Characterisation of *Aphanomyces astaci*'s virulence: from phenotype to genome

by

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Table of Content

Abstract

European crayfish species are considered keystone in freshwater ecosystems. As such, their conservation is of paramount importance to prevent biodiversity decline and loss of ecosystem function. Unfortunately, today, European crayfish species are among the most threatened crayfish species worldwide. An especially relevant threat is represented by the invasive pathogen *Aphanomyces astaci*. This oomycete, native of North America, has been one of the main causes of crayfish population declines across Europe since its first introduction 150 years ago, to the point of causing the local extinction of many populations. Over the years, several introductions of *A. astaci* strains into Europe took place through translocation of infected North American crayfish, and were followed by mass mortalities across European crayfish populations. However, in the past 20 years, more and more reports emerged of European crayfish populations surviving *A. astaci* infections or being latently infected with the pathogen. The survival of infected crayfish can be ascribed to both increased resistance of some crayfish populations and decreased virulence of some *A. astaci* strains. As the relationship between host and pathogen in Europe is changing, it is imperative to gain insights on what shapes these changes to understand the implications for the long-term coexistence of crayfish and *A. astaci* in Europe. With this thesis, I focused on the virulence of *A. astaci*, looking for mechanisms, patterns and determinants underlying the pathogen's virulence variability. In particular, by characterising the virulence of several *A. astaci* strains, I identified two possible different mechanisms of loss of virulence. I revealed that *A. astaci*'s virulence variability is not linked to variation of *in vitro* growth and sporulation, traits classically associated with a pathogen's virulence. Based on these results, I suggest that the pathogen's virulence determinants are likely its "virulence effectors", of which *A. astaci* genome is enriched. Additionally, with the present work I provided transcriptomic evidence of coevolution between *A. astaci* and European crayfish. I showed that the haplogroups based on the canonical mitochondrial markers, often used to assess *A. astaci*'s virulence to inform management actions, do not differ for some of their characterising phenotypical traits, including virulence. Finally, after experimental characterisation of virulence and assessment of its likely phenotypical determinants, i.e., sporulation and growth, the next and more comprehensive step to study the pathogen's virulence is through genomic approaches. To this aim, I provided key data for future comparative genomic studies, i.e., highly complete genome assemblies based on Nanopore (3) and Illumina reads (11). These data can be exploited in several ways, from building a pangenome of the species to a genome-wide association study (GWAS), that can offer a much deeper understanding of *A. astaci*'s virulence and adaptability. In particular, the identification of the loci associated with virulence through a GWAS has the potential to be revolutionary for the management of *A. astaci*, as it can become the basis to create a genomic tool to quickly and accurately assess the virulence of newly introduced strains, directing management actions towards the more dangerous strains.

1. General Introduction

The oomycete *Aphanomyces astaci* Shikora 1906, causative agent of the crayfish plague disease, is considered one of the worst 100 invasive species worldwide (Lowe et al., 2004). This pathogen was first brought from North America into Europe in 1859 and since then has been the cause of major losses of freshwater crayfish natural populations and farmed stocks (Cornalia, 1860; Alderman, 1996). Because of this, *A. astaci* is considered the most devastating crayfish disease known to date (Jussila et al., 2021). While the path of the first introduction into Europe remains unclear, all the subsequent introductions have been linked to translocation of infected North American crayfish species (Jussila et al., 2015). In fact, North American crayfish species are generally resistant to *A. astaci* infections and can act as healthy carriers and reservoirs of the pathogen (Jussila et al., 2015). European crayfish species are, on the other hand, extremely susceptible to *A. astaci*, and the introduction of the pathogen into a waterbody inhabited by European crayfish is often followed by mass mortality events and even local extinction of the entire crayfish population (Alderman, 1996; Jussila et al., 2015). Due to multiple translocations of infected crayfish specimens, either from North America or within Europe, and other human-mediated movements of the pathogen (e.g., through contaminated fishing equipment), *A. astaci* has now spread across the entire European continent, with a catastrophic impact on European crayfish populations (Ungureanu et al., 2020; Jussila et al., 2021).

The dramatic decline of European crayfish populations is a major concern for both conservation of biodiversity and aquaculture. The diversity of native freshwater crayfish in Europe is very limited compared to other continents, with only six crayfish species recognised as endemic, i.e., *Astacus astacus*, *Astacus pachypus*, *Pontastacus leptodactylus*, *Austropotamobius bihariensis*, *Austropotamobius pallipes* and *Austropotamobius torrentium* (Kouba et al., 2014; Pârvulescu, 2019). These species are considered keystone species and ecosystem engineers because of their significant impact on the overall biodiversity and functioning of freshwater ecosystems (Reynolds et al., 2013). While only two European crayfish species are present in the IUCN Red List as at risk of extinction (i.e., *A. astacus*, vulnerable; *A. pallipes*, endangered), all European crayfish species are considered among the most threatened crayfish species worldwide (e.g., threatened by invasive species, urbanization, climate change; Richman et al., 2015), and all are declining and nearing extinction, either on a regional or global scale (Jussila et al., 2021).

In this context, *A. astaci*, which has the potential to wipe out entire populations in a matter of weeks, is a major concern for the conservation of European crayfish species (Cerenius et al., 2009; Jussila et al., 2021). Nonetheless, much is still unknown about the pathogen and the development

of its relationship with European crayfish species. In the past 15 years, lower virulence of some *A. astaci* strains and higher resistance of some European crayfish populations have been observed, hinting to a shift in the interaction between pathogen and European host (e.g., Makkonen et al., 2012; Jussila et al., 2017, 2021). Furthermore, an increasing number of reports of latently infected European crayfish populations started to emerge (e.g., Viljamaa-Dirks et al., 2011; Kokko et al., 2012; Kušar et al., 2013; Jussila et al., 2021). However, the mechanisms underlying these examples of successful co-existence are yet to be understood. With this thesis, I aim to shed some light into the phenotypical mechanisms of adaptation of *A. astaci* to the susceptible European host, with particular focus on the loss of virulence. Additionally, I lay the basis for future comparative genomic analyses of *A. astaci* strains aimed to uncover the genomic determinants of the pathogen's virulence.

1.1. Oomycetes: pathogens of plants and animals

Commonly known as water moulds, oomycetes are fungi-like, filamentous, microbial eukaryotes (Phillips et al., 2008). Oomycetes are highly specialised for parasitic or saprophytic lifestyles, i.e., they obtain their nutrition from living organisms or dead and decaying matter, respectively (Phillips et al., 2008). They belong to the Stramenopila lineage, a diverse and species-rich group that together with Alveolata and Rhizaria forms the SAR supergroup (**Figure 1**; McGowan & Fitzpatrick, 2020). Oomycetes include some of the most devastating pathogens of plants and animals, and, as the cause of frequent disease outbreaks, represent recurring threats to both food security and biodiversity worldwide (Derevnina et al., 2016; McGowan & Fitzpatrick, 2020; Saraiva et al., 2023). Even though oomycetes are ubiquitous in both terrestrial and aquatic environments, aquatic species are much less studied compared to their terrestrial counterparts (Phillips et al., 2008; Beakes et al., 2012). As of now, our understanding of the biology of oomycetes mainly derives from the study of the plant pathogen *Phytophthora infestans*, the disease agent of the late blight in potatoes, connected to the Great Irish famine in the mid-19th century (Becking et al., 2022). However, some less-studied aquatic pathogenic oomycetes are known to cause devastating diseases among the freshwater fauna (Phillips et al., 2008). In particular, the order Saprolegniales includes two of the most dangerous aquatic oomycetes, i.e., *Saprolegnia parasitica*, pathogenic to fish, and *A. astaci*, pathogenic to freshwater crayfish (Phillips et al., 2008). *Aphanomyces astaci* is endemic in North America, where it co-exists with local crayfish host species without causing disease outbreaks (Jussila et al., 2015). Once introduced into Europe, this oomycete exhibited a very high virulence towards European crayfish species (Jussila et al., 2015).

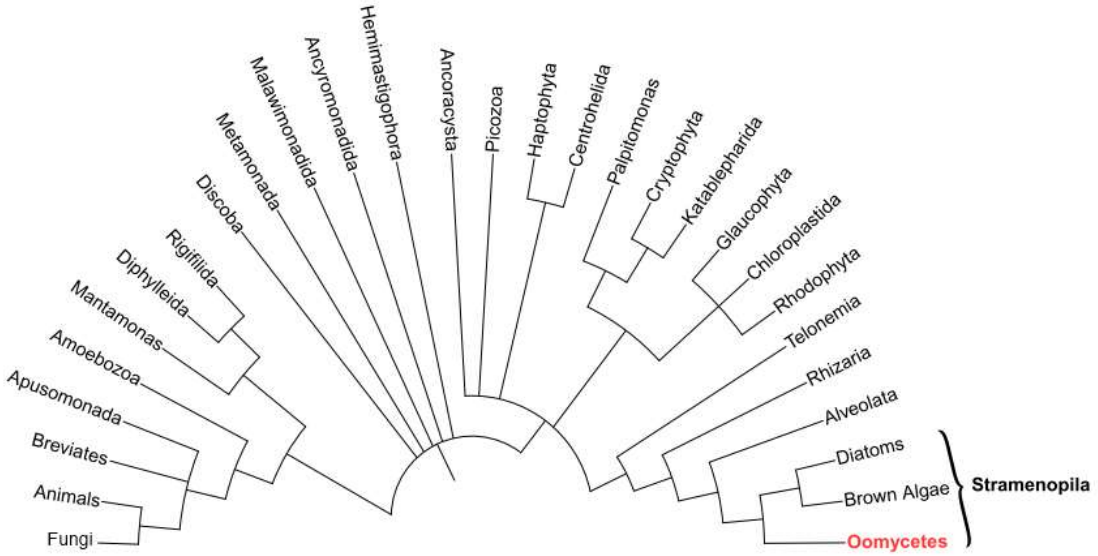

Figure 1. Oomycetes placement within a simplified phylogeny of the eukaryotes. Figure adapted from McGowan and Fitzpatrick, 2020 with permission of Elsevier.

1.2. Distribution in Europe and genetic diversity

The first wave of crayfish plague across Europe started in Italy in 1859 (Cornalia, 1860), and from there the pathogen quickly spread across the continent causing mass mortalities and collapses of European crayfish populations (Alderman, 1996). This first introduction was accidental, probably caused by wastewater from ships coming from North America and was not associated with the translocation of invasive North American crayfish (Alderman, 1996). The second wave of crayfish plague epizootics is connected to the introduction of North American crayfish species, known *A. astaci* carriers (Jussila et al., 2015). In particular, from the 1960s, large-scale introductions of the North American *Pacifastacus leniusculus* and *Procambarus clarkii* took place in Europe, with foci in Fennoscandia and Spain, respectively (Huang et al., 1994; Jussila et al., 2015). This caused the introduction of new *A. astaci* strains and new collapses of crayfish stocks (Jussila et al., 2015). At present, due to the increasing popularity of crayfish in aquaculture and pet trade, several other North American crayfish species have been introduced, increasing the risk of introducing additional strains of *A. astaci* in Europe (Faulkes, 2015).

To address the diversity of the *A. astaci* strains present in Europe, several molecular markers were developed. The first markers were based on random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) (Huang et al., 1994). This method, consisting of the random amplification of genomic DNA, allowed the grouping of *A. astaci* strains into 5 RAPD-groups: A, B, C, D, and E (Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Kozubíková et

al., 2011). These groups were congruent with those found through amplified fragment length polymorphisms (AFLPs), a more reliable method to assess the genetic diversity of *A. astaci* developed in 2014 (Rezinciuc et al., 2014). Both these methods rely on the availability of pure *A. astaci* cultures, which, however, represents a major drawback, as the isolation of *A. astaci* is particularly challenging and often unsuccessful. Furthermore, even when a pure culture is obtained, the time-consuming process of isolating *A. astaci* is not compatible with the quick genotyping of *A. astaci* strains needed for the management of the pathogen. Therefore, new markers were developed that could assess the genetic diversity of *A. astaci* directly from crayfish tissues, without the need for pure cultures: microsatellites (SSR) and genotyping based on chitinase genes, mitochondrial DNA (mtDNA), and specifically targeted nuclear loci. The microsatellites markers are able to differentiate the canonical RAPD-groups and an additional group, SSR-Up (Grandjean et al., 2014). However, microsatellite markers carry their own specific problematics, as in the case of mixed infections it is not possible to rule out cross-reactions, where the markers amplify microsatellites of co-occurring oomycetes. Moreover, the SSR-amplification fails with low amounts of pathogen's DNA, which is often the case for tissue from North American crayfish or when non-invasive sampling is utilised. Sequencing of the chitinase genes allows to distinguish between RAPD-groups A, B and D. However, due to the high amount of required nuclear DNA and the limited discerning power, it is not often used (Makkonen et al., 2012). The genotyping based on mitochondrial (mtDNA) markers, in particular the ribosomal small (rnnS) and large (rnnL) subunits, is the most widely used at the present time (Makkonen et al., 2018). It can differentiate the major RAPD-groups present in Europe (A, B, D and E), it does not need pure cultures, and it can work with low amount of DNA thanks to the multiple copies of mtDNA present in each cell. Two additional methods have been introduced for genotyping of *A. astaci* based on PCR/qPCR directed towards group specific polymorphisms in the nuclear DNA (Minardi et al., 2018; Di Domenico et al., 2021). However, as these two methods requires group specific assays, they are not suitable to identify new groups. In this thesis, classification based on mitochondrial rnnS and rnnL markers will be used.

As of today, *A. astaci* is spread across all Europe (**Figure 2**; Ungureanu et al., 2020). The introduced strains can be divided into four major haplogroups based on mitochondrial markers: haplogroups A, B, D, and E. Haplogroup A includes the strains that caused the first wave of epizootics in Europe starting 150 years ago (Huang et al., 1994; Makkonen et al., 2012). Today this haplogroup is mainly distributed in Fennoscandia and the western Balkans (Ungureanu et al., 2020). Haplogroup B was introduced through translocations of *P. leniusculus* and is widely spread across all the European countries for which data are available. *Pacifastacus leniusculus* is also the carrier of an additional genotype, RAPD-group C, which is not distinguished by the mtDNA markers and is classified as haplogroup A. Until recently this group had never been reported in

Europe, however, evidence has emerged of its presence in Ireland (Brady et al., 2024). Haplogroup D is associated with the introduction of *P. clarkii*, and is mainly distributed in Spain, with some detections in Italy and central Europe. Haplogroup E is associated with the crayfish *Faxonius limosus*, and is mainly present in central Europe (Ungureanu et al., 2020). Until recently, the analysis of North American crayfish species and their carried *A. astaci* haplogroups in Europe lead to the hypothesis that each North American crayfish species carried its own specific haplogroup, with which it shared a coevolutionary history (Grandjean et al., 2014; Jussila et al., 2015). However, in 2021, Martín-Torrijos et al. published a survey of the diversity of *A. astaci* in the USA, revealing that two or more *A. astaci* strains belonging to different haplogroups can concurrently infect the same crayfish. Furthermore, this study confirmed the origin of this pathogen in North America, revealing the great diversity of *A. astaci* in the USA, of which the haplogroups present in Europe likely represent only a small fraction (Martín-Torrijos et al., 2021).

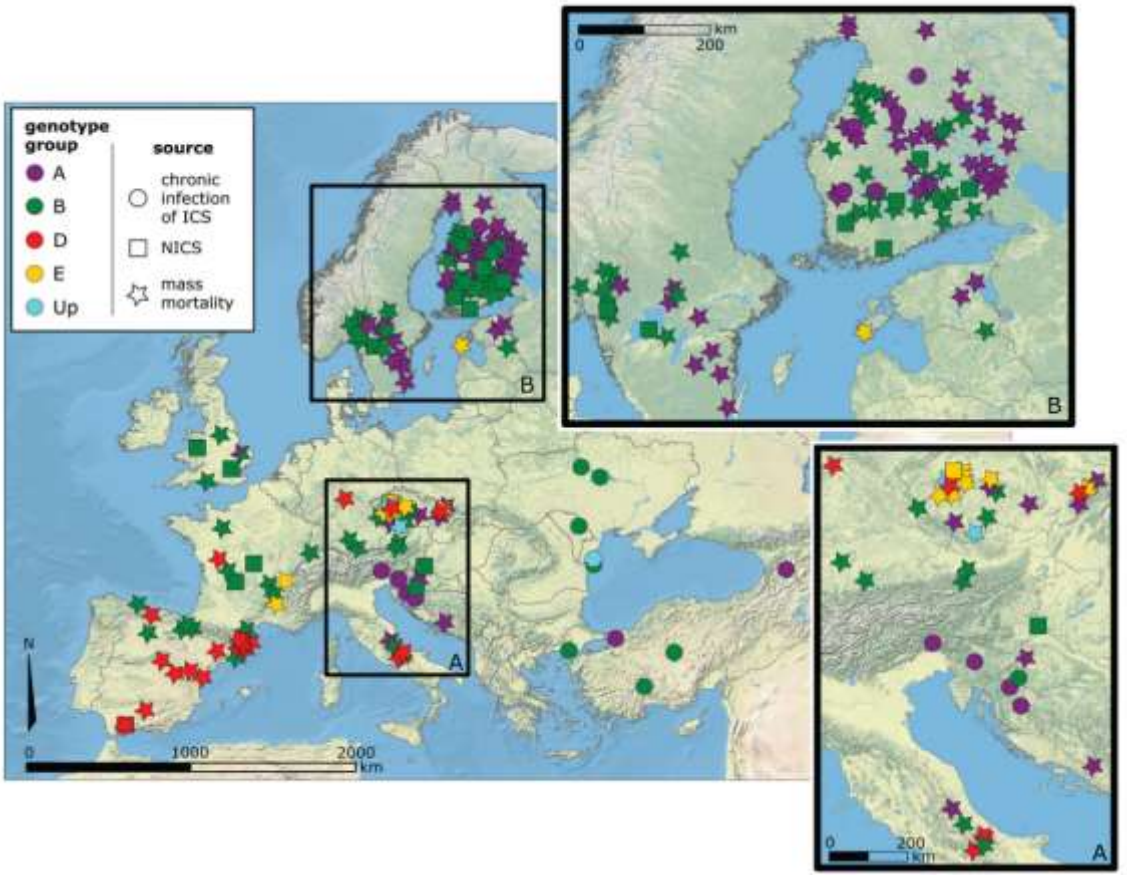

Figure 2. Distribution of the genotypes of *Aphanomyces astaci* in Europe. The symbols indicate the genotype based on mitochondrial markers or microsatellites (in the case of the Up group) and the source, i.e., American (non-indigenous crayfish species, NICS) or European (indigenous crayfish species, ICS) crayfish and chronic infections or mass mortality. Figure taken from Ungureanu et al., 2020.

1.3. *Aphanomyces astaci*'s life cycle

Aphanomyces spp. are known to reproduce both sexually and asexually. The sexual cycle is responsible for genetic variation and survival in unfavourable environments, while the asexual cycle is responsible for the dispersal of the pathogen (Diéguez-Uribeondo et al., 2009; Rezinciuc et al., 2015). Sexual reproduction has never been recorded for *A. astaci* in Europe, and the genetic stability observed across the first strains introduced into Europe supports the hypothesis of only clonal reproduction being present in this species (Huang et al., 1994). This absence of sexual reproduction is common in oomycetes pathogenic to animals (Diéguez-Uribeondo et al., 2009). However, it is also possible that, since oomycetes are heterothallic species characterised by mating types, only one mating type has been introduced, making sexual reproduction impossible in Europe (Rezinciuc et al., 2015). Unfortunately, due to the scarcity of studies on *A. astaci* in North America, it remains unclear if this pathogen can reproduce sexually.

The asexual life cycle of *A. astaci* starts with the formation of primary zoospores within specialised hyphae, the sporangia (**Figure 3**; Hardham, 2009). After being extruded from the hyphal tip, the primary zoospores round up and immediately encyst into primary cysts, structures encased in a thin cell wall. After a resting period, these structures release biflagellate secondary zoospores. Secondary zoospores represent the infective unit of the pathogen and are equipped with chemotaxis and electrotaxis (Cerenius & Söderhäll, 1984a; Rezinciuc et al., 2015). Secondary zoospores remain motile from few hours to few days depending on the temperature and environmental conditions (Oidtmann et al., 2002). If no suitable host is found, the secondary zoospores can go through the process of repeated zoospore emergence for up to three times, alternating between cyst formation and zoospore release (Cerenius & Söderhäll, 1984b). Upon contact with a suitable host, the zoospores encyst and become surrounded by sticky substances that aid the adherence to the host's cuticle (Cerenius et al., 2009). After adherence, the cysts start germinating. Germination usually successfully occurs in wounds or areas characterised by soft cuticle (e.g., abdominal cuticle and joints) (Unestam & Weiss, 1970; Nyhlén & Unestam, 1980). An infection peg forms from the zoospores and hyphae start to grow inside the cuticle (Nyhlén & Unestam, 1975). The penetration inside the cuticle of the crayfish is made possible both by enzymatic corrosion and mechanical action (Nyhlén & Unestam, 1975). After a first phase of growth inside the host, and upon specific stimuli, *A. astaci*'s hyphae grow outward from the cuticle and start to sporulate. The main stimulus that triggers the sporulation is lack of nutrients (Rezinciuc et al., 2015). In susceptible European crayfish, the main sporulation event happens at the death of the crayfish (Makkonen et al., 2013). Conversely, in resistant North American crayfish the pathogen, encapsulated within melanin, constantly produces low amounts of spores (Söderhäll & Cerenius, 1992; Strand et al., 2012). Additionally, in both North American and

European crayfish, a low-intensity sporulation is triggered during molting of the crayfish host (Strand et al., 2012). With sporulation, thus, a new cycle begins.

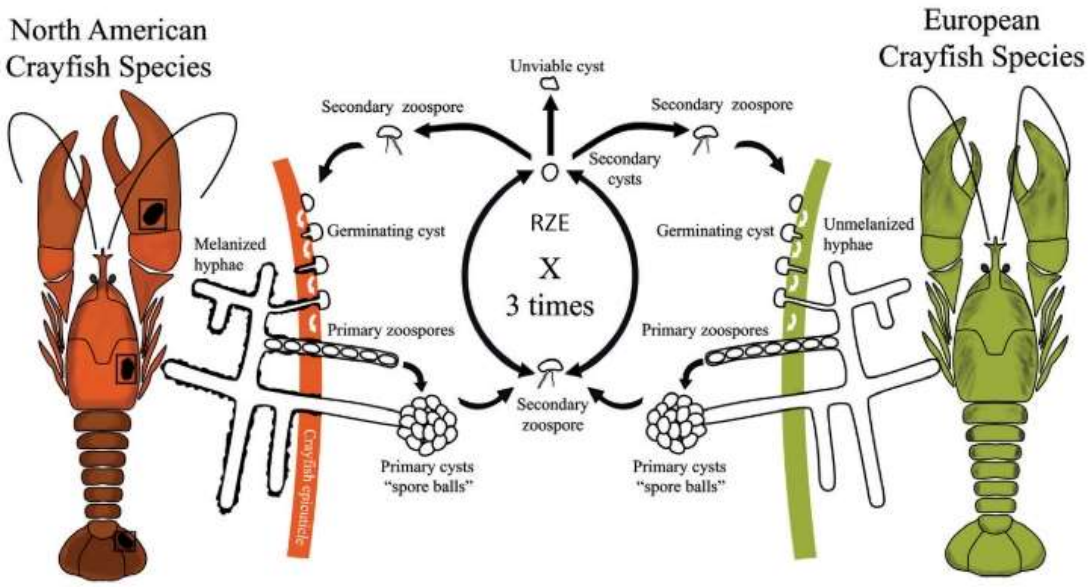

Figure 3. Life cycle of *Aphanomyces astaci*. North American crayfish are often healthy carriers of *A. astaci*, and although the pathogen is present in their cuticle, it is often encapsulated by melanin (black spots visible on the cuticle). When infecting European crayfish species, the pathogen remains mainly unmelanised. After specific stimuli (i.e., lack of nutrients), the pathogen starts the sporulating process. A sporangium is formed, with primary zoospores forming inside it and reaching the tip of the hyphae where they encyst (primary cysts) and form a cluster. From the cluster, biflagellate secondary zoospores are released. Secondary zoospores are characterised by chemotaxis and electrotaxis and can actively swim towards suitable hosts. If no suitable host is found or they encyst due to unspecific stimuli, the secondary zoospores can go through up to three cycles of repeated zoospore emergence (RZE), where they encyst and release new secondary zoospores. If the secondary zoospores find a suitable host (i.e., a crayfish), they form secondary cysts, characterised by a thin cell wall, and attach themselves to the cuticle of the crayfish, and then starts geminating and growing within the cuticle. If the host is a susceptible crayfish, where the melanisation of the pathogen is absent or insufficient, the infection usually results in the death of the crayfish. Figure reprinted from Rezinciuc et al., 2015 with permission of Taylor & Francis Group LLC – Books.

1.4. Virulence variability among *Aphanomyces astaci* strains

Before further dwelling upon the current knowledge on *A. astaci*'s virulence, for the purpose of clarity, a definition of virulence should be provided. During the years, in the general context of parasitology, several definitions of "virulence" have been proposed which include more or less explicit effects of the pathogen on the host (Ebert & Bull, 2008). In the present work, "virulence" will be defined as pathogen-mediated mortality of the host. This is the definition commonly utilised by the crayfish community in relation to an *A. astaci* infection.

As indications of virulence variability among *A. astaci* strains started to emerge, several studies have been conducted to characterise the virulence of the pathogen's strains present in Europe through infection experiments (e.g., Makkonen et al., 2012; Jussila et al., 2013; Becking et al., 2015; Viljamaa-Dirks et al., 2016). As haplogroup A and B have been present in Europe for a longer time and are the most widespread compared to the other haplogroups, they are also the most extensively studied (Huang et al., 1994; Ungureanu et al., 2020). Strains belonging to haplogroup A have been experimentally shown to be very variable in their virulence, with strains causing low/no mortality and strains causing elevated mortality (Makkonen et al., 2012, 2014). This observed virulence variability among the strains might be regarded as evidence of adaptation of *A. astaci* to its susceptible European host (Jussila et al., 2014). It is expected that after their introduction, all the pathogen strains were extremely virulent towards the European host. The observed variability could then be explained with the lower virulence evolved by some strains to adapt to the susceptible host (Jussila et al., 2014). Strains belonging to haplogroup B, on the other hand, were introduced into Europe more recently, and probably did not yet adapt to European crayfish. In fact, they have been shown to be all very virulent, with some degree of variability observed only in terms of time between exposure to the pathogen and death of the crayfish (Makkonen et al., 2012; Jussila et al., 2013).

A few studies have also been conducted on strains belonging to haplogroup D and E. However, it is important to note that straightforward comparisons of their virulence with that of haplogroups A and B cannot be done, as often the experimental design is different (e.g., cumulative tanks are used instead of single tanks), fewer strains have been analysed (especially for haplogroup E) or the virulence assessment has been conducted using different host species (i.e., *A. pallipes* instead of *A. astacus*) (e.g., Becking et al., 2015; Martínez-Ríos et al., 2022). In any case, from these experiments it emerges that both haplogroup D and haplogroup E strains seem to be very virulent towards European crayfish species.

1.5. Host-pathogen interaction: immune response vs virulence effectors

The first layer of defence of a crayfish against a pathogen is its cuticle (Rowley, 2016). The crayfish cuticle is composed by four layers: the epicuticle, constituted by a surface lipidic layer; the exocuticle which contains chitin and various calcium-rich minerals; the endocuticle, which is the thickest part and is formed by chitin and proteins; and the epidermis, the living part of the cuticle and most internal layer (Nagasawa, 2012; Rowley, 2016). When an *A. astaci*'s zoospore encounters a crayfish, it attaches to its cuticle and encysts. Even before the start of the germination process, the cyst starts producing lipases and proteinases (Cerenius et al., 2009). The lipases corrode locally the epicuticular lipid layer, while the proteinases, in conjunction with mechanical

force, help the germ tube to penetrate into the internal layers of the cuticle (Nyhlén & Unestam, 1975). After several hours from the germination of the cyst, when the young mycelium is formed, chitinases are produced to catabolise the chitinous layers of the cuticle (Cerenius et al., 2009). When the pathogen starts penetrating inside the cuticle, the innate immune response of the host gets activated after recognition of pathogen-associated molecular patterns (PAMPs) (Vazquez et al., 2009; Hauton, 2012). PAMPs represent conserved epitopes of the pathogen, that are easily accessible to the host immune system (i.e., secreted or surface molecules) (Ma & Guttman, 2008). In the case of *A. astaci*, (1,3)-β-glucan, the main component of oomycetes' cell wall, is a well-known PAMP (Cerenius et al., 2009). PAMPs are recognised by the host's pattern recognition proteins (PRPs), which activate and direct the immune response towards the point of entry of the pathogen (Hauton, 2012). The immune response is characterised by a cellular component (i.e., phagocytosis and encapsulation) and a humoral component (i.e., antimicrobial peptides and prophenoloxidase activating system), both of which are mediated by haemocytes, the invertebrates' "blood cells" (Cerenius et al., 2009; Rowley, 2016). The prophenoloxidase (proPO) activating system has been the most extensively studied defence mechanism in crustaceans, and it plays a key role in the immune response against *A. astaci* (Cerenius et al., 2003; Rowley, 2016). ProPO, the inactive form of the enzyme phenoloxidase (PO), is stored inside the haemocytes, and after interaction between PAMPs and PRPs, proPO is released from the haemocyte cytoplasm, a proteolytic cascade is triggered, and proPO is converted into its active form PO (Cerenius et al., 2008; Rowley, 2016). PO catalyses the formation of melanin, a black-brown pigment that encapsulate the pathogen preventing its further growth (Vazquez et al., 2009; Rowley, 2016). Additionally, cytotoxic quinones, intermediate products of the catalysis of melanin, have a role in inhibiting the growth of the pathogen, deactivating its proteinases and potentially killing the pathogen once encapsulated in melanin (Söderhäll and Cerenius, 1992; Vazquez et al., 2009; Rowley, 2016; Kloc et al, 2023).

After this brief overview of the general mechanisms of interaction between *A. astaci* and the host crayfish, it is important to note that while several aspects of the process are known, many others are still subject of investigation. For both the pathogen and the host, the attention of the scientific community has been mainly focused on a single enzyme or a single enzyme cascade, i.e., *A. astaci's* chitinase and the crayfish's proPO cascade. Based on the current knowledge, it is unclear if immune mechanisms different from the proPO cascade play important roles in the immune response of the crayfish against *A. astaci*. As for *A. astaci*, the genome of this pathogen is particularly enriched with virulence effectors, including molecules with a putative repression activity towards the immune response of the host (i.e., immunoglobulin A) (McGowan & Fitzpatrick, 2017). Therefore, it seems likely that several different enzymes could play a role in determining the overall virulence of *A. astaci*.

1.6. Resistant vs susceptible crayfish: how coevolution shaped the immune response against *Aphanomyces astaci*

From the evidence gained on the topic, it appears that the higher resistance of North American crayfish species to *A. astaci* infections compared to European crayfish species depends on the effectiveness of the cuticle as a barrier and on the melanisation reaction (Unestam & Weiss, 1970 Nyhlén & Unestam, 1975; Cerenius et al., 2003). It has been shown that *A. astaci* can rarely penetrate the intact cuticle of North American crayfish. In particular, the lipidic layer of the epicuticle seems to be the key to this resistance, as after its removal hyphal penetration is more easily established (Nyhlén & Unestam, 1975). Furthermore, North American crayfish are able to more efficiently encapsulate the entering pathogen in melanin compared to susceptible European crayfish (Unestam & Weiss, 1970). This difference seems to be the result of different mechanisms of activation of the proPO system, with North American crayfish constitutively expressing proPO at high levels independently from immune stimuli, while susceptible European crayfish express proPO at lower levels that can be enhanced in response to immune stimulants (Cerenius et al., 2003, 2009). These differences in the immune mechanisms of resistant and susceptible crayfish have been interpreted as evidence of coevolution between North American crayfish and *A. astaci* (Cerenius et al., 2003). North American crayfish seem to have adapted to containing the pathogen's growth through a constant activation of some components of their immune system, while the pathogen has likely responded by increasing its resistance towards the host's antimicrobial peptides produced by the proPO cascade (Cerenius et al., 2003). As a consequence, the encounter with *A. astaci* represented a too high challenge for the naïve European crayfish, resulting in the observed high virulence of *A. astaci* towards European crayfish species (Cerenius et al., 2003; Jussila et al., 2015). However, European crayfish and some pathogen's strains have now been co-existing for more than 100 years, and the increasing number of observations of latently infected European crayfish populations indicates that the relationship between the pathogen and the European host might be reaching a tentative equilibrium (e.g., Viljamaa-Dirks et al., 2011; Kušar et al., 2013; Jussila et al., 2021). As evidence of both reduced virulence of some *A. astaci* strains and increased resistance of some European crayfish populations exists, it has been hypothesised that this equilibrium is the result of coevolution taking place between pathogen and European host (Makkonen et al., 2012; Jussila et al., 2014, 2015, 2021). It is unclear what molecular changes are underlying this coevolutionary process, and as such it cannot be predicted if the acquired resistance of the host is only strain-specific, and if all strains of the pathogen, including the newly introduced ones, have the same potential of adapting to the more susceptible host.

1.7. Thesis outline

1.7.1. Aims of the study

With the present work I aimed to gain insights into the virulence variability of *A. astaci* and its adaptation to European crayfish species. In particular, I planned to detect and characterise strains with decreased virulence to better comprehend the mechanisms of virulence loss. I looked for possible influence of some phenotypical traits (i.e., sporulation and growth) on the modulation of virulence, and provided a dataset that can be exploited to identify genomic loci associated with virulence variation. Finally, I suggested future directions to exploit the produced data and to create new markers able to address pressing necessities for the management of *A. astaci*.

1.7.2. Chapters overview

In **Chapter 2**, I focused on the presumed lowered virulence of an *A. astaci* strain to obtain insights into the loss of virulence of the pathogen. To this aim, I infected the European *A. astacus* and the invasive *Procambarus virginalis* (a proxy for North American species) with two different strains of *A. astaci*, an haplogroup B strain isolated from the North American *P. leniusculus*, and an haplogroup A strain isolated from latently infected *A. astacus*. The study revealed that the strain isolated from the latently infected European crayfish lost its virulence, likely through a decreased capability of germinating or penetrating into the cuticle of the crayfish host. This reduced virulence is probably the result of the adaptation of the pathogen to its susceptible European host.

To shed some light into the host-pathogen interaction on a molecular level, in **Chapter 3** a transcriptome analysis was conducted on the hepatopancreas isolated from *A. astacus* and *P. virginalis* infected in the experiment described in **Chapter 2**. Firstly, the results of this analysis showed an activation of the immune system of *P. virginalis* after infection with the lowly virulent *A. astaci* strain. This activation provides indication that the *A. astaci* spores were able to germinate on the cuticle of the crayfish but were unable to penetrate it. Additionally, these results highlighted different responses in the two infected crayfish species to the two *A. astaci* strains. In particular, when challenged with the highly virulent *A. astaci* strain, *A. astacus* mobilised its immune system without being able to overcome the infection, while the activation of the immune system in *P. virginalis* was minimal, with the pathogen unable to cause disease in the host. In contrast, when challenged with the lowly virulent *A. astaci* strain, *A. astacus* was able to contain the pathogen without apparent mobilization of the immune system, while *P. virginalis* showed the stronger immune response. The intensity of the immune responses seems to be disconnected from the virulence of the strains, and rather related to putative previous encounters between host and similar strains. Altogether, these results provided additional evidence for the coevolution of *A. astaci* and its crayfish host, indicating that not only coevolutionary processes shaped the

relationship between North American crayfish and the pathogen, but they have also been occurring between the pathogen and European crayfish.

To gain further information on the mechanisms underlying the virulence loss of some *A. astaci* strains, in **Chapter 4** I conducted a phenotypical characterization of virulence variation of a collection of strains. Here, I identified three additional non-virulent strains. Two of these strains belonged to haplogroup B, usually considered highly virulent. While usually loss of virulence is associated with strains belonging to haplogroup A, which have been present in Europe for over a century, these results indicate that possibly haplogroup B strains are also starting to adapt to the European host. Additionally, in **Chapter 4** I characterised *in vitro* traits that might influence, and partially explain, *A. astaci*'s virulence variability (i.e., sporulation and growth). The results showed that neither *in vitro* sporulation rate nor growth rate are significantly correlated with virulence. However, when considering the variability of virulence and *in vitro* sporulation rate in the context of specific host-pathogen relationships (e.g., *A. astaci*'s haplogroup, host species, presence/absence of resistant hosts), it can be hypothesised that the selective pressure acting on each strain modulates sporulation and virulence together, selecting combinations of the traits that allows the pathogen to survive in the specific scenario. Finally, the data produced in this chapter allowed the assessment of putative phenotypical differences between two major *A. astaci* haplogroups, i.e., haplogroup A and B. The obtained results showed that there is no statistically significant difference in virulence, *in vitro* sporulation and growth rate between the two haplogroups. This lack of statistical difference has important repercussions on the management of *A. astaci*, as often, when assessing the danger posed by newly introduced strains, the haplogroup of the strain is used as a quick tool to infer its virulence.

Additionally, as the results from **Chapter 4** revealed that *in vitro* growth and sporulation are not the main determinants of *A. astaci*'s virulence variability, I worked on paving the way for future studies based on genomic analyses. In particular, in **Chapter 5** I produced three highly complete assemblies based on Nanopore long-reads for *A. astaci* strains belonging to haplogroup A, B and E, and 11 additional short-read assemblies of strains belonging to haplogroup A and B. These genomic data can be used, together with the phenotypical characterization of the strains described in **Chapter 4**, to investigate the genetic determinants of *A. astaci*'s virulence through a genome-wide association study (GWAS).

Finally, in **Chapter 6** I expanded on the findings of the previous chapters and related them to each other and to the available literature to extrapolate trends and future prospectives. In particular, based on the virulence characterisation of **Chapter 2** and **Chapter 4**, I suggested two different mechanisms of loss of virulence for *A. astaci* strains; based on **Chapter 2** and **Chapter 3** I indicated the chitinase as putative gene involved in the adaptation of *A. astaci* to European

crayfish and proposed comparative analysis of this gene based on the data presented in **Chapter 5**; based on the results of **Chapter 4**, I suggested that virulence factors might be the main determinants of *A. astaci*'s virulence, and as such they should be the focus of future research; thus, I gave indications of how to exploit the data presented in **Chapter 5** to address the virulence variability and adaptability of *A. astaci*. Finally, based on the results of **Chapter 4** I highlighted the need of new monitoring tools to successfully identify and contain the strains more relevant for the management of the pathogen, i.e., the most virulent strains. In particular, I suggested to exploit the data presented in **Chapter 4** and **Chapter 5** to identify single nucleotide polymorphisms (SNPs) associated with virulence to produce a SNP array to quickly and efficiently assess the virulence of newly introduced strains.

Chapter 2

Controlled infection experiment with *Aphanomyces astaci* provides additional evidence for latent infections and resistance in freshwater crayfish

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Abstract

For 150 years the crayfish plague disease agent *Aphanomyces astaci* has been the cause of mass mortalities among native European crayfish populations. However, recently several studies have highlighted the great variability of *A. astaci* virulence and crayfish resistance toward the disease. The main aim of this study was to compare the response of two crayfish species, the European native noble crayfish (*Astacus astacus*) and the invasive alien marbled crayfish (*Procambarus virginalis*), to an *A. astaci* challenge with a highly virulent strain from haplogroup B and a lowly virulent strain from haplogroup A. In a controlled infection experiment we showed a high resistance of marbled crayfish against an *A. astaci* infection, with zoospores from the highly virulent haplogroup B strain being able to infect the crayfish, but unable to cause signs of disease. Furthermore, we demonstrated a reduced virulence in the *A. astaci* strain belonging to haplogroup A, as shown by the light symptoms and the lack of mortality in the generally susceptible noble crayfish. Interestingly, in both marbled crayfish and noble crayfish challenged with this strain, we observed a significant decrease of the detected amount of pathogen's DNA during the experiment, suggesting that this *A. astaci* haplogroup A strain has a decreased ability of penetrating into the cuticle of the crayfish. Our results provide additional evidence of how drastically strains belonging to *A. astaci* haplogroup B and haplogroup A differ in their virulence. This study confirmed the adaptation of one specific *A. astaci* haplogroup A strain to their novel European hosts, supposedly due to reduced virulence. This feature might be the consequence of *A. astaci*'s reduced ability to penetrate into the crayfish. Finally, we experimentally showed that marbled crayfish are remarkably resistant against the crayfish plague disease and could potentially be latently infected, acting as carriers of highly virulent *A. astaci* strains.

2.1. Introduction

The causative agent of crayfish plague, *Aphanomyces astaci*, has been introduced in southern Europe in the 19th century, and quickly spread across the native crayfish stocks of most of the continent (Alderman, 1996). The colonization of Europe by the pathogen took place through two different waves (Alderman, 1996). During the first wave in the 19th century, strains belonging to haplogroup A spread throughout the continent (Huang et al., 1994), presumably without their original host (Alderman, 1996). The second wave was caused by multiple introductions of different species ofNorth American crayfish (Alderman, 1996). It is believed that each of them carried its own specific haplogroup of *A. astaci*, resulting in the introduction into Europe of three new haplogroups: B, D, and E (Huang et al., 1994; Diéguez- Uribeondo et al., 1995; Kozubíková et al., 2011; Makkonen et al., 2018; Jussila et al., 2021). North American crayfish are resistant

against *A. astaci* and can act as reservoirs for the pathogen (Unestam and Weiss, 1970; Unestam and Nylund, 1972; Alderman, 1996). Such resistance is presumably the result of a shared coevolution history in their original habitat that allowed for the establishment of a fine-tuned balance between host and parasite (Unestam, 1969). The susceptible European crayfish, however, when challenged with the new pathogen, faced disastrous crayfish plague epizootics, often resulting in the eradication of entire populations (Alderman, 1996).

In recent years, infection experiments aimed to evaluate the virulence of the different *A. astaci* strains have highlighted a considerable variance in the ability of the different haplogroups to cause the insurgence of the disease. Generally, *A. astaci* haplogroup B is classified as highly virulent, with the disease caused by this strain usually culminating in the death of all the challenged noble crayfish (Makkonen et al., 2012a, 2014; Jussila et al., 2013, 2015; Gruber et al., 2014; Becking et al., 2015). On the other hand, *A. astaci* haplogroup A has been shown to be less virulent in general, and its strains have a much more variable virulence (Makkonen et al., 2012a, 2014; Becking et al., 2015; Jussila et al., 2015). Furthermore, increased resistance during infection experiments in some populations of noble crayfish has been reported (Makkonen et al., 2014), and several reports of latently infected European crayfish populations have emerged (Jussila et al., 2011a, 2017; Viljamaa-Dirks et al., 2011; Schrimpf et al., 2012; Kusar et al., 2013; Maguire et al., 2016). In addition, the decline of some wild American crayfish populations due to crayfish plague epizootics has been observed (Jussila et al., 2014; Sandström et al., 2014) and laboratory experiments have shown that North American crayfish can be susceptible to *A. astaci* when under stressful conditions (Thörnqvist and Söderhäll, 1993; Aydin et al., 2014).

Few decades ago, yet another invasive crayfish species, the parthenogenetic marbled crayfish *Procambarus virginalis* Lyko, 2017, appeared in Europe (Chucholl et al., 2012; Lyko, 2017). It has first been spotted in 1995 in the German pet trade and has since then established numerous populations on the continent (Vogt, 2018). It evolved from *Procambarus fallax*, an American species native of Florida, after triploidization (Vogt et al., 2018). As no known primary population is present in America, it is thought that the species may have evolved in captivity in the pet trade environment (Vogt et al., 2018). *Procambarus virginalis* can act as *A. astaci* carrier, and both wild and captive specimens have been found infected with *A. astaci* (Keller et al., 2014; Mrugała et al., 2015; Makkonen et al., 2018). In two instances it was possible to genotype the strains infecting *P. virginalis* specimens, and they were identified as haplogroup D (Keller et al., 2014; Mrugała et al., 2015), characterized by elevated virulence (Martín-Torrijos et al., 2017). Therefore, we expect the marbled crayfish to be rather resistant to the crayfish plague.

With this study we aim to shed some light on the adaptation process between *A. astaci* and its new European crayfish hosts. The increasing number of reports of latently infected European

crayfish populations indicates that the continuous interaction between host and pathogen might be leading to new equilibria, balanced by an increased resistance of the crayfish and/or a decreased virulence of the pathogen (Jussila et al., 2014). We tested and compared the susceptibility of noble crayfish and marbled crayfish against a highly virulent (haplogroup B; Makkonen et al., 2019) and a lowly virulent (haplogroup A) *A. astaci* strain. The *A. astaci* haplogroup A strain has been isolated from the Finnish noble crayfish population from Lake Venesjärvi. In the last 50 years, this population has survived at least three different crayfish plague epizootics, last of which took place around the year 2000 (Jussila et al., unpublished data). Since then, the population has been slowly recovering, and the noble crayfish are now asymptomatic carriers of the pathogen. By using this *A. astaci* strain, we aim to provide additional evidence of the existence of latently infected wild noble crayfish populations. We hypothesized no mortality in both species of crayfish infected with haplogroup A. Furthermore, we hypothesized the highly virulent haplogroup B to cause the death of the noble crayfish, but no or less intense symptoms in the marbled crayfish. Finally, we expected different onset of the symptoms, with the marbled crayfish showing delayed signs of the disease compared to the noble crayfish.

2.2. Materials And Methods

2.2.1. Crayfish Species

The noble crayfish were collected from a wild population in Lake Rytky, Kuopio, Finland (62°51’22’’N, 27°25’06’’E), while the marbled crayfish were obtained from lake Singliser See, Hessen, Germany (51°3’35’’N, 9°18’18’’E; import license to Finland, ID: ESAVI/15535/04.10.12/2019, date 10.5.2019; ID: Diaari nro 842/5719/2019, date 16.4.2019). Both populations had been tested for *A. astaci* presence on previous occasions and no infections were detected (Keller et al., 2014; Jussila et al., 2017). After collection and transport from the airport in Helsinki to the University of Eastern Finland, the marbled crayfish have been placed for 3 weeks in holding tanks with no food. In the first 2 weeks the crayfish were kept at 6°C, while the third week the temperature was raised to 18°C. The water was changed once a week. The noble crayfish were kept in holding tanks at 18°C for 1 week. All holding tanks were equipped with one aeration pump to ensure adequate level of dissolved oxygen in the water. Twenty days prior to the challenge experiment, the crayfish were transferred to the individual tanks of the experimental infection system for acclimatization following a randomized system (e.g., Makkonen et al., 2019). For every crayfish, carapace length and sex were determined, and notes made on any specific features, e.g., missing limbs or injuries. Marbled crayfish produced eggs throughout both the acclimatization period and the challenge experiment. The eggs were

systematically removed prior to the challenge experiment. After the challenge, the eggs were not removed to avoid additional stress to the crayfish. During acclimatization period and challenge experiment the crayfish were given preboiled frozen sweet corn every second day. Eventual leftover corn was removed before the next feeding.

2.2.2. *A. astaci* Isolates and Zoospores Production

Two *A. astaci* strains were used for the experiment. The highly virulent *A. astaci* isolate UEF_T16B, isolated from a signal crayfish (Pacifastacus leniusculus) from Lake Tahoe, United States (39°05'30''N, 120°02'30''E), in 2013. The strain belongs to haplogroup B based on mitochondrial markers (Makkonen et al., 2019). Haplogroup B corresponds to RAPD-PCR group B (Makkonen et al., 2018). The second strain was VEN5/14 a), isolated from a noble crayfish from Lake Venesjärvi, Kankaanpää, Finland (61°4'41''N, 22°10'26''E), in 2014. The isolation of the *A. astaci* culture was successful despite the fact that the qPCR did not detect *A. astaci* DNA in the tissues of the crayfish population (Jussila et al., unpublished data). This strain belongs to haplogroup A which includes RAPD-PCR groups A and C (Makkonen et al., 2018), and likely also additional *A. astaci* strains, considering its wide geographic distribution (Martín- Torrijos et al., 2021). While no further genetic analysis of this strain has been conducted, it can be assumed it belongs to RAPD- PCR group A, as only strains belonging to RAPD-PCR groups A and B have been isolated from crayfish populations present in Finland (Viljamaa-Dirks et al., 2016).

The production of zoospores followed the method used in Makkonen et al. (2012a) with some modifications. Three pieces of agar (4 mm2 each) were cut from solid PG1 medium containing *A. astaci* hyphae and incubated in 150 mL of liquid PG1 medium at 20◦C for 1 week. Subsequently the hyphae have been finely cut with a sterile scalpel and incubated in new liquid PG1 medium at 20◦C for 3 days. At the end of the 3 days, to stimulate zoospores production, the hyphae have been washed four times with autoclaved water, and then incubated in the same water on a horizontal shaker at 18◦C for one night. For each strain twelve replicates have been produced. The density of the zoospore solution was estimated with an optical microscope (total magnification of100x) using a Bürker chamber.

2.2.3. Experimental System

The experimental infection system (RapuLatorio) consisted of individual interconnected 2 L tanks with recirculating filtered water from lake Kallavesi (Jussila et al., 2011b). The water filtration was ensured by a biological filter and a set of three 5 µm filters (Spunflow QN, Domnick Hunter Technologies Ltd., England) and two 5 µ absolute filters (Pleatflow II, Prosep Filter Systems Ltd.,

England). This system ensures that all *A. astaci* zoospores are eliminated from the circulating water (Jussila et al., 2011b). During the experiment, water pressure before the absolute filters was regularly monitored. Filters were substituted when water pressure exceeded 2 × 105 Pa. Water temperature was maintained stable by air conditioning at 18.8 ± 1.1°C. A day-night rhythm was mimicked through artificial lights, with 8 h of light and 16 h of dark. Water quality parameters (oxygen levels, temperature, conductivity, and pH) were monitored once a day. The dissolved oxygen was 93 ± 12.5% (min-max, 37– 100%). The minimum value of37% was registered on day 1 of the challenge, after the interruption of the water circulation prior to the addition of the zoospores to the tanks. The conductivity was 222 ± 8.1 µS/cm (min-max, 212–256 µS/cm), pH was 7.8 ± 0.2 (min-max, 7.2–8). The pH value was artificially lowered 24 h before the start of the challenge by three additions of 1 mL of HNO3 to the circulating water to maintain the pH value 7.8, considered adequate for the infection process (Unestam, 1966).

2.2.4. Experiment Setup and Infection

The treatment groups (*A. astaci* haplogroup A-challenged crayfish, *A. astaci* haplogroup B-challenged crayfish and controls) consisted of 20 crayfish each, for a total of 120 crayfish (60 noble crayfish and 60 marbled crayfish). During day 0 of the infection the zoospore suspension was added to the individual tanks to reach a concentration of 1000 zoospores/mL in tank water. Controls have been treated similarly by adding autoclaved water from lake Kallavesi. Prior to the addition of the zoospores, the water circulation was interrupted to maintain the concentration of zoospores constant during the infection process. Water circulation was resumed After 16 h. During the experiment, the crayfish were monitored for symptoms, either gross signs of infection (scratching, loss of balance, aimless movements of the appendages, and loss of appendages) or death, multiple times per day. Moribund crayfish were removed from the system and stored at −20°C. During the challenge, crayfish were removed from the experimental system to sample their tissues (hemolymph, hepatopancreas, and gills) as part of an overlapping experiment where tissues from alive crayfish were needed for RNA isolation and subsequent gene expression analysis (**Table 1**; Boštjančić et al., 2021). The crayfish were removed on two different dates. The first sampling took place on day 3 of the challenge. All the haplogroup B-challenged noble crayfish showing gross signs of infection were sampled (n = 19), as they were likely to die in the following days (Makkonen et al., 2012a). Five individuals were sampled for each of the other experimental groups on the same day. During the second sampling, carried out on day 21 of the experiment, five crayfish per infection group were removed from the system. As a result, ten crayfish per group were left until the end of the experiment, except the noble crayfish challenged with the strain from haplogroup B, where all crayfish were sampled on day 3 or died on day 7. The experiment lasted 45 days, after which all remaining crayfish were considered successful

survivors. We expected eventual symptoms of infection to manifest themselves within this timeframe, as other comparable infection experiments have shown (Makkonen et al., 2012a, 2014).

Table 1. Study design and sampling time point.

Species	Treatment Group	N	Sampling time point		
			Day 3	Day 21	Day 45
noble crayfish	*A. astaci* of haplogroup A	20	**5**	**5**	10
	A. astaci of haplogroup B	20	**19***	-	-
	control	20	**5**	**5**	10
marbled crayfish	*A. astaci* of haplogroup A	20	**5**	**5**	10
	A. astaci of haplogroup B	20	**5**	**5**	10
	control	20	**5**	**5**	10
total		120	44	25	50

The number of specimens (N) belonging to each experimental group is reported. During each time point specimens belonging to each treatment group were sampled and removed from the experiment. * 19 crayfish were sampled on day 3, as they were showing symptoms of crayfish plague and were expected to die in the next days.

2.2.5. DNA Extraction, qPCR and *A. astaci* DNA Quantification

To test for the presence of *A. astaci* DNA in the crayfish tissues, qPCR of the samples was conducted. Tissue samples were taken from uropods, walking legs, and abdominal cuticle. DNA extraction was conducted following a modified protocol described in Vrålstad et al. (2009). The qPCR was conducted using the assay, primers and probe developed and shared from work in progress at the Norwegian Veterinary Institute (David A. Strand, unpublished). The new and more specific assay has been used in the light of possible cross-reaction of the Vrålstad assay with another *Aphanomyces* species (Viljamaa-Dirks and Heinikainen, 2019). As for the assay described in Vrålstad et al. (2009), the primers of the Strand et al. assay target the ITS region and the two assays are, therefore, comparable. The details of the new assay are: forward primer 5'-AAC TAT CCA CGT GAA TGT ATT CTT TAT-3', reverse primer 5'-CGG CTA AGT TTA TCA GTA TGT TAT TTA-3', and probe 5'-6-FAM-AAG AAC ATC CCA GCA CAA-MGBNFQ-3'. For each reaction, the qPCR analysis was performed in 20 µL reaction volume consisting of 10 µL of TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA, United States), 500 nM of each primer, 200 nM of probe, nuclease free water, and 5 µl of DNA sample. The amplification protocol consisted of an initial warming at 95°C for 10 min and 50 cycles of denaturation phase (95°C for 15 s) and annealing phase (62°C for 60 s). PCR forming units (PFUs) were calculated following Vrålstad et al. (2009). Only samples with PFU ≥ 5 were

considered positive. PFU = 5 is the limit of detection of the assay and it represents the lowest concentration that yields a probability of false negatives <5% (Vrålstad et al., 2009).

2.2.6. Statistical Analysis

The Shapiro–Wilk test was used to assess if the PFU values in each group were normally distributed. The Levene's test was used to test the equality of variance of the PFUs values in the different groups. As for most groups the PFU values didn't follow a normal distribution and the variances among groups were not equal, the significance of the differences of the PFU values among the different experimental groups was tested with the Kruskal–Wallis test. Finally, the pairwise Wilcoxon rank- sum test was used to evaluate pairwise differences among all the experimental groups, including control groups, and across the different time points. The Benjamini–Hochberg (BH) method was used for p-value adjustment (Benjamini and Hochberg, 1995). Only biologically relevant comparisons were taken into account (e.g., comparisons between different time points of the same experimental group and comparisons between noble crayfish and marbled crayfish challenged with the same *A. astaci* strain at the same time point).

2.3. Results

2.3.1. Signs of Infection

2.3.1.1. Noble Crayfish

Among the haplogroup B-challenged noble crayfish group, all individuals showed signs of infection between day 1 and day 5 in the form of scratching of the eyes, walking legs and abdomen (**Figure 1**). The scratching of the eyes generally lasted for several seconds. Of these crayfish, 19 were removed from the experimental system on day 3, as they were considered moribund. The last crayfish of this group started showing signs of infection on day 5, and died 2 days later. Under microscopic examination, this crayfish showed a heavy presence of hyphae in its abdominal cuticle. Subsequently, the soft cuticle of this single crayfish has been used for re-cultivation of *A. astaci*. Because of this, the tissues commonly used for the qPCR were not available, and thus for this crayfish the analysis was not conducted. In the noble crayfish group challenged with haplogroup A, 11 out of 20 crayfish showed signs of infection in the form of light scratching of eyes, abdomen and walking legs, or slow, aimless movements of the walking legs with the appendages fluctuating back and forth. These signs of infection were observed between day 5 and day 31 (**Figure 1**). All crayfish belonging to this group survived until the end of the experiment.

No crayfish belonging to the control group showed signs of infection or died during the experiment.

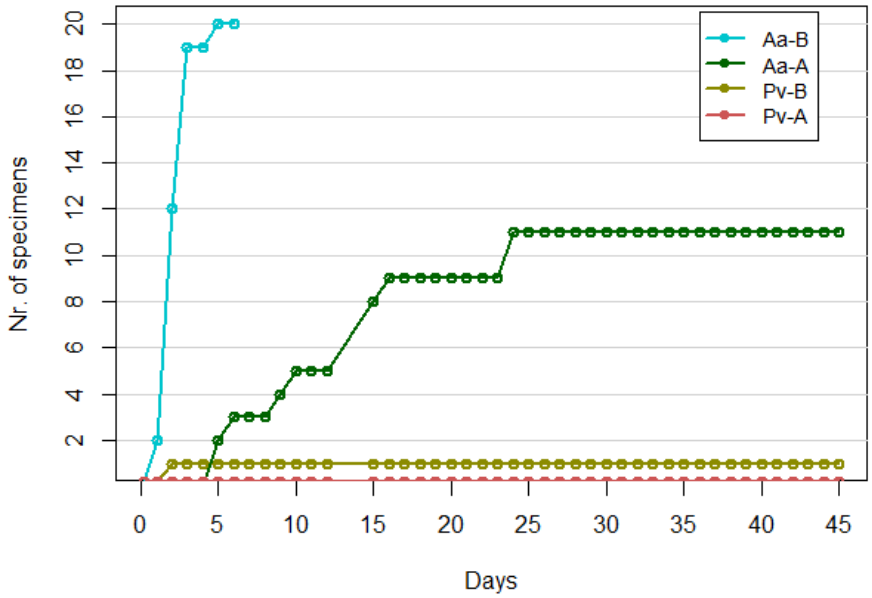

Figure 1. Cumulative number of crayfish showing gross signs of infection. Pv, marbled crayfish; Aa, noble crayfish; A, *A. astaci* of haplogroup A-challenged crayfish; B, *A. astaci* of haplogroup B-challenged crayfish.

2.3.1.2. Marbled Crayfish

Only one marbled crayfish belonging to the haplogroup B-challenged group showed signs of infection by scratching the eyes on day 2 (**Figure 1**). This particular behavior was only observed once. However, follow up observations were not possible as the crayfish was sampled the following day. As none of the control marbled crayfish were observed with similar behaviors, the scratching was considered a sign of infection. None of the crayfish belonging to the haplogroup A-challenged marbled crayfish and to the control group showed signs of infection. All marbled crayfish belonging to the three groups survived until the end of the experiment; out of these, one individual belonging to the control group molted.

2.3.2. qPCR

2.3.2.1. Noble Crayfish

In the haplogroup B-challenged noble crayfish, 15 out of 19 tested crayfish were positive for *A. astaci* DNA with the PFU values between 9 and 12949. In the haplogroup-A challenged group, *A. astaci* DNA was detected in 4 out of 20 noble crayfish (20%), with the positive samples detected only in the first time point. The PFU values of the positive samples ranged between 8 and 129. There was no significant difference in terms of *A. astaci* load between haplogroup A-

challenged and haplogroup B-challenged groups during the first sampling point (Pairwise Wilcoxon rank-sum test, n1 = 5, n2 = 19, p = 0.18, **Supplementary Tables 1**, **2**). *Aphanomyces astaci* DNA was not detected in any of the crayfish in the control groups (**Figure 2** and **Table 2**).

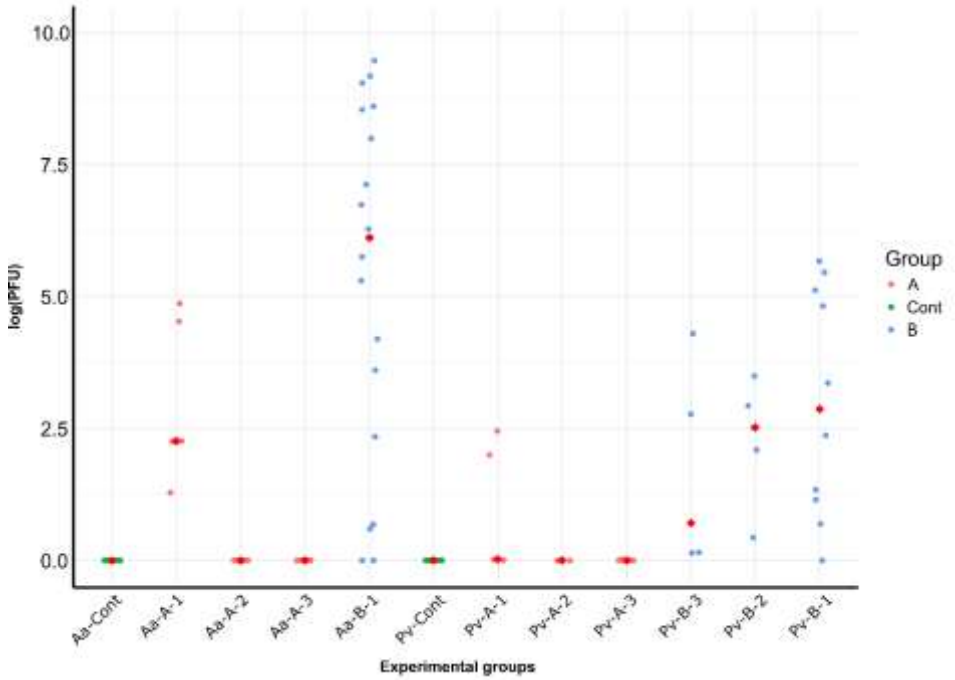

Figure 2. PFU values of *A. astaci* DNA detected in the crayfish of the different experimental groups, divided per sampling date. Medians are represented as red diamonds. Pv, marbled crayfish; Aa, noble crayfish; A, *A. astaci* of haplogroup A-challenged; B, *A. astaci* of haplogroup-B challenged; 1, first sampling point, day 3; 2, second sampling point, day 21; 3, third sampling point, day 45.

Table 2. Median of the PCR forming units (PFU) value and the range of the PFU values of each experimental group by sampling points.

Species	Group	Sampling time point	Number of samples	Positive samples	PFUs (median)	PFUs range
Noble crayfish	*A. astaci* of haplogroup A	Day 3	5	4	50.06	8.49 – 129
		Day 21	5	0	-	-
		Day 45	10	0	-	-
	A. astaci of haplogroup B	Day 3	19	15	842	9.43 – 12949
	Control	Day 3	5	0	-	-
		Day 21	5	0	-	-
		Day 45	10	0	-	-
Marbled crayfish	*A. astaci* of haplogroup A	Day 3	5	2	-	6.38 – 10.6
		Day 21	5	0	-	-
		Day 45	10	0	-	-
	A. astaci of haplogroup B	Day 3	5	2	-	15 – 72.50
		Day 21	5	4	15.55	7.11 – 31.9
		Day 45	10	6	144.50	9.7 – 289
	Control	Day 3	5	0	-	-
		Day 21	5	0	-	-
		Day 45	10	0	-	-

2.3.2.2. Marbled Crayfish

In total, *A. astaci* DNA was detected via qPCR in 12 out of 20 (60%) haplogroup B-challenged marbled crayfish, even though only one of them showed apparent behavioral signs of infection. The positive samples were detected in all time points, with two positive crayfish in the first time point, four in the second, and six in the third (**Table 2**). The PFU values of the positive crayfish ranged between 7 and 289. The only symptomatic marbled crayfish in this group tested negative in the qPCR. *Aphanomyces astaci* DNA was detected in two of the 20 marbled crayfish (10%) from the haplogroup A-challenged group, with PFU values of 6 and 11 (**Figure 2**). Both crayfish were sampled at the first time point (3 days after exposure to *A. astaci* spores). The comparison of the PFU values between haplogroup A-challenged and haplogroup B-challenged groups showed no significant difference in the first sampling point for marbled crayfish (Pairwise Wilcoxon rank-sum test, n1 = 5, n2 = 5, p = 0.27, **Supplementary Tables 1**, **2**). All crayfish from the control group were negative. All the remaining relevant comparisons between treatment groups resulted non-significant (**Supplementary Tables 1**, **2**).

2.4. Discussion

The main aim of this study was to compare the response of two crayfish species, the European native noble crayfish and the invasive marbled crayfish, to an *A. astaci* challenge with a highly virulent strain from the haplogroup B and a lowly virulent strain from the haplogroup A. We showed a high resistance of marbled crayfish against an *A. astaci* infection, with zoospores from the highly virulent haplogroup B strain being able to infect the host, but unable to cause the disease. Furthermore, we demonstrated a reduced virulence in the *A. astaci* Venesjärvi strain belonging to haplogroup A, as shown by the light symptoms and the lack of mortality in the noble crayfish. Interestingly, in both marbled crayfish and noble crayfish challenged with this strain, the pathogen DNA was only detected in the tissues of the crayfish sampled on day 3 (**Figure 2**), suggesting that this *A. astaci* haplogroup A strain has a decreased ability of penetrating into the cuticle of the crayfish and infecting the crayfish. Finally, our results prove once more how drastically the strains belonging to *A. astaci* haplogroup B and haplogroup A differ in their virulence.

2.4.1. Elevated Resistance in Marbled Crayfish

Our experiment showed a strong resistance of marbled crayfish against *A. astaci*. The crayfish were not affected by the two *A. astaci* strains used in this study, which both failed to cause

symptoms in the marbled crayfish, with only a single exception (**Figure 1**). This only symptomatic crayfish, belonging to the *A. astaci* haplogroup B-challenged group, showed a mild version of the gross signs of infection, with only briefly scratching its eyes. Although the marbled crayfish in the haplogroup B-challenged group mainly did not show gross signs of an *A. astaci* infection, the amount of *A. astaci* DNA detected in their tissues clearly showed that some of these crayfish were indeed infected (**Table 2**). This might indicate that *A. astaci* was able to germinate and penetrate into the cuticle of the crayfish, however, the marbled crayfish's immune system was capable of preventing the manifestation of the disease. This shows that the marbled crayfish has the potential to be latently infected with highly virulent strains and to act as their carrier. Interestingly, the amount of *A. astaci* DNA detected in the tissues of the marbled crayfish infected with the haplogroup B strain indicates a possible increase of the pathogen DNA over time (**Table 2** and **Figure 2**), although this increment was not statistically significant. The increase of pathogen DNA might be the result of spores attached to the cuticle of the crayfish without germinating and causing infection. However, our experimental system allowed for a constant exchange of water, with spore- free water flowing into the tanks, and contaminated water being directed from the tanks to the filters. For this reason, it is more likely that the detected increment in PFU values is the result of an active *A. astaci* infection. From the few studies where it was possible to confirm the presence of *A. astaci* in marbled crayfish specimens, it is clear that this species can withstand higher levels of infection than the one observed in this study (Keller et al., 2014; Mrugała et al., 2015). Quantitative PCR conducted on seemingly healthy specimens from a laboratory- cultured population revealed high amount of pathogen DNA in the sampled tissues ($10^4 \leq$ PFU $\leq 10^5$, Keller et al., 2014). It might be interesting in future studies to perform an even longer lasting experiment to assess the progression of the infection, and to evaluate if it would result in the manifestation of the disease.

When marbled crayfish was first discovered in open waters in Germany in 2003 (Marten et al., 2004) it was assumed to have a big invasion potential because of its high fecundity and parthenogenetic reproduction (Scholtz et al., 2003). However, since then single individuals unable to produce established populations have often been observed (Vogt, 2020). Although it is known that marbled crayfish can survive winters in Central Europe (Veselý et al., 2015), their optimal temperature for reproduction is between 20 and 25°C (Seitz et al., 2005), similar to the water temperature from Florida, where *P. fallax*, their closest relative, lives. Martin et al. (2010) already speculated that marbled crayfish might establish more successful populations in the newly invaded Madagascar, where the climate is milder. Few years later this predicted scenario has proven true, and it has been shown that in warmer climates the marbled crayfish is able to increase its range dramatically (Andriantsoa et al., 2019). In Europe, however, this species does not spread as fast and the population growth is limited, probably because of the colder temperatures (Günter

et al., 2019). It has been observed that when new populations become established in Europe, it is usually not due to the invasion potential of marbled crayfish, as is the case for red swamp crayfish (*Procambarus clarkii*) or calico crayfish (*Faxonius immunis*), but because of human mediated releases. This might change when more populations will be released in watercourses as it was observed in Slovakia and Hungary (Lipták et al., 2016; Weiperth et al., 2020). Marbled crayfish is a popular pet in the aquarium trade worldwide, and it has now established populations in at least 16 countries (Vogt, 2020), including those with warmer climate where the invasion will be more successful.

While it has been confirmed that marbled crayfish can act as *A. astaci* carrier, analysis of specimens of this species from the wild and from the aquarium trade have shown that the presence of *A. astaci* in their tissues often cannot be verified (Lipták et al., 2016, 2017; Patoka et al., 2016; Pârvulescu et al., 2017; Andriantsoa et al., 2019; Ercoli et al., 2019; Lenich, 2019; own unpublished data). No infection could be confirmed in 100 tested crayfish from Madagascar (Andriantsoa et al., 2019), 67 specimens from Slovakia (Lipták et al., 2016, 2017), four specimens from Czechia (Patoka et al., 2016), nine specimens from Romania (Pârvulescu et al., 2017), six specimens from Estonia (Ercoli et al., 2019), and 20 specimens from Germany (Lenich, 2019). On the other hand, when marbled crayfish co-exists with North American crayfish species, it is usually found to be infected. This was the case for marbled crayfish held with other North American crayfish species in common aquaria (Keller et al., 2014; Mrugała et al., 2015), or for marbled crayfish co-existing with *Faxonius limosus* in the wild (Keller et al., 2014; own unpublished data). Therefore, it can be speculated that marbled crayfish was not infected when it developed in the aquarium environment, and only becomes carrier of *A. astaci* when in contact with North American crayfish species. Because of its invasive potential and its *A. astaci* carrier status the trade with marbled crayfish is now officially forbidden by the EU Regulation 1143/2014 on Invasive Alien Species in EU countries.

It is generally expected that North American crayfish are comparatively more resistant to *A. astaci* infections than European crayfish (Svoboda et al., 2017). As shown in our study, the marbled crayfish appeared to be highly resistant to *A. astaci* infections. This resistance might be a consequence of the shared coevolution history of *A. astaci* and North American crayfish, of which marbled crayfish is a recent descendant. While marbled crayfish and its closest relative *P. fallax* share similar morphological characters, coloration and some ecological features, the triploidization in the marbled crayfish genome (a third identical copy of the *P. fallax* chromosome set, without any additional or changed DNA sequences) has led to an enhanced body size, fecundity and longevity, which contribute to its invasive capabilities (Vogt et al., 2019). More information about the resistance or eventual susceptibility of American crayfish in their natural habitat is needed to better understand the consequences of the host-pathogen coevolution process.

2.4.2. Latent Infections Due to Reduced Virulence

The tested *A. astaci* Venesjärvi strain did not cause any mortality among the challenged noble crayfish. While light gross signs of infection were observed (**Figure 1**), those appeared to be less pronounced than the ones caused by the haplogroup B strain, and did not lead to the death of any crayfish. Only four out of 20 crayfish belonging to this group were shown to be infected by *A. astaci* in our experiment. However, the qPCR assay cannot demonstrate the absence of infection, as the amount of pathogen present in the crayfish tissue might be below the detection level and the infection might be localized in different tissues to the sampled ones. It has been shown on different occasions, that haplogroup A includes strains that differ greatly in their virulence (Makkonen et al., 2012a, 2014; Becking et al., 2015; Mrugała et al., 2016; Jussila et al., 2017). For example, in Makkonen et al. (2012a) crayfish belonging to the same population and exposed to the same experimental conditions were infected with two different strains both belonging to haplogroup A. While one strain caused 100% of mortality within 19 days, the second strain failed to cause any significant increase in the death rate of the experimental crayfish. Strains belonging to haplogroup A arrived to Europe presumably without their original host (Alderman, 1996). This might have worked as selective pressure toward reduced virulence (Makkonen et al., 2012b; Jussila et al., 2015). On the other hand, a host-parasite equilibrium could be reached not only by lowered virulence of the parasite but also by increased resistance of the host. However, the noble crayfish population from Lake Rytky used in this study has already been shown, in comparable experiments, to be susceptible to *A. astaci* strains belonging to haplogroup A (Makkonen et al., 2012a). This, together with the haplogroup A strain being isolated from a latently infected population, suggests a decreased virulence of the respective *A. astaci* strain.

The lack of mortality and severe gross signs in the crayfish challenged with *A. astaci* of haplogroup A could be taken as experimental proof of the existence of wild noble crayfish populations latently infected with *A. astaci*, which then may act as carriers of the pathogen (Jussila et al., 2011a; Viljamaa- Dirks et al., 2011). A similar case of latent infection has been reported by Jussila et al. (2011a) for a noble crayfish population in Lake Mikitänjärvi. No population decline or increased mortality was observed, and the population was considered healthy until the qPCR analysis revealed that some of the specimens were infected with *A. astaci* (Jussila et al., 2011a). Unfortunately, in that instance *A. astaci* itself was not isolated, nor was it possible to identify its haplogroup (Jussila et al., 2011a). The impossibility of conducting tests on the virulence of the strain on other noble crayfish populations makes it difficult to speculate on the effective virulence of the *A. astaci* strain in this case. However, subsequent experiments showed a higher resistance of the Lake Mikitänjärvi population to both haplogroups A and B when compared to other noble crayfish populations (Makkonen et al., 2014). The two cases of latent infections of wild noble crayfish populations from Lake Mikitänjärvi and Lake Venesjärvi are just two examples of the

occasional status of equilibrium tentatively reached by European crayfish populations and *A. astaci*. In the past 20 years, cases of populations latently infected with *A. astaci* haplogroup A have been reported not only in noble crayfish, but also in other European crayfish species such as white-clawed crayfish (*Austropotamobius papilles*), stone crayfish (*Austropotamobius torrentium*), and narrow-clawed crayfish (*Pontastacus leptodactylus*) (Ungureanu et al., 2020). Interestingly, it has been observed that latent infections are not only caused by the lowly virulent *A. astaci* haplogroup A, but also by the more virulent haplogroup B (Ungureanu et al., 2020). In Europe, the situation concerning invasive crayfish species is constantly changing and new species are expected to start spreading across the continent. It might soon be the case for *Faxonius rusticus*, which is already present in the European pet trade and known for its invasive potential (Chucholl, 2012). *Faxonius rusticus* in North America carries a distinct strain of *A. astaci* belonging to haplogroup A (Panteleit et al., 2019). The introduction of additional North American crayfish species could bring new *A. astaci* strains to Europe, as demonstrated, e.g., by *F. rusticus*. It remains to be seen if the coevolution between European crayfish and specific *A. astaci* strains might eventually lead to European crayfish populations being better equipped to face infections from novel *A. astaci* strains.

Interestingly, for both species of crayfish challenged with haplogroup A strain, only the specimens sampled during the first time point tested positive in the qPCR (**Table 2** and **Figure 2**). This pattern could be explained by the detection of spores merely attached to the cuticle of the crayfish during the first sampling point. Spores unable to germinate would then detach themselves without causing an infection and get filtered away through the system, leading to negative results in the qPCR in the second and third sampling points. However, the observed pattern might also indicate a decreased capacity of this particular *A. astaci* strain of haplogroup A to penetrate the cuticle of the host. The colonization of the host by *A. astaci* starts when the spores, covered by sticky substances, attach themselves to the host surface (Cerenius et al., 2009). The germination process of the spores begins, followed by the penetration of the newly germinated hyphae into the cuticle of the crayfish (Cerenius et al., 2009). With the penetration of the hyphae, the infected host's immune system and the pathogen start interacting (Hauton, 2012). North American crayfish can, to a different level, resist the penetration of the hyphae, while native European crayfish are normally susceptible to the *A. astaci* infection. These moments of germination and penetration are crucial for the fate of the *A. astaci* infection process. The results of the transcriptome analysis of the crayfish sampled during the experiment revealed that this haplogroup A was able to trigger the immune response in the marbled crayfish 3 days after challenge (own unpublished data). This suggests that, while the spores were able to germinate, their ability to penetrate the crayfish cuticle was very limited. It is likely that the detection of the pathogen DNA in the first sampling point derives from *A. astaci* spores attached to the cuticle of the crayfish. Most of them might have

detached themselves from the host, and even those germinating failed to establish in the host, which would explain the negative qPCR results at the second and third sampling points. Further infection experiments might shed some light on the mechanisms that resulted in this speculated reduced ability of this *A. astaci* strain to penetrate into the cuticle of crayfish.

Drastically different was the response of the noble crayfish challenged with the haplogroup B strain (**Table 2** and **Figure 2**) compared to those of haplogroup A-challenged noble crayfish. The difference in PFU value between the two groups is not significant, as the variation within the groups were very high, with some of the crayfish in the haplogroup B-challenged group resulting negative despite the clear symptoms of infection. This might be the reflection of several aspects, both biological and methodological. The high within group variations might be the result of real biological differences in the resistance or sensitivity of the crayfish in the same experimental group. On the other hand, the qPCR assay is semi-quantitative. It is not possible to use the entire soft cuticle of the specimens for the qPCR. Because of this, different parts of the soft cuticles are sampled to maximize the chance of sampling cuticles containing hyphae. As a result, the amount of *A. astaci* DNA detected in the tissues is influenced by the sampling. However, in this case, the difference in the effects of *A. astaci* haplogroup A and haplogroup B on the noble crayfish are clear when considering the observed symptoms. The isolate from Lake Tahoe is highly virulent, and it caused clear symptoms of morbidity in the challenged noble crayfish, indicating a likely death of the noble crayfish in 1 or 2 days after the onset of the gross signs, as recently shown in Makkonen et al. (2019). While the *A. astaci* strain from haplogroup A used in this study was probably adapted to its new European hosts due to the absence of the original carrier (Makkonen et al., 2012b, 2018), the situation is very different for *A. astaci* belonging to haplogroup B. This haplogroup was introduced to Europe with its original host (*P. leniusculus*) which then has established numerous populations on the European continent (Alderman, 1996; Kouba et al., 2014). In this situation, scenarios where this strain wipes out the susceptible European crayfish populations would not have significant repercussion on *A. astaci*, which would still be free to circulate in the usually more resistant crayfish populations of North American origin (Jussila et al., 2015). Nonetheless, in the past decades there have been reports of populations of the native European *P. leptodactylus* latently infected with *A. astaci* strains belonging to haplogroup B (Ungureanu et al., 2020). *Pontastacus leptodactylus* is the most resistant among the native European species, but still it is considered susceptible to the crayfish plague (Svoboda et al., 2017; Jussila et al., 2020) and has been shown to suffer from mass mortalities caused by the disease (Rahe and Soylu, 1989; Timur, 1990). However, latently infected populations have been observed in Croatia (Maguire et al., 2016), Romania and Moldova (Panteleit et al., 2018), Turkey (Svoboda et al., 2014; Kokko et al., 2018), and Ukraine (Ungureanu et al., 2020). Until now, there have been no reports of noble crayfish populations resistant to *A. astaci* haplogroup B, although less

susceptible populations have been detected (Makkonen et al., 2012a). In future, it will be interesting to see if the selection of European crayfish populations resistant to *A. astaci* haplogroup A would lead to the development of resistance also toward more virulent or new haplogroups.

2.5. Conclusion

This study demonstrated the adaptation of *A. astaci* haplogroup A strain isolated from Lake Venesjärvi to their novel European hosts, supposedly due to reduced virulence. Our results indicate that this feature might be the consequence of *A. astaci*'s reduced ability to penetrate into the cuticle of crayfish. Our observations support the growing number of reports of latent infections among native European crayfish stocks, providing additional evidence that the relationship between European crayfish and *A. astaci* might be slowly heading toward an equilibrium. Finally, we empirically demonstrated that marbled crayfish are highly resistant against *A. astaci* and add evidence to the ability of this species to become latently infected and act as a carrier of highly virulent *A. astaci* strains.

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Chapter 3

Host-pathogen coevolution drives innate immune response to *Aphanomyces astaci* infection in freshwater crayfish: transcriptomic evidence

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Abstract

Background: For over a century, scientists have studied host-pathogen interactions between the crayfish plague disease agent *Aphanomyces astaci* and freshwater crayfish. It has been hypothesised that North American crayfish hosts are disease-resistant due to the long-lasting coevolution with the pathogen. Similarly, the increasing number of latent infections reported in the historically sensitive European crayfish hosts seems to indicate that similar coevolutionary processes are occurring between European crayfish and *A. astaci*. Our current understanding of these host-pathogen interactions is largely focused on the innate immunity processes in the crayfish haemolymph and cuticle, but the molecular basis of the observed disease-resistance and susceptibility remain unclear. To understand how coevolution is shaping the host's molecular response to the pathogen, susceptible native European noble crayfish and invasive disease-resistant marbled crayfish were challenged with two *A. astaci* strains of different origin: a haplogroup A strain (introduced to Europe at least 50 years ago, low virulence) and a haplogroup B strain (signal crayfish in lake Tahoe, USA, high virulence). Here, we compare the gene expression profiles of the hepatopancreas, an integrated organ of crayfish immunity and metabolism.

Results: We characterised several novel innate immune-related gene groups in both crayfish species. Across all challenge groups, we detected 412 differentially expressed genes (DEGs) in the noble crayfish, and 257 DEGs in the marbled crayfish. In the noble crayfish, a clear immune response was detected to the haplogroup B strain, but not to the haplogroup A strain. In contrast, in the marbled crayfish we detected an immune response to the haplogroup A strain, but not to the haplogroup B strain.

Conclusions: We highlight the hepatopancreas as an important hub for the synthesis of immune molecules in the response to *A. astaci*. A clear distinction between the innate immune response in the marbled crayfish and the noble crayfish is the capability of the marbled crayfish to mobilise a higher variety of innate immune response effectors.

3.1. Background

Host-pathogen interactions are models for evolutionary arms-races, thus cycles of reciprocal co-adaptation (O'Brien et al, 2013). Coevolution between hosts and pathogens is ubiquitous, often resulting in rapid evolutionary change, and is linked to the maintenance of diversity (Paterson et al., 2010; Betts et al., 2018). Pathogens impose strong selection on their hosts which try to minimize their fitness loss, e.g. by evolving resistance, while pathogens themselves are under strong selection to undermine host defences without causing the complete collapse of the host

population (Paul et al., 2004). Parasite virulence may peak after a host-jump, as the new host has not yet evolved any specific defence mechanisms (Paul et al., 2004; Geoghegan & Holmes, 2018). The theory behind host-parasite interactions is well established (Thompson, 2001, 2004), and there are ample examples for coevolutionary adaptations (Mackinnon & Read, 2004; Laanto et al., 2017). However, we are only just starting to understand the underlying genomic mechanisms and genes involved in co-adaptation processes (Ebert & Fields, 2020). Host-pathogen interactions are of high interest in conservation biology, as they not only determine the fate of invasive species, but they also affect the survival of native taxa (Strauss et al., 2012). Due to its high importance for aquaculture and management, scientists have studied the interaction between freshwater crayfish and their pathogen *Aphanomyces astaci* for over a century (Jussila et al., 2021). Still, the coevolutionary aspect of this host-pathogen interaction remains understudied.

Likely because of their coevolutionary history, North American crayfish species are generally considered resistant to the pathogen *A. astaci*, the causative agent of crayfish plague disease (Unestam, 1969; Holdich et al., 2009). It is assumed that these crayfish species are natural carriers of their specific *A. astaci* strain, usually efficiently preventing it from spreading inside their tissues through melanisation mediated encapsulation of the pathogen hyphae in the cuticle (Nyhlén & Unestam, 1980; Jussila et al., 2015). In contrast, European crayfish species do not naturally carry the pathogen and are considered susceptible to the disease (Jussila et al., 2017, 2020; Martín-Torrijos et al., 2017). Therefore, the introduction of invasive North American crayfish species into Europe, and with them of *A. astaci*, caused mass mortalities and local extinctions among European crayfish populations (Alderman, 1996). The *A. astaci* strains present in Europe can be grouped into 4 different haplogroups (Makkonen et al., 2018). Haplogroup A contains strains of unequal virulence (ranging from non-virulent to highly virulent), while haplogroups B, D and E are usually characterized by high virulence (Makkonen et al., 2012; Becking et al., 2015; Martín-Torrijos et al., 2017). Despite the high susceptibility of native European crayfish species towards the crayfish plague disease agent, latent crayfish plague infections without mass mortalities have been reported for several species infected with low virulent *A. astaci* strains of haplogroup A (Jussila et al., 2021), suggesting the presence of an ongoing dynamic coevolutionary process. However, the foundation of this naturally occurring resistance to *A. astaci* remains unclear.

Initial studies suggested that one of the main factors contributing to the resistance of North American crayfish species is the constitutively over-expressed prophenoloxidase (proPO) in the haemocytes, a key enzyme in the encapsulation of pathogens in melanin (Cerenius et al., 2003). Conversely, in European crayfish species, the expression of this enzyme is dependent on stimuli of the pathogen (Cerenius et al., 2003). Based on the current knowledge of the innate immunity

mechanisms in crustaceans, the response to pathogens comprises both cellular and humoral components, with the proPO cascade playing part in the humoral response (Hauton, 2012; Rowley, 2016; Cerenius & Söderhäll, 2018). The immune response is triggered by the pathogen-associated molecular patterns (PAMPs), such as β-(1,3)-glucan, which is one of the main constituents of the oomycetes cell wall (Jiravanichpaisal et al., 2006). These molecules are recognised by specific pattern-recognition proteins (PRPs) of the host, which can exist as soluble molecules or as associated with cell membranes. PRPs of particular relevance are lectin-like proteins, Down Syndrome Cell Adhesion Molecules (DSCAMs) and Toll-like receptors (TLRs) (Hauton, 2012; Low & Chong, 2020). The interaction between ligands and receptors leads to the activation of different molecular pathways involved in the humoral or cellular response, all of them coordinated by the core mediators of the crustacean immunity, the haemocytes. Haemocytes are crucial for the processes of phagocytosis, encapsulation and melanisation, and they are involved in delivering the molecular effectors of the humoral response, such as antimicrobial peptides and proPO, to the infection sites (Lin & Söderhäll, 2011; Rowley, 2016; Smith, 2016).

The mechanisms underlying the crayfish immune response to *A. astaci*, however, is much more complex than the simple activation of the proPO cascade, but its molecular effectors and other tissues beyond haemolymph have not received much attention. In Crustaceans, hepatopancreas represents an integrated organ of immunity and metabolism (Johnson, 1987; Rőszer, 2014). It plays a major role in pathogen clearance, antigen processing (Alday-Sanz et al., 2002; Chen et al., 2021), detoxification, and heavy metal deposition (Meng et al., 2019). It also serves as a source for immune molecules, which can be released from the epithelial cells into the haemocoel sinusoids, allowing for their rapid distribution in the haemolymph of the crayfish (Rőszer, 2014). In recent years, the involvement of the hepatopancreas in the response to various disease and environmental factors has been highlighted in crustaceans (Dai et al., 2017; Jiao et al., 2019; Meng et al., 2019; Zhang et al., 2019; Shen et al., 2020). However, its role in the immune response to *A. astaci* infection has not been clearly defined.

Through the coevolutionary transcriptomics approach, we aimed to deepen our understanding of the molecular mechanisms underlying the resistance and susceptibility of freshwater crayfish to the *A. astaci*, to unravel how coevolution is shaping the molecular response to the pathogen. By analysing gene expression profiles in the hepatopancreas, we compared the immune response of the susceptible native European noble crayfish (*Astacus astacus*) and the resistant invasive marbled crayfish (*Procambarus virginalis*) to an *A. astaci* challenge. In a controlled infection experiment, both species were infected with a highly virulent (haplogroup B, hereinafter Hap B) and a lowly virulent (haplogroup A, hereinafter Hap A) *A. astaci* strain (Francesconi et al., 2021). Previous studies focused on the early stages of the *A. astaci* infection, but the transition

from acute infection to latent infection states has not been studied. Therefore, the hepatopancreas of the crayfish was sampled during the early phase of challenge (day 3) and late phase of the challenge (day 21).

We hypothesised that the hepatopancreas is a highly relevant tissue in the immune response towards *A. astaci* infections, and we expected to detect several immune-related transcripts in all treatment groups. We expected that the gene expression profiles of the immune-related transcripts differ between the noble crayfish and the marbled crayfish, reflecting the species' different coevolutionary history with the specific *A. astaci* strain, and thus their different abilities to defend against the pathogen. Furthermore, for the susceptible noble crayfish, we expected a stronger immune response in noble crayfish challenged with the highly virulent Hap B strain compared to the less virulent Hap A strain. Conversely, we did not expect any gene expression difference among treatment groups for the resistant marbled crayfish. Lastly, we expected the latently infected crayfish to show a chronic immune response against *A. astaci*, with the presence of differentially expressed immune-related transcripts 21 days post-challenge.

The results presented in this paper deliver novel insights into the gene repertoire involved in the immune response to the *A. astaci* challenge, deepening our understanding of freshwater crayfish immunity and their interaction with the pathogen, *A. astaci*.

3.2. Materials and methods

3.2.1. Aim, design, and study setting

A controlled infection experiment was previously conducted by Francesconi et al. (2021) on the marbled crayfish and the noble crayfish. All the crayfish were acclimatised in individual tanks with circulating water for 20 days prior to the start of the experiment. The water conditions (oxygen levels, temperature, conductivity and pH) were monitored daily. The day-night rhythm was mimicked through artificial lights, with 8 h of light and 16 h of dark. All the experimental crayfish were of the similar size, the marbled crayfish (mean carapace length = 39.7 ± 2.7 mm) and noble crayfish (mean carapace length = 43.5 ± 2.3 mm). The crayfish were given every second day preboiled frozen sweet corn. The crayfish were challenged with two different strains of *A. astaci*, a highly virulent Hap B strain and a lowly virulent Hap A strain. All the challenged crayfish were infected with 1000 zoospores per mL. In total 55 individuals (30 marbled crayfish and 25 noble crayfish) were selected for RNA sequencing, with five replicates per treatment (Hap A, Hap B, control) from two time points (3d, 21d post challenge), with exception of the Hap B challenged noble crayfish group, in which all crayfish became moribund in the first days of the challenge and were therefore all sampled in the first time point. For each individual a

portion of the hepatopancreas was dissected and snap frozen in liquid nitrogen. Detailed description of the infection experiment and its results Francesconi et al. (2021). The details of the bioinformatical processing of the RNA sequencing reads and transcriptome assembly Boštjančić et al. (2022).

3.2.2. Identification of the crayfish innate immunity genes and taxonomical distribution of transcripts

We retrieved a dataset of innate immunity related genes identified in Malacostraca by Lai and Aboobaker (2017). This dataset was expanded with the selected differentially expressed genes (DEGs) identified in the Hap B challenged noble crayfish. Furthermore, we included the genes specifically related to the proPO cascade. The complete list of the used innate immunity genes and their respective sequences are available in the **Table S1** and **File S1**. Transcriptome assemblies were queried against the subset of the innate immunity related genes with BLASTn and BLASTx 2.10.1+. Hits were then inspected, their function was confirmed based on their e-value (lower than $1e^{-10}$), and the presence of the functionally important gene domains identified with a Pfam search.

3.2.3. Read mapping

All of the sample 2 × 150 bp paried-end reads (Illumina NovaSeq6000; SRA study: SRP318523, read depth: 36.8 M- 68.9 M, mean: 48.59 M) were mapped to the newly obtained reference transcriptome (Boštjančić et al., 2022) (noble crayfish TSA: GJEB00000000 and marbled crayfish TSA: GJEC00000000) using the pseudo-alignment approach implemented in Salmon 0.13.1 (Petro et al., 2017). Several "flags" were used in the Salmon mapping steps to correct the biases that might originate from sequence data: "-validateMap- pings" (Srivastava et al., 2020), "--seqBias" and "--gcBias" (Roberts et al., 2011).

3.2.4. Differential gene expression analysis

The differential gene expression analysis was conducted according to the DESeq2 protocol (Soneson et al., 2015) implemented in R with the following model design for the noble crayfish: sex (male/female) + groups (Control vs Hap A or Hap B challenge) and for the marbled crayfish: ~reproduction (yes/no) + groups (Control vs Hap A or Hap B challenge). Independent comparisons were conducted for each sampling point. Raw counts from the Salmon output were used as the input. Transcripts highly similar to the marbled crayfish and the noble crayfish mitogenome, respectively, were removed prior to the analysis based on the BLAST hits against

the mitogenome (NCBI accession number: KX279347.1 and NC_020021.1). Transcripts assigned to the bacteria and the archaea were also removed based on the DIAMOND search (see 2.2). results Counts for individual Trinity transcript isoforms were grouped to Trinity genes with the tximport R package (Soneson et al., 2015). Lowly expressed genes were filtered out: only genes with the raw counts higher/equal to 10 across at least five samples were retained. The package "Enhanced- Volcano" (Blighe et al., 2020) was used for the visualisation of the DEGs and "apeglm" for noise removal (Zhu et al., 2019). The list of DEGs was exported and their counts, log2fold changes and adjusted p-values (FDR = 0.1, p-value = 0.05) together with their respective annotations were merged. Possible overlaps between the DEGs at different time points were inspected using Venn diagrams (Ritchie et al., 2015).

3.2.5. Gene set enrichment analysis

Enrichment of the innate immunity gene sets identified in the 2.2. were conducted with ClusterProfiler (Yu et al., 2012). Based on the results of the DESeq2 analysis, for each group all genes were ranked according to the following metric: -log10(x)/sign(y), where x is the p-value and y log2 fold change. To detect the enriched gene sets we used the GSEA() function, with the p values adjusted based on Benjamini-Hochberg correction for the multiple testing (cutoff < 0.01). Graphical representation of the results was obtained using the gseaplot2() function (Yu et al., 2012).

3.3. Results and discussion

3.3.1. Immune-related transcripts in the hepatopancreas, the mediator of the crayfish immune response to *A. astaci* challenge

Genomic research on non-model organisms is faced by the challenge of annotating large sets of genes from unknown origin. This challenge is particularly evident in Crustaceans (Clark & Greenwood, 2017; Calderón-Rosete et al., 2017), which are still largely underrepresented in genomic studies. To date, only 48 out of 727 genome assemblies representing Pancrustacea belong to Crustaceans (with the remaining 679 genomes belonging to Hexapoda) (Genomes-NCBI Datasets, accessed: April 2021). Furthermore, the canonical proPO pathway, considered a core immune response mechanism in the Crustaceans (Cerenius & Söderhäll, 2004), is not represented in the KEGG database. Therefore, we conducted the annotation of the innate immunity related genes in the noble crayfish and the marbled crayfish hepatopancreas transcriptomes using a sequence and domain similarity-based approach. A total of 372 and 353

innate immune-related genes were identified through this approach in the noble crayfish and the marbled crayfish, respectively (**Figure 1**, **Table S2**, **Table S3**, **File S2**, **File S3**).

The identification of these innate immune-related genes provides a basis for future transcriptomic and genomic studies of the innate immunity in freshwater crayfish species. For example, we successfully identified members of the immune signalling Toll pathway. This pathway is conserved in most members of Malacostraca (Lai & Aboobaker, 2017) and its activation is critical for antimicrobial peptides (AMPs) expression in Hexapoda (Kawasaki & Kawai, 2014; Paro & Imler, 2016). In the noble crayfish and the marbled crayfish, we identified most of the Toll pathway-related genes as single copy (**Figure 1**). Recently, an extensive overview of innate immune-related genes has been conducted on numerous marine and freshwater Decapods (Lai & Aboobaker, 2017). The number of TLRs identified in those species ranged between 0 and 8, collocating the number of TLRs found in this study slightly above the higher value (11 in the noble crayfish and 8 in the marbled crayfish). Lastly, in the noble crayfish TOLLIP, Spätzle and Tube were detected in multiple copies (**Figure 1**).

The innate immune system in freshwater crayfish is armed with an arsenal of PRRs capable of recognising various PAMPs (Janeway & Medzhitov, 2002). The β-(1,3)-glucan receptors (often referred to as Gram-negative binding proteins (GNBPs) or lipopolysaccharide binding proteins) play a vital role in the proPO cascade activation (Cerenius & Söderhäll 2012). All GNBPs share a carbohydrate-binding β-glucanase domain as identified in this study (Lai & Aboobaker, 2017). The expansion of this family was previously reported in Decapoda (Lai & Aboobaker, 2017), and confirmed in this study with 9 GNBPs identified in the noble crayfish and 8 in the marbled crayfish (**Figure 1**). Immune molecules and pathways involved in the response to the *A. astaci* challenge are discussed in detail in the section Molecular mechanisms of the immune response to the *A. astaci* challenge.

3.3.2. Gene expression profiles of *A. astaci* challenged crayfish

3.3.2.1. Exploratory analysis of the mapping results

Mean mapping rate of the processed reads for the noble crayfish was 88.96% and for the marbled crayfish 91.98% (**Table S4**). This was followed by the principal component analysis (PCA), performed to compare the replicates of the *A. astaci* challenged crayfish with the control group. The initial results of the PCA revealed a batch effect in the noble crayfish and the marbled crayfish samples (**Figure S1**). For the noble crayfish this effect was related to the differences between male and female individuals, accounting for 21% of the variance. For the marbled crayfish, the highest level of variance (63%) was caused by the differences between reproducing

and non-reproducing parthenogenetic females (see Francesconi et al. 2021, for details). Therefore, in the down-stream differential gene expression analysis, we accounted for the sex of the noble crayfish, as well as the reproductive status of the marbled crayfish, by including them as factors in the DESeq2 analysis. After batch effect removal, the PCA analysis revealed the grouping only for the *A. astaci* Hap B challenged noble crayfish, while such grouping was revealed neither for other noble crayfish samples nor for the marbled crayfish (**Figure S1**).

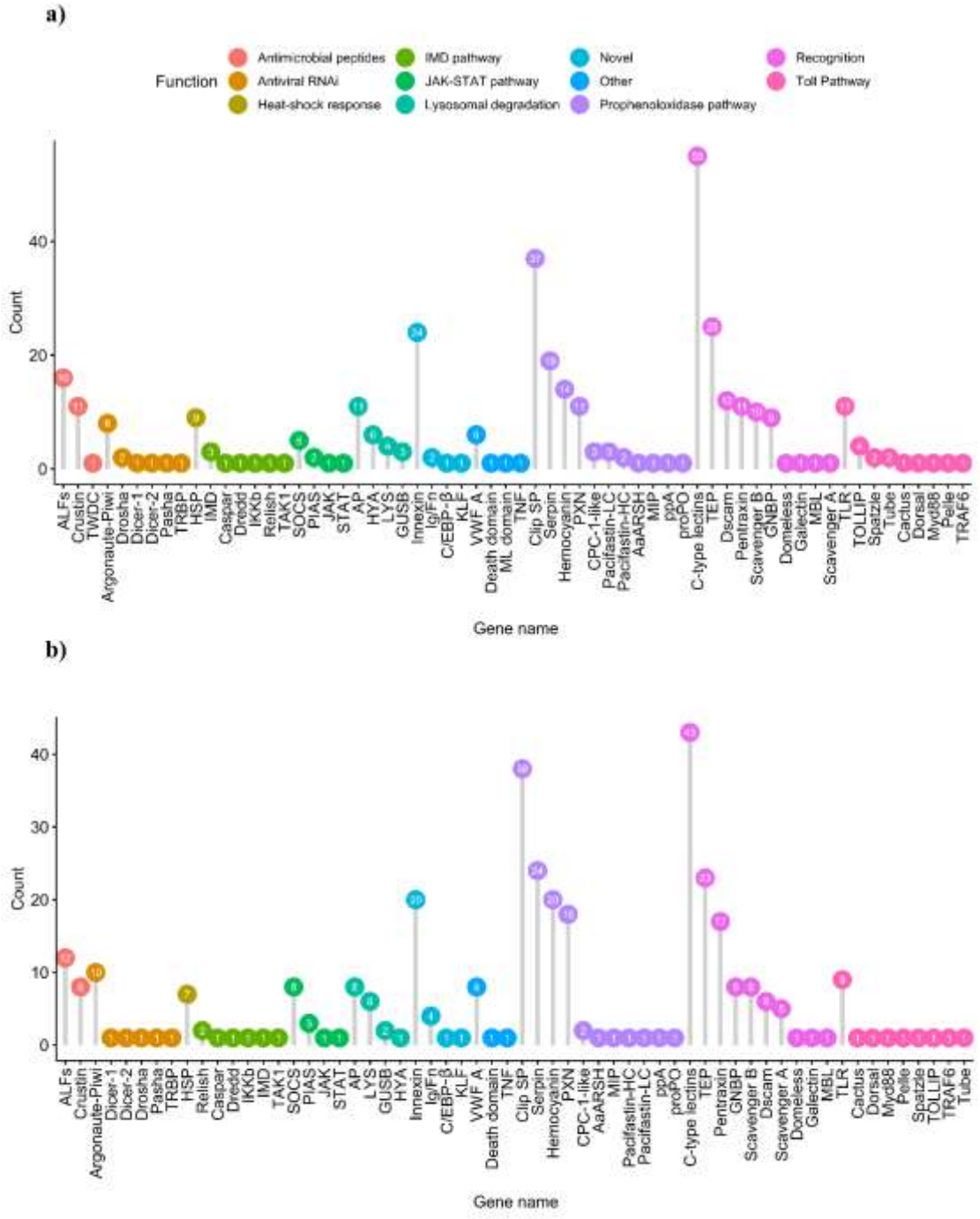


Figure 1. Genes involved in the representative immune related pathways, identified thought the similarity-based approach in (**a**) the noble crayfish and (**b**) the marbled crayfish. For all genes abbreviations are available in the Table S7.

3.3.2.2. Differentially expressed genes

In the differential gene expression analysis, 35,300 genes for the noble crayfish and 52,491 genes for the marbled crayfish were analysed after removing the genes with low gene counts. In the noble crayfish, a total of 380 DEGs (202 up-regulated and 178 down-regulated) were detected in response to the challenge with *A. astaci* across all treatments (**Figure 2**, **Table S5**). The highest number of DEGs was observed in the Hap B challenged noble crayfish 3 days post-challenge, with 243 DEGs (141 up-regulated and 102 down-regulated) (**Figure 2**), with many involved in the immune response (**Figure 3**). The lowest amount of DEGs was observed in the Hap A challenged noble crayfish 3 days post-challenge, with only 14 DEGs (7 up-regulated and 7 down-regulated) (**Figure 2**). The DEGs relevant to the innate immunity, mainly connected to the proPO cascade, were observed in the Hap B challenged noble crayfish 3 days post-challenge (**Figure 2**). In the marbled crayfish a total of 232 DEGs (102 up-regulated and 130 down-regulated) were detected in the response to the challenge with *A. astaci* across all treatments (**Figure 2**, **Table S6**). The highest number of the DEGs related to the innate immunity was observed in the Hap A challenged marbled crayfish 3 days post-challenge, with 79 DEGs (47 up-regulated and 32 down-regulated), and the highest overall number of the DEGs in the marbled crayfish was observed 21 days post-challenge with the Hap B strain, with 107 DEGs (40 up-regulated and 67 down-regulated). The lowest amount of the DEGs was observed in the Hap B challenged marbled crayfish 3 days post-challenge, with only 15 DEGs, all down-regulated (**Figure 2**, **Table S6**).

Our results indicate the absence of a chronic or a long-term immune response to the challenge with *A. astaci* in both species. The lack of the clear immune response signal 21 days post-challenge suggests that the active immune response in the hepatopancreas had already come to a halt, or was capped below the detection level of the differential gene expression analysis at the time of the second sampling (Enriched gene sets in the response to the *A. astaci* challenge). However, a chronic response could be mediated, as previously suggested in other studies, by circulating haemocytes in the haemolymph of latently infected crayfish (Cerenius et al., 2008). Future studies focused on comparing the gene expression patterns among multiple immune-relevant tissues in the crayfish might clarify this aspect.

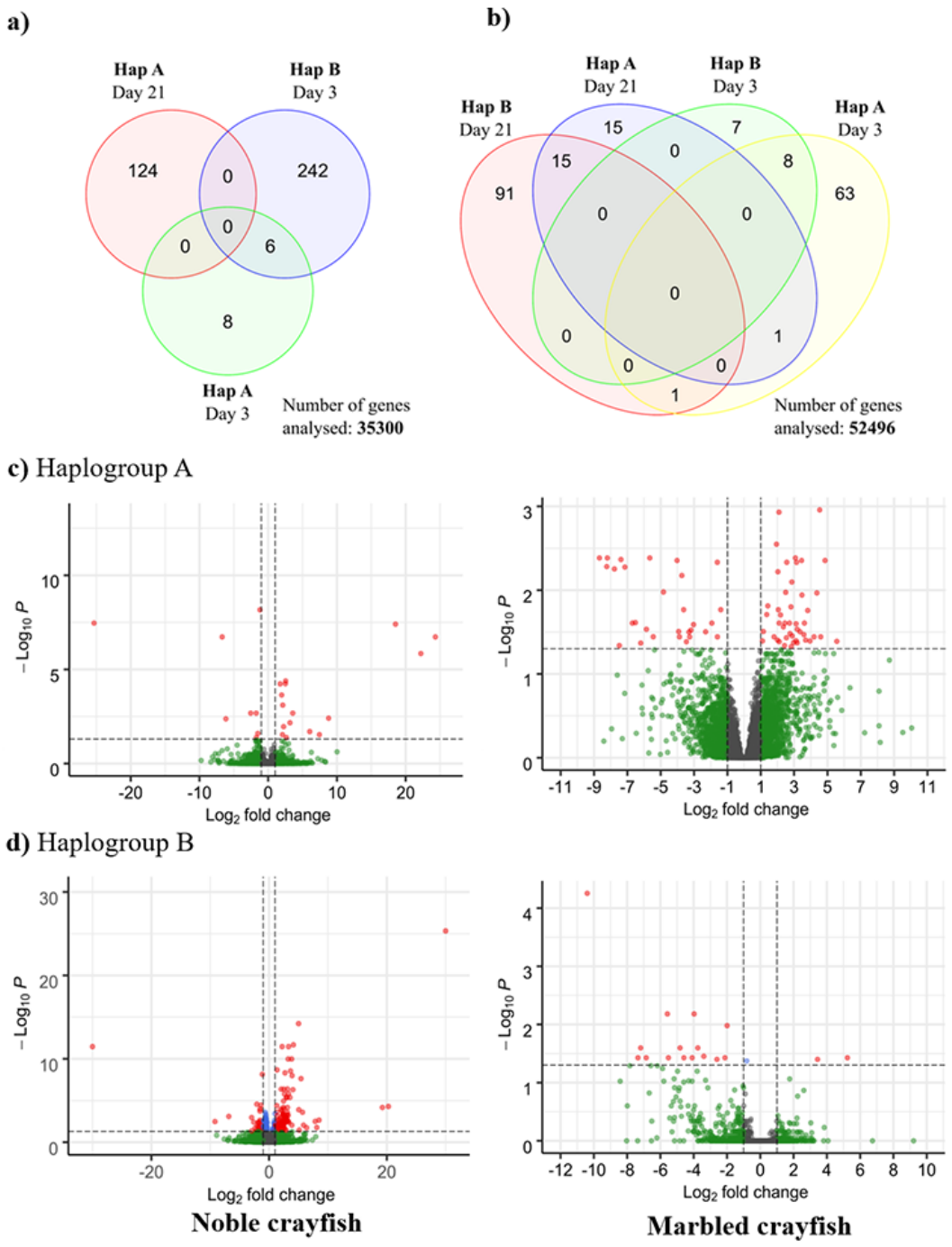


Figure 2. Results of the differential gene expression analysis. (**a**) Venn diagram representing DEGs for all treatments in the noble crayfish (**b**) Venn diagram representing differentially expressed DEGs for all treatments in the marbled crayfish. Volcano plots for the noble crayfish and marbled crayfish. (**c**) 3 days post-challenge with haplotype A, (**d**) 3 days post-challenge with haplotype B. The threshold values are represented as dashed lines (p-value = 0.05, Fold change = 2). Genes above fold change and p-value threshold are coloured red.

3.3.2.3. Enriched gene sets in the response to the *A. astaci* challenge

As a complementary approach to the differential gene expression analysis, we utilised the newly identified immune-related genes (Immune-related transcripts in the hepatopancreas, the mediator of the crayfish immune response to *A. astaci* challenge) to conduct a gene set enrichment analysis. This approach allowed us to detect moderate or minor changes in the gene expression data (Subramanian et al., 2005). For the noble crayfish, our results revealed the enrichment of AMP, proPO pathway and novel (encompassing novel genes identified in this study) gene sets in the Hap B challenged group (**Figure 4**) and recognition gene set in the Hap A challenged group 21 days post-challenge (**Figure S2**). The proPO pathway gene set was under-represented in the Hap A challenged noble crayfish 3 days post-challenge. In the marbled crayfish, AMP, proPO and recognition gene sets were enriched for the Hap B challenged group at both sampling points (**Figure S2**). Furthermore, in the Hap A challenged group, recognition and proPO gene sets were enriched (**Figure 4**). In the marbled crayfish, 21 days post-challenge with Hap A we detected no enriched gene sets. These results, in line with the differential gene expression analysis, suggest that proPO pathway, AMPs and recognition proteins, although not detected as differentially expressed, play a major role in the response to the *A. astaci* challenge. Their interplay and significance are discussed in the text further down.

3.3.3. Molecular mechanisms of the immune response to the *A. astaci* challenge

3.3.3.1. Activation of prophenoloxidase cascade

Although in both crayfish species the proPO pathway was activated, we detected a substantial difference in the immune response in the two species in the mobilisation of different effector groups and number. The activation of proPO cascade is the most explored humoral response among crustaceans (**Figure 4**) (Söderhäll & Cerenius, 1998; Cerenius & Söderhäll, 2012). Phenoloxidase (PO), synthesized in its zymogen/inactive form (proPO), is the central enzyme of the pathway. It is cleaved by its activating serine protease (ppA) into the catalytically active PO and the 20 kDA N-terminal fragment (ppA-proPO) with a strong agglutination and bacterial killing capacity (Jearaphunt et al., 2014). Activated PO is involved in the conversion of phenolic substances into the toxic quinone intermediates involved in the production of melanin, the terminal pathogen encapsulating agent of the proPO cascade (Cerenius et al., 2008).

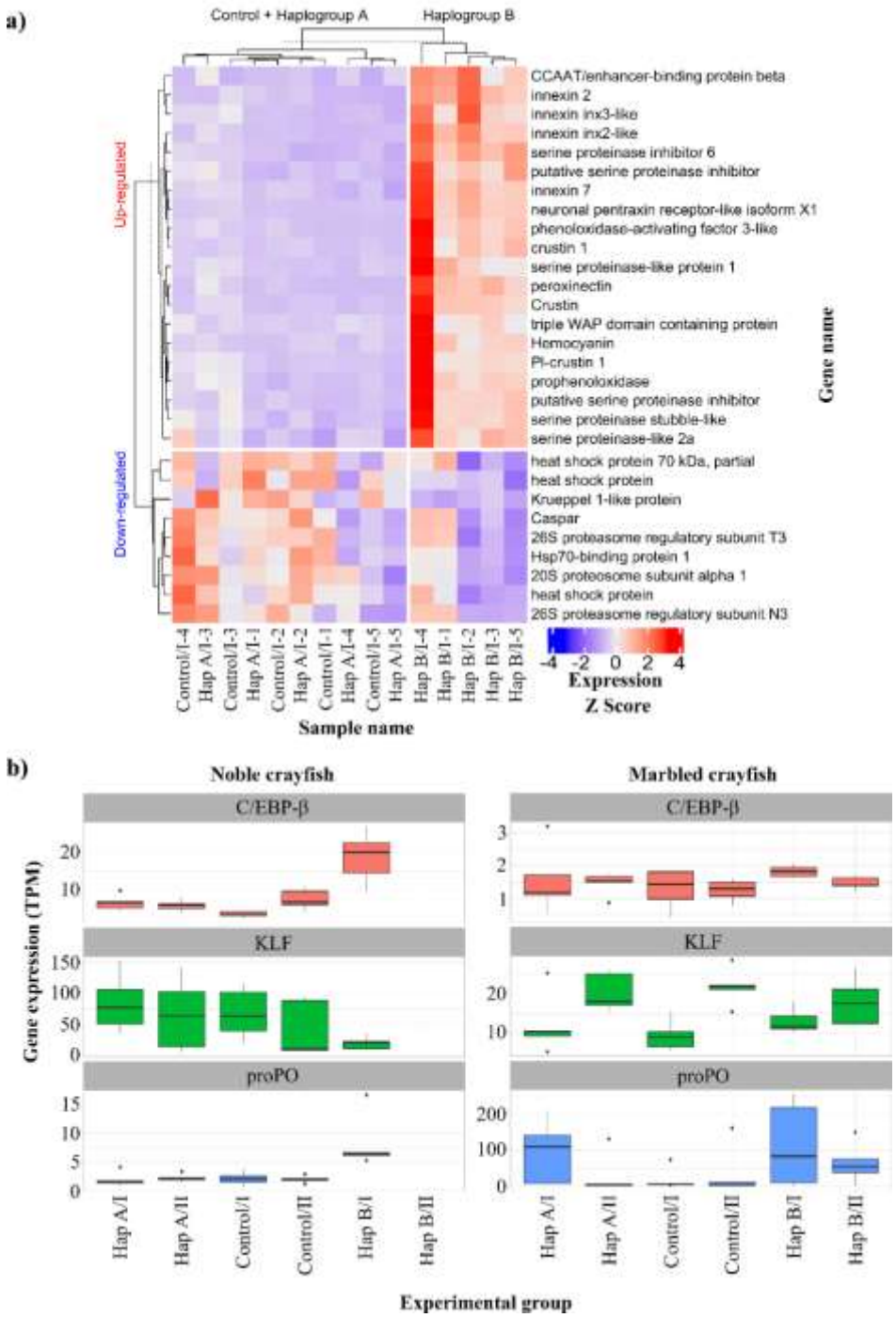
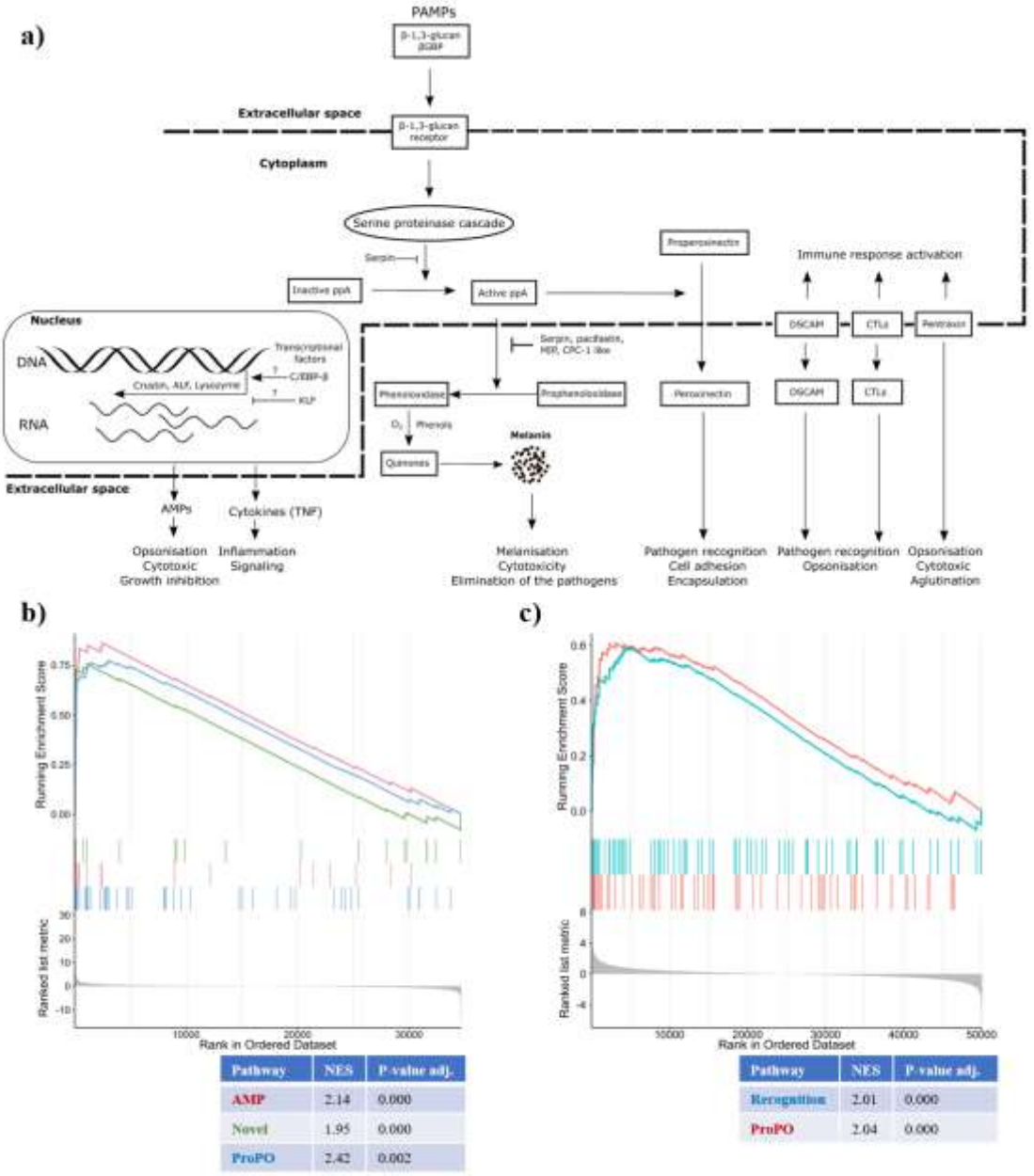


Figure 3. Heatmap of the immunity genes for each sample and treatment detected as differentially expressed in the noble crayfish (**a**) Raw counts were transformed to transcripts per million (TPM), followed by standardisation with Z-score scaling (where Z score is calculated as follows: $Z = -si-\mu/\sigma$ where -si is the gene expression for a sample in TPM, μ is mean of the expression for each gene in TPM and σ is standard deviation of the expression for each gene in TPM). Therefore, the colours in the heatmap reflect the relative expression levels between samples per each gene, with higher expression in red and lower expression in blue. Hap A, haplogroup A; Hap B, haplogroup B, I and II, first and second sampling point, respectively (3 days and 21 days post-challenge), 1–5, identifying number of the crayfish (**b**) gene expression of the prophenoloxidase (proPO), CCAAT/enhancer-binding protein beta (EBP), and Krueppel like protein (KLP) in the marbled crayfish and the noble crayfish challenged with *A. astaci*. Expression values are shown in TPM.



Pathway	NES	P value adj.
AMP	2.14	0.000
Novel	1.95	0.000
ProPO	2.42	0.002

Pathway	NES	P value adj.
Recognition	2.01	0.000
ProPO	2.04	0.000

Figure 4. Pathways involved in the freshwater crayfish immune response to *A. astaci* immune challenge, (**a**) Schematic representation of the crayfish immune response to *A. astaci* challenge (**b**) Results of the gene set enrichment analysis for the noble crayfish challenged with Hap B strain of *A. astaci* (Day 3), (**c**) results of the gene set enrichment analysis for the marbled crayfish challenged with Hap A strain of *A. astaci* (Day 3).

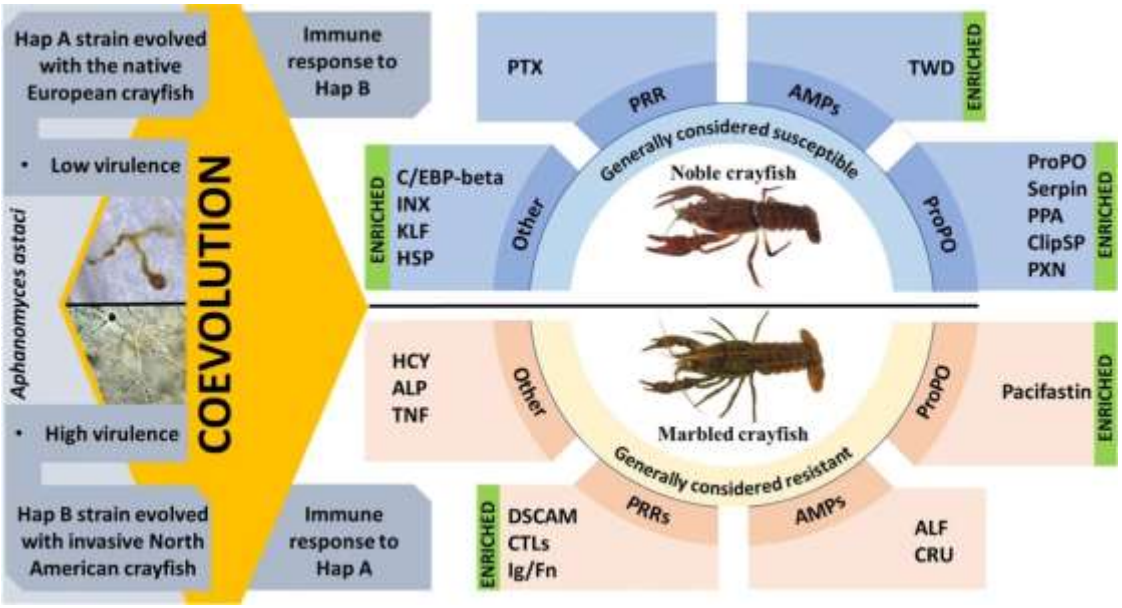


Figure 5. Graphical summary of the experimental results. The noble crayfish and the marbled crayfish were both exposed to two strains of the pathogen *A. astaci*, Hap B of high virulence and Hap A of low virulence. Both species showed immune response to *A. astaci*, although only for one strain. The immune system of the noble crayfish was activated in response to Hap B strain, while the immune system of the marbled crayfish was activated in response to Hap A strain. The utilised Hap A strain has coexisted with European noble crayfish for the past 70 years, and our results indicate that in that time frame it adapted to its new host. On the other hand, the Hap B strain, isolated from its original host in Lake Tahoe, shows a high adaptation to the invasive North American crayfish. Differentially expressed genes (DEGs) were divided in 4 groups: prophenoloxidase cascade related (ProPO), antimicrobial peptides (AMPs), pathogen recognition receptors (PRR) and Other. Enriched gene sets (based on the GSEA) were highlighted. Please refer to abbreviations for the full names of DEGs.

Alongside PO, ppA activates the formation of peroxinectin (PXN), involved in opsonisation, cell adhesion and encapsulation (Johansson et al., 1999; Lin et al., 2007). It was previously assumed, that only the mature haemocytes (granular and semigranular), which are responsible for the release of the proPO in the response to the pathogen stimulation (Söderhäll & Cerenius 1998; Cerenius & Söderhäll, 2004), are characterised by the onset of proPO expression (Cerenius & Söderhäll, 2018). Our results suggest that, alongside haemocytes, hepatopancreas is also involved in the production of the central proteins of this pathway (**Figure 3**).

In our study we observed an up-regulation of proPO, ppA and peroxinectin in the hepatopancreas of the Hap B challenged noble crayfish (**Figure 3a**, **Figure 5**), while in the marbled crayfish these genes were not differentially expressed in any treatment group. Nonetheless, our findings indicate that the expression of proPO in the hepatopancreas of both susceptible and resistant crayfish can be altered in response to the pathogen stimulation (**Figure 3**). In fact, while proPO was not differentially expressed in the marbled crayfish, the variances in the proPO expression levels (transcripts per million, TPM) were much higher in the marbled

crayfish challenged with Hap A of *A. astaci* 3 days post-challenge and Hap B of *A. astaci* 3- and 21- days post-challenge, compared to the noble crayfish challenged with Hap B of *A. astaci* (**Figure 3**). The results of the GSEA of both treatment groups of the marbled crayfish confirm the activation of the proPO pathway (**Figure 4**, **Figure S2**). Previous studies detected significant differences between the expression levels of the proPO in the haemocytes of both *A. astaci* -susceptible and -resistant crayfish (Cerenius et al., 2003). Specifically, it was observed that the expression of proPO is continuously elevated in the invasive resistant signal crayfish and the expression levels do not change in response to immune stimuli, while in the susceptible noble crayfish proPO is constitutively expressed at lower levels and its expression levels depend on the presence of the pathogen. The results of our study pointing to a modulation of the expression of proPO in response to the pathogen in the resistant marbled crayfish indicate that the basal expression levels and dynamic of activation of the proPO in the hepatopancreas and the haemocytes are likely different.

Our results indicate that in the Hap B challenged noble crayfish, several serine proteinases (Clip SPs) and serine proteinase inhibitors (serpins) were up-regulated in the response to the infection (**Figure 3**, **Table S5**), and pacifastin-HC gene was up-regulated in the Hap A challenged marbled crayfish 3 days post-challenge (**Figure 5**, **Table S6**). These genes are responsible for the spatial and the temporal control of the proPO cascade (**Figure 4**) (Cerenius et al., 2008). Excessive activation of the proPO pathway can cause damage to the host due to the production and the release of toxic quinones, therefore such inhibitory proteins are of utmost importance. In particular, the proteins involved in the proPO regulation are: pacifastin, a regulatory inhibitor of ppA (Liang et al., 1997); melanisation inhibition protein (MIP) (Söderhäll et al., 2009); caspase 1-like molecule (CPC-1-like), released concomitantly with the proPO and limits the proteolysis of proPO; and mannose-binding lectins (Jearaphunt et al., 2014). Serpins were reported to play a role in the proPO cascade inhibition (De et al., 2009). The recognition of the oomycete β-(1,3)-glucan activates the Clip SP cascade responsible for cleavage of the ppA (Cerenius & Söderhäll, 2004). The up-regulated serpins could also be involved in the inhibition of the oomycete proteinases (Bangyeekhun et al., 2001). Thus, serpins exhibit a dual role as an anti-oomycete agent and as the protectors against the proPO cascade overactivation (Rőszer, 2014; Zhao et al., 2014). This is further supported by the high number of genes encoding for the putative Clip SP (37 in the noble crayfish and 38 in the marbled crayfish) and their inhibitor serpins (19 in the noble crayfish and 24 in the marbled crayfish). The expansion of the Clip SP in Malacostraca (compared to the other Pancrustacea) was previously observed by Lai and Aboobaker (2017) with the highest number of the Clip SP (72) observed in the whiteleg shrimp. Co-expression of the proPO cascade effectors and of the proPO inhibitors in the hepatopancreas of the Hap B infected noble crayfish and of the Hap A infected marbled crayfish indicates that the proPO

cascade is highly involved in the response to the *A. astaci* challenge. Different elements of the proPO pathway seem to be activated in the marbled crayfish compared to the noble crayfish. Unfortunately, it is not possible to distinguish if this is due to real differences in the expression of the molecules involved in the proPO pathway of the two species, or if it is due to the high individual variance of the responses in the marbled crayfish. Although only one gene was annotated as the putative proPO, multiple hemocyanin (HCY) domain containing genes (14 in the noble crayfish and 20 in the marbled crayfish) were uncovered in both species (**Figure 1**). HCY is evolutionarily closely related, but distinct to the proPO (Burmester, 2002). It is believed that Crustacean HCYs can, to a certain extent, mimic the proPO functions (Cerenius & Söderhäll, 2004). Crustacean HCY is a large type-3 copper containing respiratory protein which forms hexameric structures responsible for oxygen transport (Decker et al., 2007). Alongside proPO, in the Hap B challenged noble crayfish, one of the HCY containing proteins was observed as up-regulated (**Figure 3**, **Table S5**). In the marbled crayfish challenged with the Hap A, a highly expressed HCY containing protein was also observed as up-regulated in the hepatopancreas 3 days post-challenge (**Figure 5**, **Table S6**). Unlike vertebrate hemoglobins, HCYs are cell-independent, and are solely suspended in the crayfish haemolymph (Decker et al., 2007). This means that the HCYs can be directly excreted from the hepatopancreas, where they are synthesised, to the crayfish haemolymph, without damage to the organism (Lee et al., 2004; Ward et al., 2010). On the other hand, proPO must be transported to the infection site and incorporated in the granules of semi-granular and granular haemocytes (blood cells) (Cerenius et al., 2003, 2012). Shortly after the immune challenge, a significant drop in the number of circulating haemocytes (condition termed haemocytopenia) is observed due to haemocyte mobilisation to the infection site (Ratcliffe et al., 1985; Smith, 2016). These haemocytes are mainly directly replaced during haematopoiesis from the hematopoietic tissues (Jiravanichpaisal et al., 2006). This usually occurs 12–48 hours after the initial challenge (Ratcliffe et al., 1985; Rowley, 2016). Therefore, during the period of circulating haemocyte depletion, both sensitive and resistant crayfish can rely on the components of the humoral innate immune response, such as antimicrobial peptides and HCYs, until the haemocyte replenishment. This is concordant with the observation by Decker et al. (2007) suggesting the innate immunity involvement of the high concentration of HCYs in the circulating haemolymph in tarantula (Paul et al., 1984). Finally, HCYs can be proteolytically processed, resulting in a release of AMPs, such as those belonging to the astacidin family (Choi & Lee, 2014).

3.3.3.2. Expression of pattern recognition receptors (PRRs)

We observed two up-regulated putative C-type lectins (CTLs) in the marbled crayfish, one in the *A. astaci* Hap A challenged group 3 days post-challenge and one in the *A. astaci* Hap B challenged group 21 days post-challenge (**Figure 5**, **Table S5**). Lectins are a diverse group of proteins capable of binding carbohydrate-binding domains with high specificity (Lis & Sharon, 1998). In crustaceans, lectin recognition leads to downstream activation of cellular and humoral responses such as agglutination (Jin et al., 2013), endocytosis (Shi et al., 2006), encapsulation and nodule formation (Ling & Xu, 2006), synthesis of AMPs (Vasta, 2009), antiviral activities (Zhao et al., 2009), and melanisation through the proPO cascade activation (Cerenius et al., 2010). We have identified 55 putative CTLs in the noble crayfish and 43 putative CTLs in the marbled crayfish (**Figure 1**). Among PRRs, CTLs have a major role in the innate immunity of freshwater crayfish, where they have also experienced a major increase in their diversity (Lai & Aboobaker, 2017).

Among the differentially expressed genes involved in pattern recognition we observed an up-regulated DSCAM 3 days post-challenge in the marbled crayfish challenged with *A. astaci* Hap A (**Table S6**). DSCAM is a member of the immunoglobulin (Ig) superfamily, with a similar structure in both mammalians and invertebrates. The DSCAM molecule consists of three main components, an extracellular region with several Ig and fibronectin type III domains, a transmembrane domain, and a cytoplasmic tail. Unlike its mammalian counterpart, invertebrate DSCAM exhibits hypervariability in the extracellular domains achieved through a mechanism of alternative splicing during mRNA maturation (Yamakawa, 1998; Ng et al., 2014). In total, we identified 12 putative DSCAM-encoding genes in the noble crayfish and 6 in the marbled crayfish (**Figure 1**). DSCAM molecules have been shown to be involved in the antiviral (Ng et al., 2019) and antibacterial response, mainly in the opsonisation (Cerenius & Söderhäll, 2012). It is worth noting that due to their hypervariable domain, DSCAMs are considered likely key molecules for the immunological memory in crustaceans (Low & Ching, 2020). Both CTLs and DSCAMs can exist in a membrane bound and secreted form (Chou et al., 2011; Pees et al., 2016). Therefore, CTLs and DSCAMs expressed in the hepatopancreas of crayfish can probably be excreted directly to the haemolymph upon the immune challenge, acting as a part of the humoral immune response mechanisms to the pathogen infection.

Alongside the DSCAM we observed another immunoglobulin/fibronectin (Ig/Fn) domain containing protein up-regulated 3 days post-challenge in the marbled crayfish challenged with *A. astaci* Hap A (**Table S6**). This protein shared 27% identity with the fruit fly (*Drosophila melanogaster*) protein amalgam (Ama, NCBI acc. No.: P15364.2). This amalgam-like protein was 510 amino acid (aa) long, with a molecular weight of 55.63 kDa. It contained 1–21 aa signal peptide domain, three Ig domains (67–158 aa, 166–254 aa, 257–345 aa), and a Fn domain (347–453 aa) with a cytokine receptor motive (439–443 aa). In total, we identified 2 Ig/Fn domain

containing proteins with this domain organisation in the noble crayfish and 4 in the marbled crayfish (**Figure 1**). The presence of the C-terminal Fn domain clearly distinguishes this protein form the fruit fly Ama (Liebl, 2003). Nonetheless, we can hypothesise that this protein could share the secreted nature of Ama, and its cell adhesion properties (Zeev-Ben-Mordehai et al., 2009), potentially having a role in opsonisation, and immune response mediation through its cytokine receptor motive located in the fibronectin domain.

Among the up-regulated DEGs in the Hap B challenged noble crayfish, we identified a pentraxin domain containing gene (**Table S5**, Pfam: PF00354). The protein product of this gene is 254 aa long (27.95 kDa), with a signal peptide (1–21 aa) on the N-terminus and only 55.5% identity with the neuronal pentraxin receptor-like isoform X2 from the whiteleg shrimp (XP_027224174.1, identified with Blastx). Like the most-well studied pentraxins (e.g. C-reactive protein (CRP) or serum-amyloid P component (SAP)), this pentraxin, due to its size, probably belongs to the group of short pentraxins (Du Clos, 2013). We identified 11 putative pentraxin genes in the noble crayfish and 17 in the marbled crayfish (**Figure 1**). Pentraxins (or pentaxins) represent a multifunctional and evolutionary conserved group of proteins, with a critical role in the humoral innate immune response (Mantovani et al., 2008). They can recognise a wide range of the pathogen associated molecular patterns, and serve as opsonin, cytotoxic effectors, agglutination promotors or as activators of the complement (Du Clos, 2013; Armstrong, 2015; Ma & Garret, 2018). Not much is known about the complex system of the complement in the freshwater crayfish and previously hypothesised pentraxin complement activation is most likely not mediated through the C3 component of the complement (Armstrong, 2015), as it is in vertebrates (Ma & Garret, 2018) since C3-like proteins have reportedly been lost in Pancrustacea (Lai & Aboobaker, 2017).

In endothermic animals the source of pentraxins is the liver (Pepys & Hirschfield, 2003) and in the horseshoe crab (*Limulus polyphemus*) and American lobster (*Homarus americanus*) these proteins are produced in hepatopancreas (Ng et al., 2004; Clark et al., 2013a). From there they are released to the haemolymph. Pentraxins are classical acute phase proteins. In humans, the CRP can be utilised as a marker of bacterial and fungal diseases progression (Armstrong, 2015). To best of our knowledge, this is the first time a pentraxin-domain containing protein is identified in the crayfish in the response to *A. astaci* infection. This acute protein could be a good indicator of the disease progression. The involvement of the recognition proteins in the response to the *A. astaci* challenge was further supported by the results of the GSEA (**Figure4**, **Figure S2**). Application of the acute phase proteins as the markers of the immune status has been previously proposed for the American lobster, where pentraxin-domain containing protein has been recognised as an important component of the immune response to the pathogen challenge (Clark et al., 2013a, 2013b, 2016).

3.3.3.3. Antimicrobial peptides: effectors of the innate immune response

In the noble crayfish challenged with the Hap B strain we identified three up-regulated crustins (**Table S5**). Among them, of particular interest was the DE triple whey acidic protein (TWP) domain containing crustin, identified in the noble crayfish but with no ortholog in the marbled crayfish. In the noble crayfish we identified 11 and in the marbled crayfish eight putative crustins (**Figure 1**). The crustins are part of the cationic antimicrobial peptides AMPs and have three main components: the signal peptide, the multi domain region at the N-terminus and the whey acidic protein (WAP) domain at the C-terminus. They are classified in five groups based on their structure (type I-V) (Tassanakajon et al., 2015). The crustins are mainly expressed in the crayfish haemocytes, where they can be rapidly secreted directly into the haemolymph during the immune challenge (Sricharoen et al., 2005; Smith & Dyrynda, 2015). Some crustins can also exhibit antiprotease activity, possibly inhibiting the proteases secreted by *A. astaci*, limiting the pathogen growth (Jia et al., 2008). Recently, a novel TWD containing crustin was described in the red swamp crayfish (*Procambarus clarkii*), showing antibacterial activity (Zhang et al., 2019). In the marbled crayfish challenged with the Hap B strain we identified one up-regulated crustin 21 days post-challenge (**Table S6**). The crustins may play an important role in the anti-oomycete response of the freshwater crayfish and require a closer attention in future. The TWD containing crustins might be of special interest, due to their presumed tissue wide expression profiles and participation in the host immunity throughout the whole body (Zhang et al., 2019).

Up-regulated antilipopolysaccharide factor (ALF) was identified in the Hap A challenged marbled crayfish 3 days post-challenge (**Table S6**), while DE ALFs were not detected in the noble crayfish. This suggests that ALF up-regulation might play a vital role in the resistance of the marbled crayfish towards the *A. astaci* challenge, possibly by binding to the oomycete β-1-3-glucan, hence increasing the host antimicrobial defences acting as an opsonin for the haemocytes (Tassanakajon et al., 2015). In the noble crayfish, we identified 16 putative ALFs, and in the marbled crayfish we identified 12 putative ALFs (**Figure 1**). The ALFs are small proteins with the hydrophobic N-terminal region forming, three β-sheets and three α-helices (Lai & Aboobaker, 2017), Pfam: DUF3254. They have been observed in wide range of crustaceans (Becking et al., 2020), and they are expressed in a wide range of tissues, showing growth inhibiting activity towards bacterial and fungal microorganisms, as well as opsonic activities (De La Vega et al., 2008; Sun et al., 2011). Like the crustins, they possess a signal peptide domain and can be excreted (Tassanakajon et al., 2015). The AMPs were enriched in both the noble crayfish and the marbled crayfish challenged with Hap B strain (**Figure 4**, **Figure S2**).

3.3.3.4. Innexins: involvement of the gap junction proteins in the crayfish innate immunity

Among the differentially expressed genes, we detected four up-regulated innexins (INXs) 3 days post-challenge in the Hap B challenged noble crayfish (**Table S5**). These proteins represent the subunits that compose the hemichannel of the gap junctions, and they are analogous to the vertebrate connexin subunits (Bauer et al., 2004). The gap junctions represent the sites of the direct cell to cell communications. This interaction is achieved through the formation of the plasma membrane spanning channels, with each cell contributing to one half of the channel. The mechanisms of gap-junction communications and their repercussions have long been studied in vertebrates, where they are widely distributed across tissues (Sáez et al., 2000; Neijseen et al., 2007). Although these channels were first observed in the 1950s in the noble crayfish cells, their involvement in the immunity of the freshwater crayfish species is not well understood (Furshpan & Potter, 1959). We identified 23 putative INXs in the noble crayfish and 20 putative INXs in the marbled crayfish (**Figure 1**). For comparison, 8 INXs were identified in the fruit fly, 25 in the roundworm (*Cenorabditis elegans*), 21 in the mediterranean medicinal leech (*Hirudo verbana*) and 6 in the Jonah crab (*Cancer borealis*) (Adams, 2000; Starich et al., 2001; Kandarian et al., 2012; Shruti et al., 2014). In the mud crab (*Scylla paramamosin*), Spinx2 expression was up-regulated in the hepatopancreas, the gills and the haemocytes after challenge with bacteria, and was highly expressed in the haemocytes under normal conditions (Wang et al., 2015). Although the roles of INXs in invertebrates are largely unknown, based on the current knowledge of the functions of gap junction proteins in other species, we can argue that they could be involved in the antigen processing, as well as in the metabolic and the signalling molecules trafficking (Güiza et al., 2018). This further establishes the role of the hepatopancreas as a key organ in the distribution of the immune molecules to the crayfish haemolymph (Rőszer, 2014). Further studies are needed to elucidate the roles of INXs in invertebrate immunity.

3.3.3.5. Transcriptional factors as novel components in the response to *A. astaci* challenge

Changes in the gene expression levels are controlled through a set of specific transcription factors that interact with the gene regulatory sequences, present in the promoter and enhancer regions. In the Hap B challenged noble crayfish we identified both up-regulated and down-regulated genes 3 days post-challenge, which serve as transcription factors and bona fide play vital roles in the immune response the pathogen (**Table S5**). One of these genes is a master gene expression regulator belonging to the CCAAT/enhancer-binding protein (C/EBP) family (Ramji & Foka., 2002). This family is involved in the regulation of cellular growth, differentiation and

death, as well as in haematopoiesis, and immune and inflammatory processes during various diseases (Ramji & Foka., 2002; Wang et al., 2019). The expression of the putative CCAAT/enhancer-binding protein beta (C/EBP-β), present in single copy in both the noble crayfish and the marbled crayfish, was up-regulated in the noble crayfish challenged with Hap B, while the expression levels in the marbled crayfish remained unchanged (**Figure 1**, **Figure 3**). It has been shown that the expression of the ALFm3 (member of antilipopolysaccharide factor family) in the giant tiger prawn is under the control of C/EBP-β (Kamsaeng et al., 2017). Previously it has also been shown that C/EBP-β binding sites are present in the crustin Pm7 (Amparyup et al., 2008). The interaction of the C/EBP-β and NF-κB, key transcriptional factor in Toll and IMD pathways was reported during the promotion of the inflammatory mediator's gene expression (Tsukada et al., 2011). In mice, C/EBP-β is responsible for the control of tumor necrosis factor alpha (TNFα), SAP, complement C3 component expression (Ramji & Foka., 2002). This could suggest that the putative C/EBP-β up-regulation is crucial for the acute phase of the *A. astaci* infection in the noble crayfish.

Furthermore, we detected a down-regulation of putative Krüppel 1-like factor protein (KLF1), a member of the Krüppel-like factor (KLF) family, in the noble crayfish challenged with *A. astaci* Hap B (**Table S5**, **Figure 3**). Members of KLF family are transcription factors involved in a variety of metabolic pathways and in the energetic homeostasis of various tissues (Pollak et al., 2018). KLF1 belongs to a group of KLFs which function primarily as transcriptional activators, although interaction with the transcriptional repressors has also been reported (Pollak et al., 2018). It is present in single copy in both the noble crayfish and the marbled crayfish (**Figure 1**). In the humans, KLF4 is heavily implicated in the regulation of the anti-fungal response to *Aspergillus fumigatus* and *Candida albicans* and was identified as the only transcriptional factor down-regulated during the immune challenge (Czakai et al., 2016). It has been shown that in whiteleg shrimp (*Litopenaeus vannamei*), the host LvKLF is important for the replication and gene expression of the viral pathogen (Huang et al., 2014; Liu et al., 2015). In the giant river prawn (*Macrobrachium rosenbergii*), it has been shown that the MrKLF is an important regulator of expression of four antimicrobial peptides, namely Crustin (Crus) 2, Crus8, ALF1, and ALF3 (Huang et al., 2019). Knowledge on the expression and the regulation of invertebrates KLF is lacking, therefore conclusive interpretations for the function of the putative KLF1 require further research efforts. Based on the change in the KLF1 expression levels in the noble crayfish, we might speculate that KLF1 repression is important for the activation of the immune response genes in this species. In the marbled crayfish KLF1 expression levels are unchanged during *A. astaci* challenge (**Figure 3**).

Together with the KLF1 we also detected down-regulation of the Caspar, a transcriptional suppressor homologous to the Fas-associating factor 1, in the noble crayfish challenged with *A.*

astaci Hap B (**Table S5**, **Figure 3**). This transcriptional factor has been shown to play a critical role in the fruit fly, negatively affecting its antibacterial resistance through inhibition of the IMD pathway (Kim et al., 2006). In both species the Caspar was detected in a single copy (**Figure 1**).

3.3.3.6. Other DEGs in the response to *A. astaci* challenge

Among the up-regulated DEGs in the marbled crayfish we observed several other immune related genes, such as the Tumour necrosis factor (TNF) domain-containing protein (Panther entry: PTHR15151; protein Eiger; putative cytokine) and the lysosomal enzyme putative alkaline phosphatase (AP) (**Table S6**). The cytokines, class of molecules to which TNFs belong, are heavily involved in the mediation of the immune and the inflammatory responses (Balkwill & Cytokines, 2001). They are also known activators of the extracellular trap release (ETosis), a microbicidal mechanism (Guimarães-Costa et al., 2012). TNF is also a downstream target of the above mentioned KLFs (Czakai et al., 2016). Moreover, in the fruit fly, the TNF homolog Eiger is responsible for the release of the proPO in the crystal cells (Bidla et al., 2007). The TNF is also an activator of the C/EBPβ expression and DNA binding activity (Wang et al., 2019). The implication of this gene in the regulation of anti-oomycete responses remains to be experimentally proven in future studies. Alkaline phosphatase, β-glucuronidase, lysozyme, esterases and proteases have been recognised as some of the main lysosomal enzymes in the invertebrates (Smith, 2016). Lysosomal activity has been implicated in the mechanism of antigen processing in the hepatopancreas epithelial cells and their subsequent release into the haemolymph in the giant tiger prawn (Alday-Sanz et al., 2004; Kulkarni et al., 2013; Rőszer, 2014). This observation might further establish the role of hepatopancreas in building the immune tolerance to the *A. astaci* challenge.

Interestingly, we uncovered 4 members of the heat-shock protein (HSP) family (HSP70-like, HSP-like-1, HSP-like2 and HSPBP 1) together with proteasome components (20S proteosome subunit alpha 1, 26S proteasome regulatory subunit N3 and 26S proteasome regulatory subunit T3), as down-regulated 3 days post challenge with Hap B strain in the acutely infected noble crayfish (**Table S5**, **Figure 3**). Establishing a correct protein conformation is important for the protein activity. Failure to do so could be due to a lack of molecular chaperons, such as members of the HSP family (Vabulas et al., 2010). Moreover, down-regulation of the ubiquitin mediated proteolysis proteasome genes might have led to the misfolded protein aggregation. It has been shown that HSP 70 is up-regulated in the anti-viral response to the White spot syndrome virus (WSSV) in the giant tiger prawn (Xi et al., 2009) and the red swamp crayfish (Zeng & Li, 2009). In the fruit fly, it has been shown that the HSP 27 has an antiapoptotic activity, inhibiting the

TNF-mediated cell death (Arya et al., 2007). This might suggest that during the *A. astaci* challenge, in the acutely infected noble crayfish, a tissue wide apoptosis is in progress.

3.3.4. Coevolutionary aspects of the host immune response to the pathogen challenge

Our experimental setup, consisting of the noble crayfish and the marbled crayfish challenged with *A. astaci* strains of different origin and virulence, allowed us to make inferences on coevolutionary aspects of the host immune response to the pathogen challenge (**Figure 5**). The utilized Hap B strain, characterised by high virulence, was isolated from a latently infected American invasive signal crayfish (*Pacifastacus leniusculus*) host from lake Tahoe (USA). The utilised Hap A strain, characterised by low virulence, was isolated from a repeatedly challenged, latently infected noble crayfish host population, and could have been present in this population for at least 70 years (Jussila et al., submitted manuscript). Consequently, both strains should represent extremes in the mosaic landscape of *A. astaci* strains present in Europe. The results of the infection experiment described in Francesconi et al. (2021) showed that the noble crayfish challenged with *A. astaci* Hap B have the highest amount of the pathogen DNA in their tissues, indicating that the pathogen successfully overcame the immune defences of the host. This corresponds to the high number of immune related DEGs observed in this experimental group. Furthermore, it was observed in other experiments (our unpublished experimental results) that all the noble crayfish infected with this specific Hap B strain died within 2 weeks after challenged with the parasite. On the other hand, the Hap A challenged noble crayfish contained the pathogen, without the apparent mobilisation of immune response in the hepatopancreas and were asymptomatic 45 days post-challenge (Francesconi et al., 2021). In the marbled crayfish, the Hap A challenged group showed the highest number of the immune related DEGs, while the Hap B challenged group showed no clear immune response. In fact, in the Hap B challenged marbled crayfish we observed no immune response activation based on the differential gene expression analysis, although enrichment of the proPO, AMPs and recognition gene sets suggesed a low-level mobilization of these pathways (**Figure 4**, **Figure 5**, **Figure S2**). Interestingly, the highest amount of pathogen DNA in the marbled crayfish was detected in the Hap B challenged group (Francesconi et al., 2021). This result indicates that the virulence of *A. astaci* and its ability to colonise the host's tissues are not the only factors influencing the strength of the host's immune response. In fact, one possible explanation could revolve around processes of coevolution between the crayfish and a specific strain of *A. astaci*.

It has been shown in several instances that invertebrates, although lacking an adaptive immune system, can build an immune memory, mounting an immune response of different magnitude after subsequent exposures to the same pathogen (Melillo et al., 2018; Low & Chong 2020).

Such a response could be of tolerance with a lowered immune response to known stimuli, or of potentiation with a higher immune response upon re-encounter of the same pathogen (Melillo et al., 2018). Furthermore, transgenerational immune priming, in which the immune memory is transferred to the next generations by parents exposed to the pathogen, has been observed in insects (Barribeau et al., 2016; Vilcinskas et al., 2016) and in the brine shrimp (*Artemia franciscana*) (Norouzitallab et al., 2016). While the specific mechanisms are not completely understood and are likely to be different depending on the host and the parasite, transgenerational immune priming might be the basis of the long-debated host-pathogen coevolution between North American crayfish species and *A. astaci* (Unestam 1969; Jussila et al., 2014).

It is accepted that coevolution is a dynamic and ongoing process, in which the rapid adaptation of the host to the pathogen (and vice versa) can occur over short time frames, even a few decades (Thompson, 2001). The Hap A strain was isolated from the latently infected noble crayfish in Lake Venesjärvi, Finland. The noble crayfish population in the lake faced at least 3 mass mortalities in the past 50 years until the year 2000. In 2013, the population was identified as carrier of *A. astaci* (Jussila et al., submitted manuscript). The results of our study suggest that, probably in the span of a minimum of 50 years, the Hap A strain used in this study adapted to its naïve native European host, the noble crayfish, presumably through modification of its pathogenic epitopes. This has resulted in the overall lowered virulence of the pathogen. More in general, Hap A contains the first *A. astaci* strains that arrived in Europe likely in 1859s (Alderman 1996). Therefore, it is likely that the prolonged coexistence with other European crayfish species might be leading other strains belonging to this haplogroup through the same adaptation process of the strain used in the experiment.

The noble crayfish utilised in this study come from the population inhabiting Lake Rytky. This population went through a crayfish plague epizootic in the 1980s (Jussila et al., 2020). Since then, it has recovered and there haven't been further detections of *A. astaci* presence (Jussila et al., 2020). The apparent non-activation of the immune system in the noble crayfish infected with Hap A could represent an instance of immune tolerance, in case the *A. astaci* strain that infected the population of Lake Rytky belonged to Hap A. Unfortunately, the haplogroup of that *A. astaci* strain is unknown. Therefore, it is not possible to draw conclusions on how a possible coevolution might have shaped the immune response of the crayfish from Lake Rytky to the *A. astaci* strains tested in the experiment. As Hap A of *A. astaci* adapted to the noble crayfish, the new epitopes presented by this *A. astaci* strain led to the higher expression of the diverse PRR genes in the marbled crayfish, responsible for the recognition of the pathogen and for boosting its immune response capability.

The origin of the marbled crayfish can be traced back to a recent triploidisation event occurred in *Procambarus fallax* from Florida (Gutekunst et al., 2018; 2021). To date, there are no data on the presence of *A. astaci* in Florida. However, considering the widespread distribution of *A. astaci* in the eastern USA (Martín-Torrijos et al., 2021) and the elevated resistance of the marbled crayfish to the pathogen (Francesconi et al., 2021), it is likely that *P. fallax* coevolved with some strains of *A. astaci*. The developed resistance to *A. astaci* was then inherited by the marbled crayfish. As *A. astaci* haplogroup B is only distributed in the western part of the USA, it is very unlikely that either of the marbled crayfish or the *P. fallax* encountered strains belonging to Hap B (Martín-Torrijos et al., 2021). Yet, the remarkable resistance of the marbled crayfish to the Hap B strain tested in the infection experiment indicates that the presumed coevolution of the *P. fallax* with its native *A. astaci* strain allowed the development of a broad resistance to different strains. Furthermore, in the survey of the distribution of *A. astaci* in the USA (Martín-Torrijos et al., 2021), it has been observed that different strains of the pathogen can coexist in the same population and even in the same individual. The elevated diversity of *A. astaci* in its native range and its widespread distribution would create favourable conditions for the selection of the crayfish species with a broad resistance. This is further supported by the lack of *A. astaci* epizootics in North America, even after internal translocation of the freshwater crayfish outside their natural range (Martín-Torrijos et al., 2021). Interestingly, the eastern USA is rich in strains belonging to the haplogroup A (Martín-Torrijos et al., 2021). If *P. fallax* has been subjected to multiple encounters with strains belonging to this haplogroup, this would further validate our results, indicating that the Hap A strain from Lake Venesjärvi went through quick evolutionary changes to adapt to its new noble crayfish hosts, while become less recognisable for the marbled crayfish.

It has been argued that the harm to the native European crayfish stocks by *A. astaci* would have been much more contained, if the presence of *A. astaci* in Europe resulted only from the first accidental introduction around 1850 (Jussila et al., 2021). The first mass mortalities would have led to local extinction of the crayfish populations, limiting the spread of the crayfish plague, and potentially causing the disappearance of the pathogen (Jussila et al., 2021). Unfortunately, the subsequent intentional introductions of different species of North American crayfish, and with them new haplogroups of *A. astaci*, led to an uncontrollable spread of several pathogen strains, which are now firmly established in Europe (Jussila et al., 2021). While we can conclude that since its introduction into Europe the Hap A strain used in this study went through significant evolutionary changes, the available markers cannot differentiate between this strain and other strains belonging to Hap A, whether present in Europe nor in Northern America (Martín-Torrijos et al., 2021). It is increasingly evident that while the genetic markers used until now (RAPD, mtDNA and microsatellites) allow a first general discrimination of the intraspecific diversity of

A. astaci (Huang et al., 1994; Grandjean et al., 2014; Makkonen et al., 2018), they are not reliable predictors of the virulence of the strains and of the strains' potential impact on native European crayfish and thus on freshwater ecosystems. The conservation efforts of native European crayfish would greatly benefit from a genomic approach to analyse the genome-wide intraspecific diversity of *A. astaci*. Such an approach would allow a much finer discrimination between strains, integrating information regarding virulence of the pathogen and its consequences on the freshwater ecosystem. Ultimately, this would lead to better informed and finely tuned conservation actions.

3.3.5. Study limitations

This study provides a deep insight into the innate immune response following an *A. astaci* challenge in the noble crayfish and the marbled crayfish. Transcriptomic data allowed us to explore the gene expression landscape and to identify key genes in the crayfish immunity. However, information about genomic locations and gene surroundings, which are highly influential on the gene expression profiles, are still not available. Consequently, generating first high-quality genome assemblies for the freshwater crayfish represents a priority in the field of crayfish immunity, and would allow for the future comprehensive epigenomic studies. Unfortunately, until now this has proven to be a challenging task, because freshwater crayfish genomes are often large in size and have a high proportion of repetitive DNA sequences (Gutekunst et al., 2018; Tan et al., 2020; Boštjančić et al., 2021). Furthermore, while in Decapods the role of the hepatopancreas in the immune response against pathogens has already been demonstrated, it has to be considered that the observed expression profile might be influenced by the infiltrating haemocytes (Jia et al., 2008; Rowley, 2016). In the future, this issue could be resolved by investigating additional tissues and by applying a higher resolution single cell RNA sequencing, capable of differentiating different cell populations within a tissue (Koiwai et al., 2021). Finally, the gene expression analysis in the marbled crayfish was conducted after removal of the batch effect related to the reproducing crayfish, and this could have biased our results. It has already been shown that immune related genes are over-expressed in the reproducing insects (Schwenke et al., 2017). If, similarly, reproduction in crayfish involves an up-regulation of immune related genes, the removal of the batch effect might have also removed relevant DEGs in the marbled crayfish groups. In general, differences in the gene expression caused by different sex, size, age, molt stage and the reproductive status of the experimental animals should be reduced to a minimum by selecting for the experiment animals belonging to the same cohort. This way the possible biases introduced by the removal of batch effects would be avoided.

3.4. Conclusions

Our results indicate that coevolution of the crayfish and a specific strain of *A. astaci* plays a critical role in determining the strength of the host immune response to the pathogen challenge. The lowered virulence of the Hap A strain used in this study and lowered immune response to this strain in the noble crayfish suggest that coevolution between *A. astaci* and the noble crayfish can rather rapidly occur in nature. This host-pathogen co-adaptation raise hope for the future survival of native European crayfish. Nonetheless, repeated introductions of novel *A. astaci* strains represent an overwhelming pressure for the native European crayfish populations, as is evident from the acute response of the noble crayfish to the Hap B strain. Simultaneously, it seems that the ability of the invasive marbled crayfish to mount an adequate immune response to different *A. astaci* strains is much higher, probably due to its North American origin and possible interactions of its closest relative *P. fallax* with multiple *A. astaci* strains. In the light of these results, it is now evident that future research efforts should be aimed towards elucidating the key factors in this active adaptation between the pathogen and the host. Therefore, the identified genes and pathways involved in the immune response to the pathogen *A. astaci* represent a milestone in the conservation and aquaculture efforts for the native European crayfish species. Although our understanding of the freshwater crayfish innate immune response is still limited, it is becoming clearer that multiple organs and a variety of molecular pathways play important roles. Here, we showcased the importance of the hepatopancreas as a highly relevant immune system organ in the response to the *A. astaci* challenge, for both the native noble crayfish and the invasive marbled crayfish. In the immune response of both crayfish species the activation of the proPO pathway was observed. Still, we detected a substantial difference in the immune response in the two species in the mobilisation of different groups and number of effectors. Therefore, it is crucial that future studies are not limited to the analysis of immune response in the haemolymph and to the proPO pathway, but rather consider the multifactorial nature of the innate immune response. Lastly, results provide a basis for the development of the screening assays that will allow detection of the resistant crayfish populations, a promising tool for conservation and management programs.

Abbreviations

DSCAM: Down syndrome cell adhesion molecule; proPO: Prophenoloxidase; PAMP: Pathogen associated molecular pattern; PRP: Pattern recognition proteins; TLR: Toll-like receptor; Hap B: Haplogroup B; Hap A: Haplogroup A; DE: Differentially expressed; DEG: Differentially expressed gene; AMP: Antimicrobial peptides; GNBPs: β-(1,3)-glucan receptors; ppA: Serine

protease; PXN: Peroxinectin; PO: Phenoloxidase; INX: Innexin; HCY: Hemocyanin; TPM: Transcripts per million; Clip SP: Serine proteinase; Serpin: Serine proteinase inhibitor; MIP: Melanisation inhibition protein; CPC-1-like: Caspase 1-like molecule; CTL: C-type lectin; Ig: Immunoglobulin; Fn: Fibronectin; aa: Amino acid; PTX: Pentraxin; CRP: C-reactive protein; SAP: Serum-amyloid P component; TWD: Triple whey acidic protein; WAP: Whey acidic protein; ALFs: Antilipopolysaccharide factor; C/EBP: CCAAT/enhancer-binding protein; C/EBP-β: CCAAT/enhancer-binding protein beta; KLF1: Krüppel 1-like factor protein; KLF: Krüppel-like factor; s: Crustin; TNFα: Tumor necrosis factor alpha; TNF: Tumour necrosis factor; AP: Alkaline phosphatase; ETosis: Extracellular trap release; HSP: Heat-shock .

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Chapter 4

High variation of virulence in *Aphanomyces astaci* strains lacks association with pathogenic traits and mtDNA haplogroups

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Abstract

Introduced into Europe from North America 150 years ago alongside its native crayfish hosts, the invasive pathogen *Aphanomyces astaci* is considered one of the main causes of European crayfish population decline. For the past two centuries, this oomycetes pathogen has been extensively studied, with the more recent efforts focused on containing and monitoring its spread across the continent. However, after the recent introduction of new strains, the newly-discovered diversity of *A. astaci* in North America and several years of coevolution with its European host, a new assessment of the traits linked to the pathogen's virulence is much needed. To fill this gap, we investigated the presence of phenotypic patterns (i.e., *in vitro* growth and sporulation rates) possibly associated with the pathogen's virulence (i.e., induced mortality in crayfish) in a collection of 14 *A. astaci* strains isolated both in North America and in Europe. The results highlighted a high variability in virulence, growth rate and motile spore production among the different strains, while the total-sporulation rate was more similar across strains. Surprisingly, growth and sporulation rates were not significantly correlated with virulence. Furthermore, none of the analysed parameters, including virulence, was significantly different among the major *A. astaci* haplogroups. These results indicate that each strain is defined by a characteristic combination of pathogenic features, specifically assembled for the environment and host faced by each strain. Thus, canonical mitochondrial markers, often used to infer the pathogen's virulence, are not accurate tools to deduce the phenotype of *A. astaci* strains. As the diversity of *A. astaci* strains in Europe is bound to increase due to translocations of new carrier crayfish species from North America, there is an urgent need to deepen our understanding of *A. astaci*'s virulence variability and its ability to adapt to new hosts and environments.

4.1. Introduction

Invasive species have been recognised as one of the main threats to global biodiversity, with freshwater ecosystems among the most affected by their introduction (Dudgeon et al., 2006; Polce et al., 2023). Especially problematic are invasive pathogens, which can lead to the emergence of diseases with devastating effects on native naive communities (Dudgeon et al., 2006; Chinchio et al., 2020). The oomycete *Aphanomyces astaci* Schikora 1906, causative agent of the crayfish plague, is considered one of the 100 worst invasive species worldwide (Lowe et al., 2004). The pathogen was introduced into Europe from North America for the first time in the mid-19th century (Alderman, 1996). In this first instance, the introduction was probably accidental and not associated with the simultaneous translocation of the original crayfish host (Alderman, 1996).

Afterwards, from the end of the 20th century, repeated translocations of North American crayfish species, recognised *A. astaci* carriers, for stocking or trading purposes have led to the presence of different *A. astaci* strains in Europe (Becking et al., 2022). Since its first introduction *A. astaci* has caused severe population declines and local extinctions of native crayfish populations across the continent (Alderman, 1996; Jussila et al., 2021a). Its pronounced impact on the distribution of native European crayfish, keystone species in freshwater ecosystems, had consequences far wider than the direct effect on the target crayfish species, with the potential to disrupt biodiversity and functioning of entire ecosystems (Reynolds et al., 2013; Jussila et al., 2021a). Additionally, *A. astaci* had severe effects on aquaculture, with crayfish exports drastically reducing and fishing stocks dropping by more than 90% in some areas of Europe (e.g., Germany, Spain and Scandinavia) (Jussila et al., 2021a; Becking et al., 2022).

A few factors can influence the outcome of an *A. astaci* infection as well as the success of a new habitat invasion: susceptibility level of the host, environmental factors, and pathogen's strain-specific virulence (e.g., Alderman et al., 1987; Makkonen et al., 2012; Viljamaa-Dirks et al., 2016). Significant efforts have been made to investigate the role played by the different resistance of the host species in the outcome of the infection. Since the first studies of the interaction between *A. astaci* and crayfish host, it has been observed that while native European crayfish species are widely susceptible to the pathogen, North American crayfish species are generally resistant (e.g., Unestam & Weiss, 1970; Martínez-Ríos et al., 2022) probably as a result of host-pathogen coevolution (Boštjančić et al., 2022), succumbing to the infection only when under multiple sources of stress (Aydin et al., 2014). Fewer efforts have been made to analyse the effect of the environment on *A. astaci* infections. For example, Diéguez-Uribeondo et al. (1995) revealed that some strains are better adapted to higher temperatures, likely because of the original climatic niche of the North American host species, indicating that differences in climate can affect the distribution and invasion success of *A. astaci*. Lastly, studies on the pathogen virulence have been mainly focusing on single isolated strains by directly assessing their virulence through infection experiments (e.g., Makkonen et al., 2012; Becking et al., 2015; Francesconi et al., 2021). The results of these experiments, together with observations in the wild, have led to the general assumption that the degree of virulence is associated with specific mitochondrial haplogroups (Makkonen et al., 2018). In particular, haplogroup B and D and E strains have been found to be highly virulent, while haplogroup A strains are characterised by variable but overall lower virulence (e.g., Makkonen et al., 2012; Becking et al., 2015; Francesconi et al., 2021; Martínez-Ríos et al., 2022). However, while infection experiments are important tools to assess the risk a strain poses to crayfish populations, they do not offer insights into the mechanisms behind the virulence variability across strains, as they lack a concurrent analysis of the life cycle elements that directly influence the virulence, i.e., transmission and growth.

The life cycle of *A. astaci* starts with the germination of the zoospores, i.e., infective units and transmission propagules of the pathogen, which leads to hyphal growth inside the host (Rezinciuc et al., 2015). After specific stimuli (e.g., lack of nutrients; Cerenius and Söderhäll, 1884), the hyphae start to grow outward from the cuticle of the crayfish and sporangia are formed, which release the primary cysts that develop into zoospores. At this point, the zoospores disperse into the environment and find new hosts (Rezinciuc et al., 2015). Both hyphal growth and production of zoospores are possible through the utilisation of the host resources, which leads to damage of the host's tissues and, therefore, determine the virulence of the pathogen (Frank, 1996; Pfennig, 2001). The classical theory on the evolution of virulence predicts the outcome of the interaction among virulence, growth and transmission (Frank, 1996; Pfennig, 2001). A high within-host growth and propagule production increase longevity and fitness of the pathogen. However, the subsequent increased damage to the host can reduce the survival of the host and, in turn, of the pathogen. Thus, it is expected that the maximum fitness of the pathogen is achieved through a maximised trade-off between virulence, growth and transmission (Frank, 1996; Pfennig, 2001). Because of this tight link between virulence, growth and transmission, analysing *A. astaci* growth and transmission is fundamental to deepen our understanding of the ability of this pathogen to successfully adapt to new hosts and colonise new environments.

In this study we evaluated virulence, *in vitro* growth and sporulation rates of 14 *A. astaci* strains belonging to three different haplogroups (haplogroups A, B and E) isolated from both invasive and native European crayfish species. We used these data to evaluate if sporulation and/or growth rates are correlated with virulence and can be used as virulence predictors. Furthermore, we aimed to assess if virulence, growth and sporulation rates are more similar among strains within a haplogroup than among different haplogroup. The damage caused by *A. astaci* to the European crayfish stocks has been extremely elevated with many populations disappearing because of the disease, and the introduction of new strains coming through the pet trade with alien North American crayfish into Europe is unfortunately very likely (Faulkes, 2015). Thus, it is imperative to deepen our knowledge on the determinants of *A. astaci*'s virulence, searching for patterns that can be exploited for the management of the pathogen and the conservation of endangered European crayfish.

4.2. Material and Methods

4.2.1. *Aphanomyces astaci* strains

The *A. astaci* strains used in this study were isolated from different crayfish species (both North American and European) from water bodies in North America and Europe between 2003 and 2020 (Table 1). Strains were preserved at 6°C on PG-1 agar slant cultures protected with paraffin oil (Unestam, 1969a). Prior to the tests, each culture was transferred to PG-1 agar and incubated at 18°C for 10 days. For the infection experiments and growth and sporulation rate tests, the experimental temperature was set to 18°C, which is likely to represent the optimal temperature for growth, sporulation and motility of the zoospores of most strains of *A. astaci* in our experiment (Diéguez-Uribeondo et al., 1995).

Table 1. Strain ID, original ID, place and crayfish species of isolation, haplogroup, year of isolation and GenBank accession numbers (rnnS and rnnL) of each strain used in the present study.

Strain ID	Original ID	Origin	Host	Haplo group	Year of isolation	GenBank acc. number (rnnS)	GenBank acc. number (rnnL)
HapB-1	UEF7203-6	Lake Kukkia, Finland	*Pacifastacus leniusculus*	B	2003	OR682421	OR682407
HapB-4	UEF8866-2	Lake Puujärvi, Finland	*P. leniusculus*	B	2003	OR682422	OR682408
HapB-6	UEF8147-4	Lake Pyhäjärvi, Finland	*P. leniusculus*	B	2003	OR682423	OR682409
HapB-7	KTY3-4 *	UEF Fish Research Unit, Kuopio	*Astacus astacus*	B	2008	OR682424	OR682410
HapB-8	SATR1	Lake Saimaa, Finland	*P. leniusculus*	B	2012	OR682425	OR682411
HapB-12	SATR3A1	Lake Saimaa, Finland	*P. leniusculus*	B	2012	OR682426	OR682412
HapB-13	T16A	Lake Tahoe, USA	*P. leniusculus*	B	2013	OR682427	OR682413
HapB-67	Sot 2a)	Lake Pyhäjärvi,	*P. leniusculus*	B	2018	OR682432	OR682418

		Sotkanniemi, Finland					
HapA-26	AT1D	Lake Borovniscica, Slovenia	*Austropotamobius torrentium*	A	2014	OR682428	OR682414
HapA-29	VEN5/14 b)	Lake Venesjärvi, Finland	*A. astacus*	A	2014	OR682429	OR682415
HapA-34	OI2 e)	Speyer, Germany	*Faxonius immunis*	A	2015	OR682430	OR682416
HapA-36	Or #7 a)	Trout, Normal, IL, USA	*Faxonius rusticus*	A	2016	OR682431	OR682417
HapA-78	C18a	Runkedebunk, Germany	*F. immunis*	A	2020	OR682434	OR682420
HapE-75	FL4a	Kräppelweiher, Germany	*Faxonius limosus*	E	2020	OR682433	OR682419

* This strain was isolated from an accidentally infected *A. astacus* inside the UEF Fish Research Unit facilities in Kuopio, Finland. However, the strain is likely to have been originally carried by a specimen of *P. leniusculus*.

4.2.2. Virulence assessment: infection experiments

4.2.2.1. Experimental crayfish

The infection experiments were conducted in the spring of 2021. In total, 160 1-year-old juveniles of noble crayfish (*Astacus astacus*) were obtained from breeder Helmut Jaske (Oeversee, Germany). Prior to the experiments, crayfish were sexed and carapax length was measured (Supplementary data 1 Table S1). The handling of the crayfish and the infection experiments were carried out under the permit A 19-20-003 DP issued by the Rhineland Palatinate State Agency for Consumer and Health Protection (LUA).

4.2.2.2. Experimental setup

The infection system consisted of 161 single 10 L tanks divided into 16 groups of 10 individuals each (14 infection groups and two control groups), with the exception of group HapB-1, which consisted of 11 crayfish. Crayfish were randomly assigned to each group. Due to logistic constraints, the experiments were conducted in two batches, one comprised by all strains except HapB-13 and a control (Cont1), and the other batch comprised by HapB-13 and a control (Cont2). Each tank was filled with 1 L of tap water and equipped with a mixture of gravel and sand lining to supply a gripping surface for the crayfish. The room was maintained at a stable temperature of

18°C and with a light:dark cycle of 8:16 hours. The total volume of water was exchanged every week. Crayfish were fed half a frozen pea every second day. Before the start of the experiment, crayfish were acclimatised for 10 days.

For each strain, a spore solution was produced as described in Francesconi et al. (2021). For washing the hyphae and for sporulation, filtered and autoclaved water from the artificial pond in Eußerthal, Germany (49°15'14''N, 7°57'43''E), with a pH of 7.8 was used. Before infecting the crayfish, the water in the tanks was exchanged. The spore solution obtained for each strain was then used to infect 10 crayfish with a concentration of 250 spores/mL. During the experiment, the crayfish were monitored multiple times per day to assess presence of symptoms (scratching, ataxia, autotomy) and eventual death. The infection experiments lasted for 60 days or until all the crayfish in the experimental group died. Crayfish that survived past the end of the experiment were euthanised by freezing at -80°C. No crayfish showed signs of distress during euthanasia. Crayfish tissues (abdominal cuticle, uropods, and coxa and basis of two walking legs) for the qPCR were sampled at the death of the crayfish and frozen at -20°C until needed.

4.2.2.3. DNA isolation, qPCR and *A. astaci* load quantification

Total DNA was isolated from the frozen crayfish tissues as described in Vrålstad et al. (2009). Quantitative PCR was conducted to evaluate the amount of pathogen inside the tissue of the crayfish as described in Francesconi et al. (2021) with an increased total volume of the qPCR reaction of 20 µL + 5 µL of template DNA to further dilute eventual PCR inhibitors. PCR forming units (PFUs) and agent levels were calculated based on Vrålstad et al. (2009), with only PFU values above the limit of detection of the assay (LOD) considered as positive, i.e., PFU values ≥ 5 and agent level ≥ A2.

4.2.3. Total-sporulation rate (S) and motile-sporulation rate (MS)

Sporulation tests were conducted in two batches, one comprised of HapB-1, HapB-4, HapB-6, HapB-7, HapB-13 and HapA-26 and the other comprised of HapB-8, HapB-12, HapA-29, HapA-34, HapA-36, HapB-67, HapE-75 and HapA-78. For each strain, a cube of agar with a surface area of 25 mm^2 was cut out from the outer edge of the hyphal mat and transferred onto a 10 mL cell culture plate containing 9 mL of PG-1 medium. Each strain was prepared in triplicates. Cultures were then incubated at 18°C for four days. After the incubation period, each strain was washed in 30 mL of filtered and autoclaved pond water for 2 minutes. This washing procedure was repeated three times, each time with clean water. Hyphae were then cut into pieces with a scalpel and incubated in 10 mL filtered and autoclaved pond water at 18°C for 2 days. For each

strain the density of the zoospores and of motile zoospores were estimated with an optical microscope (magnification 100x) and a Bürker chamber after 24 and 48 hours of incubation. Dry weight of the hyphal mat of each strain was measured and used to normalise the number of motile spores and total spores. Prior to weighing, the hyphal mat was dried on filter paper, and the solid agar was removed. As it was not possible to precisely estimate the weight of each individual replica due to their weight falling below the sensitivity value of the scale (i.e., 0.01 g), the three replicates of each strain were pooled before being weighed. Weight of an individual replica was then estimated as one third of the total weight. Sporulation rates (i.e., total-sporulation rate and motile-sporulation rate) were calculated as the mean number of total/motile spores*$mL^{-1}day^{-1}$.

4.2.4. *In vitro* growth rate

A cube of agar with a surface of 25 mm^2 was cut out from the outer edge of the hyphal mat and transferred onto the centre of a petri dish of 90 mm in diameter containing PG-1 agar with a thickness of around 10 mm. Each strain was prepared in triplicates. The freshly inoculated plates were incubated at 18°C for 20 days, or until the hyphal mat reached the border of the petri dish. The hyphal growth was checked and photographed every 48 hours. The pictures were then analysed with ImageJ v1.53k to determine the diameter of the hyphal mat (Schneider et al., 2012). The daily growth rate (GR) was estimated as the slope of a linear regression for the changes of area through time calculated by combining the data of the three replicates. Depending on the strain, values from 48 h to 96, 144, 192 or 240 h were considered for the statistical analysis to ensure the linearity between time and growth rate (**Supplementary data 1 Table S2**).

4.2.5. Statistical analyses

All statistical analyses were conducted in R (v4.3.1; R Core Team, 2021). Unless differently specified, the analyses were conducted by using the stats package (v4.3.1; R Core Team, 2021). When comparing means, all datasets were tested for normality using a Shapiro-Wilk test and for equality of variance with Levene's test (car package, v3.1-2; Fox and Weisberg, 2019). Based on the results of these tests, the appropriate test was then applied to infer the presence of significant difference among groups. In particular, for data normally distributed and with equal variance, we applied a t-test to search for significant differences between two groups, and an ANOVA, followed by the *post-hoc* Tukey's range test, for more than two groups (emmeans package, v1.8.7; Lenth, 2023). If data were not normally distributed and/or had no equal variance, we applied a Mann-Whitney-Wilcoxon test to check for statistically significant differences between two groups, and Kruskal-Wallis test followed by a *post-hoc* Conover-Iman test to compare more than two groups

(conover.test package, v1.1.5; Dinno, 2017). Chi-squared was used to assess statistically different distributions of categorical variables among different groups. For multiple testing, p-values were adjusted using the Benjamini-Hochberg (BH) method. For all statistical tests an α=0.5 was used. For the infection experiments, the difference in mortality among experimental groups and control groups was evaluated through Kaplan-Meier survival curves (Log Rank) through the survival package (v3.5-5; Therneau, 2023) and survminer package (v0.4.9; Kassambra et al., 2021). For the purpose of the statistical comparisons involving symptomatic and/or infected crayfish, we defined as "symptomatic" crayfish showing any of the symptoms among autotomy, ataxia and scratching. The death of a crayfish was not considered a symptom. Additionally, we defined as "infected" any crayfish with a positive PFU-value (PFU-value > 5) and/or presence of hyphae within its cuticle.

Spearman's rank correlation was computed with the rstatix package (v0.7.2; Kassambra, 2023) to assess the pairwise relationship between virulence (Vir, expressed as proportion of dead crayfish/median days to death), growth rate (GR) and sporulation rates (S, total-sporulation rate and MS, motile-sporulation rate). Correlation between year of isolation of the culture and virulence was also investigated with a Spearman's rank correlation test. The presence of grouping according to the canonical mitochondrial haplogroup classification (haplogroups based on SSU and LSU markers) was evaluated through principal component analysis (PCA) based on virulence, growth rate and sporulation rates. Prior to the PCA all data were log-transformed, scaled and centred to reduce the effect of skewness and magnitude of the variables. Statistically significant differences of the various parameters between groups were investigated through t-tests. As only one strain belonging to haplogroup E was used in the study, statistical comparisons among haplogroups were only conducted between haplogroup A and B.

Finally, to facilitate the visualisation and comparison of the global results for each strain, a rank value between 0 and 5 was assigned to each strain for each feature analysed in this paper following the key in **Table 2**.

Table 2. Key for conversion of virulence, growth rate and sporulation rates into rank values between 0 (lowest value) and 5 (highest value). Vir, virulence; I, percentage of infected crayfish based on qPCR and/or observed hyphae (%); D, percentage of dead crayfish (%); GR, growth rate (mm^2 day^{-1}); S, total-sporulation rate (spores*mL^{-1} day^{-1}); MS, motile-sporulation rate (spores*mL^{-1} day^{-1}).

Rank	Vir	I/D	GR	S	MS
0	Vir= 0	I= 0	GR= 0	S= 0	MS= 0
1	0 < Vir ≤ 0.01	0 < I ≤ 20	1 < GR ≤ 3	0 < S ≤ 1k	0 < MS ≤ 500
2	0.01 < Vir ≤ 0.03	20 < I ≤ 50	3 < GR ≤ 6	1k < S ≤ 10k	500 < MS ≤ 2.5k
3	0.03 < Vir ≤ 0.05	50 < I ≤ 70	6 < GR ≤ 9	10k < S ≤ 50k	2.5k < MS ≤ 10k

4	0.05 < Vir ≤ 0.1	70 < I ≤ 90	9 < GR ≤ 12	50k < S ≤ 100k	10k < MS ≤ 15k
5	Vir > 0.1	I = 100	GR > 12	S > 100k	MS > 15k

4.2.6. Haplotype network analysis

For each strain used in our analyses, we determined the haplogroup according to Makkonen et al. (2018). For the DNA isolation from *A. astaci* hyphal tissue, three cubes of hyphae-containing PG-1 agar (4 mm^2 each) were cut from the stock culture and grown in 100 mL of PG-1 medium for 7 days at 18°C. Next, hyphal tissue was dried on filter paper and stored at -20°C until further processing. The high-salt DNA isolation protocol was adapted from Aljanabi and Martinez (1997) as follow: for each sample, 450 µL SEB (2 mM EDTA (pH 8), 10 mM Tris-HCl (pH 8), 0.4 M NaCl), 100 µL SDS (10%) and 5 µL Proteinase K (10 mg/mL) were added to the tissue inside an Eppendorf tube. The tube was vortexed and incubated for 1 h at 60°C with shaking (550 rpm). Then, the samples were frozen for 30 min at -20 °C and subsequentially, thawed at room temperature. This was followed by addition of 350 µL NaCl (5 M) and centrifugation for 30 min at 13 000 x g. The supernatant (600 µL) was transferred into a new tube, and DNA was precipitated with 600µL of ice-cold isopropanol for 30 min. The samples were centrifuged at 4° C for 20 min at 13.000 x g, and supernatant was removed. To clear the pallet, 200 µL of ice-cold ethanol (70%) was added to the pellet and the samples were centrifuged at 4° C for 10 min at 13.000 x g. Residual ethanol was removed and the pallet was dissolved in 100 µL of DNAse-free H2O.

Haplotyping was conducted based on the mitochondrial ribosomal small (rnnS) and large (rnnL) subunits with the primers described in Makkonen et al. (2018). PCR reactions were conducted with GoTaq Green Master Mix (Promega, USA) in 25 µL reaction volume according to the manufacturer's instructions. PCR products were visualised in 1% w/v agarose gel stained with MIDORI Green Xtra dye (NIPPON Genetics EUROPE, DE). Individual bands were cut out of the gel and purified using the column-based PCR und Gel extraction Mini Prep Kit (Genaxxon bioscience, DE) according to the manufacturer's instructions. PCR products were Sanger-sequenced on the Applied Biosystems 3730 (Applied Biosystems, USA).

The obtained rnnS and rnnL sequence chromatograms were analysed in Geneious Prime (v2023.1.1) and nucleotide sequences were aligned using Mafft (v7.310). For the network analysis, the rnnS and rnnL sequences obtained in this study and additional *A. astaci* sequences obtained from GenBank were used (**Table 1**, **Supplementary data 1 Table S3**). The median-joining haplotype network was visualized in PopArt v1.7 (Bandelt et al., 1999).

4.3. Results

4.3.1. Virulence assessment: infection experiment

One crayfish died in Cont1 at day 40 of the infection experiment, while no crayfish died in Cont2. Nine strains caused an increased mortality in the experimental crayfish compared to the control groups (Log-rank test, $p < 0.05$; **Figure 1**, **Table 3**). Out of these, four strains caused 100% of mortality (HapB-4, HapB-8, HapA-36, HapA-78). The most virulent strain was HapB-4, which killed all the crayfish within seven days. Five strains (HapA-26, HapA-29, HapA-34, HapB-67, HapB-7) did not cause an increased mortality in the experimental groups compared to the control. Out of these, for two strains (HapA-26 and HapA-29) no deaths were observed. One crayfish died in each of the groups of HapB-7 and HapB-67, while three crayfish died in the HapA-34 group. When considering the experimental groups with a significant increase in mortality, the median number of days from the start of the experiment to the death of the crayfish varied greatly among the strains, ranging from 6 to 31 (**Figure 1**, **Table 3**). There was no correlation between virulence and year of isolation of the strain (Spearman's rank correlation, $r(12)=-0.07$, $p=0.815$; **Table 4**).

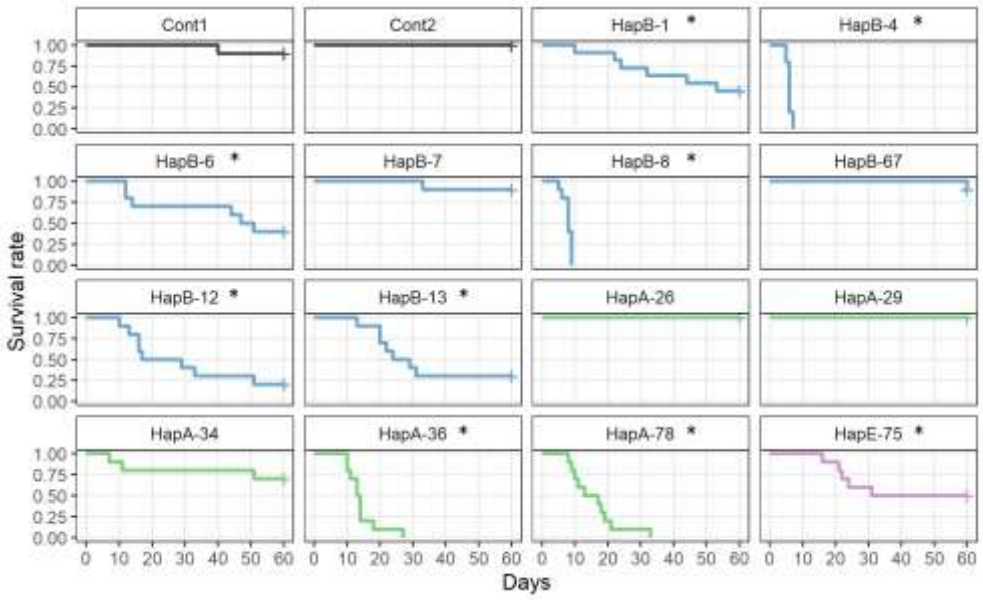

Figure 1. Survival curves indicating the proportion of surviving crayfish through time in each experimental group. Cont1 represents the control group for all strains apart from HapB-13, while Cont2 is the control group for HapB-13. Asterisks (*) indicate groups with survival rates significantly different from their respective control group.

Table 3. Summary of the results of the infection experiment. For each strain are reported: proportion of dead crayfish, Log-rank p-value of the Kaplan–Meier survival curves compared to control group, median days of death of the crayfish, virulence (Vir) calculated as proportion of death crayfish/median days to death, proportion of *A. astaci* positive crayfish (qPCR), agent level, and proportion of crayfish showing symptoms.

Strain ID	Proportion of dead crayfish	P-value (log-rank test)	Median days until death (range)	Vir	Proportion of *A. astaci* positive crayfish (qPCR)	Agent level	Proportion of crayfish showing symptoms
HapB-1	0.55	0.03	31.2 (10-55)	0.02	0.73	A1-A4	0.36
HapB-4	1	<0.001	6 (5-7)	0.17	1	A4-A5	0.5
HapB-6	0.6	0.02	30 (12-51)	0.02	1	A2-A4	0.4
HapB-7	0.1	0.97	33	0	0.1	A0-A4	0.1
HapB-8	1	<0.001	7.9 (5-9)	0.12	1	A3-A5	0.5
HapB-12	0.8	<0.001	23.1 (10-51)	0.05	1	A2-A5	0.7
HapB-13	0.7	0.002	22.7 (13-31)	0.03	1	A1-A5	0.4
HapB-67	0.1	0.97	60	0	0.8	A0-A3	0
HapA-26	0	0.32	ND	0	0.2	A0-A2	0
HapA-29	0	0.32	ND	0	NA	NA	0
HapA-34	0.3	0.27	23 (7-51)	0.03	0.7	A0-A5	0
HapA-36	1	<0.001	14.4 (10-27)	0.07	1	A3-A4	0.9
HapA-78	1	<0.001	15.9 (8-33)	0.07	1	A2-A5	0.8
HapE-75	0.5	0.03	22.8 (16-31)	0.02	0.7	A0-A4	0.2

Due to technical problems, it was not possible to analyse the cuticles isolates from the crayfish belonging to the HapA-29 infection group. Therefore, qPCR and microscopic analysis were conducted only on 15 experimental groups. The qPCR analysis for *A. astaci*'s load within the crayfish cuticle revealed that 99 out of the 131 infected crayfish were positive for *A. astaci* presence, with agent levels ranging from A2 to A5. The remaining 32 crayfish were negative, with agent level A0 (no detection of *A. astaci*) or A1 (PFU-value below the LOD) (**Table 3**, **Supplementary data 1 Table S4**). Only for strain HapB-13, the PFU values detected in the tissues of the dead crayfish were statistically different from the PFU values in the tissues of the alive crayfish at the time of sampling (Wilcoxon-Mann-Whitney, p=0.03; **Supplementary data 2 Figure S1**).

Most of the crayfish that died during the experiment showed symptoms of *A. astaci* infection (n=48, 63.1%; **Table 3**, **Supplementary data 1 Table S4**). Within each experimental group, the percentage of symptomatic crayfish was between 50% and 100%, with the only exception being the experimental crayfish infected with HapE-75 (n=2, 20%). Most crayfish exhibited the first

symptoms within two days before their death (n=37; 77%; **Supplementary data 2 Table S1**), with no significant difference among experimental groups (Kruskal-Wallis rank sum test, $\chi^2(9)=15.36$, p-value=0.08). The most frequent symptom was autotomy of the limbs (either walking legs or claws), with more than half of the crayfish that ultimately died presenting this symptom (n=41, 52.6%). The second most frequent symptom was ataxia (n=21, 26.9% of the total dead crayfish). Only two crayfish were observed while scratching (belonging to HapB-1 and HapB-7; **Supplementary data 2 Table S1**). Five crayfish out of 49 (9.4%) survived after showing symptoms. These crayfish belonged to the groups infected with HapB-7, HapE-75, HapB-6 (n=2) and HapB-8. Four strains did not cause symptoms in the challenged crayfish (HapB-67, HapA-26, HapA-29, HapA-34). Considering the infected crayfish (PFU-value > 5; agent level ≥ A2) among the different groups, the PFU-values and the presence of hyphae in the cuticle are not correlated with the insurgence of symptoms before death (respectively: t-test, $t(70)=0.627$, p-value=0.532; Chi-squared, $\chi^2(1)=0.16$, p-value=0.688; Table 4, **Supplementary data 1 Table S4**). Furthermore, for the infected crayfish there is no correlation between virulence and insurgence of symptoms (Spearman's rank correlation, r=0.5, p-value=0.07, **Table 4**).

Table 4. Summary table reporting the p-values of the correlation tests done in this study. The p-values represents the statistical significance of Spearman's rank correlation unless differently indicated. The cut-off considered was α= 0.05. Vir, virulence; GR, growth rate; MS, motile-sporulation rate; Sy, crayfish showing symptoms before their death; Sur, survival of the experimental crayfish; PFU, PFU-values representing the pathogen load inside the tissues of the crayfish; Hyph, observed hyphae in the cuticle of the crayfish; M-Hyph, observed melanised hyphae in the cuticle of the crayfish.

1st variable	2nd variable	p-value
Vir	Year	0.815
Vir	GR	0.469
Vir	S	0.811
Vir	MS	0.576
Vir	Sy	0.07
GR	S	0.483
GR	MS	0.378
GR	PFU	0.3
S	MS	0.004*
Sy	PFU	0.532^{+}
Sy	Hyph	0.688^{++}
M-Hyph	Sur	0.615^{++}

($^{+}$) t-test comparing mean PFU-value of symptomatic vs asymptomatic crayfish; ($^{++}$) Chi-squared comparing binomial variables, i.e., presence/absence of symptoms in the crayfish vs presence/absence of hyphae in the crayfish cuticle, presence/absence of symptoms in the crayfish vs survival/death of the crayfish; (*) indicates significantly different values.

Microscopical analysis of the abdominal cuticle of the experimental crayfish revealed that, among all the crayfish exposed to *A. astaci* (n=131), 61% of them had observable hyphal growth within their abdominal cuticle, and in 24% of the cases the hyphae presented some degree of melanisation. When hyphae were observed inside the cuticle of the crayfish, their partial melanisation was not connected to changes in the likelihood of survival of the crayfish compared to crayfish with complete absence of melanised hyphae (Chi-squared, $\chi^2(1)=0.25$, p-value=0.615).

4.3.2. Total-sporulation rate (S) and motile-sporulation rate (MS)

All strains produced spores under the experimental conditions (**Figure 2a**, **Supplementary data 2 Table S2**). However, only nine strains out of 14 produced motile spores (**Figure 3b**). The Kruskal-Wallis test revealed statistically significant differences between at least two groups both for S (H(13)=32, p=0.003) and MS (H(8)=17, p=0.03) of the strains. The results of the pairwise Conover-Iman test are reported in **Tables S3** and **S4** of the **Supplementary data 2**. For most of the strains, the S values are highly variable among the three replicates, with SD values > 10000 (**Supplementary data 2 Table S2**, **Figure 2a**). The MS also appears to be variable among the replicates of the strains (**Supplementary data 2 Table S2**, **Figure 2b**). Strains HapA-26, HapB-8, HapA-78, HapB-12 were among the strains with both the highest S and MS. Strain HapB-7 had a high S but was among the strains with the lowest MS. Overall, S and MS were significantly positively correlated (Spearman's rank correlation, r(12)=0.72, p=0.004; **Table 4**). There is no significant correlation between Vir and sporulation rates (S, Spearman's rank correlation, r(12)=0.07, p=0.811; MS, Spearman's rank correlation, r(12)=0.16, p=0.576; **Table 4**).

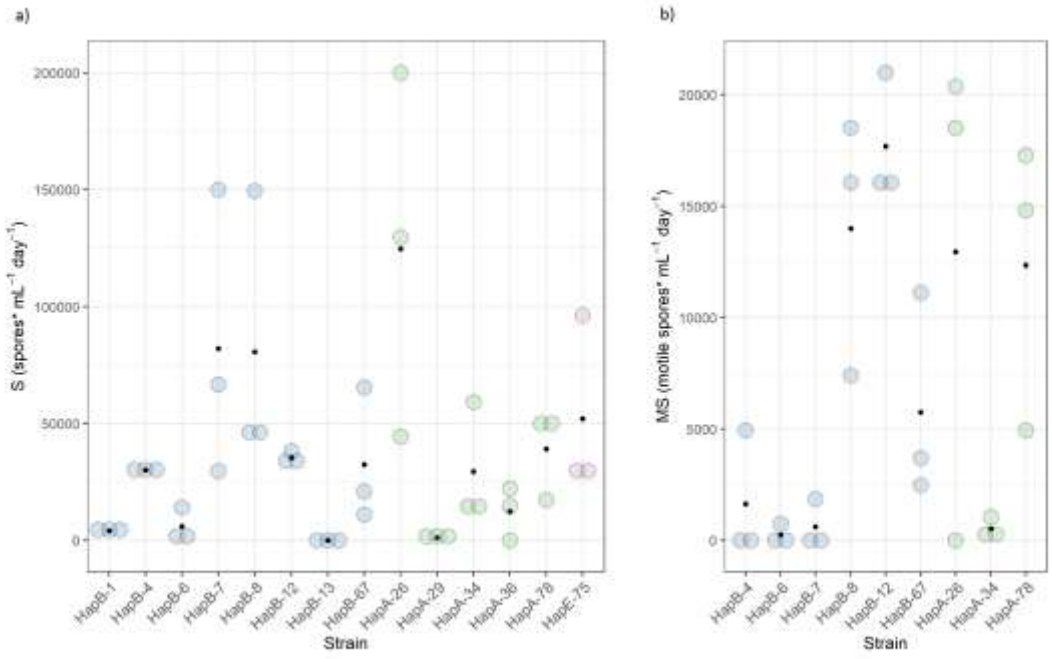

Figure 2. a) Daily total-sporulation rate (S) for all 14 *A. astaci* strains. **b**) daily motile-sporulation rate (MS) for the 9 strains that produced motile spores. Three circles of the same colour represent the three replicates of one strain. Black dots represent the mean value for each strain.

4.3.3. *In vitro* growth rate (GR)

For all strains, the linear regressions fit well the data on the increments of areas, with adjusted R^2 ranging between 0.910 and 0.997 and p-values < 0.001 (**Supplementary data 2 Table S5** and **Table S6**). ANOVA showed the presence of significant differences among groups ($F(13)=130.0$, $p<0.001$). The results of the *post-hoc* Tukey's range test are shown in **Figure 3** and **Supplementary materials, Table S11**. Finally, GR is not significantly correlated with S (Spearman's rank correlation, $r(12)=-0.2$, $p=0.483$), MS (Spearman's rank correlation, $r(12)=-0.26$, $p=0.378$), PFU-values (Spearman's rank correlation, $r(11)=0.313$, p-value=0.3), nor Vir (Spearman's rank correlation, $r(12)=0.21$, $p=0.469$; **Table 4**).

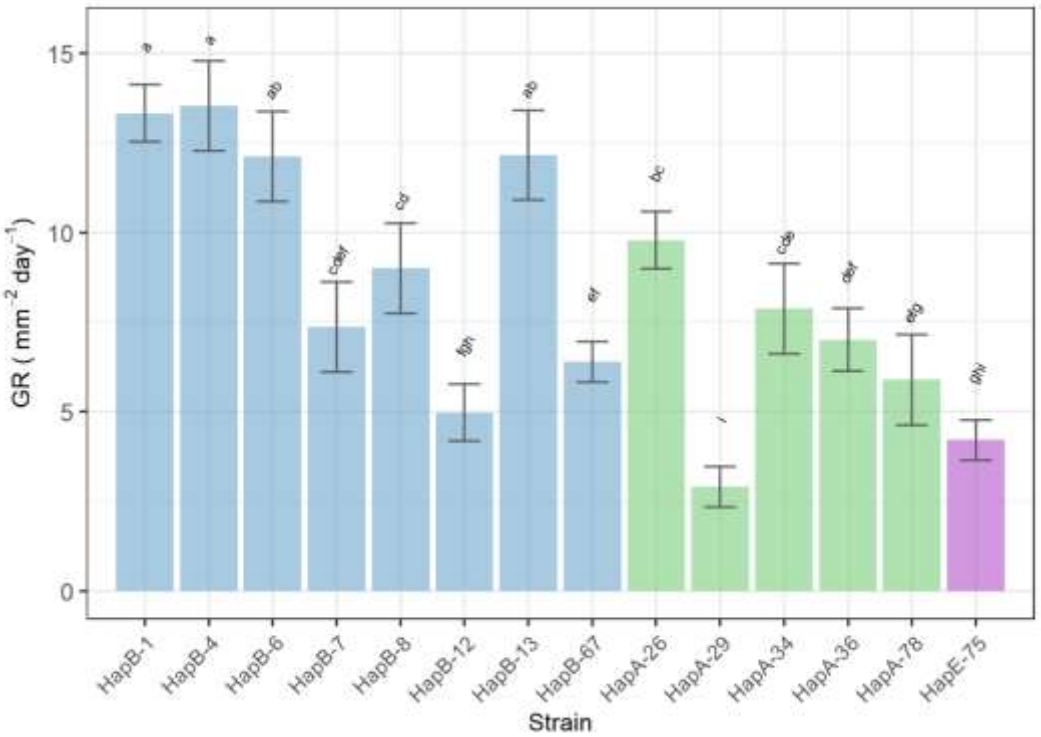

Figure 3. Growth rate (mm^2 day^{-1}) of each strain calculated as the slope of a linear regression for the changes of area through time calculated by combining data of the three replicates. Letters represent statistically significant differences based on Tukey's range test. Whiskers represent 95% confidence interval. GR, growth rate.

4.3.4. Statistical analysis after haplogrouping and haplotype network analysis

After grouping the strains by haplogroup, no statistically significant difference was detected between haplogroup A and haplogroup B in terms of Vir (Mann-Withney-Wilcoxon, W=16, p-value=0.6), GR (t-test, t(11)=-2, p-value=0.1), S (Mann-Withney-Wilcoxon, W=182, p-value=1) and MS (Mann-Withney-Wilcoxon, W=179, p-value=0.9) (**Table 5**). The PC analysis shows that PC1 (mainly influenced by S and MS) and PC2 (mainly influenced by GR and Vir) together explain 78.3% of the differences among strains. No grouping based on the genetic signature shown in the network analysis can be recognised among the strains in the PCA analysis (**Figure 4a-b**). A summary of the characteristics of each strain transposed into ranks from 0 to 5 is shown in **Figure 5**.

Table 5. Means or medians and respective standard deviation (SD) or range for total-sporulation rate (S), motile-sporulation (MS), growth rate (GR) and virulence (V) of haplogroup A and haplogroup B.

		Median	Range	Mean	SD
S	Hap-A	17284	0 - 150000	-	-
	Hap-B	29012	0 - 200000	-	-
MS	Hap-A	0	0 - 20370	-	-
	Hap-B	370	0 - 20988	-	-
Vir	Hap-A	0.0273	0 - 0.0741	-	-
	Hap-B	0.0263	0.00167 - 0.16667	-	-
GR	Hap-A	-	-	6.70	± 2.55
	Hap-B	-	-	9.86	± 3.35

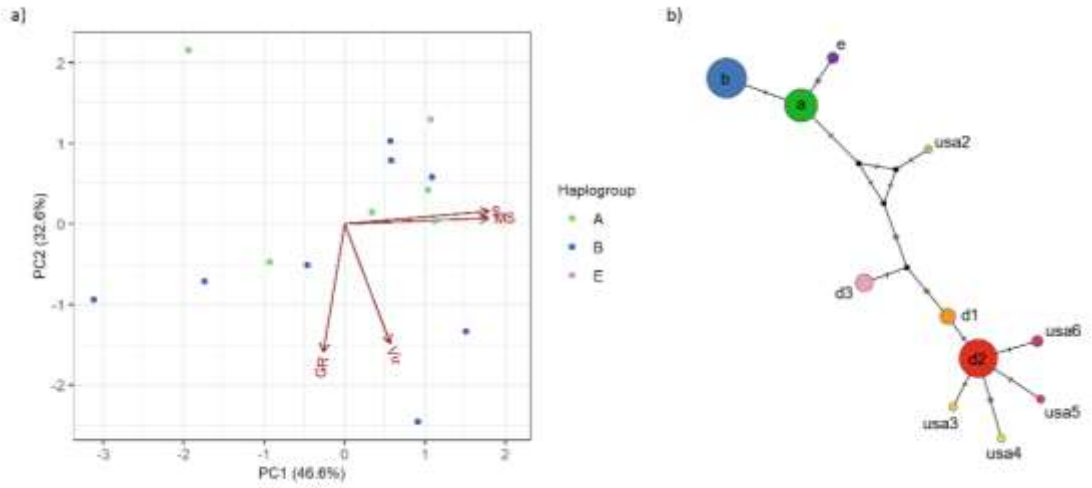

Figure 4. a) PC analysis. Vir, virulence; GR, growth rate; S, total-sporulation rate; MS, motile-sporulation rate. **b**) Network analysis based on concatenated mitochondrial ribosomal small (rnnS) and large (rnnL) subunits of *Aphanomyces astaci*. Circle represents different haplotypes. Circle areas are proportional to the number of specimens sharing that haplotype. Hatch marks indicate the number of mutations separating haplotypes. Haplotypes a, b and e belong to haplogroup A, B and E, respectively.

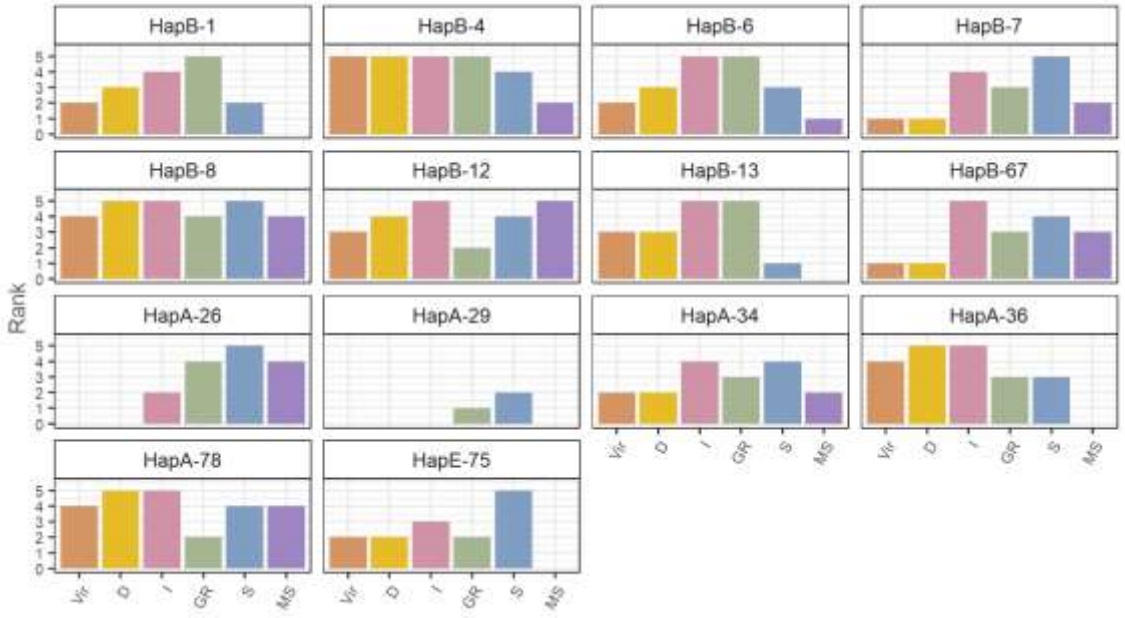

Figure 5. Rank of the main parameters measured for each strain. Vir, virulence; D, percentage of dead crayfish; I, percentage of infected crayfish (based on qPCR and/or observed hyphae); GR, growth rate; S, total-sporulation rate; MS, motile-sporulation rate. For HapA-29 the percentage of infected crayfish (I) is not available. For rank classification, see Table 2.

4.4. Discussion

Aphanomyces astaci is one of the most studied pathogens of aquatic invertebrates due to its catastrophic impact on crayfish stocks, both in the wild and in aquaculture. Despite the relevance of this pathogen, systematic studies analysing its virulence along with the virulence-influencing factors have been lacking. To bridge this gap, in this study we analysed virulence and *in vitro* growth and sporulation rates of 14 *A. astaci* strains. Our results show a great variability across the strains of all the analysed parameters, with the exception of the total-sporulation rate. Additionally, neither *in vitro* growth rate nor total/motile-sporulation rates have a statistically significant correlation with virulence. Finally, none of the analysed parameters is significantly different across haplogroups. These results indicate the presence of complex interactions between pathogen, host and environment influencing the virulence evolution of each strain.

4.4.1. High virulence variability across *A. astaci* strains

The infection experiments showed a high variability in the virulence of the strains towards the susceptible *A. astacus* (**Figure 1**, **Table 3**). This variability is very common for strains belonging to haplogroup A (Makkonen et al., 2014), and our results showcase this feature, with the mortality

caused by the strains ranging from 0% to 100% (**Figure 1**). The only strain in this experiment belonging to haplogroup E, HapE-75, is characterised by an intermediate virulence, with 50% of mortality. Unfortunately, a systematic comparison with other studies is not possible, as strains belonging to this haplogroup have been seldomly tested. Among the least virulent strains appear two strains belonging to haplogroup B (HapB-7 and HapB-67) (**Figure 1**). This is quite surprising, as haplogroup B strains are typically characterised by high virulence (Makkonen et al., 2014; Becking et al., 2015). This result is probably not connected to the prolonged cultivation in laboratory conditions, as there is no significant correlation between year of isolation and virulence, and one of the two strains (i.e., HapB-67) was isolated only three years before the infection experiments (**Table 1**, **Table 4**). A decreased virulence of *A. astaci* is usually connected to the circulation of the strain among susceptible crayfish species in the absence of their original North American crayfish hosts (Jussila et al., 2015; Francesconi et al., 2021). However, in this case, both strains were circulating within a resistant population of the North American *P. leniusculus* prior to their isolation, and it is therefore unclear what environmental selective pressure might have caused the loss of virulence.

Based on qPCR and microscopic analysis, almost all strains completed, at least to a certain degree, the first phases of the infection process (i.e., attachment and germination of the zoospores into the host) (**Table 3**, **Supplementary data 1 Table S4**). This indicates that the difference in the overall virulence for these strains is likely dependent on their growth through the cuticle and inside the host and/or overcoming the host's immune defence. An exception to this germination efficiency is represented by strain HapA-26, that was able to attach and start growing in only 30% of the exposed crayfish. Similarly, in Francesconi et al. (2021), another strain belonging to the haplogroup A isolated from Lake Venesjärvi, Finland, was hypothesised to have a low ability to germinate on the host. These two strains (HapA-26 and the strain from Lake Venesjärvi) are likely descendants of the first Haplogroup A strains that were introduced into Europe around 150 years ago without their original host species (Alderman, 1996), and they might have partially lost their virulence to adapt to the new European hosts by decreasing their capability of attaching and germinating on the host (Francesconi et al., 2021).

Interestingly, most of the infected crayfish showed the first symptoms of infection within two days before their death and, in total, only 9.4% of the symptomatic crayfish were able to successfully fend off the pathogen and survive the infection (**Figure 5**, **Supplementary data 2 Table S1**). Unsurprisingly, observations of melanised hyphae in the cuticle of the crayfish were not connected to the survival of infected specimens, indicating that an attempt of the crayfish immune system to contain the pathogen was present but unsuccessful. This lack of an efficient melanisation of the pathogen has often been observed in susceptible crayfish, and is considered one of the main reasons for the susceptibility of European crayfish to *A. astaci* (Cerenius et al.,

2003). Furthermore, the lack of effective melanisation and the quick death of the crayfish after insurgence of symptoms are in accordance with the recent paper from Boštjančić et al. (2023). Here, it was found that within 24 h from the first symptoms, crayfish infected with *A. astaci* lost most of their circulating haemocytes (main effectors of the cellular immune response in crustaceans and responsible for the melanisation process; Söderhäll, 2016), leaving the crayfish defenceless against the growing pathogen.

4.4.2. *In vitro* sporulation rate does not correlate with virulence

Our data suggest that there is no correlation between the strain's normalised sporulation rate, both for total and motile spores, and the virulence of the strain. Furthermore, our results highlight a certain uniformity in the normalised total-sporulation rate of each strain, with most strains producing a high number of spores (ranks 4 and 5), while the motile-sporulation rate was much more variable (**Figure 2a-b**). Our results confirm the conclusions of a previous study where the motility of zoospores was found to be uncorrelated with virulence (Unestam, 1969b). It has to be considered, however, that the *in vitro* measurements of motile zoospores production represent static pictures of a parameter that for its own nature is not static. Zoospore production is a cyclic process in which the alternation between non-motile propagules (cysts) and motile propagules (zoospores) occurs at a dynamic pace (Rezinciuc et al., 2015). Therefore, our observed differences could be the result of the spores being in different stages of such cycle. However, it is clear that the variability in the zoospore production within and among the strains of our study is high, either because of the different sporulation rates or because of the different timing of the production.

While from our results it is not possible to draw patterns equally applicable to all strains in terms of virulence and sporulation rates, our data highlight how the phenotypes of different *A. astaci* strains represent potential contrasted responses to specific host-pathogen co-adaptations. For example, strain HapA-26 is characterised by the highest sporulation rate of our collection (both in terms of total spores and motile spores) and caused no mortality among the experimental crayfish (**Figure 1**, **Figure 2a-b**, **Supplementary data 2 Table S3** and **S4**). It has been shown that in susceptible crayfish infected with *A. astaci*, the main release of spores happens after the crayfish death (Makkonen et al., 2013). Consequently, a strain with very low virulence would greatly benefit from releasing a high number of spores when the host moults or dies for unrelated causes. Additionally, as it appears that this strain has a reduced capability of germinating and/or penetrating into the crayfish tissues, a high sporulation rate can maximise the number of contacts with suitable hosts.

Conversely, another non virulent strain, HapA-29, has one of the lowest sporulation rates among the tested strains and produced no motile spores (**Figure 1**, **Figure 2a-b**). This strain has been

coexisting with native European crayfish populations in Lake Venesjärvi, Finland, for at least the past 50 years (Jussila et al., 2021b). After the first two decades characterised by three distinct mass mortalities among the crayfish, this strain and the new European host seem to have coevolved to reach an equilibrium (Francesconi et al., 2021; Jussila et al., 2021b). In this scenario, the occasional spores released after the natural death of a crayfish might be sufficient for the strain to circulate inside the population. However, it cannot be excluded that this interaction is still evolving and the pathogen, unable to propagate at sufficient levels, is destined to disappear from the population.

Interestingly, two of the most virulent strains of our collection (HapB-4 and HapB-8) have high total-sporulation rates (**Figure 1**, **Figure 2**). While it might seem counterintuitive, as highly virulent strains with high dispersion capacity might wipe-out their host and cause their own extinction (Jussila et al., 2015), this apparent contradiction can be explained by the co-introduction of the pathogen and the resistant North American host in the waterbodies (i.e., *P. leniusculus* translocated into Lake Puujärvi and Lake Saimaa; respectively Makkonen et al., 2012; Jussila et al., 2016). In general, in resistant latently infected crayfish that tend to maintain a low pathogen prevalence among their populations and low pathogen growth within their tissues (e.g., Maguire et al., 2016; Mojžišová et al., 2022), *A. astaci* could benefit from maximising its sporulation at the moulting or death of the host to insure further transmission. This trait, which would be detrimental for the pathogen when circulating only among susceptible crayfish (leading to the host population wipe-out and therefore pathogen suicide), is not negatively selected if North American and European crayfish species coexist, due to the resistant host acting as a pathogen reservoir, allowing for maximum dispersal capacity and virulence towards the susceptible host. All these different situations highlight how different ecological and evolutionary circumstances can shape *A. astaci* phenotypes. While in theory virulence and sporulation are undoubtedly connected, several other factors can influence these phenotypes and weaken their link.

4.4.3. *In vitro* growth rate does not correlate with virulence

Our results indicate that the *in vitro* growth rate of *A. astaci* is rather variable among different strains (**Figure 3**), and, similarly to the sporulation rate, is not statistically correlated with the virulence (**Table 4**). This lack of correlation between virulence and growth might be quite surprising based on the theory on the evolution of virulence, which directly connects the growth of the pathogen inside the host and the exploitation of host resources with tissues deterioration and increased probability of the host's death (Pfennig, 2001). Congruous with this expected connection, a first study conducted in 1969b by Unestam on three *A. astaci* strains found that the faster *in vitro* growing strains were also more virulent. In a later study from Viljamaa-Dirks et al.

(2016) on four *A. astaci* strains, while it was not possible to detect significant correlation between growth rate and virulence, a general trend linking higher *in vitro* growth rate to higher virulence was observed. This discrepancy between our results and the results of Unestam (1969b) and Viljamaa-Dirks et al. (2016) could depend on the bigger sample size used in our study, which includes a higher variety of strains (i.e., in terms of country of isolation and host crayfish species) and therefore a higher phenotypical diversity. The *in vitro* growth rate does not account for some of the fundamental stages of the infection process, such as attachment to the host, germination and interaction with the host's immune defences (Rezinciuc et al., 2015). Thus, different efficiency of the strains to move through these stages might render the *in vitro* growth rate less predictive of the strain's growth inside the crayfish and, therefore, of its virulence.

The lack of correlation between *in vitro* growth and virulence observed in this study could, however, also be indicative of a real biological signal. In fact, the notion of the virulence of the pathogen being independent from its growth is theoretically sound. While faster growing pathogens exploit and damage the host faster compared to slower growing pathogens (Pfennig, 2001), the final virulence is often the result of several factors contributing in a relative fashion (Casadevall and Pirofski, 2001). Pathogens are often armed with several virulence factors, such as toxins or enzymes, that induce damage to the host. In this case, the virulence would be more likely dependent on the invasion capacity of host tissues or of interference with the host defences (Casadevall and Pirofski, 2001). Additionally, the expression of factors related to virulence requires expenditures (i.e., energy and resources) and it represents, therefore, a "fitness cost" (Diard and Hardt, 2017). In a recent study, it has been found that the genome of *A. astaci* is particularly enriched with genes encoding for putative virulence factors (i.e., proteins containing pathogenicity-related domains) compared to other related oomycete species (McGowan and Fitzpatrick, 2017). The expression of these virulence factors undoubtedly has a high energetic cost, and it might barely be covered by the resources extracted from the host tissues. In such cases, the virulence itself is not determined by the growth of the pathogen, but it is the growth of the pathogen that is modulated by the preferential allocation of resources toward the expression of virulence factors (Laine and Barrès, 2013; Diard and Hardt, 2017; Kim et al., 2020). As our knowledge on *A. astaci* genome deepens, it will be interesting to investigate if and how potential variability in the virulence factor arsenal and of its expression in different *A. astaci* strains affect the growth of the strains.

4.4.4. Haplogroups are inefficient predictors of *A. astaci* pathogenic phenotypes

Our results show that *A. astaci*'s virulence, *in vitro* sporulation rates and growth rate are not correlated to the strain's mitochondrial haplogroup (**Table 5**). In fact, none of these factors is

significantly different between haplogroup A and B. Furthermore, the PCA does not show the presence of any recognisable grouping based on genetic markers (**Figure 4a**). Unfortunately, not much literature is available on sporulation and growth rates of the different strains. In a study from 2016 on a limited sample size, Viljamaa-Dirks et al. found a statistically significant difference between the growth rate of strains belonging to haplogroup A and B, with the latter growing faster. Many more studies have been conducted to investigate the virulence of *A. astaci* strains, and it is found that the virulence of *A. astaci* haplogroup B is generally higher compared to that of haplogroup A (e.g., Makkonen et al., 2012; Becking et al., 2015; Viljamaa-Dirks et al., 2016). In contrast, our study did not reveal a difference in the virulence of the analysed groups of strains, which might be explained by the origin of investigated strains, stochastic effects or coevolution with the new host. In particular, our analysis includes both strains isolated in Europe and in the US. A recent study from Martín-Torrijos et al. (2021) showed that the diversity of *A. astaci* in Europe represents only a minimal fraction of the entire diversity of the pathogen in its original range in North America. While these new strains isolated from North America can still be grouped by using the canonical mitochondrial markers, these markers, developed to address the limited diversity of *A. astaci* strains in Europe, are probably not suited to detect the full range of intraspecific diversity of this pathogen. Furthermore, in the 1980s during the second invasion wave, *A. astaci* was mainly introduced into Europe through translocation of its invasive crayfish host species (mainly *P. leniusculus*) for restocking purposes (Theissinger et al., 2022), and the source of the crayfish has mostly been the same (e.g., Lake Tahoe) (Jussila et al., 2015). This presumably resulted in the introduction of a limited number of pathogen strains which might have suffered from founder effects and genetic drift. However, in the last decades, a wider variety of invasive North American crayfish species has been introduced through the pet trade, likely causing the diffusion of more diverse strains of *A. astaci* in Europe (Faulkes, 2015; Mrugała et al., 2015; Panteleit et al., 2017). These "newer" strains have been less extensively studied. Unfortunately, the diversity of *A. astaci* in Europe is likely to increase due to the popularity of crayfish in the pet trade (Faulkes, 2015), rendering the assessment of the strains' phenotypes through canonical mitochondrial markers less and less accurate.

Finally, the discrepancy between the results of this study and the general observation of an association between *A. astaci* haplogroups and the levels of virulence observed in previous studies is likely partially also a consequence of ongoing coevolutionary processes between the pathogen and its new European host. After the first studies on North American crayfish species and *A. astaci*, it quickly became apparent that the coexistence between the two is the result of centuries of coevolution (Unestam, 1969b). A growing body of evidence, including the rising number of research studies documenting latent infections in European crayfish populations (e.g., Viljamaa-Dirks et al., 2011; Maguire et al., 2016; Francesconi et al., 2021) and data indicating host-

pathogen co-adaptation (Boštjančić et al., 2022), indicate that a similar process is taking place between *A. astaci* and European crayfish species. This process is likely to have selected *A. astaci* with reduced virulence in areas without the original North American host. Since the pathogen *A. astaci* is subjected to a new host-pathogen environment, i.e., availability of host refugees, strain competition and successive invasion of foreign strains, we might observe a highly variable and dynamic process of coevolutionary change.

4.5. Concluding remarks

Each *A. astaci* strain is characterised by a unique combination of virulence, sporulation rate and growth rate, independent from their mitochondrial haplogroup (**Figure 7**). As similarly to other pathogenic oomycetes *A. astaci* seems to solely reproduce asexually (Diéguez-Uribeondo et al., 2009), it can be assumed that the strains evolve independently acting as separated lineages and progressively diverging from each other. Based on this premise, the translocation of *A. astaci* from the US to Europe likely acted as a catalyst for the differentiation of the strains. In the span of almost two centuries after its introduction into Europe, *A. astaci* was able to colonise most of the continent, inhabiting a variety of different habitats (Ungureanu et al., 2020). Furthermore, *A. astaci* has spread across populations of four European native crayfish species, each characterised by different levels of resistance to the pathogen (e.g., Kokko et al., 2012; Caprioli et al., 2013; Kušar et al., 2013; Svoboda et al., 2017). Evidence of coevolution between the new host species and the pathogen have already emerged (Francesconi et al., 2021; Jussila et al., 2021b; Boštjančić et al., 2022). The different combinations of environment and host species, with the additional variable of the presence or absence of the original carrier crayfish species, modulate the selective pressure acting on different *A. astaci* strains. The result is a combination of phenotypes that is specifically assembled for the environmental challenges faced by each strain. Thus, measuring pathogenicity-related traits of *A. astaci* does not allow to predict the strain specific virulence. Finally, additional insights would certainly arise from comparative genomic analyses of the varying *A. astaci* strains, which would offer more direct connections between the genetic variation and phenotypic plasticity of the differentially adapted *A. astaci* strains.

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Chapter 5

De novo assemblies of three *Aphanomyces astaci* strains based on Nanopore long-reads and 11 additional short-read assemblies

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Abstract

Objectives:

The oomycete *Aphanomyces astaci* is considered one of the worst invasive species worldwide. Endemic in North America, for the past 150 years *A. astaci* has caused severe decline and even local extinctions of European crayfish populations, with disastrous effects on the overall biodiversity of freshwater ecosystems. However, in the last two decades, reports of more resistant European crayfish populations and less virulent *A. astaci* strains have emerged. This occasional new equilibrium could be the key of a future stable co-existence between host and pathogen. Therefore, exploring the mechanisms underlying the virulence variation of *A. astaci* is pivotal to understand the future possible scenarios of co-existence between pathogen and host in Europe.

Data description:

Here we present three highly complete genome assemblies based on Nanopore long-reads of strains belonging to different pathogen's haplogroups (A, B and E), and 11 additional short-read genome assemblies of strains belonging to haplogroup A and B. These data can pave the way for future comparative genomic studies aimed at uncovering the determinants of the remarkable adaptability of *A. astaci* and of its virulence variation. These studies have the potential to provide revolutionary tools for the management of this invasive pathogen.

5.1. Objective

Aphanomyces astaci, etiological agent of the crayfish plague disease, is considered one the 100 worst invasive species worldwide (Alderman, 1996; Lowe et al., 2004). This oomycete is endemic in North America, and since it was first introduced into Europe 150 years ago, *A. astaci* caused countless disease outbreaks among European crayfish populations (Alderman, 1996; Jussila et al., 2021a). The crayfish plague disease is mostly fatal towards European crayfish species, and it caused the local extinction of entire populations, leading to dramatic collapses of European crayfish stocks across the continent, both in the wild and in the aquaculture context (Alderman, 1996; Jussila et al., 2021a). As European crayfish are considered keystone species within freshwater ecosystems, the decline and eradication of their populations has the potential to compromise the biodiversity and functioning of freshwater ecosystems (Reynolds et al., 2013). In the past decade, numerous reports of European crayfish populations surviving *A. astaci* infections have emerged (Viljamaa-Dirks et al., 2011; Jussila et al., 2021b, 2017). While this is likely partially due to increased resistance of the crayfish populations (Makkonen et al., 2012; Gruber et al., 2014), several infection experiments have provided evidence regarding the virulence loss of some *A. astaci* strains (Makkonen et al., 2012; Francesconi et al., 2021), likely a consequence of the pathogen adapting to its susceptible European host (Jussila et al., 2015;

Francesconi et al., 2021). As the variability of *A. astaci* strains in Europe is bound to increase due to new introductions of carrier North American crayfish species within the pet trade and aquaculture, it is imperative to understand the genetic determinants of the virulence variation of this pathogen (Faulkes, 2015; Mrugała et al., 2015). Here, we provide three genome assemblies based on Nanopore long-reads of *A. astaci* strains belonging to haplogroup A, B and E, and 11 additional short-reads assemblies of strains belonging to haplogroup A and B. This data is expected to contribute to future molecular studies aimed to identify single nucleotide polymorphisms associated with phenotypical traits of importance for the management of the pathogen (i.e., virulence and sporulation).

5.2. Data description

The *A. astaci* samples are part of a collection including strains isolated from infected crayfish between 2003 and 2020 (for details, see **Table S1**, Appendix). For each strain, after three days of growth in liquid medium, genomic DNA was extracted following a modified phenol/chloroform protocol (for details, see Appendix). For strain HapB-8, HapA-34 and HapE-75, RNA was also isolated from the tissue using the NucleoSpin RNA Kit (Macherey Nagel) according to the manufacturer's protocol. The quantity of both DNA and RNA was measured with Quantus™ Fluorometer (Promega, Madison, Wisconsin, USA).

5.2.1. Illumina sequencing

For all the strains, with the exception of HapB-8, DNA was sequenced as paired-end libraries (350 bp) using the platform NovaSeq PE150 (Novogene, UK). The RNA was sequenced as poly-A enriched libraries on the same platform. For both DNA and RNA sequencing, Illumina reads were trimmed with Trimmomatic v0.39 (Bolger et al., 2014). Before assembly, the DNA-sequencing trimmed reads were deduplicated using dedupe.sh v 38.87.

5.2.2. Genome assembly of Illumina short-reads

Read assembly was carried out with ABySS v. 4.2.1 with the parameters k=90, kc=2 and B=4G (Simpson et al., 2009). Contigs below 200 bps of length were removed with *reformat.sh*. Scaffolding was carried out with RagTag v. 2.1.0 using Nanopore HapB-8 assembly as reference (Alonge et al., 2022). Assemblies were then purged from contigs below 500 bps with *reformat.sh* and deduplicated with Purge Haplotigs. Assembly statistics and completeness were checked though Quast v5.0.2 and BUSCO v5.3.2 using the lineage stramenopiles_odb10 (for details, see **Table S2**, Appendix).

5.2.3. Transcriptome assembly

The assembly of the transcriptome was obtained through Trinity v2.1.1 (Grabherr et al., 2011). Additionally, available *A. astaci* RNA-seq data (accession numbers: SRX236908, SRX236910, SRX236909) were similarly assembled to be used as additional evidence for genome annotation.

5.2.4. Nanopore sequencing, genome assembly and annotation

HapB-8 was sequenced only using Oxford Nanopore long-reads. For strain HapA-34 and HapE-75 Oxford Nanopore reads were produced in addition to Illumina short-reads. For HapB-8 strain the library by ligation was prepared using Nanopore Q20+ Early Access Kit (SQK-Q20EA), while the libraries for strains HapA-34 and HapE-75 were prepared with the Nanopore Q20+ Native Barcoding Kit 24 (SQK-NBD112.24). The three strains were sequenced on FLO-MIN112 flowcells on a MinION Mk1B. The Nanopore reads were called and demultiplexed using Guppy v5.0.6. The reads that passed the Guppy quality check were filtered to retain reads of quality above Q10 and length above 10 kb using NanoFilt v2.6 (De Coster et al., 2018). Reads belonging to strain HapB-8 were assembled using Canu v2.2 (Koren et al., 2017), deduplicated using Purge Haplotigs v1.1.2 (Roach et al., 2018), and then polished using Medaka v1.2.1. Reads belonging to strain HapA-34 and strain HapE-75 were assembled in combination with the respective paired-end short reads using MaSuRCA v4.0.9 with Flye as long-reads assembler (Zimin et al., 2013). The resulting genomes where deduplicated using Purge Haplotigs. The quality of the final assembly was estimated with Quast v5.0.2 and BUSCO v5.3.2. Final statistics are reposted in **Table S2** (Appendix).

5.2.5. Genome annotation

Annotation of the Nanopore HapB-8 genome assembly was conducted using a hybrid approach with MAKER2 v3.01.04 [22], using both *ab initio* gene prediction and an evidence-based gene individuation. Final statistics are reposted in **Table S3** (Appendix).

5.3. Limitations

- The mitochondrial reads were not eliminated before assembling of the nuclear genomes, and might slightly impact the final size of the assemblies and of the gene content.
- RNA-seq data were produced from hyphae grown in laboratory conditions. Therefore, products of genes that are only expressed during the sporulation phase, within the spores, or during an active infection are not represented.

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6. General Discussion

The devastating effects of the introduction of *Aphanomyces astaci* into Europe on the indigenous freshwater fauna have been recorded for the past 150 years (e.g., Cornalia, 1860; Alderman, 1996; Ungureanu et al., 2020). The picture these reports painted has been extremely bleak, with mass mortalities within European crayfish populations observed across the whole continent (Alderman, 1996). The crayfish plague disease agent eradicated several crayfish populations, caused the dramatic decline of crayfish stocks (both farmed and in the wild), and is one of the main factors contributing to the risk of extinction of all European crayfish species (Alderman, 1996; Holdich et al., 2009; Jussila et al., 2021). However, in the past couple of decades, an increasing number of reports emerged indicating the existence of latently infected European crayfish populations, pointing to an evolution of the relationship between pathogen and new host (e.g., Viljamaa-Dirks et al., 2011; Kokko et al., 2012; Kušar et al., 2013; Maguire et al., 2016). The existence of infected but healthy European crayfish populations is due to both an increased resistance of some European crayfish populations and the decreased virulence of some *A. astaci* strains (Makkonen et al., 2012; Jussila et al., 2015, 2016). The present work is dedicated to understanding the mechanisms underlying the virulence variation observed in *A. astaci*, with particular focus on the loss of virulence, and to propose the development of new monitoring tools that leverage the genetic determinants of virulence variation of this pathogen.

In particular:

- In section ***6.1.*** I addressed the findings of **Chapter 2** and **Chapter 4** where, through experimental characterization of the virulence of *A. astaci*'s strains, I identified four strains with reduced virulence. Special attention was paid to the possible mechanisms underlying the loss of virulence;
- In section ***6.2.*** I analysed possible phenotypic determinants of the virulence variability of *A. astaci* strains. Based on the results from **Chapter 4**, I looked for a correlation between virulence and *in vitro* growth and sporulation;
- In section ***6.3.*** I addressed the findings of **Chapter 2** and **Chapter 3** to gain transcriptomic evidence of coevolution between the pathogen and European crayfish species. I then focused on the chitinase enzyme as a putative molecule responsible for the loss of virulence of some *A. astaci* strains. Furthermore, I suggested the exploitation of the data presented in **Chapter 4** and **Chapter 5** to assess the possible effect of coevolution on the chitinase genes;
- Section ***6.4.*** is based on the results from **Chapter 4**, where I looked at possible phenotypic differences between strains belonging to haplogroup A and haplogroup B.

The considered phenotypic traits are virulence and *in vitro* growth and sporulation. In the light of the results, I questioned the suitability of the available genotyping markers to address the full range of *A. astaci*'s genetic diversity;

- In section ***6.5.*** I presented the genomic data provided in **Chapter 5**, including genome assemblies of *A. astaci* strains based on Nanopore long-reads (3) and Illumina short-reads (11). I then suggested how to exploit these data, alone or in combination with the characterisation provided in **Chapter 4**, to better understand the adaptability and virulence variability of *A. astaci*. Finally, in section ***6.5.1.*** I specifically considered the contribution of these genomic data for an improved management of *A. astaci*.

6.1. The adaptation of *Aphanomyces astaci* to susceptible hosts: hints of different mechanisms leading to loss of virulence

The high variability in virulence documented in **Chapter 2** and **Chapter 4** suggests adaptation by the pathogen as a response to its naive European host. When *A. astaci* was first introduced into Europe, its virulence was extremely elevated (Alderman, 1996; Jussila et al., 2015). Therefore, the observed virulence variation when infecting susceptible crayfish indicates loss of virulence of the pathogen, likely to adapt to the new host. The results presented in **Chapter 2** and **Chapter 4** offer insights into the mechanisms underlying the loss of virulence of some *A. astaci* strains, with possible different mechanisms evolving based on the haplogroup. In summary, through infection experiments, I identified five strains that caused no mortality or very low mortality throughout the experimental period. As the crayfish used in the experiments were observed to be susceptible to other strains, I excluded that their survival was due to increased resistance of the crayfish, and instead concluded that I identified non-virulent strains. These strains are: the haplogroup A Venesjärvi strain (**Chapter 2**) and HapA-26, HapA-29, HapB-7 and HapB-67 (**Chapter 4**). The Venesjärvi strain tested in **Chapter 2** and HapA-29 from **Chapter 4** are likely the same strain, as they were isolated from the same crayfish, and there are no reasons to suspect the presence of multiple strains circulating in the lake inhabited by the infected crayfish. Therefore, in the following paragraphs, the Venesjärvi strain and HapA-29 will be discussed as one.

Based on several infection experiments involving haplogroup A strains, it has been suggested that some strains might have lost efficiency in moving through the first stages of infection, i.e., attachment, germination and the initial penetration of the host cuticle (Makkonen et al., 2012; 2014; Jussila et al., 2014). The results presented in **Chapter 4** appear to be consistent with the proposed hypothesis. I showed that HapA-26 is not virulent, with all the exposed crayfish surviving until the end of the experiment and showing no symptoms of infection. The cuticle of

the experimental crayfish was analysed microscopically, to observe the presence of hyphae or spores attached to its surface, and through qPCR, to assess the pathogen load within it. These analyses showed that among the crayfish infected with HapA-26 only two were positive for the presence of *A. astaci* in their cuticle. This is a clear indication of a decreased capability of the spores to infect the crayfish, probably because of problems in the attachment, germination or penetration of the cuticle. Furthermore, the two crayfish infected by this strain exhibited a very low pathogen load in their tissues, and no apparent hyphal growth was observed. This suggests that, while in some cases some spores retained the ability of attaching, germinating and penetrating the host's cuticle, the hyphae could not effectively colonise the host, probably due to an inability to contrast the host immune defence (i.e., the mechanical barrier represented by the cuticle or/end the inducible immune response).

The haplogroup A Venesjärvi strain, tested in **Chapter 2**, seems to have lost its virulence through a similar process. The strain only caused light signs of infection in the experimental *Astacus astacus* and caused no mortality. Thanks to the unconventional experimental setup, it was possible to gain some more precise insights into the mechanism of virulence loss of this strain. In fact, while usually crayfish are only tested for the presence of *A. astaci* at their death or at the end of the experiment, here, part of the crayfish was removed from the experimental system before the end of the experiment and subsequently tested. It was possible to observe that for both species of crayfish (i.e., *A. astacus* and *Procambarus virginalis*), only crayfish collected during the first sampling point three days post-challenge were positive for the presence of *A. astaci*. Crayfish sampled 21 and 45 days post-challenge were, instead, negative. While negative results do not necessarily indicate complete absence of infection, the discernible trend highlights a reduction in the pathogen load over time. This trend could be explained by spores attached to the cuticle that, unable to germinate or penetrate into the crayfish, ultimately detached themselves and were washed away by the circulating water of the experimental system. The transcriptome data analysed and presented in **Chapter 3** indicate that the immune system of *P. virginalis* was active three days post-challenge. This suggests a successful germination of the spores and a subsequent attempt of the pathogen to penetrate into the cuticle of the crayfish, which is when the interaction between pathogen and immune system of the host starts (Vazquez et al., 2009). The light symptoms of infection observed in *A. astacus* also support this hypothesis. Common signs of *A. astaci* infection, which usually lead to the death of the crayfish, are prolonged scratching of the abdominal cuticle and of the eyes, aimless movements of the walking legs (ataxia), loss of limbs and paralysis (Jussila et al., 2013; Boštjančić et al., 2023). Scratching and ataxia were both observed in some of the crayfish infected with the Venesjärvi strain. These symptoms were, however, much less pronounced than those observed in the crayfish infected with the highly virulent haplogroup B strain tested in the same experimental setting (**Chapter 2**). The scratching

is likely associated with the discomfort and itching that the germinating and growing pathogen causes to the host crayfish. Therefore, observation of these symptoms in the crayfish challenged with the Venesjärvi strain is an additional indication of the successful germination of the strain and of the attempts to penetrate into the host. Thus, based on the transcriptomic data and the described symptoms, it can be speculated that the spores were able to attach and germinate, but were mostly unable to efficiently penetrate into the cuticle of the crayfish. The spores ended up being washed away in the following days. Therefore, it seems that HapA-26 and the Venesjärvi strain have lost their virulence through similar, or even identical, processes, with a reduced capability of germinating/penetrating into the host.

While in the case of the above-mentioned haplogroup A strains, HapA-26 and the Venesjärvi strain, it can be hypothesised that the main cause of the virulence loss is the inability to infect the host, this does not seem to be the process through which all strains decrease their virulence. In **Chapter 4** two additional strains with reduced virulence were identified which belonged to haplogroup B (HapB-7 and HapB-67). In these two experimental groups, based on microscopic and qPCR analysis of the crayfish cuticle, almost all crayfish resulted infected, with hyphae clearly visible within the cuticles and agent levels mostly between A0 and A3. Here, the spores of the two strains were clearly able to germinate and penetrate the cuticle to a certain degree. It seemed, however, that the strains were not able to continue to grow, stopped by the cuticle or by the host inducible immune defence.

Considering these four examples of non-virulent strains, it seems that the loss of virulence might be achieved through different mechanisms depending on the haplogroup, with haplogroup A strains unable to germinate/penetrate and haplogroup B strains able to germinate and penetrate but unable to colonise the host. One of the factors influencing the development of different mechanisms to reduce virulence is certainly the history of the haplogroups in Europe. The analysed haplogroup A strains with reduced virulence (i.e., HapA-26 and the Venesjärvi strain) are likely descendants of the first strains arrived in Europe 150 years ago (Alderman, 1996). The two strains were isolated from Slovenia and Finland, respectively. Both these countries were reached by the first wave of crayfish plague epizootic and the only North American invasive crayfish in their territory is *Pacifastacus leniusculus*, that in Europe has ever only been associated with *A. astaci* haplogroup B and C (Kouba et al., 2014; Jussila et al., 2017; Ungureanu et al., 2020). Therefore, these two strains have been adapting to the new European host and habitat for over a century. On the other hand, strains belonging to haplogroup B have been introduced into Europe starting from the late 1960s (Huang et al., 1994; Jussila et al., 2015). Thus, the time available to haplogroup A and B to evolve and adapt to the new European host and environment has been very different. Additionally, during the first wave of crayfish plague in Europe, the strains were introduced without a resistant host, in contrast to the haplogroup B strains that were

introduced concomitantly with the resistant *P. leniusculus* (Alderman, 1996; Jussila et al., 2015). This led to vastly different selective pressures on the two haplogroups. The less virulent haplogroup A strains, less likely to cause the extinction of the host and therefore of the pathogen itself, were selected. In contrast, haplogroup B strains had in *P. leniusculus* a continuous reservoir, which mitigated the selective pressure to reduce their virulence (Jussila et al., 2014, 2015).

Interestingly, it has been observed that *P. leniusculus* is not well adapted to its new European environmental conditions, and several reports have emerged of stressed *P. leniusculus* populations showing symptoms of crayfish plague disease and facing mass mortalities (Jussila et al., 2014). Furthermore, the status of chronic carrier of *A. astaci* seems to have made *P. leniusculus* populations in Europe susceptible to new diseases (i.e., the eroded swimmeret syndrome, caused by the concomitant infection by *A. astaci* and *Fusarium* species) (Edsman et al., 2015; Jussila et al., 2021). Based on these findings, it is evident that the introduction of *A. astaci* alongside *P. leniusculus* may not enable the crayfish host to serve as a reservoir for *A. astaci*, especially in cases where the pathogen´s virulence remains elevated. This would result in an increased selective pressure towards *A. astaci* haplogroup B, which similarly to what has happened to the strains belonging to haplogroup A, would favour a lower virulence.

Based on these considerations, we could expect that as the selective pressure on haplogroup B strains increases, and with the prolonged presence of *A. astaci* haplogroup B in Europe, these strains might also lose part of their ability to geminate/penetrate into the host. However, at this stage, it is unclear if the observed loss of germination/penetration efficiency and of colonising the host are different steps of the same process, or are truly different processes of adaptation. Furthermore, we cannot discern if the different mechanisms of loss of virulence in the strains belonging to haplogroup A and B are solely due to the different selective pressure and evolution time in Europe, or also due to intrinsic genomic differences between the haplogroups (e.g., genes relevant for the virulence distributed in more or less dynamic parts of the genome; Derevnina et al., 2016). Comparative genomic studies of different *A. astaci* strains, enabled by the data presented in **Chapter 5**, will likely provide answers to these questions. Additionally, further insights might arise by analysing the evolution and adaptation of the other *A. astaci* haplogroups present in Europe.

6.2. *Aphanomyces astaci*'s virulence variability does not depend on *in vitro* growth and sporulation: are virulence factors its main determinants?

The trade-offs theory on the evolution of virulence among pathogens postulates that different components of the pathogen's fitness, such as growth and transmission, are tightly connected to virulence, and their evolution is interconnected (Frank, 1996; Cressler et al., 2015). The fitness of the pathogen increases with higher within-host growth and propagule production. However, as the pathogen grows and reproduces, it consumes host resources, causing damage to the host's tissues and ultimately incrementing the probability of the host death. The higher probability of host death, in turn, decreases the fitness of the pathogen itself (Frank, 1996; Pfennig, 2001; Cressler et al., 2015). From this derives the hypothesis that maximal fitness is achieved through a well-regulated trade-off between pathogen's growth and transmission, and virulence (Frank, 1996; Pfennig, 2001; Jussila et al., 2015).

The influence of sporulation and hyphal growth on the virulence variation in *A. astaci* strains has not received much attention by the scientific community. Until now, only two studies have been conducted on the topic. Both Unestam (1969) and Viljamaa-Dirks et al. (2016) observed a trend of positive correlation between growth of the pathogen and its virulence. However, due to the small sample size (i.e., three and four strains respectively) and similar origin of the strains, these results are rather inconclusive. However, in depth analysis of the correlation between sporulation, growth and virulence could have repercussions on both our understanding of the pathogen adaptability and the pathogen management. The adaptation of *A. astaci* to the new crayfish hosts has been the key for its success in Europe. In particular, it is assumed that the current observed virulence variability is a consequence of part of the strains adapting to the new host through decreased virulence. Insights on the relationship between virulence and connected phenotypes such as sporulation and growth could help predict the virulence of the newly introduced strains, supporting the management of this invasive pathogen.

The results presented in **Chapter 4** showed that neither *in vitro* growth nor sporulation of the pathogen are correlated with its virulence. These results might seem surprising based on the trade-offs theory on the evolution virulence (Frank, 1996; Pfennig, 2001; Cressler et al., 2015). A plausible explanation for this lack of correlation between virulence and *in vitro* growth could revolve around possible inconsistencies between *in vitro* and *in vivo* measurements of growth. In particular, the *in vitro* growth rate does not account for some of the fundamental stages of the infection process, such as attachment to the host, germination and interaction with the host's immune defences (Rezinciuc et al., 2015). Thus, different efficiency of the strains to move through these stages might render the *in vitro* growth rate less predictive of the strain's growth inside the crayfish and, therefore, of its virulence. However, the lack of correlation between virulence with *in vitro* growth and sporulation might also be indicative of a real biological signal.

While it is undoubtedly true that the pathogen's growth and transmission cause damage to the host (Pfennig, 2001), the final pathogenicity is often the result of several factors contributing to a pathogen´s virulence in a relative fashion (Casadevall & Pirofski, 2001). Therefore, in some cases, the influence of the growth and transmission on the overall virulence of the pathogen might be negligible, with the virulence mainly depending on the production of so-called "virulence factors", such as toxins or enzymes that disrupt the tissues, overcome the immune defences or compromise the metabolism of the host (Casadevall & Pirofski, 2001).

To explain the relationship between growth, sporulation and virulence factors, it is useful to look at the trade-offs theory in terms of "economy" of the resources (Frank, 1996; Casadevall & Pirofski, 2001; Schmid-Hempel, 2009). The pathogen has a limited amount of resources, extracted from the host, at its disposal. Therefore, the investment of resources into one fitness component can be detrimental for another. This leads to the resources being preferentially directed towards components that give higher fitness benefits (Frank, 1996; Schmid-Hempel, 2009). Thus, the expression of the virulence factors has a "fitness cost" which is bound to subtract resources from other parameters that are commonly associated with the fitness of the pathogen, such as growth and transmission (Diard & Hardt, 2017). Therefore, expression of these virulence factors might not be compatible with a simultaneous high speed of growth or elevated propagule production. In a study from 2017, McGowan & Fitzpatrick found that *A. astaci*'s genome is particularly enriched of genes encoding for putative virulence factors (i.e., proteins containing virulence-related domains) compared to other related oomycete species. In particular, *A. astaci*'s genome presents an expansion of important virulence factors such as Immunoglobulin A peptidases (hydrolytic enzymes with a putative function in suppressing the host immune response) and enzymes involved in the catabolism of the cellular components (e.g., peptidases, proteases and glycoside hydrolases). Because of its elevated number of genes coding for virulent factors and based on the lack of correlation between virulence and *in vitro* growth and sporulation, we can assume that for *A. astaci* the expression of these genes is what determines the virulence rather than its hyphal growth or propagule production. Additionally, in the case of the sporulation, it should also be considered that the main sporulation event is triggered when the host crayfish is moribund (Makkonen et al., 2013). It is therefore likely that the nutrient uptake that takes place to support the spore formation happens only when the host health is already fatally compromised. Finally, based on these considerations, I hypothesised that the virulence variability observed across *A. astaci* strains might depend on the pathogen's virulence factors, which might have different affinity for the host's target molecules or might be coded by genes having different expression or different copy number.

6.2.1. *Aphanomyces astaci*'s sporulation: why does its variability matter?

While it is not possible to draw a pattern of correlation between sporulation and virulence that is common to all the analysed strains, the results suggest that sporulation and virulence might be modulated together by the specific selective pressure acting on each strain. For example, two of the tested strains, HapA-26 and HapB-8, have both very high sporulation rates but opposite virulence (i.e., no mortality and 100% of mortality caused, respectively). These two strains clearly adapted to their introduction into a new environment in different ways. As mentioned previously, strain HapA-26, which was isolated from a latently infected European crayfish population in Slovenia (Jussila et al., 2017), has probably been co-existing with European crayfish for more than a century. The lack of virulence of this strain observed in **Chapter 4** indicates that HapA-26 adapted to its susceptible host by reducing its virulence. As the death of the host is the trigger for the main sporulation event of *A. astaci* (Makkonen et al., 2013), a lowly virulent strain might need to release a high number of spores to ensure its propagation, maximising the chance of transmission when the host moults or dies for unrelated causes. Additionally, as the strain seems to have a reduced capability of germinating and/or penetrating into the host, a high sporulation rate can increase the number of contacts with suitable hosts.

Conversely, strain HapB-8 has a very high virulence and sporulation rate. This combination of phenotypes might be very problematic for a pathogen, as it has the potential to wipe out the host population and, with it, cause its own extinction (Jussila et al., 2015). However, it must be pointed out that the virulence determination carried out in **Chapter 4** is based on the interaction of the pathogen with susceptible European crayfish. North American crayfish are, on the other hand, mainly resistant even to the most virulent strains. Strain HapB-8 was introduced into Lake Saimaa, Finland, with its original North American host *P. leniusculus* (Jussila et al., 2016). As in populations of resistant, latently infected crayfish the *A. astaci* prevalence tends to be low (e.g., Maguire et al., 2016; Mojžišová et al., 2022), *A. astaci* could benefit from maximising its sporulation at the moulting or death of the host to insure further transmission. This trait, coupled with the high virulence, effectively led to the eradication of the *A. astacus* population in Lake Saimaa (Jussila et al., 2016). However, as the lake was also inhabited by the resistant *P. leniusculus*, which acted as a pathogen reservoir, the combination of high virulence and high sporulation rate was not negatively selected. These two examples show that a relationship between *A. astaci*'s sporulation and virulence exists. However, this relationship is not one of causation, where a higher sporulation rate causes an increased mortality of the host and, therefore, a higher virulence of the strain. Sporulation and virulence are, instead, modulated together by the selective pressure, which favours combinations of these two traits that are adequate to the survival of the pathogen in specific scenarios.

When looking for signs of adaptation of *A. astaci* to its European host, the measured parameter has been the virulence itself, i.e., the capacity of a strain to cause mortality in crayfish. The sporulation potential of the strains has never received much attention. However, the aggressiveness of a pathogen is determined by both its virulence and capacity of transmission (Casadevall & Pirofski, 2001). Furthermore, as discussed in the previous paragraphs, sporulation might have a key role in shaping the way virulence variates in response to different selective pressures. Therefore, ignoring the variability of the sporulation potential of different strains might lead to an underestimation of the extent of the changes *A. astaci* faced to adapt to the new host. Obtaining additional understanding regarding the variability of sporulation as an adaptive mechanism could offer fresh perspectives in addressing the *A. astaci* challenge.

6.3. Evidence of coevolution of *Aphanomyces astaci* with the European host: is the future a bit brighter?

Since the end of the 1960s, it has been hypothesised that behind the resistance of North American crayfish to the disease caused by *A. astaci* was the coevolution between the two organisms (Unestam, 1969; Unestam & Weiss, 1970). Today, this theory is widely accepted by the scientific community, mainly supported by the evidence of different mechanisms of activation in North American and European crayfish species of the prophenoloxydase cascade, one of the main effectors of the immunity in invertebrates (Cerenius et al, 2003; 2009). The coevolutionary arms-race between host and pathogen shaped the virulence of the first and the resistance of the latter in an on-going process that constantly maintains balance (Brockhurst et al., 2014). In this context, when *A. astaci* was first introduced into Europe, it met a suitable and naïve host with no specific defence against it (Cerenius et al., 2003; Jussila et al., 2015). The observations of latently infected populations in the last decade and the virulence variability among *A. astaci* strains raised speculations of coevolutionary processes taking place between the pathogen and the new European hosts (Jussila et al., 2014, 2016). While there is still much unknown about this process, knowledge of what drives the coevolution would be of fundamental importance to formulate educated predictions for the future of the interactions between *A. astaci* and crayfish and coherent management actions.

The work presented in **Chapter 2** and **Chapter 3** offers molecular evidence for the adaptation of the pathogen to the susceptible host. In particular, *P. virginalis* was remarkably resistant to both haplogroup A (the Venesjärvi strain) and B of *A. astaci*. However, the experimental crayfish showed a clear activation of the immune response towards *A. astaci* Venesjärvi strain, while no clear immune response was detected when exposed to the highly virulent haplogroup B strain. As

described in **Chapter 2**, the Venesjärvi strain seems to have lost its ability to penetrate inside the host after decades of co-existence with *A. astacus* populations. This reduced virulence is presumably accompanied by a change in the pathogen's epitopes (i.e., part of the molecule recognised by the immune system of the host). These changes in the epitopes presented by *A. astaci* led in *P. virginalis* to a higher expression of pattern recognition receptors, PRRs, when compared to the haplogroup B-challenged *P. virginalis*. In fact, PRRs, which are responsible for the recognition of the pathogen, are connected to the coevolutionary history of the pathogen with its host (Hauton, 2012). Although invertebrates lack an adaptive immune system, they are able to develop an immune memory, altering the intensity of their immune response after consecutive encounters with the same pathogen (Melillo et al., 2018). Such response could either be of tolerance, with a lower immune response to known stimuli, or potentiation with a higher immune response after re-encountering the same pathogen (Melillo et al., 2018). As transgenerational immune priming, where the immune memory is transferred vertically, has been observed in invertebrates (Barribeau et al., 2016; Norouzitallab et al., 2016), it is possible that this mechanism is at the base of coevolution between crayfish and *A. astaci*. In this context, the high expression of PRRs in *P. virginalis* challenged with the Venesjärvi strain would indicate that the crayfish has never before encountered an *A. astaci* strain with those epitopes.

While *P. virginalis* is not a North American crayfish species, its evolutionary history overlaps, until very recently, with that of *Procambarus fallax* from Florida (Gutekunst et al., 2021). In fact, *P. virginalis* originated from a recent triploidisation event that occurred in *P. fallax* in the context of the pet trade in Europe (Chucholl, 2013; Lyko, 2017; Gutekunst et al., 2021). While there are no data relative to the presence of *A. astaci* in Florida, due to the widespread distribution of different haplotypes of *A. astaci* in the eastern USA (Martín-Torrijos et al., 2021), it is likely that *P. fallax* coevolved with one or more *A. astaci* strains. The developed broad resistance of *P. fallax* towards *A. astaci* was then inherited by *P. virginalis*. If we assume that *P. virginalis* (in the ancestral form of *P. fallax*) has met and coevolved with *A. astaci* haplogroup A (as this haplogroup is very widely distributed in the eastern USA; Martín-Torrijos et al., 2021), the immune response detected in *P. virginalis* against *A. astaci* haplogroup A would indicate that in the decades of coevolution with its European host, this strain changed its epitopes enough to be mostly unrecognisable by the immune system of the *P. virginalis*.

For the purpose of this speculation, it is important to note that *P. fallax* probably never encountered any *A. astaci* strain belonging to haplogroup B, as this haplogroup seem to only be distributed in western USA (Martín-Torrijos et al., 2021). If the above hypothesis about the reason for the presence of an immune response against haplogroup A is correct, it might then seem counterintuitive that the *P. virginalis* showed no immune response toward *A. astaci* haplogroup B, which was never before encountered by the crayfish nor by its progenitor. However, if the

changes in the epitopes of *A. astaci* haplogroup A are connected to the loss of virulence, we can expect that the mutational distance of these epitopes between the strains of *A. astaci* that were introduced into Europe and any strain that never left the USA is much bigger than the mutational distance between any two strains in the USA (or strains in Europe that did not yet adapt to the European host). The genomes of several oomycetes are known to evolve very quickly, mainly due to highly dynamic regions enriched with repeats and transposable elements (Derevnina et al., 2016). Genes associated with virulence are often found in such fast-evolving genomic regions, enabling the pathogen´s quick adaptation to environmental changes and new hosts (Derevnina et al., 2016). Furthermore, the fast proliferation of oomycetes that reproduce asexually (like in the case of *A. astaci*) increases the chances of spontaneous mutations, partially compensating for the lack of sexual recombination (Derevnina et al., 2016). Considering these premises, it is fair to hypothesise that in the span of 150 years in Europe, the epitopes of *A. astaci* haplogroup A changed enough to be perceived as a never-before encountered pathogen.

A candidate molecule that could have changed its epitope is the chitinase enzyme. This enzyme is probably connected to the virulence, as it has a role in the catabolism of the crayfish cuticle, and it has accumulated mutations likely to adapt to the new host (Makkonen et al., 2012). In a paper from 2012, Makkonen et al. studied the diversity of three chitinase genes belonging to the GH18 family across different *A. astaci* strains isolated in Europe. Here, it was found that the chitinase genes were more polymorphic within strains belonging to haplogroup A than within strains belonging to haplogroup B. This difference in polymorphisms is likely a consequence of the more recent introduction of haplogroup B strains into Europe compared to haplogroup A strains, and of the different selective pressures acting on them. It has been suggested that the abundance of polymorphisms is connected to the virulence variability of the strains. In fact, in the strains belonging to haplogroup A, one of the mutations in the CHI2C gene caused a translation stop codon in the catalytic domain, which might indicate loss of function in one of the chitinase genes of this haplogroup (Makkonen et al., 2012). Therefore, based on its variability in Europe within haplogroup A and on its connection with the virulence of the pathogen, the chitinase is a good putative molecule for the modified epitopes that are not recognised by *P. virginalis* in the results described in **Chapter 3**. Unfortunately, in the cited study, the analysed haplogroup A strains likely all derived from the first *A. astaci* introduction in the 19th century. As none of the haplogroup A strains recently introduced into Europe was included, it is not possible to confirm the connection between presence in Europe and increased variability of the chitinase genes. Therefore, it is not possible to confirm if the chitinase contains the epitopes not recognised by the *P. virginalis* in **Chapter 2** and **Chapter 3**. However, genomic data from haplogroup A strains recently introduced into Europe and strains directly isolated from the USA presented in **Chapter 5** can help shed some light into this issue. Leveraging these data with respect to the virulence

characterisation in **Chapter 4**, the analysis of the chitinase genes diversity can provide a quick and inexpensive way to obtain important insights on the role of this enzyme regarding the observed virulence variation and adaptability of *A. astaci*, and on the coevolution with the crayfish hosts. Furthermore, as the diversity of *A. astaci* in America is starting to gain more attention (Martín-Torrijos et al., 2021, 2023), new strains that never reached Europe will start to be available for molecular investigations, providing more information regarding the putative role of the chitinase, and of other molecules, in the evolution of *A. astaci* in Europe.

Finally, independently from the enzymes and other molecules involved in the adaptation of *A. astaci* to the European host, the reduced virulence of some of the pathogen's strains raises hope for the future co-existence of the crayfish and the pathogen in Europe. Other than the direct positive aspect of some pathogen strains not compromising the health and the viability of crayfish populations, the circulation of non-virulent strains might promote a partial immunization of the infected populations. As discussed previously in this section, repeated encounters with the same pathogen can lead to the development of an immune memory of that encounter, i.e., a natural "priming" of the host innate immune system (Melillo et al., 2018). It can then be speculated that infections with lowly virulent *A. astaci* strains might prime the crayfish, which, in turn, might become more resistant towards other more virulent pathogen strains. This very process could be at the base of the increased resistance observed in some European crayfish populations. The discovery of more resistant European crayfish populations and of lowly virulent *A. astaci* strains is still relatively new (i.e., the past 15 years; Makkonen et al., 2012; Martín-Torrijos et al., 2017; Martínez-Ríos et al., 2022). This, unfortunately, implies that this new type of interaction between European crayfish and pathogen, where the host does not ultimately die, are yet to be studied in depth. Future studies focused on the molecular mechanisms of the host-pathogen interaction could help understand the direction of this interaction in Europe, allowing for educated guesses of what to expect in the years to come.

6.4. Different but not that different: lack of significant phenotypic differences between *Aphanomyces astaci*'s haplogroups

For the most part of the last two decades, it has been widely accepted by the scientific community that different haplogroups of *A. astaci* are characterised by different virulence. In particular, infection experiments showed that haplogroup B appeared to always be extremely virulent towards European crayfish (or at least towards the more susceptible *A. astacus*), while haplogroup A has been observed to be more variable but, overall, less virulent (e.g., Makkonen et al., 2012, 2019; Becking et al., 2015; Jussila et al., 2015; Viljamaa-Dirks et al., 2016). In the study presented

in **Chapter 4**, where we focused on the phenotypical differences among haplogroup A and B, we demonstrated that not only *in vitro* sporulation and growth are not statistically different between these two haplogroups, but also virulence is not statistically different. While these results might be surprising when compared to the available literature, the lack of a clear difference in virulence between haplogroups can be explained by several factors, i.e., sampling biases, diverse origin of the analysed strains, coevolution, or inadequacy of the available genotyping tools applied to determine the haplogroups. Before the development of sensitive molecular method to detect the pathogen in crayfish tissues (i.e., qPCR directed towards the ITS region of *A. astaci*; Vrålstad et al., 2009), what attracted the attention of scientists to a new *A. astaci* strain was a mass mortality event within a crayfish population. This led to a clear sampling bias towards more virulent strains, which have also been the most studied. The scientific community has been able to overcome this bias only in the last 15 years, when the new molecular detection method allowed the identification of lately infected populations, increasing the probability of detecting and isolating less virulent strains of *A. astaci*. This is the case of two of the strains used in **Chapter 4**, which were isolated from latently infected populations. The discrepancy between our results and the literature is also likely the consequence of the higher diversity of strains used in our study compared to most of the literature on the topic. Recently, a survey conducted on the diversity of *A. astaci* in eastern USA highlighted a remarkable genetic diversity of the pathogen in its original range, with several newly identified haplotypes (Martín-Torrijos et al., 2021). However, most of the strains belonging to haplogroup A and B present in Europe have a similar origin (Jussila et al., 2015; 2016). The first introduction of *A. astaci* into Europe resulted in the spread of haplogroup A across most of continental Europe and Fennoscandia (Jussila et al., 2015; 2016). Until very recently, this first introduction was probably the only source of haplogroup A strains in Europe (Jussila et al., 2016). On the other hand, the introduction of haplogroup B into the continent was mainly part of a controlled re-stocking effort with *P. leniusculus* operated by the authorities, and the main source of the strains can be traced back to Lake Tahoe and Lake Hennessey (USA) (Jussila et al., 2015). As a result, the most studied strains belonging to haplogroup B are probably quite homogeneous in their traits. The more recently-introduced strains originating from different parts of the USA and carried by different crayfish species have started to be introduced through the pet trade in the past 20 years (Faulkes, 2015; Mrugała et al., 2015; Panteleit et al., 2017). However, these new haplogroups and strains are less widespread and, as such, much less studied (Ungureanu et al., 2020). Among the strains analysed in **Chapter 4**, we included strains isolated directly from the USA and at least two of the more recently-introduced strains, likely including a greater variability of genotypes/traits than what has been studied in the existing literature. Finally, the coevolution between pathogen and European crayfish, and between pathogen and North American crayfish in Europe, represents an ongoing adaptation process that is probably adding new variability among the pathogen strains at a speedy pace (Makkonen et al., 2012; Jussila et al., 2014). It can be

predicted that, with the passing years, as the strains continue to adapt to their new environment, more and more variability will be detected across the strains.

Another reason underlying the lack of phenotypic differences between *A. astaci* haplogroups is likely the unsuitability of the used genotyping markers to address the real diversity of this pathogen. As the mitochondrial markers rnnS (ribosomal-small subunit) and rnnL (ribosomal-large subunit) are the most used markers to genotype *A. astaci* strains (Makkonen et al., 2018), they will be the focus of this paragraph. These mitochondrial markers present several advantages compared to the other available markers: contrary to RFLP and AFLP they do not need pure *A. astaci* cultures, and can be directly used to genotype the pathogen from the infected crayfish tissues or environmental samples (Huang et al., 1994; Rezinciuc et al., 2014); they are more sensitive compared to nuclear DNA markers (i.e., chitinase genes and microsatellites) and work with relatively low amount of target DNA due to several copies of mtDNA being present in every cell (Makkonen et al., 2012; Grandjean et al., 2014). However, these markers present a core problem: they were developed based on the diversity of *A. astaci* in Europe (Makkonen et al., 2018). As the diversity of *A. astaci* in Europe is bound to increase, both because of new introduction from North America and because of coevolution with the new European host and the translocated North American host, the mitochondrial markers are probably going to become less and less efficient in detecting the full range of intraspecific diversity of *A. astaci*. Furthermore, the mtDNA are known to underestimate the diversity of *A. astaci* compared to the nuclear markers, as they are not capable of differentiating between RAPD-groups A and C (both grouped into haplogroup A), or between the microsatellite groups SSR-Up and SSR-A (both grouped into haplogroup A) (Grandjean et al., 2014; Makkonen et al., 2018). As a result, the phenotypic diversity across strains is probably much higher compared to the genotypic diversity detected by the mtDNA markers, which contributes to the lack of statistical differences of the virulence between haplogroup A and B detected in our analysis. Therefore, there is a clear need for an advanced genomic monitoring tool to assess the *A. astaci* diversity across Europe.

6.5. Characterization of the *Aphanomyces astaci* strains and their virulence: can genomic approaches open new horizons?

Until now, significant efforts were made by the scientific community to obtain an extensive experimental characterization of the virulence of different strains (including the work presented in **Chapter 4**), and to study specific enzymes that might affect virulence (i.e., chitinase genes; Anderson & Cerenius, 2002; Makkonen et al. 2012). The next and more comprehensive step to understand the basis of *A. astaci's* virulence variability is by genomic approaches. In **Chapter 5**

I produced three highly complete and contiguous genome assemblies of *A. astaci* strains, belonging to haplogroup A, B and E, based on Nanopore long-reads, alone or in combination with short-reads. As all the other publicly available assemblies of *A. astaci* genomes are based on Illumina reads (NCBI GenBank, last accessed 11 January 2024), these first assemblies of *A. astaci* strains that use Nanopore long-reads are bound to provide a better resolution of long stretches of repetitive elements compared to the available genome assemblies. Repetitive regions have been found to be key elements in the adaptability and virulence variation of some pathogenic oomycetes (Torres et al., 2020). These regions are prone to mutations, genomic rearrangements, duplication, and silencing, and can be the cause of a certain degree of genomic variability on which the selective pressure can act (Derevnina et al., 2016; Frantzeskakis et al., 2019; Torres et al., 2020). In future research, these Nanopore assemblies, with their higher resolution of the repetitive areas, could draw the attention towards more dynamic genomic regions, providing prime gene candidates connected to the adaptability and virulence variability in *A. astaci* (Jiang & Tyler, 2012; Derevnina et al., 2016; Engelbrecht et al., 2021).

In addition to the Nanopore assemblies, in **Chapter 5** I provided genome assemblies based on Illumina reads for 11 *A. astaci* strains of different origins (i.e., isolated from different waterbodies and crayfish species and/or in different years). These data, together with the already available genomes, can be used to build the backbone of an *A. astaci* pangenome. Genomic studies on a collection of strains can provide important insights both on the evolution and the virulence determinants of the pathogen (Sundin et al., 2016). The multiple-genome alignment at the base of the pangenome can inform us on what genes constitute the core genome, identical to all strains, and what genes constitute the accessory genome. Identifying the accessory genome, which is characterised by genes present only in a subset of strains, might be particularly insightful, as it often contains genes that codify for effectors correlated with the virulence of the pathogen (Croll & McDonald, 2012). The pangenome might also provide explanations for the phenotypic variability observed in *A. astaci* (e.g., the growth and sporulation variability observed in **Chapter 4**). In combination with phenotypic characterisation of the traits of interest, it could provide indications of specific variants that are correlated with the observed phenotypes. From a pangenome we can also gain information on the presence of dynamic genomic areas affected by positive selection, indication of variants that increase the fitness of the strain (McCann et al., 2013). Finally, these data can be used to investigate hints of sexual reproduction in *A. astaci*. While sexual reproduction has never been observed in this pathogen, two studies found possible indications for sexual recombination in the high number of single nucleotide polymorphisms (SNPs) detected within *A. astaci* genomes and in the variability observed within the chitinase genes (Makkonen et al., 2012; Jussila et al., 2016). Additionally, through a preliminary analysis of the data presented in **Chapter 5**, we also found possible indication of sexual reproduction in

the high number of observed heterozygosity compared to the expected heterozygosity in two *A. astaci* strains (i.e., HapA-26 and HapA-29; own unpublished results). Further analysis of these data might give us insights into the possibility of sexual reproduction for *A. astaci*.

Finally, the dataset presented in **Chapter 5** can be used to conduct a genome-wide association study (GWAS) to determine SNPs associated with virulence in *A. astaci.* In general, GWAS aims to identify genetic variants with significantly different frequencies between specimens with different phenotypes (Uffelmann et al., 2021). This type of analysis can be very insightful when investigating complex traits, such as virulence, that are expected to be polygenic (Visscher et al., 2017). In fact, GWASs have already been successfully employed to gain insights into the molecular mechanisms of pathogens' virulence (i.e., Dalman et al., 2013; Shakouka et al., 2022; Chen et al., 2023). The genomic data presented in **Chapter 5**, in combination with the phenotypic characterisation conducted in **Chapter 4,** constitute the first step towards a GWAS aimed to unveil the genomic loci correlated with the observed virulence variability in *A. astaci*.

Before conducting a GWAS on *A. astaci*, it is important to be aware of the challenges that it might pose. Firstly, due to the laborious task of isolating *A. astaci* strains and testing their virulence in a standardised way, the analysis is necessarily limited by a small sample size. As a GWAS evaluates tens of thousands to hundreds of thousands of SNPs, a small sample size is not able to counteract the large number of multiple comparisons which inflate the false positives rate (Hong & Park, 2012). Additionally, a small sample size increases the number of false negative rate, and therefore reduces the statistical power of the analysis (Hong & Park, 2012). Furthermore, this type of analysis is unlikely to detect the influence on the studied traits of rare alleles or SNPs with weak effect size, i.e., weak association between genetic variant and phenotype (Bartoli & Roux, 2017). However, even if the analysis might not detect all the loci correlated with virulence variability, we are likely to observe meaningful results if SNPs have a strong effect size (Hong & Park, 2012; Bartoli & Roux, 2017). Therefore, we can expect to gain important new insights that can draw the attention towards specific families of genes or biological processes. Further problems could arise if the linkage disequilibrium (LD) is high (Bartoli & Roux, 2017). High LD, which indicates the non-random association between alleles, can make the interpretation of the results quite tricky, as it is likely to result in a noisy background of SNPs associated with the phenotypic trait not because of causality, but because they are inherited together with the causally-associated SNPs (Allen et al., 2021). To date, we do not have an estimate of LD for *A. astaci*, however, high LD has often been observed for pathogenic species, especially when the pathogen reproduces asexually, as is the case for *A. astaci* (Bartoli & Roux, 2017; Demirjian et al., 2023). With that being said, even in the presence of a high LD, when there is evidence of phenotypic convergence, i.e., independent evolution of the same phenotypic trait across different lineages (e.g., the loss of virulence emerging within haplogroup A and B of *A. astaci*), GWASs can still be

effective (Allen et al., 2021). Furthermore, subsequent analyses (i.e., fine-mapping methodologies) can be successfully used to identify SNPs that are likely causally associated with the trait of interest (Schaid et al., 2018).

6.5.1. The prospective of new markers

European crayfish species are considered keystone species, and as such they influence the functioning and biodiversity of the ecosystem they inhabit (Reynolds et al., 2013). In particular, they have a significant impact on the food web by feeding on aquatic vegetation and invertebrates, and by being important food resources for other animals (Reynolds et al., 2013). Therefore, the extinction of European crayfish species, even only on a population level, has a wide range of repercussions that affect ecosystems as whole. Unfortunately, European crayfish species are threatened by several factors, such as climate change, pollution, habitat fragmentation, and invasive species (Holdich et al., 2009; Jussila et al., 2021). As *A. astaci* can quickly eradicate entire crayfish populations, especially when weakened by other factors, the management of this pathogen needs to be at the centre of programs for the conservation of European crayfish species and the preservation of aquatic ecosystems. As there are no efficient treatments to control the disease once a crayfish population has been infected (Rezinciuc et al., 2015), it is crucial to prevent the further spread of the pathogen.

Aphanomyces astaci is already widespread across most of Europe (Ungureanu et al., 2020), and the task of containing the further spread of each strain seems nothing short than unrealistic. However, as some of the pathogen's strains appear to be losing their virulence, partially or completely, not all strains need to be a priority for the management of the species, which should, instead, focus on the most virulent strains. Therefore, strain-specific assessments of the virulence would provide indications of the most relevant strains for management. However, to be able to exploit virulence assessments for systematic monitoring of the strains, the assessment itself needs to be precise and quick. As discussed in section ***6.4.***, the mitochondrial markers, or the other available markers, cannot fulfil this role, as the association between genotype and virulence, that has been so often observed, is collecting an increasing number of exceptions. On the other hand, infection experiments, which have been fundamental to characterise virulence in research settings, are too laborious and time-consuming to be used routinely. Therefore, new genomic tools are needed that allow to quickly infer the dangerousness of a newly-introduced strain.

In **Chapter 5** I presented genomic data that can be used to create new markers associated with the virulence. As discussed in section ***6.5.*** a GWAS that exploits these data can identify SNPs associated with the degree of virulence, following a binomial key of "high" and "low" virulence potential. These SNPs can represent the basis to create a SNP array available for monitoring.

These arrays contain specific probes, oligonucleotides containing the identified and selected SNPs associated with the specific trait (LaFramboise, 2009). Single strand DNA isolated from the tested sample hybridise with the probe if complementary, i.e., if the sample contain the specific SNP variant. Based on the pattern of hybridization, it will be possible to assess the virulence potential of an *A. astaci* strain. Furthermore, as the protocol is based on PCR amplification of genomic SNPs, it has the potential to be sensitive enough to test environmental samples (i.e., water). Currently, genotyping of *A. astaci* strains is severely limited by the elevated amount of pathogen DNA needed to successfully apply the different genotyping methods (Mojžišová et al., 2022). This implies the need to collect from the to-be-tested-crayfish more tissue than what is compatible with a non-destructive sampling procedure, i.e., the crayfish needs to be killed. However, killing the crayfish is not always possible, especially when monitoring endangered populations. In this context, markers that are able to genotype and characterise *A. astaci* strains by using environmental DNA would provide a feasible solution. Therefore, overall, the SNP array would represent a quick, sensitive and cheap tool to assess the dangerousness of *A. astaci* strains, allowing extended screening of waterbodies and ensuring speedy and focused decisions within management programs of *A. astaci*.

7. General conclusions

The methodological advancements of the last 15 years, such as the development of a sensitive qPCR assay capable of detecting low loads of *Aphanomyces astaci* DNA within crayfish tissues, have enabled the discovery of infected but healthy European crayfish populations (Vrålstad et al., 2009). The possible co-existence between *A. astaci* and European crayfish opened new, and brighter, scenarios for the future of the European crayfish species (Jussila et al., 2014). The interaction between pathogen and its European host is clearly evolving. Field and experimental evidence points to the adaptation of both involved parties, with some European crayfish populations acquiring a higher resistance to the pathogen, and some *A. astaci* strains lowering or losing their virulence (e.g., Viljamaa-Dirks et al., 2011; Kokko et al., 2012; Makkonen et al., 2012; Kušar et al., 2013; Maguire et al., 2016). It seems, therefore, that pathogen and European host are coevolving. However, very little is known about the genetic and phenotypic processes underlying the coevolution within this specific host-pathogen system. Crayfish are keystone species within freshwater ecosystems, and their conservation is vital to preserve biodiversity and functioning of the ecosystems they inhabit (Reynolds et al., 2013). Currently, *A. astaci* is one of the main threats to crayfish survival, and, unfortunately, this pathogen is here to stay (Jussila et al., 2021). Attempts to eradicate or limiting the spread of the pathogen have been mainly unsuccessful, and *A. astaci* has now colonised most of Europe (Ungureanu et al., 2020). However, the key of more efficient and focused management strategies for this invasive pathogen might hide within a deeper understanding of the processes underlying the evolution of the relationship between *A. astaci* and European crayfish.

With this in mind, the present work is dedicated to further the knowledge on the adaptation of *A. astaci* to its European host. In particular, I focused on understanding the processes underlying the loss of virulence of some *A. astaci* strains. I identified four different non-virulent strains belonging to two of the major *A. astaci* haplogroups, i.e., haplogroup A and B. The careful assessment of symptoms, transcriptomic evidence, within-host hyphal growth and pathogen load, led to the identification of possible mechanisms of loss of virulence. In particular, it appears that strains belonging to haplogroup A lost their efficiency to move through the very first stages of infection, with lower efficiency in germinating on the host and in starting the penetration of the cuticle. On the other hand, strains belonging to haplogroup B seem to be able to germinate and efficiently start the penetration process without, however, being able to successfully colonise the host. It is unclear if these different mechanisms of loss of virulence represent different stages of the same adaptation process, or different adaptation responses. Characterisation of more non-virulent strains, especially belonging to different haplogroups, might help gain insights into the potential diversity of the mechanisms of loss of virulence.

In an effort to identify possible phenotypic determinants of *A. astaci*'s virulence, I revealed that traits such as *in vitro* sporulation and growth are not statistically correlated with virulence, and, therefore, cannot explain the virulence variability observed among *A. astaci* strains. While a biological correlation between these traits is likely to exist, there are other factors that might play a more significant role in determining the virulence of *A. astaci*. In particular, the expression of the large pool of effector genes *A. astaci* possesses, which is likely incompatible with high growth and sporulation rates due to limited resources availability, might be what ultimately determines the virulence of the pathogen.

Through the present work, I provided transcriptomic evidence of coevolution taking place between European crayfish and *A. astaci*. In particular, the analysed data indicate an activation of the immune response of the invasive *Procamabrus virginalis* when challenged with a non-virulent *A. astaci* haplogroup A strain. This strain had co-existed with the susceptible *Astacus astacus* for at least 50 years in Lake Venesjärvi, and apparently adapted to the new host through reduced virulence. Based on this and on the likely interaction history of *P. virginalis* with other haplogroup A strains, I suggested that the Venesjärvi strain changed its epitopes to the extent of not being recognised by the immune system of *P. virginalis*. Therefore, it provoked an immune response compatible with a first encounter between host and pathogen. A putative enzyme that could have undergone these changes is the chitinase, which has been linked to both the pathogen's virulence and its adaptation in Europe.

Additionally, I showed that the two major *A. astaci* haplogroups, A and B, do not statistically differ in some of their key phenotypic traits, i.e., virulence and *in vitro* growth and sporulation. These results have direct repercussions on the management of *A. astaci*. It is, in fact, common practice to predict the virulence of a newly detected strains through assessments of the haplogroup of the strain. Here, I demonstrated that the correlation between virulence and haplogroup is not accurate, and new genetic markers are urgently needed to support management decisions.

Finally, based on the literature and the results obtained here, I suggested to focus the efforts to understand *A. astaci*'s virulence on comparative genomic studies. For this specific purpose, I provided highly complete genome assemblies based on Nanopore reads for three strains belonging to haplogroup A, B and E. Additionally, I provided 11 genome assemblies based on Illumina reads of strains belonging to haplogroup A and B. The Nanopore assemblies are bound to provide a better resolution of repetitive elements than the publicly available assemblies based on Illumina reads. As repetitive elements have been shown to have an impact on the adaptation and virulence variability of some oomycetes, these Nanopore assemblies can provide significant insights into the possible effect of repetitive elements on *A. astaci*'s virulence. Additionally, all the provided assemblies, together with the ones publicly available, can represent the backbone of an *A. astaci*

pangenome. The multi-genome alignment at the base of the pangenome can provide insights on the evolution, adaptation and possible sexual reproduction of *A. astaci*. Finally, the genomic data of **Chapter 5** and the phenotypic characterization of **Chapter 4**, can be used to conduct a GWAS to uncover the putative genomic loci correlated to virulence. The success of this kind of studies has the potential to revolutionize our understanding of the virulence of the pathogen and also the effectiveness of the available management tools. In particular, a GWAS will open up the possibility of creating SNP arrays based on genomic SNPs correlated with specific traits of *A. astaci*, such as virulence. Such SNP array could be used with high sensitivity on environmental DNA samples, overcoming the problematic relative to collecting and killing the crayfish for testing. Therefore, the SNP array would allow a quick, easy and efficient characterization of the strains based on a trait that is relevant for the management of the pathogen. This, in turn, would enable to pinpoint the most environmentally dangerous strains, i.e., the most virulent, and would allow quicker and more informed decision making.

Overall, in this work I chose to pursue phenotypic and genomic approaches, with a main focus on the pathogen's virulence, to uncover the processes defining the changes in the interaction between *A. astaci* and crayfish in Europe. However, these are not the only promising approaches. Further insights might come from the exploration of potential epigenetic changes influencing the virulence. Epigenetic changes are defined as stable and heritable gene expression changes that depend on alterations of the chromosome rather than of the DNA sequence (Berger et al., 2009). Epigenetic changes have been shown to play important roles in host-pathogen coevolution, as they have been connected to variation in virulence and rapid adaptation to the host in some oomycetes of the genus *Phytophthora* (Kasuga & Gijzen, 2013; Rojas-Rojas & Vega-Arreguín, 2021). While the possible epigenetic aspect of the adaptation of *A. astaci* to European crayfish has never been taken into account, this approach could bring new information and fresh prospectives to the study of the host-pathogen relationship. Finally, when approaching the study of a host-pathogen relationship, much more comprehensive information can be obtained by addressing the adaptation mechanisms that take place in both the host and the pathogen (Näpflin et al., 2019). The existence of both susceptible and more resistant European crayfish populations to *A. astaci* infections provide a great opportunity to uncover the genetic variants correlated with increased resistance in the crayfish. This type of evidence can further increase our understanding of the coevolutionary aspect of the relationship between host and pathogen. This approach is currently hindered by the lack of a reference genome for the host crayfish. However, as efforts are being made to produce a reference genome for *A. astacus*, it might soon be possible to concomitantly address the reciprocal adaptation of *A. astacus* and *A. astaci*.

To conclude, the present work represents a significant step forward in understanding the evolution of the relationship between *A. astaci* and European crayfish. Additionally, it lays the basis for

future work that can further our understanding of *A. astaci*'s virulence variability, with repercussions on the management of the pathogen and conservation of the crayfish species. However, much is still unknown and significant efforts need to be made to unravel the coevolutionary processes taking place between *A. astaci* and European crayfish species. On the other hand, in last few years the study of the crayfish immunity and the pathogen's virulence has gained more vigour, and more and more attention has been paid to the conservation of European crayfish species. Thus, we can expect important discoveries in the next years, and a brighter future for the co-existence of *A. astaci* and crayfish in Europe.

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Appendix

Status and author contributions of publications included in the thesis

Curriculum vitae

Declaration

Supplementary materials

Status and author contributions of publications included in the thesis

Chapter 2

C. Francesconi, J. Makkonen, A. Schrimpf, J. Jussila, H. Kokko, K. Theissinger (2021). Controlled infection experiment with *Aphanomyces astaci* provides additional evidence for latent infections and resistance in freshwater crayfish. Frontiers in Ecology and Evolution 9:647037. doi: 10.3389/fevo.2021.647037.

K.T. Conceptualisation; C.F., J.M., K.T. Investigation; C.F. Formal analysis; J.M., J.J., H.K., A.S., C.F Methodology; K.T. Funding acquisition; J.M., J.J., H.K., K.T. Supervision; A.S. provided the expertise on the handling of the samples; C.F., J.M., J.J., H.K., A.S., K.T Validation; C.F. Visualisation; C.F. Writing - original draft; C.F., K.T., J.J., J.M., A.S., H.K. Writing - review & editing. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Chapter 3

L.L. Boštjančić*, **C. Francesconi***, C. Rutz, L. Hoffbeck, L. Poidevin, A. Kress, J. Jussila, J. Makonnen, B. Feldmeyer, M. Bálint, O. Lecompte, K. Theissinger (2022). Host-pathogen coevolution drives innate immune response to *Aphanomyces astaci* infection in freshwater crayfish: transcriptomic evidence. BMC Genomics. doi: 10.1186/s12864-022-08571-z

K.T., C.F., J.J., J.M. Conceptualisation; Lj.L.B., A.K., C.R. Data curation; Lj.L.B., C.F., C.R., L. H, L.P. Formal analysis; K.T., M.B. Funding acquisition; C.F., J.J., J.M., K.T. Investigation; Lj.L.B., O.L., C.R., L. H, L.P., B.F. Methodology; K.T. Project administration; K.T., O.L., M.B. Resources; A.K., Lj.L.B, C.R. Software; O.L., K.T., M.B. Supervision; O.L., K.T., K.S., C.F., Lj.L.B. Validation; Lj.L.B. Visualisation; Lj.L.B., C.F. Roles/Writing - original draft; Lj.L.B., C.F., K.T., O.L., C.R., L.H., L.P., A.K., J.J., J.M., K.S., B.F., M.B. Writing - review & editing. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Chapter 4

C. Francesconi, L.L. Boštjančić, L. Bonassin, L. Schardt, C. Rutz, J. Makkonen, K. Schwenk, O. Lecompte, K. Theissinger (Submitted manuscript). High variation of virulence in *Aphanomyces astaci* strains lacks association with pathogenic traits and mtDNA haplogroups. Journal of Invertebrate Pathology.

C.F., J.M, K.T., Lj.L.B. Conceptualisation; C.F. Data curation; C.F. Formal analysis; K.T., O.L. Funding acquisition; Lj.L.B., C.F., Investigation; Lj.L.B., C.F., L.S. Methodology; K.T., O.L. Project administration; K.S., K.T. Resources; C.F. Software; K.S., K.T., O.L. Supervision; C.F., C.R., J.M., L.B., Lj.L.B., L.S., O.L., K.S., K.T. Validation; C.F. Visualisation; C.F. Writing - original draft. C.F., C.R., J.M., L.B., Lj.L.B., L.S., O.L., K.S., K.T. Writing - review & editing. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Chapter 5

C. Francesconi, L.L. Boštjančić, L. Bonassin, L. Schardt, S. Ploch, M. Thines, K. Schwenk, O. Lecompte, K. Theissinger (Manuscript in preparation). *De novo* assemblies of three *Aphanomyces astaci strains* based on Nanopore long-reads and 11 additional short-read assemblies

C.F., O.L., K.T., Lj.L.B. Conceptualisation; C.F. Data curation; C.F. Formal analysis. K.T., O.L. Funding acquisition; Lj.L.B., Lj.L.B., C.F., L.S., M.T., O.L, S.P Methodology; K.T., O.L. Project administration; K.S., K.T. Resources; C.F. Software; K.S., K.T., O.L. Supervision; C.F., L.B., Lj.L.B., L.S., S.P., M.T., K.S., K.T., O.L. Validation; C.F. Writing - original draft.

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Curriculum vitae

Caterina Francesconi

Education

Since 2019	PhD student in Molecular Ecology Institute for Environmental Sciences • University of Kaiserslautern-Landau, Germany
2015 - 2017	Master's Degree in Biomolecular and Environmental Sciences Department of Chemistry, Biology and Biotechnology • University of Perugia, Italy 110/100 *cum laude*
2011 - 2015	Bachelor's Degree in Biological Sciences Department of Chemistry, Biology and Biotechnology • University of Perugia, Italy 110/100 *cum laude*

Training

May 2022 – June 2022	Visiting researcher at the Laboratory for Biology and Molecular Genetics Department for Biochemical Engineering, Faculty of Food Technology and Biotechnology Science, University of Zagreb, Croatia.
December 2021 – December 2021	Visiting researcher at the Laboratory of Histology, Faculty of Science, University of Zagreb, Croatia
November 2021 – December 2022	Visiting researcher at the LOEWE Centre for Translational Biodiversity Genomics Senckenberg Biodiversity and Climate Research Centre, Frankfurt am Main, Germany.
August 2019 – October 2019	Visiting researcher at the Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland.
January 2018 – October 2018	Erasmus internship at the Laboratory of Conservation Genetics Institute for Environmental Sciences, University of Kaiserslautern-Landau, Germany.

Publications

- L.L. Boštjančić, P. Dragičević, L. Bonassin, **C. Francesconi**, A. Tarandek, C. Rutz, O. Lecompte, K. Theissinger (Submitted manuscript). Role of transcriptional factors C/EBP and Kr-h1 under immune challenge in *Astacus astacus* (Linnaeus, 1758). Aquaculture.
- L. Bonassin, L.L. Boštjančić, **C. Francesconi**, J. Paetsch, C. Rutz, O. Lecompte, L. Pârvulescu, K. Theissinger (Submitted manuscript). Conservation genomics of endemic crayfish species *Austropotamobius bihariensis* populations using reduced representation sequencing. BMC Ecology and Evolution.
- **C. Francesconi**, L.L. Boštjančić, L. Bonassin, L. Schardt, C. Rutz, J. Makkonen, K. Schwenk, O. Lecompte, K. Theissinger (Submitted manuscript). High variation of

virulence in *Aphanomyces astaci* strains lacks association with pathogenic traits and mtDNA haplogroups. Journal of Invertebrate Pathology.

- A. Thielsch, **C. Francesconi**, L.L. Boštjančić, C. Leeb, K. Theissinger (Accepted manuscript). The functional role of Daphnia in the host-pathogen interaction of crayfish and the crayfish plague disease agent (*Aphanomyces astaci*). Journal of Invertebrate Pathology.
- L.L. Boštjančić, **C. Francesconi**, L. Bonassin, S. Hudina, R. Gračan, I. Maguire, C. Rutz, A. Beck, A. Dobrović, O. Lecompte, K. Theissinger (2023). Temporal dynamics of the immune response in *Astacus astacus* (Linnaeus, 1758) challenged with *Aphanomyces astaci* Schikora, 1906. Fish and Shellfish Immunology. doi:10.1016/j.fsi.2023.109185
- C. Rutz, L. Bonassin, A. Kress, **C. Francesconi**, L.L. Boštjančić, D. Merlat, K. Theissinger, O. Lecompte (2023). Abundance and Diversification of Repetitive Elements in Decapoda Genomes. Genes. 14(8):1627. doi:10.3390/genes14081627.
- L.L. Boštjančić*, **C. Francesconi***, C. Rutz, L. Hoffbeck, L. Poidevin, A. Kress, J. Jussila, J. Makonnen, B. Feldmeyer, M. Bálint, O. Lecompte, K. Theissinger (2022). Host-pathogen coevolution drives innate immune response to *Aphanomyces astaci* infection in freshwater crayfish: transcriptomic evidence. BMC Genomics. doi: 10.1186/s12864-022-08571-z
- L.L. Boštjančić*, **C. Francesconi***, C. Rutz, L. Hoffbeck, L. Poidevin, A. Kress, J. Jussila, J. Makonnen, B. Feldmeyer, M. Bálint, O. Lecompte, K. Theissinger (2022). Dataset of the *de novo* assembly and annotation of the marbled and noble crayfish hepatopancreas transcriptomes. BMC Research Notes. doi: 10.1186/s13104-022-06137-6
- **C. Francesconi**, J. Makkonen, A. Schrimpf, J. Jussila, H. Kokko, K. Theissinger (2021). Controlled infection experiment with *Aphanomyces astaci* provides additional evidence for latent infections and resistance in freshwater crayfish. Frontiers in Ecology and Evolution 9:647037. doi: 10.3389/fevo.2021.647037.
- **C. Francesconi**, M. Pirvu, A. Schrimpf, R. Schulz, L. Parvulescu, K. Theissinger (2021). Mating strategies of invasive versus indigenous crayfish: multiple paternity as driver for invasion success? Freshwater Crayfish. doi: 10.1101/2021.05.28.445155.
- J. Jussila, **C. Francesconi**, K. Theissinger, H. Kokko, J. Makkonen (2021). Is *Aphanomyces astaci* losing its stamina: a latent crayfish plague disease agent from lake Venesjärvi, Finland. Freshwater Crayfish. doi: 10.5869/fc.2021.v26-2.139.

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Participation In International Conferences

- **C. Francesconi**, L.L. Boštjančić, L. Bonassin, L. Schardt, C. Rutz, J. Makkonen, O. Lecompte, M. Bálint, K. Schwenk, K. Theissinger. Characterisation of pathogenicity factors of a collection of *Aphanomyces astaci* strains. 5th Croatian Symposium On Invasive Species. November 27-28 2021, Zagreb, Croatia. (Oral presentation).
- **C. Francesconi**, L.L. Boštjančić, L. Bonassin, J. Makkonen, C. Rutz, B. Feldmeyer, M. Bálint, K. Schwenk, O. Lecompte, K. Theissinger. Whole-genome single nucleotide polymorphism analysis of an *Aphanomyces astaci* strain collection: searching for the genomic determinants of virulence – International Association of Astacology (IAA) Conference, September 5-8 2023, Pavia, Italy. Book of abstracts, p. 42. (Oral presentation).

- **C. Francesconi**, L.L. Boštjančić, L. Bonassin, J. Makkonen, S. Ploch, M. Thines, C. Rutz, A. Kress, O. Lecompte, K. Schwenk, K. Theissinger. Exploring *Aphanomyces astaci*'s virulence: variability and genomic determinants – International Association of Astacology (IAA) Conference, June 20-25 2022, Hluboka nad Vltavou, Czech Republic. Book of abstracts, p. 42. (Oral presentation).
- **C. Francesconi**, L.L. Boštjančić, L. Bonassin, J. Makkonen, A. Schrimpf, J. Jussila, H. Kokko, K. Schwenk, K. Theissinger. *Aphanomyces astaci* vs freshwater crayfish: a story of death, resistance and coevolution. 4th Croatian Symposium On Invasive Species. November 29-30 2021, Zagreb, Croatia. Book of abstracts, p. 44. (Oral presentation).
- **C. Francesconi**, M. Pîrvu, A. Schrimpf, R. Schulz, L. Pârvulescu, K. Theissinger. Mating strategies of invasive versus indigenous crayfish: multiple paternity and parthenogenesis as driver for invasion success? – International Association of Astacology (IAA) Conference August 27-31 2019, Gotland, Sweden. Book of abstracts, p. 17. (Poster).

Awards

- Special prize for innovation for "Whole-genome single nucleotide polymorphism analysis of an *Aphanomyces astaci* strain collection: searching for the genomic determinants of virulence" – IAA Pavia 2023
- Award for best poster presentation for "Mating strategies of invasive versus indigenous crayfish: multiple paternity and parthenogenesis as driver for invasion success?" – IAA Gotland 2019

Teaching experience

- Seminar "Phylogenetic and Population genetic analysis" held in February and September 2021 and September 2022, University of Kaiserslautern-Landau.

Courses

- Analysis of RNA sequencing data with R/Bioconductor - Physalia-courses. June 15-19 2020.
- Introduction to genome-wide association studies (GWAS) - Physalia-courses. June 21-25 2021.
- Genome assembly and annotations – Physalia-courses. March 14-18 2022.

Declaration

I here declare that the thesis entitled "Characterisation of *Aphanomyces astaci*'s virulence: from phenotype to genome" is the result of my own work, except where otherwise indicated.

All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective publication.

I have not used AI (Artificial Intelligence) tools while writing this work.

This work has not been submitted in any form for any scientific examination in Germany or any other country.

I am aware of and understand that violation of the aforementioned points may result in a revocation of the PhD title as well as in legal consequences.

Supplementary material – Chapter 2

Supplementary Table S1. Pairwise comparisons using Wilcoxon rank-sum test with continuity correction (without p-value correction). Aa, noble crayfish; Pv, marbled crayfish; A, *A. astaci* haplogroup A; B, *A. astaci* haplogroup B; Cont, control; 1, first sampling point, day 3; 2, second sampling point, day 21; 3, third sampling point, day 45.

	Aa-Cont	**Aa-A-1**	**Aa-A-2**	**Aa-A-3**	**Aa-B-1**	**Pv-Cont**	**Pv-A-1**	**Pv-A-2**	**Pv-A-3**	**Pv-B-1**	**Pv-B-2**
Aa-A-1	1.5e-06	-	-	-	-	-	-	-	-	-	-
Aa-A-2	-	0.00749	-	-	-	-	-	-	-	-	-
Aa-A-3	0.17911	0.00071	0.57161	-	-	-	-	-	-	-	-
Aa-B-1	1.5e-07	0.13543	0.00251	8.2e-05	-	-	-	-	-	-	-
Pv-Cont	-	1.5e-06	-	0.17911	1.5e-07	-	-	-	-	-	-
Pv-A-1	1.5e-06	0.09524	0.00749	0.00117	0.02290	1.5e-06	-	-	-	-	-
Pv-A-2	-	0.00749	-	0.57161	0.00251	-	0.00749	-	-	-	-
Pv-A-3	0.17911	0.00071	0.57161	1.00000	8.2e-05	0.17911	0.00297	0.57161	-	-	-
Pv-B-1	1.5e-06	0.22222	0.00749	0.00071	0.05490	1.5e-06	0.22222	0.00749	0.00071	-	-
Pv-B-2	1.5e-06	0.69048	0.00749	0.00071	0.06453	1.5e-06	0.05556	0.00749	0.00071	0.5476	-
Pv-B-3	1.2e-06	0.95305	0.00539	0.00038	0.05677	1.2e-06	0.12920	0.00539	0.00038	0.25441	0.67865

Supplementary Table S2. Pairwise comparisons using Wilcoxon rank-sum test with continuity correction (p-value adjustment method: Benjamini–Hochberg). Aa, noble crayfish; Pv, marbled crayfish; A, *A. astaci* haplogroup A; B, *A. astaci* haplogroup B; Cont, control; 1, first sampling point, day 3; 2, second sampling point, day 21; 3, third sampling point, day 45.

	Aa-Cont	**Aa-A-1**	**Aa-A-2**	**Aa-A-3**	**Aa-B-1**	**Pv-Cont**	**Pv-A-1**	**Pv-A-2**	**Pv-A-3**	**Pv-B-1**	**Pv-B-2**
Aa-A-1	7.5e-06	-	-	-	-	-	-	-	-	-	-
Aa-A-2	-	0.01249	-	-	-	-	-	-	-	-	-
Aa-A-3	0.22389	0.00194	0.61244	-	-	-	-	-	-	-	-
Aa-B-1	4.4e-06	0.18467	0.00602	0.00035	-	-	-	-	-	-	-
Pv-Cont	-	7.5e-06	-	0.22389	4.4e-06	-	-	-	-	-	-
Pv-A-1	7.5e-06	0.13605	0.01249	0.00304	0.03713	7.5e-06	-	-	-	-	-
Pv-A-2	-	0.01249	-	0.61244	0.00602	-	0.01249	-	-	-	-
Pv-A-3	0.22389	0.00194	0.61244	1.00000	0.00035	0.22389	0.00686	0.61244	-	-	-
Pv-B-1	7.5e-06	0.26667	0.01249	0.00194	0.08515	7.5e-06	0.26667	0.01249	0.00194	-	-
Pv-B-2	7.5e-06	0.71429	0.01249	0.00194	0.09443	7.5e-06	0.08515	0.01249	0.00194	0.61244	-
Pv-B-3	7.5e-06	0.96920	0.01155	0.00142	0.08515	7.5e-06	0.18028	0.01155	0.00142	0.29931	0.71429

Supplementary Table S3. qPCR results.

Species	*A. astaci* haplogroup	Sampling date	ID Number	C_t-value	PFU	GroupID
Marbled crayfish	A	13-set	**19**	45,05	0,02	Pv-A-1
			46	36,52	6,38	Pv-A-1
			69	47,62	0,00	Pv-A-1
			72	45,44	0,02	Pv-A-1
			75	35,76	10,60	Pv-A-1
		01-ott	**9**	55,00	0,00	Pv-A-2
			16	55,00	0,00	Pv-A-2
			80	55,00	0,00	Pv-A-2
			93	55,00	0,00	Pv-A-2
			115	55,00	0,00	Pv-A-2
		24-ott	**109**	55,00	0,00	Pv-A-3
			25	55,00	0,00	Pv-A-3
			37	55,00	0,00	Pv-A-3
			53	55,00	0,00	Pv-A-3
			5	55,00	0,00	Pv-A-3
			28	55,00	0,00	Pv-A-3
			49	55,00	0,00	Pv-A-3
			89	55,00	0,00	Pv-A-3
			110	55,00	0,00	Pv-A-3
			62	44,52	0,03	Pv-A-3
Noble crayfish	A	13-set	**83**	32,06	129,00	Aa-A-1
			27	36,07	8,61	Aa-A-1
			33	32,57	91,50	Aa-A-1
			50	36,09	8,49	Aa-A-1
			70	37,83	2,62	Aa-A-1
		01-ott	**43**	55,00	0,00	Aa-A-2
			15	55,00	0,00	Aa-A-2
			40	55,00	0,00	Aa-A-2
			68	55,00	0,00	Aa-A-2
			87	55,00	0,00	Aa-A-2
		24-ott	**92**	55,00	0,00	Aa-A-3
			97	55,00	0,00	Aa-A-3
			116	55,00	0,00	Aa-A-3
			114	55,00	0,00	Aa-A-3
			58	55,00	0,00	Aa-A-3
			65	55,00	0,00	Aa-A-3
			90	55,00	0,00	Aa-A-3
			106	55,00	0,00	Aa-A-3
			7	55,00	0,00	Aa-A-3
			117	47,44	0,00	Aa-A-3
Marbled crayfish	Control	13-set	**6**	55,00	0,00	Pv-Cont
			20	55,00	0,00	Pv-Cont
			23	55,00	0,00	Pv-Cont
			61	55,00	0,00	Pv-Cont
			71	55,00	0,00	Pv-Cont
		01-ott	**32**	55	0,00	Pv-Cont
			91	55,00	0,00	Pv-Cont
			100	55,00	0,00	Pv-Cont
			102	55,00	0,00	Pv-Cont
			120	55,00	0,00	Pv-Cont
		24-ott	**17**	55,00	0,00	Pv-Cont
			39	55,00	0,00	Pv-Cont
			59	55,00	0,00	Pv-Cont
			104	55,00	0,00	Pv-Cont
			95	55,00	0,00	Pv-Cont
			112	55,00	0,00	Pv-Cont
			42	55,00	0,00	Pv-Cont
			52	55,00	0,00	Pv-Cont
			85	55,00	0,00	Pv-Cont
			98	55,00	0,00	Pv-Cont
Noble crayfish	Control	13-set	**14**	55,00	0,00	Aa-Cont
			31	55,00	0,00	Aa-Cont
			34	55,00	0,00	Aa-Cont
			79	55,00	0,00	Aa-Cont

			86	55,00	0,00	Aa-Cont
		01-ott	**10**	55,00	0,00	Aa-Cont
			21	55,00	0,00	Aa-Cont
			76	55,00	0,00	Aa-Cont
			108	55,00	0,00	Aa-Cont
			119	55,00	0,00	Aa-Cont
		24-ott	**3**	55,00	0,00	Aa-Cont
			22	55,00	0,00	Aa-Cont
			55	55,00	0,00	Aa-Cont
			66	55,00	0,00	Aa-Cont
			81	55,00	0,00	Aa-Cont
			18	55,00	0,00	Aa-Cont
			44	55,00	0,00	Aa-Cont
			51	55,00	0,00	Aa-Cont
			73	55,00	0,00	Aa-Cont
			101	55,00	0,00	Aa-Cont
Marbled crayfish	B	13-set	**13**	32,91	72,50	Pv-B-1
			29	39,22	1,03	Pv-B-1
			45	42,04	0,15	Pv-B-1
			60	41,93	0,17	Pv-B-1
			78	35,25	15,00	Pv-B-1
		01-ott	**8**	40,17	0,54	Pv-B-2
			30	36,35	7,11	Pv-B-2
			77	35,65	11,40	Pv-B-2
			103	35,00	17,70	Pv-B-2
			111	34,13	31,90	Pv-B-2
		24-ott	**26**	39,26	1,00	Pv-B-3
			47	34,34	27,80	Pv-B-3
			64	32,14	123,00	Pv-B-3
			88	35,89	9,70	Pv-B-3
			4	55,00	0,00	Pv-B-3
			11	37,72	2,82	Pv-B-3
			35	31,19	233,00	Pv-B-3
			67	31,69	166,00	Pv-B-3
			84	38,13	2,15	Pv-B-3
			96	30,87	289,00	Pv-B-3
Noble crayfish	B	13-set	**99**	55,00	0,00	Aa-B-1
			105	30,21	451,00	Aa-B-1
			1	35,94	9,43	Aa-B-1
			2	26,61	5111,00	Aa-B-1
			12	31,42	199,00	Aa-B-1
			36	39,29	0,98	Aa-B-1
			38	33,06	65,60	Aa-B-1
			41	25,23	12949,00	Aa-B-1
			48	29,96	533,00	Aa-B-1
			54	28,72	1235,00	Aa-B-1
			56	55,00	0,00	Aa-B-1
			57	39,55	0,82	Aa-B-1
			63	25,67	9648,00	Aa-B-1
			74	29,28	842,00	Aa-B-1
			82	33,96	35,70	Aa-B-1
			94	25,86	8473,00	Aa-B-1
			107	26,52	5435,00	Aa-B-1
			113	30,74	314,00	Aa-B-1
			118	27,42	2959,00	Aa-B-1
		16-set	**24**	-	-	Aa-B-1

Supplementary Table S4. Symptoms observed each day. The table shows all the noble crayfish belonging to both *A. astaci* haplogroup A and B challenged groups and the only marbled crayfish that showed symptoms during the experiment. Aa-A, *A. astaci* haplogroup A challenged noble crayfish; Aa-B, *A. astaci* haplogroup B challenged noble crayfish; Pv-B, *A. astaci* haplogroup B challenged marbled crayfish; S, scratching; L, aimless movements of the legs.

		Specimen ID																																								
		Aa-A																				Pv-B	Aa-B																			
Date	Days post challenge	7	15	27	33	40	43	50	58	65	68	70	83	87	90	92	97	106	114	116	117	60	1	2	12	24	36	38	41	48	54	56	57	63	74	82	94	99	105	107	113	118
10-set	1																														S	S										
11-set	2																					S			S		S			S		S		S			S	S	S		S	S
12-set	3																						S	S	S		S/L	S	S	S	S	S	S	S/L	S	L	S	S		S	S	
13-set	4																																									
14-set	5	S							S																	S																
15-set	6	S				S / L																																				
16-set	7																																									
17-set	8																																									
18-set	9															S																										
19-set	10		S			S																																				
20-set	11																																									
21-set	12																																									
22-set	13																																									
23-set	14																																									
24-set	15		S							S	S			L																												
25-set	16	S	S				L																																			
26-set	17	S					S																																			
27-set	18																																									
28-set	19																																									
29-set	20										L			S																												
30-set	21																																									
01-ott	22																																									
02-ott	23																																									
03-ott	24																S				S																					
04-ott	25																																									
05-ott	26																S																									
06-ott	27																																									
07-ott	28																																									
08-ott	29																																									
09-ott	30																																									
10-ott	31															S																										
11-ott	32																																									
12-ott	33																																									
13-ott	34																																									
14-ott	35																																									
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21-ott	42																																									
22-ott	43																																									
23-ott	44																																									
24-ott	45																																									

Supplementary material – Chapter 3

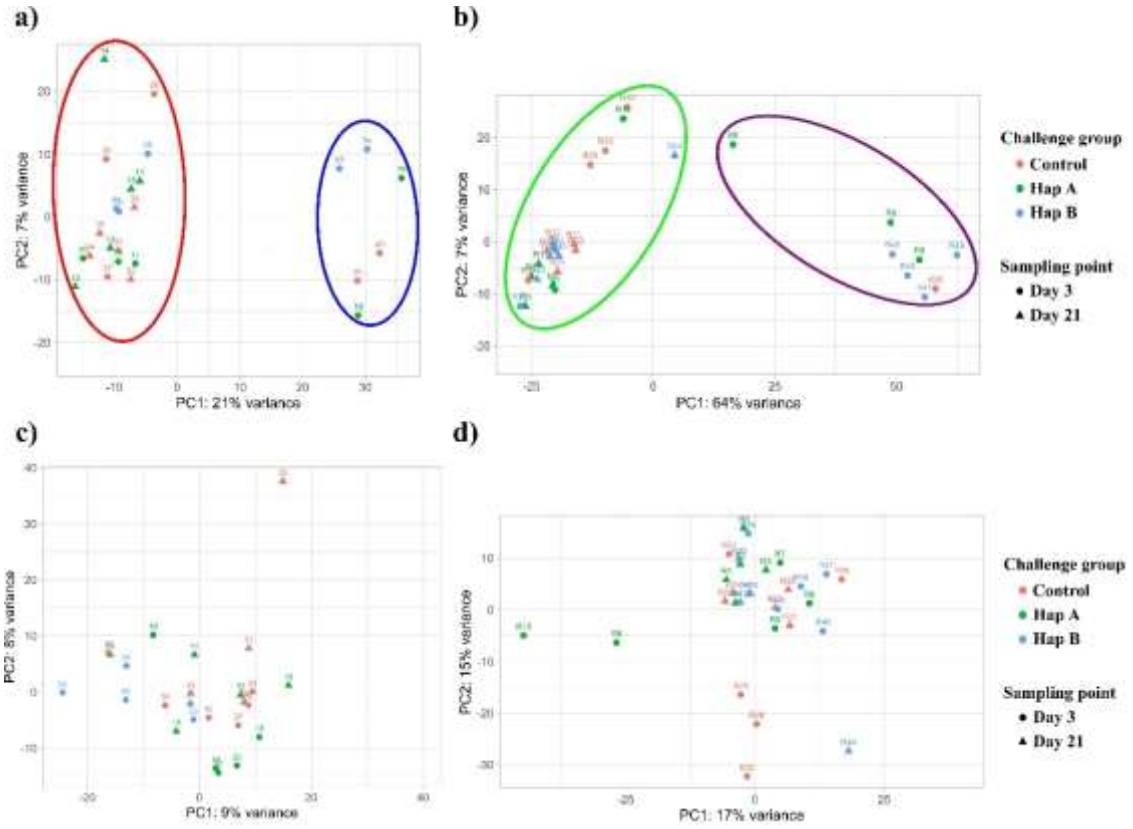
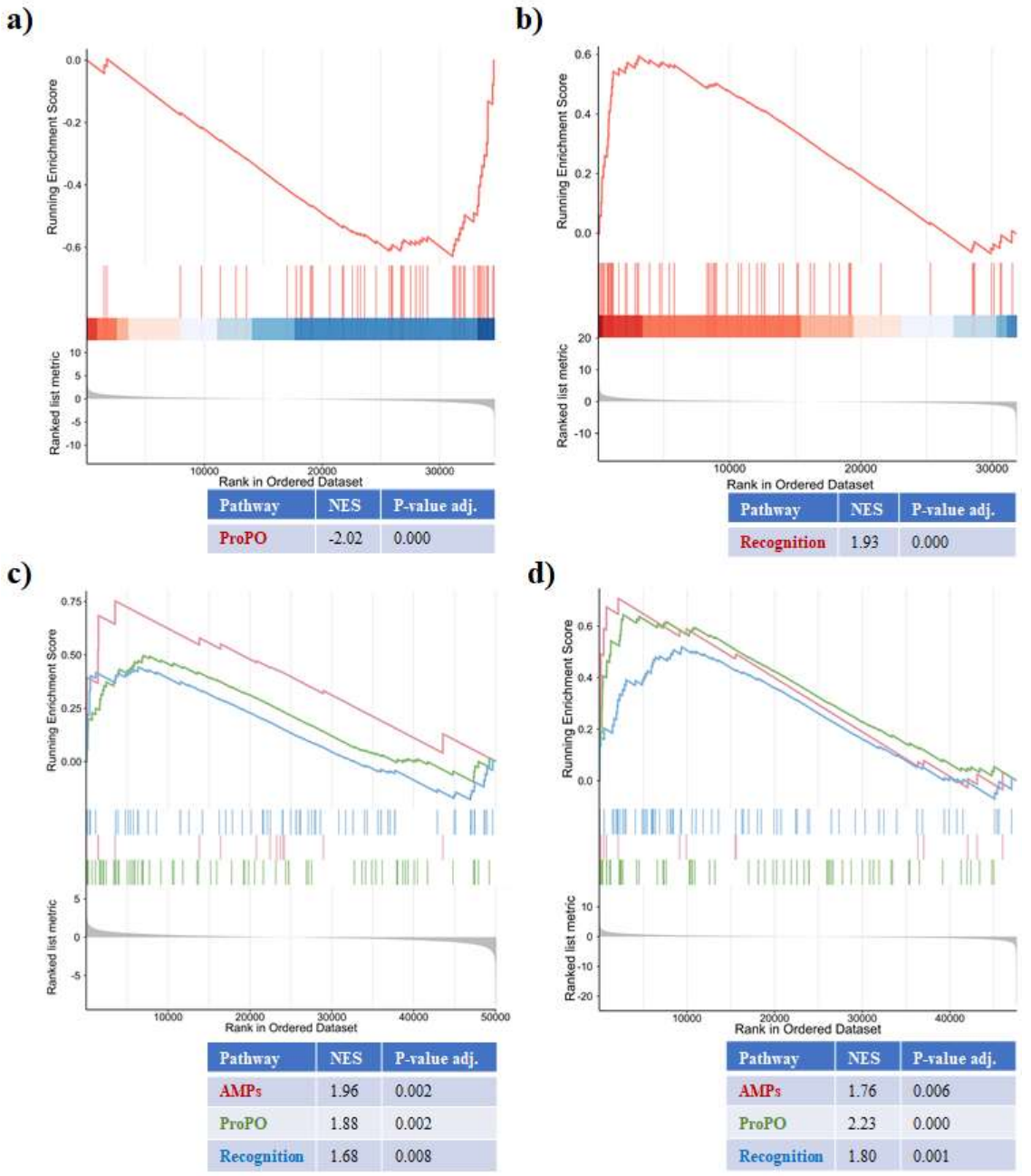


Figure S1. Results of the principal component analysis (PCA) analysis for (a) noble crayfish and (b) marbled crayfish on the rlog transformed datasets, indicating batch effect related to differences between males (blue) and females (red) in noble crayfish and reproduction (reproducing- green, non-reproducing- purple) in marbled crayfish. The PCA with batch effect removal using removeBatchEffect() function implemented in limma R package (Ritchie et al., 2015) for (c) noble crayfish and (d) marbled crayfish.



Pathway	NES	P-value adj.
ProPO	-2.02	0.000

Pathway	NES	P-value adj.
Recognition	1.93	0.000

Pathway	NES	P-value adj.
AMPs	1.96	0.002
ProPO	1.88	0.002
Recognition	1.68	0.008

Pathway	NES	P-value adj.
AMPs	1.76	0.006
ProPO	2.23	0.000
Recognition	1.80	0.001

Figure S2. Results of the Gene set enrichment analysis for (a) Hap A challenged noble crayfish (Day 3), (b) Hap A challenged noble crayfish (Day 21), (c) Hap B challenged marbled crayfish (Day 3), (d) Hap B challenged marbled crayfish (Day 21). Adjusted p- values, and Normalized enrichment scores (NES) are shown. AMPs- antimicrobial *peptides, ProPO- prophenoloxidase pathway.*

The remaining Supplementary material of Chapter 3 are available only digitally at https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-022-08571-z due size or extension of the files incompatible with a printed version.

Additional supplementary materials are:

Tables S1. List of sequences used in the BLAST analysis for identification of the innate immunity genes in noble and marbled crayfish and their respective gene accession numbers.

Table S2. Innate immunity genes identified through the BLAST search with their respective match length, %identity, e- values and Dammit! annotations in the noble crayfish.

Table S3. Innate immunity genes identified through the BLAST search with their respective match length, %identity, e- values and Dammit! annotations in the marbled crayfish.

Table S4. Raw and post pre-processing Illumina sequence data statistics and mapping results of the read pseudo-alignment with Salmon against the de novo assembled transcriptome assemblies for noble crayfish and marbled crayfish.

Table S5. List of differentially expressed genes in the response of the noble crayfish to the challenge with *A. astaci*.

Table S6. List of differentially expressed genes and their respective annotations in the response of the marbled crayfish to the challenge with *A. astaci*.

File S1. FASTA sequences used in the BLAST analysis for identification of the innate immunity genes in noble crayfish and marbled crayfish.

File S2. FASTA sequences of the innate immunity related transcripts identified through the BLAST analysis in the noble crayfish.

File S3. FASTA sequences of the innate immunity related transcripts identified through the BLAST analysis in the marbled crayfish.

Supplementary material – Chapter 4

Supplementary data 1

Table S1. For each crayfish, the experimental group, crayfish ID, carapax length (mm) and sex are reported.

Group	Crayfish ID	Carapax length (mm)	Sex
Cont1	1	28,43	F
	2	25,59	M
	3	27,65	M
	4	25,66	F
	5	24,92	M
	6	23,64	M
	7	22,4	F
	8	21,25	F
	9	24,2	M
Cont2	161	25	F
	162	26	F
	163	24	F
	164	27	M
	165	28,5	F
	166	28,2	F
	167	25,2	F
	168	23	F
	169	26,5	M
	170	26	F
HapB-1	111	23	F
	112	24,5	M
	113	26,2	F
	114	25,4	M
	115	24,3	F
	116	26,3	F
	117	27	M
	118	24,5	F
	119	23,5	F
	120	23	M
	125a	25,29	M
HapB-4	151	25,21	F
	152	19,34	M
	153	25,52	F
	154	20,47	F
	155	22,65	M
	156	30,42	M
	157	25,31	M
	158	21,59	F
	159	20,03	F
	160	23,3	F
HapB-6	121	24,59	F
	122	24,15	M
	123	24,15	M
	124	25,5	F
	125b	NA	NA
	126	23,06	F
	127	28,34	M
	128	26,55	M
	129	20,24	F
	130	25,14	M
HapB-7	31	22,59	F
	32	25,43	M
	33	24,23	M
	34	27	F
	35	19,56	F
HapB-7	36	22,31	M
	37	25,93	F
	38	20,96	F
	39	26,38	F
	40	20,76	M
HapB-8	141	22,68	M
	142	22,67	M
	143	21,8	M
	144	18,56	F
	145	20,46	M
	146	20,4	M
	147	21,18	M
	148	23,91	M
	149	22,09	F
	150	22,95	F
HapB-12	131	21,87	F
	132	24,34	M
	133	25,95	F
	134	22,02	M
	135	22,86	M
	136	23,2	M
	137	21,44	F
	138	21,9	F
	139	24,26	M
	140	22,52	M
HapB-13	181	26	M
	182	25,5	M
	183	25	F
	184	25	F
	185	26,5	F
	186	25	F
	187	24	F
	188	27	F
	189	25	F
	190	27,5	F
HapB-67	21	24,08	M
	22	27,19	F
	23	25,3	M
	24	25,08	M
	25	24,03	F
	26	22,63	M
	27	23,03	M
	28	25,52	F
	29	22,2	F
	30	19,95	F
HapA-26	41	23,32	M
	42	24,42	M
	43	26,93	F
	44	25,05	F
	45	25,62	M
	46	25,41	F
	47	21,28	M
	48	25,33	F
	49	25,57	M
	50	20,04	M
HapA-29	51	26,58	M
	52	25,53	M
	53	27,07	M
	54	25,61	M
	55	24,55	F
	56	20,04	F
	57	25,83	M
	58	25,56	M
	59	26,17	M
	60	26,81	M
HapA-34	101	31	M
	102	62	M
	103	29	M
	104	26	M
	105	26,1	M
	106	23,5	F
	107	25,9	M
	108	26	M
	109	28,7	M
	110	24,5	M
HapA-36	10	21,84	F
	11	26,23	F
	12	24,38	M
	13	27,13	F
	14	26,32	F
	15	24,87	F
	16	29,99	F
	17	26,24	M
	18	21,76	F
	19	26,5	M
	20	20,96	F
HapA-78	61	24,48	F
	62	25,2	F
	63	25,98	F
	64	25,67	M
	65	23,62	M
	66	20,18	F
	67	23,29	M
	68	23,62	M
	69	27,02	F
	70	23,58	M
HapE-75	91	20,26	M
	92	21,34	M
	93	21,29	F
	94	26,19	M
	95	21,91	M
	96	21,32	M
	97	23,73	M
	98	25,26	M
	99	22,42	F
	100	23	F

Table S2. Radius (mm), area (mm^2) and increment of areas (mm^2) are reported for the three replicates of each strain in the different time points.

Strain ID	Time (h)	Radius (mm)	Area (mm^2)	Increment of Area (mm^2)
HapB-1	48	10,81	366,77	366,77
		10,25	330,06	330,06
		9,49	282,93	282,93
	96	20,34	1299,72	932,95
		20,82	1361,14	1031,08
		20,29	1292,71	1009,77
	144	30,28	2880,46	1580,73
		30,82	2984,11	1622,97
		30,51	2924,38	1631,68
	192	40,39	5125,04	2244,58
		40,98	5275,86	2291,75
		40,46	5141,55	2217,17
HapB-4	48	9,54	285,92	285,92
		9,59	288,62	288,62
		8,92	249,97	249,97
	96	19,68	1216,13	930,21
		19,63	1209,95	921,33
		18,23	1044,05	794,09
	144	29,85	2798,29	1582,16
		29,29	2695,18	1485,23
		29,30	2697,02	1652,97
HapB-6	48	10,82	367,45	367,45
		12,22	468,74	468,74
		11,87	442,64	442,64
	96	21,33	1429,33	1061,87
		21,07	1394,03	925,29
		20,47	1316,39	873,75
	144	30,12	2849,15	1419,82
		31,05	3028,81	1634,78
		31,06	3030,77	1714,37
HapB-7	48	11,12	388,47	388,47
		11,19	393,03	393,03
		9,84	304,19	304,19
	96	18,01	1018,44	629,97
		18,15	1034,34	641,31
		16,27	831,62	527,43
	144	25,78	2087,12	1068,68
		25,18	1991,87	957,53
		25,31	2011,70	1180,08
HapB-8	48	10,66	357,00	357,00
		11,14	389,52	389,52
		10,78	365,08	365,08
	96	19,33	1173,85	816,86
		19,37	1178,11	788,59
		19,40	1182,37	817,29
	144	27,43	2362,89	1189,03
		28,18	2493,89	1315,78
		27,54	2382,74	1200,38
HapB-12	48	11,33	403,28	403,28
		10,86	370,52	370,52
		11,44	411,15	411,15

Strain ID	Time (h)	Radius (mm)	Area (mm^2)	Increment of Area (mm^2)
HapA-26	48	10,35	336,21	336,21
		10,29	332,32	332,32
		9,55	286,22	286,22
	96	16,94	901,52	565,31
		17,09	917,56	585,24
		17,18	926,71	640,49
	144	26,54	2212,85	1311,32
		26,78	2253,05	1335,49
		26,30	2172,18	1245,47
	192	35,04	3857,25	1644,40
		35,09	3867,16	1614,11
		35,07	3863,86	1691,68
HapA-29	48	8,42	222,73	222,73
		8,65	234,79	234,79
		8,26	214,08	214,08
	96	13,39	562,84	340,11
		12,81	515,12	280,33
		13,54	575,53	361,44
	144	19,07	1141,89	579,05
		18,04	1021,84	506,72
		19,07	1141,89	566,36
	192	23,24	1696,77	554,88
		23,48	1731,99	710,15
		23,46	1729,04	587,15
	240	28,61	2571,49	874,73
		28,02	2465,65	733,65
		27,96	2455,97	726,93
HapA-34	48	9,91	308,22	308,22
		9,99	313,53	313,53
		9,44	279,96	279,96
	96	16,55	860,49	552,27
		16,54	859,45	545,92
		15,69	772,89	492,93
	144	24,41	1871,14	1010,65
		24,66	1910,45	1051,00
		24,48	1881,89	1109,00
HapA-36	48	10,55	349,34	349,34
		10,11	320,79	320,79
		10,91	373,59	373,59
	96	18,08	1026,38	677,04
		18,00	1017,88	697,08
		17,69	983,12	609,52
	144	25,33	2015,67	989,30
		25,13	1983,97	966,09
		25,51	2044,42	1061,30
	192	32,16	3249,24	1233,57
		33,22	3465,92	1450,24
		NA	NA	NA
HapA-78	48	10,37	337,84	337,84
		10,27	331,35	331,35
		10,06	317,94	317,94

	96	17,80	994,82	591,54
		17,36	946,23	575,72
		17,84	999,86	588,71
	144	24,64	1907,35	912,53
		23,98	1805,79	859,55
		24,41	1871,91	872,05
	192	30,39	2901,42	994,07
		30,51	2923,42	1117,63
		31,09	3035,65	1163,74
HapB-13	48	9,71	296,20	296,20
		9,14	262,16	262,16
		9,40	277,30	277,30
	96	18,44	1067,67	771,46
		17,93	1009,97	747,81
		17,79	994,26	716,97
	144	28,35	2524,97	1457,30
		27,86	2438,44	1428,46
		27,89	2443,69	1449,43
HapB-67	48	10,78	364,74	364,74
		10,42	341,10	341,10
		10,30	333,29	333,29
	96	18,57	1082,78	718,04
		17,69	983,12	642,01
		18,02	1019,57	686,28
	144	26,13	2145,01	1062,23
		25,47	2037,22	1054,10
		25,53	2047,63	1028,06
	192	32,68	3355,16	1210,16
		31,53	3123,18	1085,97
		32,53	3324,43	1276,81
	240	39,94	5010,22	1655,06
		39,35	4864,51	1741,33
		39,12	4807,81	1483,38

	96	17,95	1011,66	673,83
		16,90	896,74	565,39
		17,03	911,13	593,19
	144	24,55	1893,44	881,78
		23,55	1741,59	844,86
		24,39	1868,84	957,72
HapE-75	48	10,09	319,84	319,84
		9,42	278,48	278,48
		9,50	283,53	283,53
	96	15,85	788,74	468,90
		15,62	766,01	487,53
		15,45	749,42	465,89
	144	22,48	1586,90	798,16
		22,03	1524,68	758,67
		21,80	1493,01	743,59
	192	29,21	2680,48	1093,58
		27,40	2357,72	833,04
		27,29	2339,68	846,67
	240	35,14	3878,19	1197,71
		32,42	3301,99	944,27
		33,10	3441,96	1102,28

Table S3. Strain ID, GeneBank Accession number for mitochondrial ribosomal small (rnnS) and large (rnnL) subunits, and reference of each strain used in the network analysis.

	GeneBank Accession nr.		
Strain ID	**rnnS**	**rnnL**	**Reference**
AUT2	MF150011.1	MF150010.1	Makkonen et al., 2018
StGm9	MF150013.1	MF150012.1	Makkonen et al., 2018
GiSt5	MF150015.1	MF150014.1	Makkonen et al., 2018
IvoOkt13	MF150017.1	MF150016.1	Makkonen et al., 2018
AP03	MF973121.1	MF975950.1	Makkonen et al., 2018
EviraK047/99	MF973122.1	MF975951.1	Makkonen et al., 2018
Kv1	MF973123.1	MF975952.1	Makkonen et al., 2018
L1	MF973124.1	MF975953.1	Makkonen et al., 2018
UEF VEN5/14	MF973125.1	MF975954.1	Makkonen et al., 2018
PI	MF973126.1	MF975955.1	Makkonen et al., 2018
SAP-Malaga5	MF973128.1	MF975957.1	Makkonen et al., 2018
SAP-Pamplona1	MF973129.1	MF975958.1	Makkonen et al., 2018
UEF 7203	MF973130.1	MF975959.1	Makkonen et al., 2018
UEF 7204-4	MF973131.1	MF975960.1	Makkonen et al., 2018
UEF 7208	MF973132.1	MF975961.1	Makkonen et al., 2018
UEF 8140-1	MF973133.1	MF975962.1	Makkonen et al., 2018
UEF 8147-4	MF973134.1	MF975963.1	Makkonen et al., 2018
UEF 8866-2	MF973135.1	MF975964.1	Makkonen et al., 2018
UEF AT1D	MF973136.1	MF975965.1	Makkonen et al., 2018
UEF KTY3-4	MF973137.1	MF975966.1	Makkonen et al., 2018
UEF OI-1B	MF973138.1	MF975967.1	Makkonen et al., 2018
UEF OI-1D	MF973139.1	MF975968.1	Makkonen et al., 2018
UEF OI-2E	MF973140.1	MF975969.1	Makkonen et al., 2018
UEF SATR1	MF973141.1	MF975970.1	Makkonen et al., 2018
UEF SATR2	MF973142.1	MF975971.1	Makkonen et al., 2018
UEF T2B	MF973143.1	MF975972.1	Makkonen et al., 2018
UEF T16-JR25A	MF973144.1	MF975973.1	Makkonen et al., 2018
UEF T16-JR27P	MF973145.1	MF975974.1	Makkonen et al., 2018
UEF T16K	MF973146.1	MF975975.1	Makkonen et al., 2018
Upor4	MF973147.1	MF975976.1	Makkonen et al., 2018
Li10	MF973148.1	MF975977.1	Makkonen et al., 2018
CE19/01-1	MW346522.1	MW346542.1	Martin-Torrijos et al 2021
CE19/06-10	MW346516.1	MW346536.1	Martin-Torrijos et al 2021
CE19/04-58	MW346519.1	MW346539.1	Martin-Torrijos et al 2021
CE19/04-57	MW346520.1	MW346540.1	Martin-Torrijos et al 2021
CE19/06-2	MW346513.1	MW346533.1	Martin-Torrijos et al 2021
CE19/06-23	MW346516.1	MW346532.1	Martin-Torrijos et al 2021
CE19/06-3	MW346510.1	MW346530.1	Martin-Torrijos et al 2021
CE19/04-55	MW346521.1	MW346541.1	Martin-Torrijos et al 2021
CE19/04-59	MW346518.1	MW346538.1	Martin-Torrijos et al 2021
CE19/C-136	MW346494.1	MW346481.1	Martin-Torrijos et al 2021
CE19/C-135	MW346495.1	MW346482.1	Martin-Torrijos et al 2021
CE19/C-127	MW346498.1	MW346485.1	Martin-Torrijos et al 2021
CE19/C-138	MW346492.1	MW346479.1	Martin-Torrijos et al 2021
CE19/C-140	MW346491.1	MW346478.1	Martin-Torrijos et al 2021
CE19/C-150	MW346487.1	MW346474.1	Martin-Torrijos et al 2021
CE19/06-4	MW346509.1	MW346529.1	Martin-Torrijos et al 2021
CE19/04-61	MW346517.1	MW346537.1	Martin-Torrijos et al 2021
CE19/07-3	MW346504.1	MW346524.1	Martin-Torrijos et al 2021
CE19/06-15	MW346515.1	MW346535.1	Martin-Torrijos et al 2021
CE19/07-8	MW346503.1	MW346523.1	Martin-Torrijos et al 2021
CE19/C-141	MW346490.1	MW346477.1	Martin-Torrijos et al 2021
CE19/C-128	MW346497.1	MW346484.1	Martin-Torrijos et al 2021
CE19/C-145	MW346489.1	MW346476.1	Martin-Torrijos et al 2021
CE19/06-19	MW346514.1	MW346534.1	Martin-Torrijos et al 2021
CE19/06-27	MW346511.1	MW346531.1	Martin-Torrijos et al 2021

CE19/07-19	MW346508.1	MW346528.1	Martin-Torrijos et al 2021
CE19/07-21	MW346507.1	MW346527.1	Martin-Torrijos et al 2021
CE19/-137	MW346493.1	MW346480.1	Martin-Torrijos et al 2021
CE19/07-23	MW346506.1	MW346526.1	Martin-Torrijos et al 2021
CE19/07-28	MW346505.1	MW346525.1	Martin-Torrijos et al 2021
CE19/C-130	MW346496.1	MW346483.1	Martin-Torrijos et al 2021
CE19/C-147	MW346488.1	MW346475.1	Martin-Torrijos et al 2021
CJ1	MG905008.1	MG905000.1	Martin-Torrijos et al 2018
JPN-P10	MG905009.1	MG905001.1	Martin-Torrijos et al 2018
JPN-P11	MG905010.1	MG905002.1	Martin-Torrijos et al 2018
JPN-P12	MG905011.1	MG905003.1	Martin-Torrijos et al 2018
JPN-P13	MG905012.1	MG905004.1	Martin-Torrijos et al 2018
JPN-P1	MG905013.1	MG905005.1	Martin-Torrijos et al 2018
JPN-D4	MG905014.1	MG905006.1	Martin-Torrijos et al 2018
JPN-A1	MG905015.1	MG905007.1	Martin-Torrijos et al 2018

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Supplementary data 2

Table S4. Summary table of the observed symptoms. For each experimental group are indicated: the number of crayfish observed scratching (S), the number of crayfish that experienced autotomy (A, loss of limbs), crayfish showing ataxia/unresponsiveness (A/U), crayfish showing symptoms that died during the experiment (CS-D), crayfish showing symptoms that survived until the end of the experiment (CS-S), number of dead crayfish (nD), and median of the days to first symptom (M-days).

Strain ID	S	A	A/U	CS-D	CS-S	nD	M-days (range)
HapB-1	1	3	4	5	0	7	1 (0-20)
HapB-4	0	4	4	5	0	10	1 (0-1)
HapB-6	0	2	2	3	2	6	1 (0-3)
HapB-7	1	1	0	1	1	1	0
HapB-8	0	4	4	5	0	10	2 (1-3)
HapB-12	0	6	2	6	1	8	1,5 (1-14)
HapB-13	0	4	2	6	0	7	2 (0-3)
HapA-26	0	0	0	0	0	0	NA
HapA-29	0	0	0	0	0	0	NA
HapA-34	0	0	0	0	0	3	NA
HapA-36	0	9	2	9	0	10	2 (1-7)
HapB-67	0	0	0	0	0	1	NA
HapA-78	0	6	1	7	0	10	1 (0-3)
HapE-75	0	2	0	1	1	5	1 (1)
Total	**2**	**41**	**21**	**48**	**5**	**78**	

Table S5. Mean values ± standard deviation (SD) of the total-sporulation rate (S) and motile-sporulation rate (MS) of each strain.

	S		MS	
Strain ID	**Mean**	**SD**	**Mean**	**SD**
HapB-1	4074,1	± 3291,9	-	-
HapB-4	30041,2	± 1885,8	1646	± 2851
HapB-6	5925,9	± 7066,2	247	± 428
HapB-7	82098,8	± 61651,2	617	± 1069
HapB-8	80658,4	± 59520,2	13992	± 5834
HapB-12	35390,9	± 3968,6	17695	± 2851
HapB-13	61,7	± 53,5	-	-
HapB-67	32510,3	± 28935,6	5761	± 4674
HapA-26	124691,4	± 77895,3	12963	± 11264
HapA-29	1234,6	± 2138,3	-	-
HapA-34	29453,3	± 25879,1	529	± 529
HapA-36	12345,7	± 11315,0	-	-
HapA-78	39094,7	± 18898,7	12346	± 6533
HapE-75	52003	± 38380,6	-	-

Table S6. Pairwise comparison of total-sporulation rates (S) of all the strains. For each comparison, the test statistic and p-value are shown. P-values were adjusted using Benjamini–Hochberg (BH) method. Statistally significant differences between pairs are indicated by an asterisk (*).

		HapB-1	**HapB-12**	**HapB-13**	**HapA-26**	**HapA-29**	**HapA-34**	**HapA-36**	**HapB-4**	**HapB-6**	**HapB-67**	**HapB-7**	**HapE-75**	**HapA-78**
HapB-12	t-statistic	-3,01126												
	p-value	0.0083*												
HapB-13	t-statistic	0,917716	3,928975											
	p-value	0,2383	0.0013*											
HapA-26	t-statistic	-4,67462	-1,66336	-5,59234										
	p-value	0.0004*	0,0905	0.0003*										
HapA-29	t-statistic	0,774323	3,785581	-0,14339	5,448943									
	p-value	0,2631	0.0018*	0,4639	0.0002*									
HapA-34	t-statistic	-2,09354	0,917716	-3,01126	2,581078	-2,86787								
	p-value	0,046*	0,2317	0.0086*	0.0194*	0.0107*								
HapA-36	t-statistic	-0,51622	2,495042	-1,43393	4,158404	-1,29054	1,577325							
	p-value	0,3468	0.0231*	0,1298	0.0010*	0,16	0,1042							
HapB-4	t-statistic	-2,38033	0,63093	-3,29804	2,294292	-3,15465	-0.286786	-1,86411						
	p-value	0,0284*	0,311	0.0055*	0,0335	0.0069*	0,4256	0,0663						
HapB-6	t-statistic	-0,11471	2,896543	-1,03243	4,559905	-0,88904	1,978826	0,401501	2,265613					
	p-value	0,465	0.0103*	0,2142	0.0005*	0,2378	0,0547	0,3835	0,0348					
HapB-67	t-statistic	-2,20826	0,803002	-3,12597	2,466364	-2,98258	-0,11471	-1,69204	0,172071	-2,09354				
	p-value	0,0385*	0,2567	0.0069*	0.0240*	0.0086*	0,4702	0,0873	0,4574	0,047				
HapB-7	t-statistic	-4,1584	-1,14715	-5,07612	0,516215	-4,93273	-2,06486	-3,64219	-1,77808	-4,04369	-1,95015			
	p-value	0.0011*	0,1916	0.0003*	0,3512	0.0003*	0,0478*	0.0024*	0,0755	0.0011*	0,0569			
HapE-75	t-statistic	-3,24069	-0,22943	-4,1584	1,433932	-4,01501	-1,14715	-2,72447	-0,86036	-3,12597	-1,03243	0,917716		
	p-value	0.0061*	0,4443	0.0012*	0,1276	0.0011*	0,1947	0.0147*	0,244	0.0072*	0,2175	0,2349		
HapA-78	t-statistic	-3,18333	-0,17207	-4,10105	1,491289	-3,95765	-1,08979	-2,66711	-0,803	-3,06862	-0.975074	0,975074	0,057357	
	p-value	0.0067*	0,4628	0.0010*	0,1195	0.0013*	0,2059	0.0163*	0,2601	0.0077*	0,2228	0,2261	0,4773	
HapB-8	t-statistic	-4,21576	-1,2045	-5,13348	0,458858	-4,99009	-2,12222	-3,69955	-1,83543	-4,10105	2,007505	-0,05736	-0,97507	-1,03243
	p-value	0.0012*	0,1808	0.0003*	0,3651	0.0003*	0,0453*	0.0021*	0,0688	0.0011*	0,0527	0,4826	0,2295	0,2209

Table S7. Pairwise comparisons of the motile-sporulation rate (MS) among the 8 strains that produced motile spores. For each comparison, the test statistic and p-value are shown. P-values were adjusted using Benjamini–Hochberg (BH) method. Statistally significant differences between pairs are indicated by an asterisk (*).

		HapB-12	**HapA-26**	**HapA-34**	**HapB-4**	**HapB-6**	**HapB-67**	**HapB-7**	**HapA-78**
HapA-26	t-statistic	1,078273							
	p-value	0,231							
HapA-34	t-statistic	3,383546	2,305273						
	p-value	0.0149*	0,0374*						
HapB-4	t-statistic	3,383546	2,305273	0					
	p-value	0.0199*	0,0399*	0,5					
HapB-6	t-statistic	3,792546	2,714273	0,409	0,409				
	p-value	0.0240*	0.0284*	0,4266	0,4419				
HapB-67	t-statistic	1,859091	0,780818	-1,52446	-1,52446	-1,93346			
	p-value	0,0794	0,3081	0,1241	0,1303	0,0731			
HapB-7	t-statistic	3,643819	2,565546	0,260272	0,260272	-0,14873	1,784727		
	p-value	0.0167*	0,0292*	0,4487	0,4631	0,4543	0,0864		
HapA-78	t-statistic	0,929545	-0,14873	-2,454	-2,454	-2,863	-0,92955	-2,71427	
	p-value	0,2627	0,4677	0,0316	0,034*	0.0266*	0,2737	0.0320*	
HapB-8	t-statistic	0,669272	-0,409	-2,71427	-2,71427	-3,12327	-1,18982	-2,97455	-0,26027
	p-value	0,3412	0,4124	0.0233*	0.0256*	0.0211*	0,2042	0.0244*	0,4351

Table S8. Growth rates (GR) calculated for each strain as the slope of a linear regression for the changes of area through time calculated combining data of the three replicas. SE, standard error; CL, interval of confidence.

Strain ID	**GR**	**SE**	**95% CL**
HapB-1	13,32	0,402	12,53 - 14,12
HapB-4	13,53	0,635	12,27 - 14,78
HapB-6	12,12	0,635	10,86 - 13,38
HapB-7	7,36	0,635	6,11 - 8,62
HapB-8	9,01	0,635	7,75 - 10,26
HapB-12	4,97	0,402	4,18 - 5,77
HapB-13	12,15	0,635	10,89 - 13,41
HapB-67	6,39	0,284	5,83 - 6,96
HapA-26	9,78	0,402	8,99 - 10,58
HapA-29	2,92	0,284	2,35 - 3,48
HapA-34	7,88	0,635	6,62 - 9,14
HapA-36	7,01	0,439	6,14 - 7,87
HapA-78	5,89	0,635	4,64 - 7,15
HapE-75	4,22	0,284	3,66 - 4,78

Table S9. F-statistic (F), degrees of freedom (Df), p-value and adjusted R2 (adj. R2) for each strain.

Strain ID	F	Df	p-value	adj. R^2
HapB-1	4298	10	1,66E-14	0,9974
HapB-4	584,7	7	5,27E-08	0,9865
HapB-6	185,1	7	2,73E-06	0,9584
HapB-7	81,4	7	4,19E-05	0,91
HapB-8	714	7	2,64E-08	0,989
HapB-12	359,1	10	3,64E-09	0,9702
HapB-13	482	7	1,02E-07	0,984
HapB-67	355	13	8,05E-11	0,962
HapA-26	315,4	10	6,84E-09	0,9662
HapA-29	163,9	13	9,59E-09	0,9208
HapA-34	116	7	0,000013	0,9353
HapA-36	300,4	9	3,2E-08	0,9677
HapA-78	255,1	7	9,16E-07	0,9695
HapE-75	167	13	8,56E-09	0,9222

Table S10. Pairwise comparisons between growth rate (GR) of the strains. P-values resulted from the post-hoc Tukey's range test.

	Hap B-1	**HapB -12**	**HapB-13**	**HapA-26**	**HapA -29**	**HapA-34**	**Hap A-36**	**HapB-4**	**HapB-6**	**HapB-67**	**HapB -7**	**Hap E-75**	**Hap A-78**
Hap B-12	3,06E -14												
Hap B-13	0,954	1,75E-13											
Hap A-26	5,69E -07	4,92E-12	0,105										
Hap A-29	3,06E -14	0,0040 6	3,15E-14	3,09E-14									
Hap A-34	3,39E -09	0,0122	0,0004 34	0,393	5,99E-09								
Hap A-36	1,38E -13	0,0505	6,62E-08	0,0006 28	1,6E-10	0,997							
Hap B-4	1	7,97E-14	0,959	0,0001 7	3,06E-14	4,24E-07	5,58E -12						
Hap B-6	0,943	1,8E-13	1	0,117	3,15E-14	0,0005 03	8,18E -08	0,951					
Hap B-67	3,09E -14	0,196	1,4E-11	2,12E-08	1,82E-12	0,677	0,996	1,55E-13	1,8E-11				
Hap B-7	8,99E -11	0,0966	3,75E-05	0,0873	2,54E-07	1	1	2,41E-08	0,0000 44	0,9812 33			
Hap E-75	3,06E -14	0,96	7,68E-14	8,39E-14	0,0813	5,14E-05	3,81E -05	3,11E-14	8,13E-14	2,58E-05	0,0011 3		
Hap A-78	1,64E -13	0,994	1,41E-08	7,39E-05	0,0028 1	0,623	0,975	4,2E-12	1,7E-08	0,9999 8	0,9328 5	0,482	
Hap B-8	5,75E -06	3,18E-05	0,0392	0,999	1,16E-12	0,993	0,358	0,0001 36	0,0437	0,0178 67	0,8590 2	2,22E -08	0,043 8

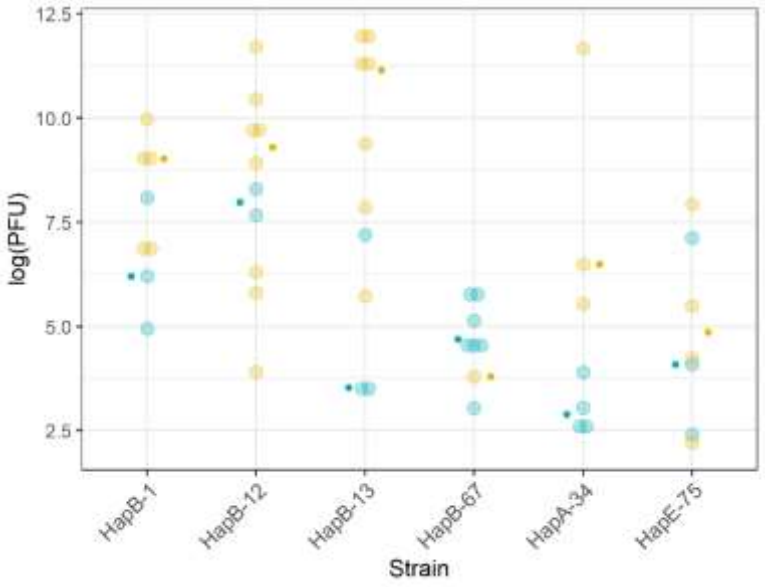

Figure S4. Comparison between the log-transformed PFU values of the crayfish that survived (blue circles) and the ones that died during the experiment (yellow circles) for each experimental group excluding the groups with no deaths and 100% of deaths. Dots represent the median value.

The remaining Supplementary material of Chapter 4 will be made available only digitally as part of the published paper in the journal of choice due size of the file incompatible with a printed version.

Additional supplementary materials are:

Supplementary data 1, Table S11. Table reporting for each crayfish in the infection experiment the following parameters: experimental group (Strain), Ct values resulted from the qPCR conducted to detect *A. astaci* in the crayfish tissues in duplicates (Rep1_Ct, Rep2_Ct), corresponding PFU values (Rep1_PFUs, Rep2_PFUs), the mean PFU value and their standard deviation (SD), the agent level (A0-A7), whether the crayfish succumbed during the experiment (Died; F, survived; T, succumbed), whether hyphae or melanised hyphae were observed in the crayfish abdominal cuticle during microscopical analysis (F, not present; T, present), whether the crayfish showed any symptom during the experiment (N, no symptoms; S, symptoms).

Supplementary material – Chapter 5

Material and methods

Samples

Aphanomyces astaci pure cultures were isolated from infected crayfish belonging to both North American and European species from waterbodies both in North American and Europe. The sampling was conducted between 2003 and 2020 (**Table S1**).

Table S1. *Aphanomyces astaci* strain ID, place and crayfish species of isolation, haplogroup, year of isolation and GenBank accession numbers (rnnS and rnnL) of each strain used in the present study.

Strain ID	Origin	Host	Haplogroup	Year of isolation	GenBank acc. number (rnnS)	GenBank acc. number (rnnL)
HapB-1	Lake Kukkia, Finland	*Pacifastacus leniusculus*	B	2003	OR682421	OR682407
HapB-4	Lake Puujärvi, Finland	*P. leniusculus*	B	2003	OR682422	OR682408
HapB-6	Lake Pyhäjärvi, Finland	*P. leniusculus*	B	2003	OR682423	OR682409
HapB-7	UEF Fish Research Unit, Kuopio	*Astacus astacus*	B	2008	OR682424	OR682410
HapB-8	Lake Saimaa, Finland	*P. leniusculus*	B	2012	OR682425	OR682411
HapB-12	Lake Saimaa, Finland	*P. leniusculus*	B	2012	OR682426	OR682412
HapB-13	Lake Tahoe, USA	*P. leniusculus*	B	2013	OR682427	OR682413
HapB-67	Lake Pyhäjärvi, Sotkanniemi, Finland	*P. leniusculus*	B	2018	OR682432	OR682418
HapA-26	Lake Borovniscica, Slovenia	*Austropotamobius torrentium*	A	2014	OR682428	OR682414
HapA-29	Lake Venesjärvi, Finland	*A. astacus*	A	2014	OR682429	OR682415
HapA-34	Speyer, Germany	*Faxonius immunis*	A	2015	OR682430	OR682416
HapA-36	Trout, Normal, IL, USA	*Faxonius rusticus*	A	2016	OR682431	OR682417
HapA-78	Runkedebunk, Germany	*F. immunis*	A	2020	OR682434	OR682420
HapE-75	Kräppelweiher, Germany	*Faxonius limosus*	E	2020	OR682433	OR682419

* This strain was isolated from an accidentally infected *A. astacus* inside the UEF Fish Research Unit facilities in Kuopio, Finland. However, the strain is likely to have been originally carried by a specimen of *P. leniusculus*.

Samples preparation and genomic DNA (gDNA) isolation

For the production of *A. astaci* hyphal tissue for the gDNA isolation, three cubes of hyphae-containing solid PG-1 medium (4 mm^3 each) were cut from the stock culture and put to grow in a petri dish containing 20 mL of liquid PG-1 medium. For each strain, 20 petri dishes were prepared. After an incubation period of 3 days at 18° C, the hyphal tissue was dried on filter paper for 30 seconds, the solid PG-1 medium cubes were removed, and the tissue was stored at -80° C until further processing.

In preparation for the gDNA isolation, the hyphal tissue was grinded in liquid nitrogen using mortar and pestle, and then collected in 50 mL falcon tubes. The DNA isolation protocol was the following: for each sample, 9 mL of lysis buffer (10 mM Tris/HCl pH 8.0, 50 mM EDTA, 0.5% SDS, 0.5% Triton X-100, 0.5% Tween 20) and 200 µl of 20 mg/mL proteinase K were added to the 50 mL falcon tube containing the frozen grinded hyphae. After vortexing softly, the tissue was incubated at 65°C for 1 hour, with gentle shaking every 10 minutes. The samples were then cooled on ice for 5 minutes. Afterwards, 9 mL of phenol/chloroform were added to the falcon tubes, and after inverting the tubes several times, the tubes were centrifuged at 3.900g for 5 minutes. The supernatant was transferred to a new 50 mL tube and equal amount of chloroform/isoamyl alcohol (Roti C/I, Carl Roth) was added. Samples were then centrifuged as above. Supernatant was transferred to a new 50 mL falcon tube and 10mg/mL RNAse A was added in a 1:100 ratio. Tubes were then incubated at 37°C for 15 minutes. Afterwards, Roti C/I was added in a 1:1 ratio. After centrifugation at 3.900g for 5 minutes, 5 mL of supernatant were transferred to a 15 mL falcon tube. 2.5 mL of Ammoniumacetate 7.5M and 7.5 mL of ice-cold isopropanol were added. The mixture was gently inverted few times and set aside at room temperature for 5 minutes. The tubes were centrifugated for 10 min at 3.900 g. Supernatant was discarded, 1 mL of ice-cold 70% ethanol was added, and the tubes were centrifuged for 5 minutes at 3.900 g. The supernatant was discarded and the wash with ethanol was repeated a second time. The supernatant was discarded, and the pellet was resuspended in 70 µL of warm TE (heated at 65° C). Isolated gDNA was stored at -80°C until further use.

Genome assembly statistics

Final statistics of the genome assemblies were estimated with Quast v5.0.2 and BUSCO v5.3.2 (**Table S2**).

Table S2. Summary table reporting for each genome assembly of a *Aphanomyces astaci* strain: Strain ID, type of reads used for the assembly (I, Illumina reads; N, Nanopore reads), length of the assembly (Mb), number of scaffolds, N50 (bp), L50 and BUSCO score based on the lineage stramenopiles_odb10.

Strain ID	Reads	Length (Mb)	Scaffolds	N50 (bp)	L50	BUSCO
HapB-1	I	62.5	2413	502732	38	C:99.0%[S:99.0%,D:0.0%],F:0.0%,M:1.0%
HapB-4	I	62.3	2379	528434	38	C:100.0%[S:100.0%,D:0.0%],F:0.0%,M:0.0%
HapB-6	I	62.5	2536	500910	39	C:100.0%[S:100.0%,D:0.0%],F:0.0%,M:0.0%
HapB-7	I	62.5	2498	490310	39	C:100.0%[S:100.0%,D:0.0%],F:0.0%,M:0.0%
HapB-8	N	80	227	626550	41	C:100.0%[S:92.0%,D:8.0%],F:0.0%,M:0.0%
HapB-12	I	62.5	2498	509815	39	C:100.0%[S:100.0%,D:0.0%],F:0.0%,M:0.0%
HapB-13	I	62.5	2489	516293	38	C:100.0%[S:100.0%,D:0.0%],F:0.0%,M:0.0%
HapB-67	I	67.2	3452	557294	39	C:97.0%[S:97.0%,D:0.0%],F:3.0%,M:0.0%
HapA-26	I	55.8	2878	454514	37	C:98.0%[S:97.0%,D:1.0%],F:2.0%,M:0.0%
HapA-29	I	54.3	1500	460621	37	C:100.0%[S:100.0%,D:0.0%],F:0.0%,M:0.0%
HapA-34	I; N	71.4	540	218556	95	C:100.0%[S:99.0%,D:1.0%],F:0.0%,M:0.0%
HapA-36	I	59.1	2632	468937	39	C:99.0%[S:99.0%,D:0.0%],F:1.0%,M:0.0%
HapA-78	I	62.8	3434	504888	39	C:100.0%[S:99.0%,D:1.0%],F:0.0%,M:0.0%
HapE-75	I; N	73.2	562	207981	116	C:100.0%[S:97.0%,D:3.0%],F:0.0%,M:0.0%

Genome annotation

A first run of Maker was conducted with options "est2genome=1" and "proteome2genome=1" using as evidence the available proteomes of *A. astaci* (proteome ID: UP000019040), *Saprolegnia parasitica* (proteome ID: UP000030745) and *Saprolegnia diclina* (proteome ID: UP000030762) (maker_opts.ctl, protein) and the transcriptomes assembled in this study (maker_opts.ctl, est). Additionally, an existing annotation file (accession number: GCA_003546765.1) was used as model_gff. After the first run of MAKER2, agat v0.9.1 was used to select from the obtained annotation file annotations with AED≤0.1 (*agat_sp_filter_feature_by_attribute_value.pl*) to obtain only correct genes, and among those the longest isoforms were retained (*agat_sp_keep_longest_isoform.pl*). We then extracted the protein sequences from the filtered annotations (*agat_sp_extract_sequences.pl*) and blasted them against each other using blastp v2.12.0+ to individuate redundant proteins. Afterwards, we filtered the proteins to retain only non-redundant ones (*agat_sp_filter_by_mrnaBlastValue.pl*, default values). From the obtained annotation (converted to a simple genbank format, *gff2gbSmallDNA.pl*), we used Augustus v3.4.0 to randomly select 150 genes (*randomSplit.pl*) and used these to train *ab initio* gene predictor augustus (in order: *etraining*, *augustus* and *optimize_augustus.pl*). Afterwards we re-trained

augustus with the meta-parameters automatically generated in the previous run (*etraining*, *augustus*). Finally, MAKER2 was run again using the generated gene-prediction model (*maker_opts.ctl*, *augustus_species*) (**Table S3**). Functional annotations of the genome were obtained by blasting the protein sequences obtained from MAKER2 to the Pfam 35.0 database using InterProScan v5.60-92.0.

Table S3. Statistics relative to the annotation of the genome assembly based on Nanopore long-reads of strain HapB-8 of *Aphanomyces astaci.*

Number of gene models (bp)	**25813**
Average gene length (bp)	1929,2
Number of exons	98541
Average number of exons per gene model	3,8
Average exon length (bp)	366,4
Number of transcripts	25813
Average number of transcripts per gene model	1
Number of gene models less than 200bp length	59
BUSCO (stramenopiles_odb10)	C:99.0%[S:92.0%,D:7.0%],F:0.0%,M:1.0%