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Crayfish plague dilemma: how to be a courteous killer?

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Aphanomyces astaci is among the most serious pathogens affecting European aquatic ecosystems. We demonstrate that both virulence of *A. astaci* isolates and resistance of native European crayfish stocks vary notably. Some native European crayfish stocks latently carry crayfish plague, indicating adaptation and contemporary co-evolution between host and pathogen. The earliest introduced *A. astaci* genotypes have adapted to novel, susceptible native European crayfishes, likely under an evolutionary pressure to maintain a necessary host population as an essential habitat. Then, highly virulent genotypes that were introduced together with their original American hosts, have more resistant host populations present in Europe. This creates a dilemma for *A. astaci*: whether to increase virulence to better utilize invasive American hosts or to reduce virulence to better utilize the native European hosts. All *A. astaci* genotypes are potent killers, but they already show lowered virulence similarly to previous examples of virulence evolution in novel pathogens.

Introduction

Crayfish plague (*Aphanomyces astaci*) was introduced to Europe during the late 19th century (Souty-Grosset *et al.* 2006) which resulted in a catastrophic series of disease epidemics eradicating most of the native European freshwater crayfish (Holdich 2002). The disease entered the last remaining untouched corners of the continent, such as Turkey (year 1984), the British Isles (year 1981) and Norway (year 1974), roughly 100 years after its arrival in Europe. Since the first introduction of *A. astaci* to Europe, four additional genotypes have invaded the continent, together with their North American host crayfish species. Currently, five different *A. astaci* genotypes have been recognized (Huang *et al.* 1994, Diéguez-Uribeondo *et al.* 1995, Kozubíková *et al.* 2011).

The relationship between *A. astaci* and its native European hosts has been dynamic and rather intensively studied (Souty-Grosset *et al.* 2006), yet the co-evolution of the different genotypes with their hosts has so far been largely ignored. Until recently, the relationship has generally been dictated by the high killing rate of the pathogen among the host population and the low resistance of the hosts (Edgerton *et al.* 2004). Accordingly, the prevailing theories also used in the management actions of crayfish stocks have been based on the assumption of crayfish plague epidemics leading to aquatic ecosystems devoid of native European crayfish (Westman 2000).

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Most *A. astaci* genotypes have spread together with introductions of crayfish of North American origin. The introductions have been either accidental or controlled with the purpose to recreate harvestable crayfish stocks using novel species (Edgerton *et al.* 2004). Also, attempts to produce disease-free stocklings by artificial incubation have taken place (Nylund and Westman 1992), but even the tested sophisticated hatchery systems have faced problems. *A. astaci* has been shown to travel from infected broodstock to offspring even via artificially incubated eggs (Makkonen *et al.* 2010).

The *Aphanomyces* genus consists of 35–40 described species ranging from specialized plant or animal parasites to saprotrophic species growing on decaying organic material (Johnson *et al.* 2002, Dieguez-Uribeondo *et al.* 2009). The genus *Aphanomyces* appears to be the first lineage to diverge, and the most ancestral group, among the Saprolegniales (Oomycetes) (Petersen and Rosendahl 2000), with *Aphanomyces* forming a basal clade with *Plectospira* and *Pachymetra*. *Aphanomyces* comprises three distinct lineages: plant parasitic, animal parasitic and saprophytic or opportunistic parasitic species (Dieguez-Uribeondo *et al.* 2009).

The life cycle of *Aphanomyces astaci* comprises the swimming zoospore stage, encysted spore stage and a period of hyphal growth within the host species (Dieguez-Uribeondo *et al.* 2006). The zoospores are produced within the hyphae which protrude into the ambient water and release primary spores that encyst and germinate (Svensson & Unestam 1975, Cerenius & Söderhäll 1984a, 1985). If a suitable surface is not found, a repeated zoospore emergence (RZE) allows the *A. astaci* spores to try and locate a suitable host up to three times (Cerenius and Söderhäll 1984, 1985). Once established on a suitable host surface, the spores germinate (Nyhlén and Unestam 1975) and with the aid of proteases and chitinases, the hyphae penetrate the host cuticle and the growth in the host starts (Unestam & Weiss 1970). The final outcome is the release of spores from the infected host (Söderhäll & Cerenius 1987). The quantity of the released spores depends on the severity of the infection, moribund noble crayfish have been reported to release up to 3.2 million spores (Makkonen *et al.* 2012c) while infected signal crayfish seem to constantly release a low number of spores (Strand *et al.* 2012).

When *A. astaci* hyphae penetrate through the crayfish body cavity, β-1,3-glucans of its cell wall are recognized by the crayfish blood cells via the non-self recognition system. This launches the prophenoloxidase system (proPO-system) which is the main defense reaction that the crayfish possesses against *A. astaci* infection (Söderhäll and Cerenius 1998). In this reaction, the pathogen is first encapsulated by semigranular blood cells. Then, a layer of granular blood cells is aggregated around the capsule and the proPO-system is activated during degranulation of the granular cells (Unestam and Nylund 1972). The final outcome in the activation of the proPO-system is a pathogen surrounded by melanin. Although the pathogens growth and dispersal are restricted, it is still alive (Söderhäll and Cerenius 1999).

In this paper, we summarize the conclusions based on the studies that have been carried out at the University of Eastern Finland on the relationship between crayfish plague (*A. astaci*) and its host crayfish species during the last decade. We also make references to the studies of other groups, when they are available within the defined topic.

Crayfish plague and crayfish co-evolution

As-genotype adaptation to novel hosts

The As-genotype *A. astaci* was introduced to Europe in the late 1800s likely accidentally (Aldermann 1996, Souty-Grosset *et al.* 2006) along with crayfish that had either been transferred in ballast waters or imported to be used for ornamental purposes. The first arrival of the North American crayfish disease occurred in southern Europe, in Italy. Regardless of that, the first arrived host species of *A. astaci* remains unknown, the first introduced *A. astaci* did not have an original North American host species habitat in Europe (Alderman 1996). This led to a repeated series of host jumps during its spread through European aquatic ecosystems (Makkonen *et al.* 2012a).

Due to originally high virulence towards native European crayfish (Unestam & Weiss 1970, Unestam 1972), rapidly spreading *A. astaci* destroyed most of the suitable habitat for itself by killing all the hosts (Ninni 1865, Hofer 1904), and consequently it is likely there was then a high selection pressure for reduced virulence and thus capability to sustain habitat in areas nearly devoid of suitable hosts (Makkonen *et al.* 2012a, 2012b). In central Europe, where the structure of the water courses is commonly simple and thus do not allow refuges, the suitable habitat for *A. astaci* disappeared rapidly (Alderman 1996).

Lively crayfish trade in central Europe and trappers moving equipment and crayfish between waterbodies contributed to the spreading of the disease (Alderman 1996) and were likely one of the key factors in sustaining the disease for the first decades since its European invasion. After *A. astaci* was introduced to the Nordic countries, especially to Finland and Sweden, the situation changed. Many of the new water courses in the Nordic countries were so complex and structurally fragmented, that numerous native crayfish stocks, subpopulations of the noble crayfish, were able to survive for long periods (Bohman and Edsman 2011). Interestingly, anecdotal and indirect evidence has remained on how crayfish populations used to recover after an *A. astaci* epidemic. Those cases led to the formulation of the hypothesis according to which crayfish plague may chronically infect the European crayfishes (Fürst 1995).

The original host crayfish species was likely introduced only in small numbers with the As-genotype. Given that the chitinase gene sequence of the As-genotype resembles that of strains found in *Procambarids* rather than that found on other studied possible host species, such as *Pacifastacus leniusculus* (Makkonen *et al.* 2012a), the original host species of the As-genotype could have been a *Procambarid*. However, in historical records available, *Procambarid* species are not known to have been introduced when the first *A. astaci* strain arrived. Anyhow, in the light of the current evidence, there is no reason to assume that the originally arrived As-genotype disease would have been adapted to infect the signal crayfish (*Pacifastacus leniusculus*). This has significant repercussions for the subsequent virulence evolution in this disease strain. Arguably, the century of co-evolutionary history between the As-genotype and its varying hosts has been one of the key factors differentiating the contemporary As-genotype from the other *A. astaci* RAPD genotypes (PsI, PsII, Pc and Or) (Kozubíková *et al.* 2011), that were later introduced during massive introductions of North American crayfish species.

Recently, it has been demonstrated that the noble crayfish is capable of surviving experimental *A. astaci* infection under laboratory conditions (Makkonen *et al.* 2012b, 2014). The noble crayfish individuals survived *A. astaci* infection after intense inoculations with either the PsI or As-genotype (Jussila *et al.* 2011a, Makkonen *et al.* 2012b). The noble crayfish have been able to fully survive infections especially with a particular As-Kemijoki isolate (UEFT2B) extracted from wild crayfish from the Kemijoki in the northernmost distribution range of *A. astacus* in Europe. In addition, two individual noble crayfish survived in one experiment after being infected with highly virulent PsI-genotype (UEF8866-2) isolated from the Puujärvi epidemics in 1996. These laboratory trials lasted for up to three months, thus indicating that an instant mass mortality would not have occurred within a single growing season typical to the northern European climate. In some trials, the whole experimental group survived (Makkonen *et al.* 2012b, 2014), not just a few individuals.

Previously, it had been suggested and reported that zoospores of *A. astaci* isolates could lose their motility, possibly resulting in the loss of their virulence, when maintained for long periods in laboratories (Unestam 1969), but we have not observed this among our isolates. Bearing this in mind, in our laboratory, As-Kemijoki *A. astaci* isolate, which has been shown to be the least virulent of the tested isolates, has been maintained for a rather short period of time, i.e. no more than 6 years.

The qPCR analysis of *A. astaci* (Vrålstad *et al.* 2009), carried out in our laboratory, showed that, for certain isolates, the level of *A. astaci* DNA detected in surviving experimentally-infected crayfish is very low, even at the detection level of A0, i.e. no detection. This might suggest that the differences in isolate virulence

Fig. 1. Noble crayfish (*Astacus astacus*) from the Kemijoki infected with As-genotype *Aphanomyces astaci* during the year 2006 acute *Aphanomyces astaci* epidemic showing melanisation and resulting exoskeleton erosion.

in this case could be due to the inability of the zoospores to attach on or penetrate through the crayfish exoskeleton. In most cases, the low agent levels have been associated with those As-genotype isolates that were unable to cause high mortality in our experiments (Makkonen *et al*. 2012b, 2014).

In our laboratory experiments, we have demonstrated that zoospore dose is very crucial for the induction of the infection and its progress (Makkonen *et al*. 2014). The intensity of pathogen attack, i.e. density of the zoospores in the ambient water, could also be a factor dictating the disease progress in the susceptible crayfish host. Normally, we have been using zoospore densities reflecting the intensities of acute *A. astaci* epidemic in nature (Makkonen *et al*. 2012c) and clearly exceeding the spore density which would occur as a result of a chronic infection in nature (Strand *et al*. 2012). In the case of experimental infection using the As-genotype from the Kemijoki epidemic (Makkonen *et al*. 2012b), we found variable mortality among the tested noble crayfish populations, quite often ranging from a very low to zero mortality.

In the original, and to our knowledge northernmost, As-genotype epidemic in the Kemijoki during 2006, the mortality was high but the epidemic progressed rather slowly, and some subpopulations of the Kemijoki noble crayfish have probably survived (P. Muje, Lappi Fishery Center, pers. comm.). During the first wave of the epidemic, the noble crayfish showed increased melanization (Fig. 1) frequently indicating a slow progress of the infection (our own observation). The As-genotype isolate from the Kemijoki may also be adapted to cooler conditions due to the geographic location of and transmission route to the Kemijoki in the northernmost range of *A. astacus*. Further research is needed to reveal if the disease faces a trade-off between virulence and cold-tolerance, and what in general is the mechanism of the decreased virulence in this particular strain.

PsI-genotype virulence

The highly virulent *A. astaci* isolate PsI-Puujärvi normally causes rapid 100% mortality among the noble crayfish stocks (Makkonen *et al*. 2012b, 2014) with an exception of low survival during a laboratory trial of limited length (Jussila *et al*. 2011a).

The high mortality typically caused by the PsI-genotype shows that one of the reasons for complete eradication of the As-genotype habitat, i.e. native European crayfish, would be the presence of the PsI-genotype *A. astaci's* carrier host North American signal crayfish in Europe. This invasive, non-native species has been purposely introduced to Finland and elsewhere in Europe in massive numbers (Souty-Grosset *et al*. 2006). The presence of the signal crayfish allows the PsI-genotype of *A. astaci* to sustain a permanent habitat within the distribution of the native Euro-

pean crayfish. It also serves as a reservoir and vector for the dispersal of the PsI-genotype disease along with the signal crayfish introductions.

So far, the tested PsI-genotype from the Puujärvi epidemic has caused 100% mortality among all the tested Finnish noble crayfish populations in a series of experiments in our laboratory (Makkonen *et al*. 2012b, Gruber *et al*. 2014, and our unpubl. data), with one exception (Jussila *et al*. 2011). In our experiments, the mortality started a few days to a week after initial inoculation, and 100% mortality was reached within a week. The Mikitänjärvi noble crayfish being an exception to this with postponed mortality and a slower dying rate (Makkonen *et al*. 2014). The tested PsI-genotype from the Puujärvi epidemic has been detected to be more virulent than any of the As-genotypes in our experiments even when the inoculation spore dose was 1000× higher for the rather virulent As-genotype from the Kivesjärvi epidemic (Evira6462/06) (Makkonen *et al*. 2014).

In the light of the high virulence of the PsI-genotype, the evolutionary pressure for the PsI-genotype to adapt to more susceptible host crayfish has been low, and is expected to remain low as long as they have their relatively resistant original hosts present. However, it is difficult to predict how the host–parasite co-evolution will proceed between the signal crayfish and its specific, native parasite, the PsI-genotype *A. astaci*. These results emphasize the importance of preventing additional introductions and spread of invasive crayfishes in Europe to minimize interactions between the multiple stressors of climate change and invasive species, while suggesting candidate regions for the debatable management option of assisted colonization, as has been suggested by Capinha *et al*. (2013).

The signal crayfish (*P. leniusculus*) was originally introduced to Europe, because it was thought that the co-existence with *A. astaci* in North America would have produced stocks immune to *A. astaci* infection (Westman 2000). Later it was discovered that this is not the case (Thörnqvist and Söderhäll 1993, Souty-Grosset *et al*. 2006, Jussila *et al*. 2013). In Finland, the first reported *A. astaci* epidemic among the signal crayfish population occurred in Puujärvi (Karjalohja) in the year 1996, when an acute epidemic took place in a mixed noble-crayfish and signal-crayfish population. The outcome was that the noble crayfish were eradicated and the signal crayfish population collapsed to a low density (Edgerton and Jussila 2004). Later, the signal crayfish population partially recovered and is now diseased and a symptomatic carrier of *A. astaci* (O. Kilpinen, Puujärvi water pollution control association, pers. comm.). Similar progress of *A. astaci* epidemics occurred in Pyhäjärvi (Säkylä), where the signal crayfish population collapsed several times during this century, i.e. in 2004 and 2009 (M. Jori, Pyhäjärvi institute, pers. comm.). To date, we have information showing that in Finland roughly 10% of the introduced signal crayfish stocks have collapsed to a low population density at least once, with similar data existing on population failures in Sweden (Sahlin *et al*. 2010 and L. Edsman, Swedish University of Agricultural Sciences, pers. comm.). The reasons for the population collapses may be and likely are multiple, but acute outbreak of *A. astaci* induced disease has normally been one of the main factors (authors' unpubl. data).

The signal crayfish was introduced to Lake Saimaa during the mid-1990s and the growing population has since been infected, or was originally infected but did not show gross symptoms (melanisation, lost limbs, eroded exoskeleton). The population partially collapsed in 2007. Since then, the Lake Saimaa signal crayfish population has been heavily infected with *A. astaci* with 60%–80% of the catch showing gross symptoms (Fig. 2) and half of the commercial catch being commercially substandard (Jussila *et al*. 2013). Also, the production is rather low as indicated by low catch per unit of effort (CPUE) of standard market size crayfish being smaller than 2. The chronic epidemic in the Lake Saimaa signal crayfish is among the worst of those reported in Finland, possibly due to the stock being parasitized also by *Psorospermium haeckeli* (Thörnqvist and Söderhäll 1993, Jussila *et al*. 2013).

Latent infections

The hypothesis of chronic crayfish plague infections (Fürst 1995, Jussila *et al*. 2011b) was developed to describe the situation where re-

Fig. 2. Signal crayfish (*Pacifastacus leniusculus*) from Lake Saimaa infected with PsI-genotype *Aphanomyces astaci*. (**A** and **B**) shell erosion, and (**C**) the melanised and eroded swimmerets.

introductions of native European crayfish failed after *A. astaci* infection or a suspected *A. astaci* epidemic had eradicated the original crayfish population. A considerable proportion of the re-introductions failed due to repeated crayfish plague epidemics (Erkamo *et al*. 2010). The conclusion was that *A. astaci* could have remained present and infective in the aquatic system, with several potential alternatives for such survival (Fürst 1995).

The diagnostic methods did not allow detection of low-level infection until the development of sensitive qPCR methods improved the situation (Vrålstad *et al*. 2009). There are now a number of reports on latent infection cases in Finland (Jussila *et al*. 2011b, Viljamaa-Dirks *et al*. 2011). There is also a novel method for detection of the spores from water samples (Strand *et al*. 2011), thus enabling the detection of at least chronically infected signal crayfish populations (Strand *et al*. 2012).

We have recently detected a very low level *A. astaci* infection in a commercially productive noble crayfish population in Mikitänjärvi (Jussila *et al*. 2011b). The infection rate is low and also *A. astaci* agent level in the infected noble crayfish individuals is low (Jussila *et al*. 2011b, Makkonen *et al*. 2014). The positive detection was from noble crayfish that had been collected from crayfish trapper's holding cages each year during 2009–2011. Furthermore, the infection prevalence increases due to stress experienced by the noble crayfish and up to 100% of the stressed Mikitänjärvi noble crayfish tested positive for *A. astaci* DNA. Furthermore, there have been discoveries of latent crayfish plague infections among other Finnish noble crayfish populations by other Finnish research institutions.

Mikitänjärvi is the upmost lake in the Luvanjoki system and downstream crayfish populations have also been tested. So far, the Luvanjärvi crayfish population has tested negative (n = 10, all negative) while one population from the Nuottijoki further downstream has tested positive (n = 9, three tested positive). The sample sizes were rather small, normally 10–30 crayfish, and a much larger sample size would be needed to support speculations of healthy downstream populations (Shrimpf *et al*. 2013). It should still be noted that one crayfish population downstream from Mikitänjärvi has until now proved negative.

The possible variation in the virulence of different *A. astaci* strains has long been discussed (Huang *et al*. 1994, Edgerton *et al*. 2004), with our recent publications supporting these theories (Makkonen *et al*. 2012b, 2014). The isolation of the Mikitänjärvi *A. astaci* has so far failed, which also shows that this disease agent differs from other tested *A. astaci* infections, of which the strains have successfully been isolated from infected crayfish (Viljamaa-Dirks and Heinikainen 2006). The Mikitänjärvi noble crayfish show some minor gross symptoms, i.e. mainly melanisation, similarly to some of the *A. astaci* infected signal crayfish populations.

We showed that the Mikitänjärvi noble crayfish seem to be more resistant against both PsI- and As-genotypes of *A. astaci* (Makkonen *et al*. 2014). The improvement in the disease resistance is shown in at least 3.5 times slower development of the mortality when infected with an *A. astaci* strain that is known to cause a high mortality rate under laboratory conditions. Even with the slower progress of the experimental epidemic, all the PsI-Puujärvi inoculated crayfish

died, while most of the As-genotype inoculated survived. This could indicate that the low level *A. astaci* infection might prime the Mikitänjärvi noble crayfish immune system, and could thus be preparing the host crayfish to better combat disease attacks.

The case of the Turkish narrow-clawed crayfish (*Astacus leptodactylus*), which have been recovering from As-genotype *A. astaci* epidemics and acting as carriers showing gross symptoms, has been reported by two research groups (Kokko *et al.* 2012, Svoboda *et al.* 2012). It has been claimed that the narrow-clawed crayfish might be more resistant against *A. astaci* (Unestam 1969) and the epidemics in Turkey show that this could be the case. Also, the indications for decreased susceptibility of the narrow-clawed crayfish to the disease have been reported elsewhere (Schikora 1906, Kossakowski 1973, Alderman *et al.* 1987). On the other hand, the epidemics in Turkey started in 1984, roughly 100 years after *A. astaci* arrived in Europe (Souty-Grosset *et al.* 2006). This could have allowed *A. astaci* enough time to evolve into a less virulent form before infecting crayfish in Turkish waters, even though the time frame for evolutionary changes is short (but *see* Reznick *et al.* 1997), at least according to traditional thinking, but could have included numerous *A. astaci* generations. The evolutionary pressure during the spread of *A. astaci* among central European native crayfish species and populations is likely to have been strong which, also due to several possible host jumps, could have lead to a very rapid and effective selection of less virulent characters.

In the case of the Turkish narrow-clawed crayfish recovering from the *A. astaci* epidemics, it has been suggested that the recovery does not appear to have been assisted by man-made introductions (Harlioğlu 2004, 2008). Thus, the populations have recovered because of the reproduction by those crayfish either resistant enough to survive the acute infection or that were able to avoid the infection, similarly to what has been debated for the case of Nordic chronic crayfish plague cases (Fürst 1995).

In further scientific discussion of chronic or latent infections, we suggest that when discussing individual crayfish being positive for *A. astaci* for long periods of time without the crayfish population suffering mass mortalities nor showing gross symptoms; the term 'chronic crayfish plague infection' should be substituted with 'latent crayfish plague infection'. The term 'latent' would be more in line with the idea of symptom-free infection. We suggest that chronic crayfish plague infection should refer only to sparse population or aquatic ecosystem level cases, when the crayfish plague disease agents remain in the crayfish population causing continuously gross symptoms and increased mortality, but not mass mortality. Indeed, in most cases the infected signal crayfish populations are chronically infected and not expected to become free of the disease by natural means.

Conclusions

First, there is strong evidence indicating that significant differences exist in the *A. astaci* virulence both between PsI- and As-genotypes and within genotypes. Very low virulent *A. astaci* isolates in the As-genotype, which may even act as latent infection agents, have been discovered in laboratory trials. These isolates resulted in no mortality at all in the studied populations in the experiments lasting from one to three months.

Second, the results also indicate that certain native European crayfish stocks vary in their resistance, the most data coming from the Finnish noble crayfish populations tested.

Third, the crayfish plague and native European crayfish seem to have already reached a sustainable co-existence in some cases. The two reported cases from Finland, together with several more cases so far detected, but not reported, have confirmed some of the aspects of the chronic crayfish plague infections. These cases should in the future be referred to as latent infections, due to low mortality rates and fewer gross symptoms in the infected native European crayfish.

Fourth, PsI-genotype has still been seen to be highly virulent. The reasons for its different status compared to the As-genotype may be due to its original host species being present in Europe in massive numbers. This fact has decreased, if not removed, the selection pressure, i.e. necessity to co-evolve with the native European crayfish hosts, since there seems to be plenty of suit-

able habitat for the PsI-genotype in the form of introduced North American crayfish stocks. This possibility makes the PsI-genotype, and possibly also other recently introduced *A. astaci* genotypes, significantly worse alien invaders than the old, by now less virulent As-genotype.

There are several direct and indirect indications of increased resistance towards *A. astaci* in native European crayfish stocks. Our laboratory experiments have revealed that some of the Finnish noble crayfish stocks seem to be able to survive *A. astaci* infection with a rather low mortality rate, at least for a short period of time (1–3 months). There are also recent reports of Turkish narrow clawed crayfish populations surviving and recovering from *A. astaci* epidemics.

The prospects for the conservation of native European crayfish lies largely in the prevention of the spread of both alien crayfish and their diseases, especially crayfish plague. Even with the indications of lowered virulence of certain *A. astaci* isolates and increased resistance towards *A. astaci* infections among native European crayfish populations, it is of utmost importance that the spread of both alien crayfish and their diseases is prevented.

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