact (see section in Box 2 on "Data Deficient" species). Finally, we edited the scenarios of Kumschick and colleagues [25] to resolve some terminological ambiguities in respect of our classifications, and to ensure that the scenarios are aligned with the mechanisms of impact identified in the GISD.

Instead of using the impact mechanisms and scenarios to produce an overall numerical impact score for a species, we use the scenarios to assign a species to one of five sequential categories of impact: in ascending order of impact, these categories are Minimal (ML), Minor (MI), Moderate (MO), Major (MR), and Massive (MA) (Figure 2; Box 2). The process of categorisation would involve collation of all available evidence on impact for the members of a taxon from all regions to which the taxa have been introduced (or from the focal region where relevant), and using that evidence to inform expert opinion on the category of impact indicated. The impact category to which a species is assigned is that corresponding to the highest level of deleterious impact identified from any of the impact mechanisms (Box 2; Table 1). Listing of a species in a higher category explicitly assumes that there is evidence that the species has had a greater deleterious impact on some aspect of an environment in which it is alien than a species in a lower category of impact. Impact rating should be considered in the absence of management, but our approach may contribute to a process of prioritising species for management (e.g., if a new incursion by a high impact species is detected), as is required by Aichi target 9 of the Convention on Biological Diversity's Strategic Plan 2020 (www.cbd.int/sp/targets/rationale/target-9). We would expect some species to move between categories in successive categorisation processes, at the most trivial level from Not Evaluated (NE) into one of the evaluated categories (Figure 2), but subsequently from No Alien Population (NA) to an alien category (Data Deficient [DD], or one of Minimal (ML), Minor (MI), Moderate (MO), Major (MR), or Massive (MA) if introduced into the wild beyond its natural range limits), and potentially then between different categories of alien impact. Species whose alien status is uncertain can be identified as cryptogenic (CG) within any of the impact categories (Box 2).

Box 1. A Glossary of Key Definitions

Alien species: a species moved by human activities beyond the limits of its native geographic range into an area in which it does not naturally occur. The movement allows the species to overcome fundamental biogeographic barriers to its natural dispersal. Common synonyms are exotic, introduced, non-indigenous, or non-native [50].

Environmental impact: a measurable change to the properties of an ecosystem by an alien species [2]. Our definition means that our scheme applies to all ecosystems—whether largely natural or largely managed by humans—but explicitly considers only effects that have impacts on the native biota or the ecosystem processes that derive from that environment. The same alien species may also have impacts on human societies and economies [37], but these represent additional and complex dimensions of impacts [51–56], and one should avoid conflating environmental with non-environmental impacts.

Deleterious impact: an impact that changes the environment in such a way as to reduce native biodiversity or alter ecosystem function to the detriment of the incumbent native species—as indicated by a change in importance or abundance following invasion. This is similar to the "adverse effect" concept [57]. This definition intentionally excludes societal judgments regarding the desirability or value of aliens, although our assumption is that the classification will be used as a mechanism to prevent impacts that are judged to be "negative" by those concerned.

Impact mechanisms: categories into which different types of alien species impact are classified. The IUCN GISD identifies 13 such categories; a list of these impact mechanisms is given in Figure 1.

Propagule pressure: a composite measure of the number of individuals that are released or escape into a region to which they are not native. It incorporates estimates of the absolute number of individuals involved in any one release/escape event (propagule size) and the number of discrete such events (propagule number) [58].

Residence time: the length of time that an alien species has been in its introduced range [59].

Uncertainty

There are likely to be many cases where uncertainty exists about the correct categorisation of a species in terms of the magnitude of its impacts, even for species for which data is considered adequate (Box 2; Table 1). Consequently, it will be sensible to include an estimate of the degree of uncertainty attached to all categorisations, so that the degree of confidence in every classification is explicitly made clear. Only epistemic or reducible uncertainty (i.e., uncertainty due to data quality) is of importance for the

Impact mechanism

1. Competition
2. Predation
3. Hybridisation
4. Disease transmission
5. Parasitism
6. Poisoning/Toxicity
7. Bio-fouling
8. Grazing/Herbivory/ Browsing
9. Rooting/Digging
10. Trampling
11. Flammability
12. Interaction with other invasive species
13. Other

Schinus terebinthifolius

Imperata cylindrica

Adelges piceae

Impact outcomes

ENVIRONMENTAL IMPACT OUTCOMES

Ecosystem/Habitat

Modification of hydrology/water regulation or purification and quality/soil moisture
Primary productivity alteration
Modification of nutrient pool (e.g. soil N availability) and fluxes (e.g. litter decomposition)
Modification of natural benthic communities
Modification of food web (includes trophic cascades, plant-pollinator interactions, natural enemies - biocontrol)
Reduction in native biodiversity
Unspecified ecosystem modification
Habitat degradation
Habitat or refugia replacement/loss
Physical disturbance
Modification of fire regime
Modification of successional patterns
Soil or sediment modification: erosion
Soil or sediment modification: accretion/bioaccumulation
Soil or sediment modification: modification of structure
Soil or sediment modification: modification of pH, salinity or organic substances
Other (specify)

Species/ population

Population size decline
Species range change (i.e. contraction, expansion, shift)
Reduces/inhibits the growth of other species
Alteration of genetic resources: changes in gene pool/selective loss of genotypes
Indirect mortality
Plant/animal health
Interference with reproduction

SOCIO-ECONOMIC IMPACT OUTCOMES

Damage to agriculture (food, fuel and fibre)
Damage to forestry (food, fuel and fibre)
Damage to aquaculture/mariculture/fishery
Reduce/damage livestock and products (food, fibre, labour...)
Human health (diseases, allergies, injuries, toxicity)
Human nuisance
Modification of landscape
Damage to infrastructures
Damage to ornamentals (gardens, golf courses...)
Modification of cultural, educational, aesthetic, religious and ornamental values
Alteration of recreational use and tourism
Impact on trade/international relations
Limited access to water, land and other
Other economic impact (damages to properties)

Figure 1. Impact scheme of the Global Invasive Species Database, implemented by the IUCN Species Survival Commission (SSC) Invasive Species Specialist Group. The GISD stores detailed information on more than 800 invasive alien species, including on the impacts they cause. The GISD has recently been redesigned, and all information has been re-classified in order to improve the searching functionalities of the database. The schema developed for the revised GISD has allowed all species stored in the database to be coded in respect of the direct mechanisms by which their impacts occur (e.g., predation), and by the outcomes of those impact mechanisms on the environment or on human activities. For example, the grass *Imperata cylindrica* (Poales: Poaceae) almost doubles litter biomass in invaded locations, which increases potential fuel for fires (impact mechanism coded as flammability, and impact outcome as modification of fire regime). The plant *Schinus terebinthifolius* (Sapindales: Anacardiaceae) is a bio-fouling agent, forming dense thickets in gullies and river bottoms, with the ultimate effect of changing the hydrology of river streams of invaded freshwater bodies (mechanism coded as bio-fouling, and impact outcome described as modification of hydrology). The insect *Adelges piceae* (Hemiptera: Adelgidae) releases a toxin causing stress to trees, which eventually die. The impact outcome of *A. piceae* is described in GISD as damage to forestry, with its mechanism of impact coded as poisoning/toxicity, but it can also be coded as having an environmental impact on plant/animal health, as it has been here. In the table, mechanisms and outcomes are reported in two separate columns, and the three examples of the connections between mechanisms and outcomes are shown. Impact outcomes in the GISD database can be environmental or socio-economic, but our categorisation scheme of species in terms of the magnitudes of their impacts (Figure 2; Table 1) concerns only the former.
doi:10.1371/journal.pbio.1001850.g001

proposed classification. Uncertainty related to variation in impacts in space or time (stochasticity or irreducible uncertainty) is not considered because only the highest impact reported is considered. We acknowledge that there are different ways to characterise uncertainty, but we suggest for practical purposes a categorisation of uncertainty into three levels—high, medium, and low confidence—based on approaches used by the Intergovernmental Panel on Climate Change (IPCC) [27] and European and Mediterranean Plant Protection Organization (EPPO) [28,29]. Further details are given in Tables S1 and S2 and Text S1.

Discussion

What follows is a condensed version of our Discussion for the general reader: we encourage those with a more specific interest in the subject to read the full version, available as Text S2.

There are abundant examples of alien species having deleterious environmental impacts that alter the structure, function, or dynamics of the ecosystem concerned. The need to prioritise management responses to these impacts (or the objectively quantified risk of such threats) provides a strong impetus to develop a standardised system by which impacts can be rigorously quantified and compared in terms of their magnitudes. However, there is no commonly employed method of quantifying and ranking impacts on biodiversity and ecosystems [30]. Regulatory bodies have attempted to develop a variety of different

Table 1. Impact criteria for assigning alien species to different categories in the classification scheme (Box 2).

Impact Class	Massive (MA)	Major (MR)	Moderate (MO)	Minor (MI)	Minimal (ML)
Categories should adhere to the following general meaning	*Causes at least local extinction of species, and irreversible changes in community composition; even if the alien species is removed the system does not recover its original state*	*Causes changes in community composition, which are reversible if the alien species is removed*	*Causes declines in population densities, but no changes in community composition*	*Causes reductions in individual fitness, but no declines in native population densities*	*No effect on fitness of individuals of native species*
Competition (1)	Competition resulting in replacement or local extinction of one or several native species; changes in community composition are irreversible	Competition resulting in local or population extinction of at least one native species, leading to changes in community composition, but changes are reversible when the alien species is removed	Competition resulting in a decline of population size of at least one native species, but no changes in community composition	Competition affects fitness (e.g., growth, reproduction, defence, immunocompetence) of native individuals without decline of their populations	Negligible level of competition with native species; reduction of fitness of native individuals is not detectable
Predation (2)	Predators directly or indirectly (e.g., via mesopredator release) resulting in replacement or local extinction of one or several native species (i.e., species vanish from communities at sites where they occurred before the alien arrived); changes in community composition are irreversible	Predators directly or indirectly (e.g., via mesopredator release) resulting in local or population extinction of at least one native species, leading to changes in community composition, but changes are reversible when the alien species is removed	Predators directly or indirectly (e.g., via mesopredator release) resulting in a decline of population size of at least one native species but no changes in community composition	Predators directly or indirectly (e.g., via mesopredator release) affecting fitness (e.g., growth, reproduction) of native individuals without decline of their populations	Negligible level of predation on native species
Hybridisation (3)	Hybridisation between the alien species and native species is common in the wild; hybrids are fully vigorous and fertile; pure native species cannot be recovered by removing the alien, resulting in replacement or local extinction of native species by introgressive hybridisation (genomic extinction)	Hybridisation between alien species and native species is common in the wild; F1 hybrids are vigorous and fertile, however offspring of F1 hybrids are weak and sterile (hybrid breakdown), thus limited gene flow between alien and natives; individuals of alien species and hybrids discernible from pure natives, pure native populations can be recovered by removing the alien and hybrids.	Hybridisation between alien species and native species is regularly observed in the wild; hybrids are vigorous, but sterile (reduced hybrid fertility),limited gene flow between alien and natives, local decline of populations of pure native species, but pure native species persists	Hybridisation between alien species and native species is observed in the wild, but rare; hybrids are weak and never reach maturity (reduced hybrid viability), no decline of pure native populations	No hybridisation between alien species and native species observed in the wild (prezygotic barriers), hybridisation with a native species might be possible in captivity
Transmission of diseases to native species (4)	Transmission of diseases to native species resulting in replacement or local extinction of native species (i.e., species vanish from communities at sites where they occurred before the alien arrived); changes in community composition are irreversible	Transmission of diseases to native species resulting in local or population extinction of at least one native species, leading to changes in community composition, but changes are reversible when the alien species is removed	Transmission of diseases to native species resulting in a decline of population size of at least one native species, but no changes in community composition	Transmission of diseases to native species affects fitness (e.g., growth, reproduction, defence, immunocompetence) of native individuals without decline of their populations	The alien species is not a host of diseases transmissible to native species or very low level of transmission of diseases to native species; reduction of fitness of native individuals is not detectable
Parasitism (5)	Parasites or pathogens directly or indirectly (e.g., apparent competition) resulting in replacement or local extinction of one or several native species (i.e., species vanish from communities at sites where they occurred before the alien arrived); changes in community composition are irreversible	Parasites or pathogens directly or indirectly (e.g., apparent competition) resulting in local or population extinction of at least one native species, leading to changes in community composition, but changes are reversible when the alien species is removed	Parasites or pathogens directly or indirectly (e.g., apparent competition) resulting in a decline of population size of at least one native species but no changes in community composition	Parasites or pathogens directly or indirectly (e.g., apparent competition) affecting fitness (e.g., growth, reproduction, defence, immunocompetence) of native individuals without decline of their populations	Negligible level of parasitism or disease incidence (pathogens) on native species, reduction of fitness of native individuals is not detectable

Table 1. Cont.

Impact Class	Massive (MA)	Major (MR)	Moderate (MO)	Minor (MI)	Minimal (ML)
Poisoning/ toxicity (6)	The alien species is toxic/ allergenic by ingestion, inhalation, or contact to wildlife or allelopathic to plants, resulting in replacement or local extinction of native species; changes in community composition are irreversible	The alien species is toxic/allergenic by ingestion, inhalation, or contact to wildlife or allelopathic to plants, resulting in local or population extinction of at least one native species (i.e., species vanish from communities at sites where they occurred before the alien arrived), leading to changes in community composition, but changes are reversible when the alien species is removed	The alien species is toxic/allergenic by ingestion, inhalation, or contact to wildlife or allelopathic to plants, resulting in a decline of population size of at least one native species, but no changes in community composition (native species richness)	The alien species is toxic/allergenic by ingestion, inhalation, or contact to wildlife or allelopathic to plants, affects fitness (e.g., growth, reproduction, defence, immunocompetence) of native individuals without decline of their populations	The alien species is not toxic/allergenic/ allelopathic, or if it is, the level is very low, reduction of fitness of native individuals is not detectable
Bio-fouling (7)	Bio-fouling resulting in replacement or local extinction of one or several native species (i.e., species vanish from communities at sites where they occurred before the alien arrived); changes in community composition are irreversible	Bio-fouling resulting in local or population extinction of at least one native species, leading to changes in community composition, but changes are reversible when the alien species is removed	Bio-fouling resulting in a decline of population size of at least one native species, but no changes in community composition	Bio-fouling affects fitness (e.g., growth, reproduction, defence, immunocompetence) of native individuals without decline of their populations	Negligible level of bio-fouling on native species; reduction of fitness of native individuals is not detectable
Grazing/ herbivory/ browsing (8)	Herbivory resulting in replacement or local extinction of one or several native plant species (i.e., species vanish from communities at sites where they occurred before the alien arrived); changes in community composition are irreversible	Herbivory resulting in local or population extinction of at least one native plant species, leading to changes in community composition, but changes are reversible when the alien species is removed	Herbivory resulting in a decline of population size of at least one native species, but no changes in community composition	Herbivory affects fitness (e.g., growth, reproduction, defence, immunocompetence) of individual native plants without decline of their populations	Negligible level of herbivory on native plant species, reduction of fitness on native plants is not detectable
Chemical, physical, or structural impact on ecosystem (9, 10, 11)	Many changes in chemical, physical, and/or structural biotope characteristics; or changes in nutrient and water cycling; or disturbance regimes; or changes in natural succession, resulting in replacement or local extinction of native species (i.e., species vanish from communities at sites where they occurred before the alien arrived); changes (abiotic and biotic) are irreversible	Changes in chemical, physical, and/or structural biotope characteristics; or changes in nutrient cycling; or disturbance regimes; or changes in natural succession, resulting in local extinction of at least one native species, leading to changes in community composition, but changes are reversible when the alien species is removed	Changes in chemical, physical, and/or structural biotope characteristics; or changes in nutrient cycling; or disturbance regimes; or changes in natural succession, resulting in a decline of population size of at least one native species, but no changes in community composition	Changes in chemical, physical, and/or structural biotope characteristics; or changes in nutrient cycling; or disturbance regimes; or changes in natural succession detectable, affecting fitness (e.g., growth, reproduction, defence, immunocompetence) of native individuals without decline of their populations	No changes in chemical, physical, and/or structural biotope characteristics; or changes in nutrient cycling; or disturbance regimes; or changes in natural succession detectable, or changes are small with no reduction of fitness of native individuals detectable

Table 1. Cont.

Impact Class	Massive (MA)	Major (MR)	Moderate (MO)	Minor (MI)	Minimal (ML)
Interaction with other alien species (12)	Interaction of an alien species with other aliens (e.g., pollination, seed dispersal, habitat modification) facilitates replacement or local extinction of one or several native species (i.e., species vanish from communities at sites where they occurred before the alien arrived), and produces irreversible changes in community composition that would not have occurred in the absence of the species. These interactions may be included in other impact classes (e.g., predation, apparent competition) but would not have resulted in the particular level of impact without an interaction with other alien species	Interaction of an alien species with other aliens (e.g., pollination, seed dispersal, habitat modification) facilitates local or population extinction of at least one native species, and produces changes in community composition that are reversible but would not have occurred in the absence of the species. These interactions may be included in other impact classes (e.g., predation, apparent competition) but would not have resulted in the particular level of impact without an interaction with other alien species	Interaction of an alien species with other aliens (e.g., pollination, seed dispersal, habitat modification) facilitates a decline of population size of at least one native species, but no changes in community composition; changes would not have occurred in the absence of the species. These interactions may be included in other impact classes (e.g., predation, apparent competition) but would not have resulted in the particular level of impact without an interaction with other alien species	Interaction of an alien species with other aliens (e.g., pollination, seed dispersal) affects fitness (e.g., growth, reproduction, defence, immunocompetence) of native species' individuals without decline of their populations; changes would not have occurred in the absence of the species. These interactions may be included in other impact classes (e.g., predation, apparent competition) but would not have resulted in the particular level of impact without an interaction with other alien species	Interaction of an alien species with other aliens (e.g., pollination, seed dispersal) but with minimal effects on native species; reduction of fitness of native individuals is not detectable

These categories are for species that have been evaluated, have alien populations (i.e., are known to have been introduced outside their native range), and for which there is adequate data to allow classification (see Figure 2). Classification follows the general principle outlined in the first row. However, we specifically outlined the different mechanisms through which an alien species can cause impacts in order to help assessors to look at the different aspects and to identify potential research gaps. Numbers next to different impact classes reference the numbering of impacts in the classification of impact mechanisms in the GISD (Figure 1).
doi:10.1371/journal.pbio.1001850.t001

schemes [31–33], but a unified standard classification does not exist. Indeed, the lack of a standard metric, coupled with data deficiencies, is likely a major reason why risk assessments rarely include quantitative evaluations of impact [34]. We believe that our proposed classification scheme (Figure 2; Table 1; Box 2) provides a pragmatic solution to some of these needs. It also has the attractive quality that it follows a similar approach to the already widely adopted Red Listing approach to categorising extinction risk, and so could theoretically be quickly integrated with existing practices and policies across the globe. It aligns with mechanisms of impact identified in the IUCN GISD (Figure 1), and hence can be used in conjunction with that important database. The interlink between the IUCN GISD and Red List may also permit a more structured application of the present scheme to the evaluation of the impact of alien species on species assessed in the Red List.

Our scheme overcomes the problems that arise from the fact that there is no standard metric of impact, or method of quantifying it. By relating quantitative studies to a set of standardised semi-quantitative scenarios enhanced by descriptions, we can identify and rank mechanisms of impact indicated by the evidence provided. Although there is often a significant degree of uncertainty surrounding the impact of any given alien species, both because of measurement error and subsequent translation of what a quantitative trait measure means in terms of actual environmental change, the broad separation of our categories in terms of the level of impact they represent means that impacts can be classified with a good degree of confidence [24]. Furthermore, our scheme includes a mechanism for appending estimates of uncertainty to each categorisation (Text S1). Similar issues of uncertainty pertain to the IUCN Red List criteria and categories (albeit that they are often overlooked), but while the precise categorisation of some species is the subject of considerable debate [35], there is little doubt that the Red List functions as an effective and credible guide to the threat of extinction and as a valuable trend indicator over time [36]. We hope that our categorisation scheme will come to be viewed in the same light.

In contrast to the previous use of such scenarios to estimate overall impact [24,26,37], here they are simply used to identify the mechanism by which a species has its highest impact. A lack of data on some mechanisms can affect estimates of overall impact, but does not prevent the classification of a species under our scheme, if information is available on other mechanisms of impact. Our categorisation scheme is therefore effective with less available data than required to assess the overall impact of a species.

A lack of information on some mechanisms of impact may lead to a species being placed in a lower impact category than might otherwise be the case. However, in many cases, it will be difficult to distinguish whether an alien is the driver of environmental changes, or simply a "passenger" responding to the same driver as the natives [38]. Synergistic interactions between alien species and other stressors are also possible—and perhaps increasingly common—but difficult to anticipate [39]. This suggests that categorisation will be cautious: an alien is likely to be assigned to a high impact category if it is associated with significant change, even if it is not the main driver. This is a sensible situation under the precautionary principle, where benefit of the doubt should not be given to the alien. However, our system is intended to be dynamic, allowing for updates as new or more reliable data become available, and as the documented impact

Box 2. Description of the Categories in the Impact Classification Scheme

The relationship between categories is shown in Figure 2. A species is considered to have a given level of impact (**MA**, **MR**, **MO**, **MI**, or **ML**) when the best available evidence indicates that it has previously had impacts in a region to which it is not native that meet any of the relevant criteria presented in Table 1. Species are categorised by the most severe impact recorded under any impact mechanism (Table 1), as follows:

Massive (MA) A species is considered to have **Massive** impacts when it *leads to the replacement and local extinction of native species, and produces irreversible changes in the structure of communities and the abiotic or biotic composition of ecosystems*. Note that "local" refers to the typical spatial extent over which the original native communities can be characterised.

Major (MR) A species is considered to have **Major** impacts when it *causes the local or population extinction of at least one native species, and leads to reversible changes in the structure of communities and the abiotic or biotic composition of ecosystems*, and has no impacts that cause it to be classified in the **MA** impact category.

Moderate (MO) A species is considered to have **Moderate** impacts when it *causes declines in the population densities of native species, but no changes to the structure of communities or to the abiotic or biotic composition of ecosystems*, and has no impacts that would cause it to be classified in a higher impact category.

Minor (MI) A species is considered to have **Minor** impacts when it *causes reductions in the fitness of individuals in the native biota, but no declines in native population densities*, and has no impacts that would cause it to be classified in a higher impact category.

Minimal (ML) A species is considered to have **Minimal** impacts when it is *unlikely to have caused deleterious impacts on the native biota or abiotic environment*. Species that have been evaluated under the categorisation process but for which impacts have not been assessed in any study should not be classified in this category, but rather should be categorised as **Data Deficient**.

Data Deficient (DD) A species is categorised as **Data Deficient** when the best available evidence indicates that it has individuals existing in a wild state in a region beyond the boundary of its native geographic range, but either there is inadequate information to classify the species with respect to its impact, or insufficient time has elapsed since introduction for impacts to have become apparent. It is expected that all introduced species will have an impact at some level, because by definition an alien individual in a new environment has a non-zero impact. However, listing a species as **Data Deficient** recognises that current information is insufficient to assess that level of impact.

No Alien Populations (NA) A species is categorised as **No Alien Populations** when there is no reliable evidence that it has or had individuals existing in a wild state in a region beyond the boundary of its native geographic range. We assume that absence of evidence is evidence of absence in this case, as it is impossible to prove that a species has no alien individuals anywhere in the world. Species with individuals kept in captivity or cultivation in an area to which it is not native [60] would be classified here. A species could currently have no individuals existing in a wild state in a region beyond the boundary of its native geographic range because it has died out in, or has been eradicated from, such an area. In these cases, there should be evidence relating to impact that causes it to be classified in one of the impact categories (**ML**, **MI**, **MO**, **MR**, **MA**), or alternatively no evidence of impact, which would cause it to be classified as **Data Deficient**.

Not Evaluated (NE) A species is **Not Evaluated** when it has not yet been evaluated against the criteria, as is also the case in the IUCN Red List [23].

Cryptogenic (CG) Cryptogenic is not a category within the scheme presented in Figure 2, but rather a label to be applied to those taxa for which it is unclear, following evaluation, whether the individuals present at a location are native or alien [61]. This is a particular problem in the marine realm, for cosmopolitan plants and for many stored product arthropod pests, for which the native geographic ranges are unknown. Cryptogenic taxa may have deleterious impacts where they occur [62,63]. We suggest on the basis of the precautionary principle that cryptogenic species are evaluated as if they were aliens, but that their impact categorisation is modified by the **CG** label (e.g., for a cryptogenic species with **Major** impact: *Genus species* **MR [CG]**).

history of a species unfolds through space and time [40–42]. In fact, the classification scheme could in practice serve to identify knowledge gaps for invaders for which there is currently little or no information.

The use of standardised scenarios allows analysis of a wide range of factors relating to impact, such as correlates of magnitude, variation, and temporal and spatial change. The category of impact to which an alien species is assigned can increase or decrease as more deleterious impacts are discovered, if the alien species is subsequently identified as a passenger rather than a driver of change, or if environmental influences change. The protocol can also be applied with minor modification to impacts at a range of spatial scales, allowing national, regional, and global categorisation of impacts. It complements and can inform national assessment schemes in which species are assigned to different lists [43–45] depending on whether they are species with a low risk of impact ("white list," ML, or perhaps MI in this scheme), of assumed or uncertain impact ("grey list"), or have measurable impacts of concern ("black list," corresponding to MO, MR, or MA) on environments. In all of these respects, the scheme is analogous to the IUCN Red List [46]. Another similarity with the IUCN Red List approach is that some impact listings, as with some threat listings, are likely to be context dependent. For example, a relatively widespread taxon may be classified as at high risk of extinction in some national Red Lists if the species is locally rare or threatened (e.g., the country is near the range edge). Similarly, an alien impact that is observed in one area of the introduced range may not occur elsewhere, or may not be as important elsewhere: invasiveness, and by extension impact, is a characteristic of a population rather than a species [2,47]. Overall, the assessment of impacts at more restricted scales may predominantly depend on evidence of impacts elsewhere (which may be subject to higher error, given context-dependent variation), whereas at large scales, information on impacts will increasingly derive from the focal region.

All of this highlights the importance of ensuring that the impacts of aliens on populations and communities are measured at an appropriate spatial scale, taking into account the typical spatial size at which original native communities can be characterised (termed the "local scale" here). Studies at very restricted spatial scales (i.e., patches of 10s or 100s of square metres)

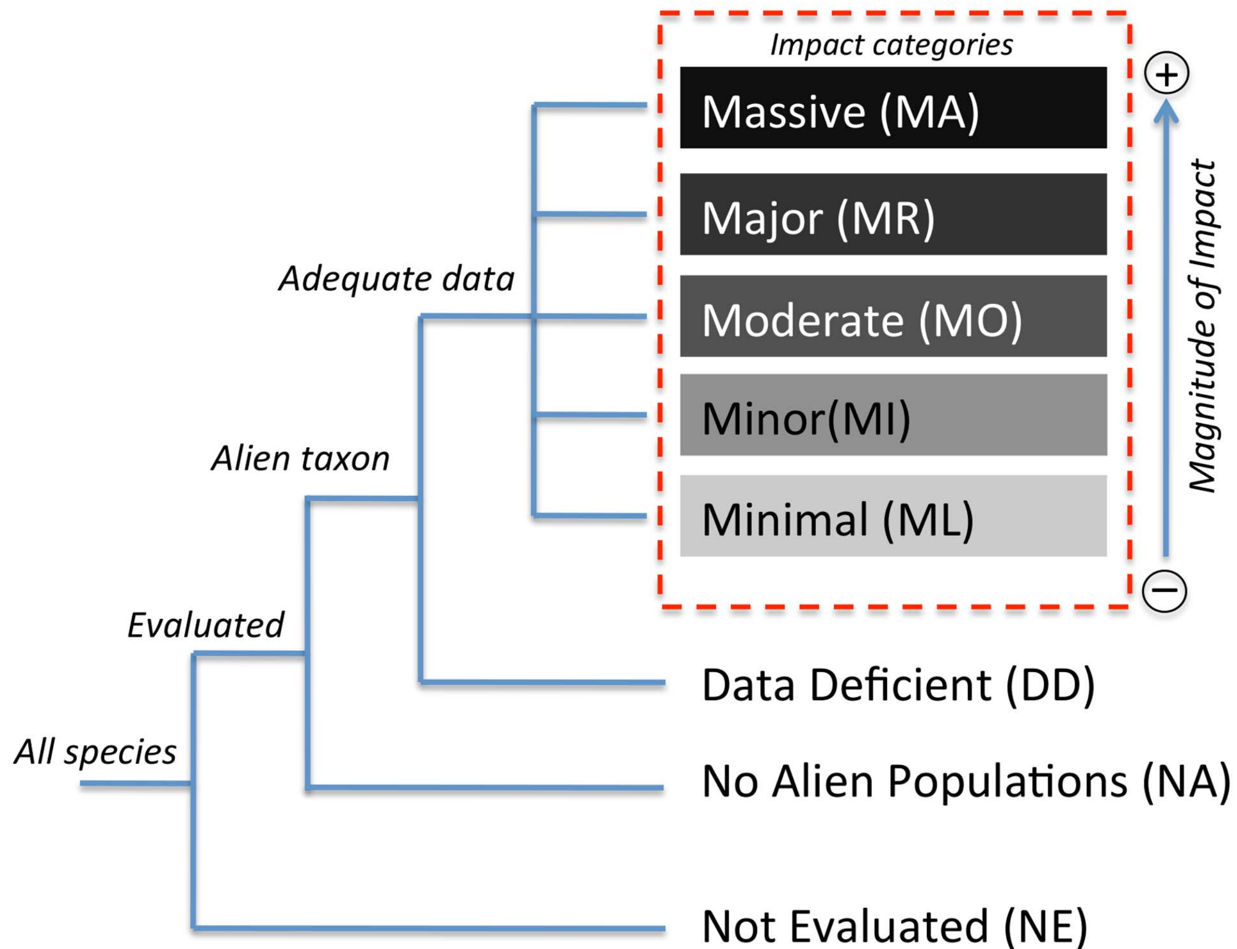

Figure 2. The different categories in the alien species impact scheme, and the relationship between them. Descriptions of the categories are provided in Box 2. The CG category is not represented in this diagram as CG taxa may be found in any category.
doi:10.1371/journal.pbio.1001850.g002

might overestimate impacts if extrapolated to larger scales, while studies at extensive spatial scales (i.e., regional or national) might underestimate them. For example, an alien species might be shown in a field experiment to exclude natives from areas the size of experimental plots, and perhaps even to extirpate natives from entire habitat patches, without having a significant effect on community diversity (e.g., because of the influence of spatial dynamics, refugia, or rescue effects). In this case, it is likely that populations of some natives would have declined (e.g., competitors or food species) in the habitats in which the alien species occurs, without resulting in local extinctions: the appropriate classification under our scheme would therefore be MO in this case (Table 1). This approach has the benefit of identifying impacts demonstrated in very small habitat patches that may be a cause for greater concern in the future.

One shortcoming of the proposed classification scheme is that it is not designed to be predictive by itself. For example, it cannot be applied to species with no previous history of alien populations (if evaluated, these species cannot be classified other than NA), and, as recorded impacts usually accrue with population growth, species that have not been introduced for long (short residence times; Box 1) or not introduced in large numbers (low propagule pressures; Box 1) are likely to receive a low rating. Nevertheless, the scheme could provide predictive information on the likely magnitude of impacts of a species, if it is phylogenetically or functionally similar to a species that has known impacts as an alien on the native biota or abiotic environment [33], or if there is a mechanistic understanding of how impacts might progress. This may be helpful given that a history of impact elsewhere is currently often considered to be the best available predictor of the impact potential of an alien species [40,48,49], but is of no use for predicting impacts of species with no alien populations. Such species could be assessed under our scheme, but with their categorisation assigned a high level of uncertainty. We do not advocate that such approaches substitute for the precautionary principle in cases of species with unknown impacts, but they may nevertheless help to understand which species may be most damaging if introduced. A future development of the scheme would be to include an estimate of potential impact for such species.

Supporting Information

Figure S1 The relationship between the overall potential environmental impact score and the impact category to which the species is assigned under our classification scheme, for data on alien mammals in Europe (from [9]). Environmental impact score is the sum of the impacts over the six categories given by Kumschick and colleagues (39). Species are assigned to impact category on the basis of the largest impact value in any of the six categories. Note that Kumschick and colleagues (39) do not score impacts under several of the classes listed in Table 1. The analysis is confined to impacts recorded for species in their alien ranges in Europe (indicating the scalable nature of our approach): a global analysis might shift some species to higher impact categories. Note that the data points have been jittered to improve visibility. Impact score and category are clearly positively related, but some species can have higher scores than other species in higher categories.
(TIF)

Figure S2 Relationship between the overall environmental impact of European alien plants (the median score across all assessed classes of impact. Note that not all classes of impact in Table 1 were assessed) and the impact classification assigned under our scheme (defined by the highest score achieved in any of the impact classes). Species with names indicated have, compared to their average impact across the classes assessed, a disproportionally strong impact in one individual class. While their high impact may be overlooked when assessing the overall impact, it is captured by our suggested classification scheme under which species are assigned on the basis of maximum, not average, impact. For example, *Cortaderia selloana* exerts a strong impact (**MA**) on ecosystem processes, its impacts in other classes being **MO** at most. Note that data points have been jittered to improve visibility. Based on M. Vilà, Z. Marková, P. Pyšek, J. Pergl (unpublished data) following the impact assessment methodology of [10].
(TIF)

Table S1 Guidance regarding the use of the confidence rating (modified from the EPPO pest risk assessment decision support scheme [2,64]).
(DOCX)

Table S2 Suggested distribution of likelihoods (in percent) of the impact of alien species being in a certain category depending on the confidence of the assessment. Probability distributions follow a standardised beta distribution with parameters α and β. The histogram below the table provides a pictorial representation of the same probabilities.
(DOCX)

Text S1 Categorising uncertainty.
(DOCX)

Text S2 Full version of the Discussion.
(DOCX)

Acknowledgments

This study is a joint effort of the working group sImpact that formed at a workshop at sDiv, the Synthesis Centre within the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig. We thank Ben Collen and Christian Lexer for comments that improved the manuscript.

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