ORIGINAL RESEARCH

Temporal dynamics of the immune response in *Astacus astacus* (Linnaeus, 1758) challenged with *Aphanomyces astaci* Schikora, 1906

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Abstract

One of the main drivers of biodiversity loss in freshwater ecosystems are alien invasive species. In Europe, pathogen *Aphanomyces astaci* Schikora, 1906, is considered as one of the most problematic invasive species, as its introduction caused the severe decimation of the European freshwater crayfish stocks. The most affected are the populations of noble crayfish, keystone species native to European freshwaters. Unfortunately, even after decades of research, we do not understand the temporal dynamics of the noble crayfish immune response during *Ap. astaci* infection. Here, we studied the changes in the immune response of the noble crayfish during a time course challenge with a highly virulent strain of *Ap. astaci*. We recorded gross symptoms of the disease, changes in the total haemocyte count (THC), gene expression profiles of putative immune response regulators and pathogen load. Additionally, we conducted a preliminary histological analysis of the pathogen dissemination in host tissues. Based on the occurrence of symptoms we propose three stages in the crayfish plague disease progression: asymptomatic stage, symptomatic stage, and finally death of infected individual. Furthermore, based on the qPCR analysis we could differentiate three *Ap. astaci* growth phases: initial lag phase, followed by exponential growth phase and finally sporulation phase. We observed that all measured immune response parameters were significantly correlated to the observed increase in the pathogen load (qPCR). Altogether, our results point to the absence of a successful immune response in the noble crayfish to a challenge with a highly virulent strain of *Ap. astaci*. The noble crayfish immune system was not able to suppress the growth of the intruding pathogen. In general, our observations have to be considered in the context of the specific combination of crayfish plague pathogen virulence and disease resistance of the challenged crayfish population, which defines the temporal dynamics of their interaction.

Keywords: noble crayfish, crayfish plague, biodiversity conservation, transcriptional factors

Abbreviations

C/EBP-CCAAT/enhancer-binding protein

EF1α-elongation factor 1 α

HIS-hepatosomatic index

Kr-h1-Krüppel homolog-1 protein

PFU-PCR forming units

proPO-prophenoloxidase

qPCR-quantitative PCR

THC-total haemocyte count

WSSV-white spot syndrome virus

Introduction

Conservation of the freshwater habitats and the vast biodiversity within them is a global priority [1]. The introduction of invasive alien species has been recognised as the major contributor to the overall biodiversity loss in freshwater ecosystems [1]. Translocations of invasive aquatic crustaceans and their associated pathogens has inflicted a significant economic burden, estimated to 271 million US$ worldwide in the period between 2000 and 2020 [2]. Concurrently, it is estimated that the management costs solely for the invasive signal crayfish *Pacifastacus leniusculus* (Dana, 1852) amounted to 103.9 million US$ [2]. The signal crayfish is also a carrier of a highly virulent strain of the oomycete pathogen *Aphanomyces astaci* Schikora, 1906 [3]. This invasive horror combo, paired with the poor decision making of the management authorities, led to the decimation of the native European freshwater crayfish species [4,5]. In particular, vulnerable noble crayfish, *Astacus astacus* (Linnaeus, 1758), populations have experienced a significant decline due to *Ap. astaci* introduction, with an estimated loss of 40-50% of total populations across Europe, and up to 78% in Scandinavia [6]. It is therefore not surprising that the conservation status of the noble crayfish is considered inadequate and deteriorating across Europe, according to the Art 17 of the European Union Habitats Directive. A resolution of this crisis caused by the introduction of invasive crayfish and pathogens associated with them is still not in sight, and introductions of the new alien crayfish species into European freshwaters are still ongoing [5]. Continuous pressure from novel invasive species hinders the ability of the European crayfish species to adapt to the invasive pathogen and *vice versa* [7].

Co-evolutionary processes played a significant role in shaping the host-pathogen relationship between the freshwater crayfish and *Ap. astaci* [8]. Resistant North American crayfish, main carriers and transmitters of the pathogen, are often latently infected with no visible signs of the disease. Conversely, native European crayfish are highly susceptible to the pathogen infection [9]. After a century of dramatic coexistence, numerous resistant or latently infected noble crayfish populations have been identified either in the wild [7,10,11] or under experimental conditions [12–14]. Survival of the noble crayfish challenged with *Ap. astaci* depends on the strain [12–14] and pathogen infection dose [12,15]. Gradual reduction in virulence of some *Ap. astaci* strains could partially explain the increased survival rates among the *Ap. astaci* challenged noble crayfish [16]. On the other hand, the molecular mechanisms of the increased immune resistance of some noble crayfish populations in the context of overall high susceptibility to the pathogen, remains elusive.

Over the last 50 years, our understanding of the disease caused by *Ap. astaci* and its interaction with the immune system of the noble crayfish has significantly advanced. Initial studies showed that the crayfish epicuticle represents the first and foremost barrier to the pathogen progression [17]. This was followed by an observation of a strong reaction of the crayfish haemocytes towards *Ap. astaci* [18]. Encapsulation, followed by the degranulation of granular haemocytes, and the deposition of melanin on the hyphal cell wall was later recognised as the main component of the prophenoloxidase (proPO) system [19]. Low expression of the proPO transcripts in the noble crayfish, compared to the invasive signal crayfish, was soon suggested as a significant factor of its susceptibility towards *Ap. astaci* [20]. The increase of the proPO activity and its following gradual decrease during the *Ap. astaci* challenge is accompanied by the drop of the total haemocyte count, and increased reactive oxygen species production in the noble crayfish [12]. Similarly, in our recent study, we observed alteration of the expression of multiple noble crayfish genes, in the hepatopancreas during a challenge with *Ap. astaci* [8]. Therefore, it seems likely that the hepatopancreas is an important hub for the synthesis of immune response molecules [8,21]. Changes in the hepatopancreas size (hepatosomatic index) reflect the crayfish condition, nutritional status, its energy reserves as well as its immune status [22,23]. Similarly, changes in the total haemocyte count reflect changes in the immune status of the crayfish, and can be significantly altered during immune stimulation or pathogen challenge [12,24]. This suggests that a much broader gene network of interaction partners from multiple tissues is mobilised during the immune response of the noble crayfish to *Ap. astaci*.

Mounting an effective immune system response is, in part, dependant on the capability of the host to recognise the intruding pathogen and to timely activate its response mechanisms. To achieve pathogen clearance, this activation of the immune reaction has to be of sufficient intensity and duration [25]. This modulation is achieved through the activation of transcriptional factors, which interact with the gene regulatory sequences present in the promoter and enhancer gene regions to finetune gene expression. The putative transcriptional regulators CCAAT/enhancer-binding protein (*C/EBP*) and Krüppel homolog-1 protein (*Kr-h1*) have been linked to the immune response of the noble crayfish to *Ap. astaci* challenge [8]. Changes in their expression might be critical for mounting a successful immune response. At present, it is unknown if the unsuccessful immune response in the noble crayfish is a consequence of a weak and/or untimely immune response to the challenge with *Ap. astaci*.

Here we explored the dynamics of the *Ap. astaci* infection process and the noble crayfish immune response under controlled experimental conditions. To this end, we conducted a time course experiment where we infected noble crayfish with a lethal dose of *Ap. astaci* zoospores from the highly virulent strain. During the progression of the infection, we recorded gross symptoms of the disease, hepatosomatic index (HSI) and total haemocyte count (THC), as well as the gene expression profiles of transcriptional factors possibly involved in the immune response (*C/EBP* and *Kr-h1*). In parallel, we monitored the increase of *Ap. astaci* pathogenic load (qPCR) inside the crayfish and conducted a pilot histological analysis of the pathogen disseminations within the host tissues (hepatopancreas, gills, abdominal muscle and heart). This allowed us to address the following research questions: **Are there defined stages of the crayfish plague disease? Does the onset of gross symptoms of infection happen before or after the immune system activation? How does the *Ap. astaci* growth rate change during the infection of the susceptible noble crayfish?** Answers to these questions can shed light on the interaction between the highly virulent pathogen and its susceptible host, with respect to how and when the immune system of the noble crayfish fails to efficiently respond to the challenge with highly virulent strain of *Ap. astaci*.

Materials and Methods

Crayfish

In total, 62 noble crayfish were obtained from the breeder Helmut Jaske (Oeversee, Germany). The crayfish breed was previously tested and confirmed negative for carrying the crayfish plague disease agent. All crayfish used in the experiment were 3-year-old intermoult sexually mature males. Carapace lengths and mass of each individual crayfish are available in the **Supplementary table 1**. Crayfish were held individually in 5 L tanks, with gravel on the bottom and 2 L of tap water. Room temperature was kept constant at 18°C and a light/dark cycle of 8/16 hours was maintained. The initial acclimatisation period before the start of the experiment was 17 days. Water exchange was conducted weekly, and crayfish were fed with one frozen green pea every 2 days. All animal experiments were conducted according to the German animal Welfare Act (TierSchG) and Animal Welfare-Experimental Animal Ordinance (TierSchVersV), under the permit number: F 153/Anz.200 issued on the 21.10.2021 with the approval of the Regierungspräsidium Darmstadt.

Aphanomyces astaci and zoospore production

The *Ap. astaci* isolate SATR1 was used for the infection experiment. This strain was isolated from the signal crayfish from Lake Saimaa, Suomi (Finland), in 2012 and it belongs to the haplogroup B lineage (genotype: PSI) [26]. Strains belonging to the haplogroup B are usually characterised as highly virulent and this strain is known to induce 100% mortality in the noble crayfish (our unpublished results). Production of zoospores followed the protocol from [13]. Briefly, three cubes of agar (9 mm^2) were cut from the solid PG1 medium plate with actively growing culture and incubated in 150 ml of liquid PG1 medium in Erlenmeyer flasks for 14 days at 18 °C (six replicates). Three replicates of the culture were subsequently pooled and washed four times (hourly) with 700 mL of autoclaved water. After the fourth wash, hyphae were incubated overnight in 800 mL of autoclaved water at 18°C (two replicates). The density of the zoospore solution was estimated with an optical microscope (total magnification of 100x) using a Bürker chamber.

Design of the infection experiment

Upon the end of the 17-day acclimatisation period, the crayfish were challenged with 1000 zoospores/mL of *Ap. astaci*. Crayfish were randomly assigned to an infection group (N = 32) and a control group (N = 30). All crayfish were monitored during the experiment 2 times a

day (for 30 min) for the symptoms of *Ap. astaci* infection. We recorded the occurrence of characteristic symptoms of infection, i.e. scratching ("walking on stilts"), behavioural abnormalities (non-responsiveness to external stimuli), loss of balance, or missing limbs [27]. To assess the dynamics of the infection we sampled the haemolymph and tissues from the crayfish at 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 192 h after the challenge. Due to the occurrence of mortality at 216 h in our experiment, we chose 192 h as the last sampling point for the analysis of immune response parameters. At all sampling points, samplings were made from three individuals (representing biological triplicates), with at least three control and three infected crayfish sampled simultaneously. Additionally, as a control for the mortality induced by the *Ap. astaci* strain (later in text referred to as mortality group), six crayfish from infected group and five crayfish for control group were kept until death (infected) or euthanised 5 days post-death of the last infected crayfish (control group). Crayfish in the mortality group were not included in the analysis of immunological parameters, due to the post-mortem degradation of the tissues. Details about of the assignment of each crayfish to infected or control group, as well as the sampling group (timepoints 6-92 h or mortality) are available in the **Supplementary table 1**.

Total haemocyte count

At each timepoint from each crayfish (control and infected group) 100 µL of haemolymph was drown from the dorsal cephalotoracic cavity, using a 1 mL disposable sterile syringe (Injekt® F, B.Braun, Germany) and 25 mm (26 G) disposable sterile needle (Sterican®, B.Braun, Germany). Haemolymph was immediately transferred to 1.5 mL tube containing 400 µL of cold crayfish anticoagulant [22]. Total haemocyte count was immediately estimated with an optical microscope (total magnification of 100x) using a Bürker chamber. No immediate mortalities were induced by haemolymph sampling in infected or control group. Post haemolymph sampling, individual crayfish were euthanised and we proceeded with sampling of the tissues (see below).

Before subjecting to euthanasia and tissue sampling

Before subjecting to euthanasia all crayfish were placed at -20 °C for 20 to 25 minutes to induce non responsiveness to external stimuli [12,28,29]. Crayfish were checked every 5 min and any signs of stress were recorded: uncontrolled movement of the limbs, tail flicking, loss of limbs, scratching. All experimental crayfish did not show any signs of distress during this process. This was followed by euthanasia with swift splitting of the ventral nerve cord as per international guidelines for humane killing of crayfish [30] and immediate sampling of the tissues for histological analysis. Tissue samples were taken at the same time from abdominal

cuticle, uropod and walking legs for the detection of *Ap. astaci* load using the qPCR method (described below) [31]. Upon opening the cephalothorax of the crayfish, the whole hepatopancreas was carefully removed and wet weight of hepatopancreas was recorded. The hepatosomatic index was calculated according to [22]. For the histological analysis, heart muscle, one lobe of the hepatopancreas, one arch of gill (above fifth pereopod) and a cross section through the third abdominal segment were sampled. For the analysis of the gene expression, we sampled the remaining lobe of the hepatopancreas which was immediately frozen in liquid nitrogen and then stored at -80 °C until RNA extraction.

Expression of the immune response genes in the hepatopancreas

Total RNA was isolated using the NucleoSpin RNA Mini Kit for RNA purification (Macherey-Nagel, Germany). RNA quality was assessed on the NanoVue Spectrophotometer, and RNA quantity with the QuantiFluor RNA System on the Quantus platform (Promega, USA). For the cDNA synthesis, 0.5 µg of total RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturers protocol. Target loci for the qPCR were CCAAT/enhancer-binding protein (*C/EBP*) and Krüppel homolog-1 (Kr-h1; identified as putative Krüppel 1-like factor protein in Boštjančić et al 2022), previously observed to be differentially expressed in the noble crayfish challenged with *Ap. astaci* [8]. Elongation factor one alpha (*EF1α*) was used as a housekeeping gene. Target-specific primers were synthesised for the amplification of *C/EBP* and *EF1α* from [23]. For the quantification of *Kr-h1* expression, the following primer pair was designed: forward primer 5'-AGTGTGAGGTGTGCGGTAAG-3' (Kr-h1-F), reverse primer 5'-GGCAGTACTCACAGGTGTATGG-3' (Kr-h1-R), with 96 % amplification efficiency. Sequences of *C/EBP* and *Kr-h1* transcripts are deposited under the GenBank accession numbers GJEB01120680.1 and GJEB01090533.1, respectively. For the quantitative reverse transcription PCR (RT-qPCR), target loci were amplified in 10 µL reactions with iTaq Universal SYBR Green Supermix (Bio-Rad, USA), with 1 µL of 10 x diluted input cDNA template on the CFX Opus 96 Real-Time PCR (Bio-Rad, USA) according to the manufacturer's specifications. All samples were run in duplicates with the standard deviation of cycle threshold values < 0.5. The difference in the gene expression values between the samples was calculated according to the delta-delta Ct method ($2^{-\Delta\Delta CT}$) [32].

Evaluation of the *Ap. astaci* infection load

The load of *Ap. astaci* in the crayfish tissues (see above) was assessed following the DNA extraction protocol and quantitative PCR assay described in [31] for all 62 experimental noble crayfish (including mortality group). Quantitative PCR assay was conducted according

to [13] with following modifications: DNA concentration was measured using the QuantiFluor dsDNA (Promega, USA), and the concentration of each sample was normalised to 10 ng/µL before the qPCR assay. The total reaction volume of the qPCR assay was 25 µL, consisting of 12.5 µL of 2x TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA, United States), 500 nM of each primer, 200 nM of MGB probe, nuclease free water, and 5 µL of DNA sample. All samples were run in duplicates and corresponding PFU (PCR forming units) values were calculated according to the [31] and were used as approximation of pathogen load inside the crayfish tissues.

Pilot histological analysis of *Ap. astaci* disseminations in host

Tissue samples from 25 infected crayfish (excluding mortality controls) were analysed to detect the presence of *Ap. astaci* hyphae. Upon tissue sampling (described above), all tissues for histological analysis were immediately placed in Bouin's fixative solution (Carl Roth, Germany) and processed for routine embedding technique in Paraplast media (Sherwood Medical, USA). Tissues embedded in paraplast blocks were sectioned at 7– 8 µm using a rotary microtome (Shandon Finesse 325, Thermo Fisher Scientific, USA). Sections were stained following standard protocols with Grocott staining (Biognost, Croatia) to highlight *Ap. astaci* hyphae. Histological slides were analysed under the Nikon Eclipse E600 light microscope equipped with digital camera AxioCam ERc5s and ZEN2 lite software (Carl Zeiss Microscopy GmbH, Germany). To analyse presence or absence of *Ap. astaci* infiltrating hyphae within each specimen, 15 randomly selected slides extracted from various sampling points were examined for each tissue per animal.

Statistical analysis and data visualisation

All statistical analyses were conducted in R 4.2.2. [33]. All datasets were tested for normality using Shapiro–Wilk test, and appropriate statistical tests were applied (t-test for normal distributed data, and a non-parametric Wilcoxon rank sum test for non-normally distributed datasets). For multiple testing, we used Benjamini–Hochberg (BH) method for p-value adjustment with the significance level $\alpha = 0.05$. Data visualisation was conducted with the ggplot2 [34] and ggtree [35] packages. Final editing of the resulting graphics was done in Inkscape 1.2 [36].

Results

Outcomes of the infection experiment

All infected crayfish in the mortality group (n=6) died from the infection with the highly virulent strain of *Ap. astaci*, between 216 h and 240 h post challenge (**Figure 1A**). The first gross symptoms of infection were observed at 96 h post challenge in the form of behavioural changes and autotomy of pereiopods and chelipeds. Behavioural changes included scratching of the abdominal cuticle, eyes and walking legs, and were usually followed by loss balance (ataxia) and non-responsiveness to external stimuli (from 120 h). In the crayfish with autotomy, the fracture plane was located between the ischium and the basis of the limb (**Figure 1B**). Among the crayfish in the mortality group, 5/6 showed autotomy of at least one limb prior to death. In the most extreme case, autotomy of 6 limbs was observed in one dead crayfish (**Figure 1B**). None of the controls for mortality showed symptoms or died during the experiment.

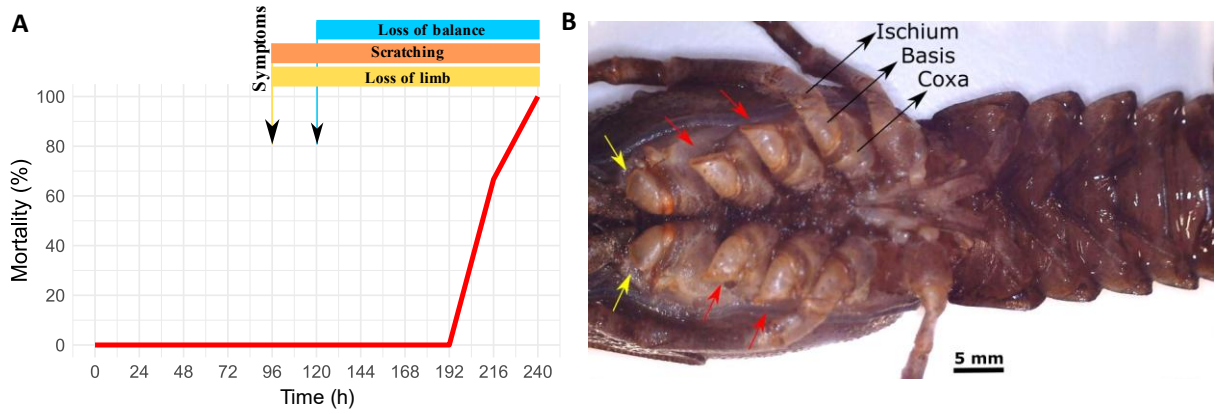

Figure 1. Gross symptoms of an *Ap. astaci* infection in the noble crayfish. (**A**) Cumulative number of dead crayfish (n= 6), and onset of symptoms of infection: loss of limbs, scratching of the abdominal cuticle, eyes and walking legs, loss of balance (ataxia) and death. (**B**) Moribund noble crayfish showing loss of pereiopods (red arrows) and chelipeds (yellow arrows). The fracture planes are located between the ischium and basis of the limbs.

Changes in hepatosomatic index (HSI) and total haemocyte count (THC)

The average HSI among the noble crayfish was 4.59 (SD ± 0.59) for the control crayfish and 4.66 (SD ± 0.86) for the infected crayfish, with no statistical difference (t-test, p-value > 0.05) between the two groups, nor between different timepoints (**Figure 2A**). The average THC among the control crayfish was 1.27 x 10^5 cells/mL (SD ±8.86 x 10^4). A significant THC

increase was recorded 96 h (t-test, p-value = 0.028) followed by a decrease at 120 h (t-test, p-value = 0.012) after challenge with *Ap. astaci*, with an average of 4.78 x 10^5 cells/mL (SD ± 2.36 x 10^5) and 6.96 x 10^4 cells/mL (SD ± 2.31 x 10^3), respectively (**Figure 2B**).

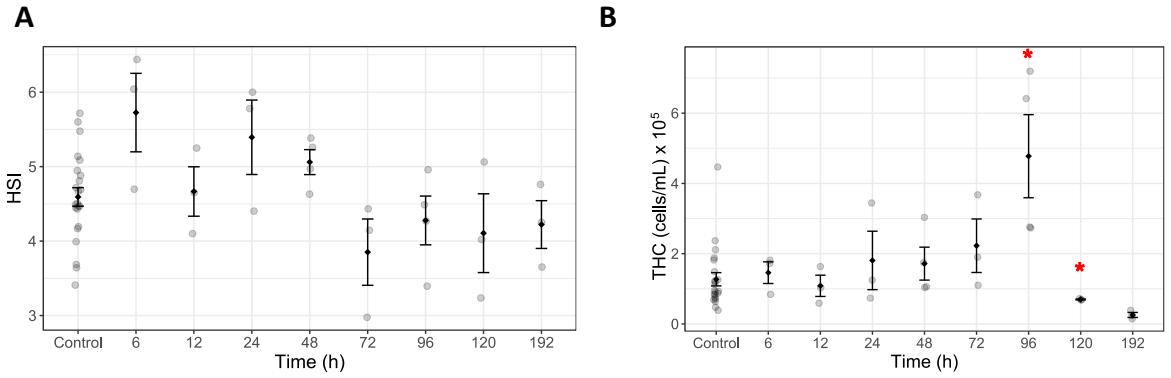

Figure 2. Changes in (**A**) hepatosomatic condition index-HSI and (**B**) total haemocyte count-THC in the noble crayfish circulation after challenge with highly virulent strain of *Ap. astaci*. Timepoints with statistically significant difference in gene expression from the control are marked with an asterisk (*), error bars indicate the standard error of the mean.

Changes in the gene expression of *C/EBP* and *Kr-h1*

Analysis of the *C/EBP* and *Kr-h1* gene expression in the hepatopancreas showed that the expression of these genes changes during the course of the infection experiment. Specifically, down regulation of *C/EBP* was observed at 12 h, significant up-regulation of the *C/EBP* was observed at 192 h (t-test, p-value = 4.6 x 10^{-4}) (**Figure 4A**), while a down-regulation in the gene expression of *Kr-h1* was observed at 120 h (t-test, p-value = 2.8 x 10^{-5}) and 192 h (t-test, p-value = 1.5 x 10^{-4}) after the challenge with *Ap. astaci* (**Figure 4B**).

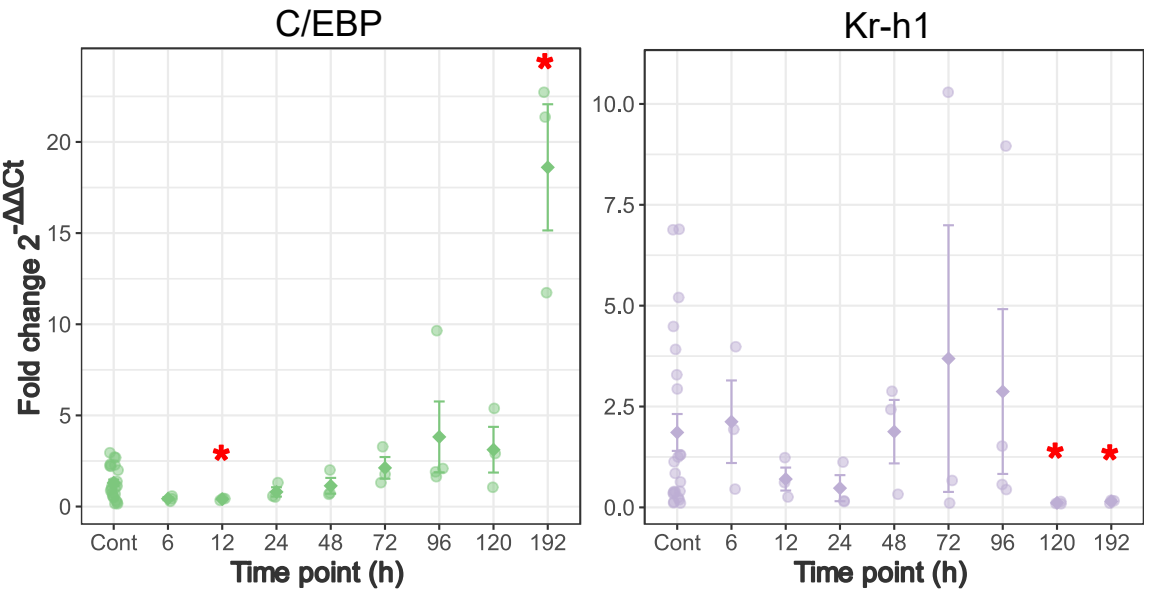

Figure 3. Differential gene expression analysis results of CCAAT/enhancer-binding protein (*C/EBP*) (**A**) and Krüppel homolog-1 protein (*Kr-h1*) (**B**). Timepoints with statistically significant difference in gene expression compared to the control are marked with an asterisk (*), error bars indicate standard error of the mean.

Changes in the *Ap. astaci* pathogen load during the noble crayfish challenge

Among the challenged noble crayfish 31 out of 32 were positive for the presence *Ap. astaci* in their tissues (PCR forming unit, PFU > 5). Three distinct *Ap. astaci* growth phases were observed in the infected crayfish: initial lag phase up to 96 h (average PFU = 3.67 x 10^3, SD ± 4.13 x 10^3) followed by the exponential growth phase (average PFU = 6.4 x 10^4, SD ± 7.4 x 10^4) and finally the putative sporulation phase in the dead crayfish of the mortality group (average PFU = 1.32 x 10^6, SD ± 8.30 x 10^5) (**Figure 5A**). *C/EBP* expression was significantly positively correlated (Spearman correlation test) to the pathogen load (PFU) (ρ = 0.61, p-value = 0.001). Conversely, *Kr-h1* expression (ρ = -0.60, p-value = 1.3 x 10^{-3}), THC (ρ = -0.42, p-value = 0.04) and HSI (ρ = -0.42, p-value = 0.03) were significantly negative correlated with the pathogen load (PFU) in the challenged noble crayfish (**Figure 5B**).

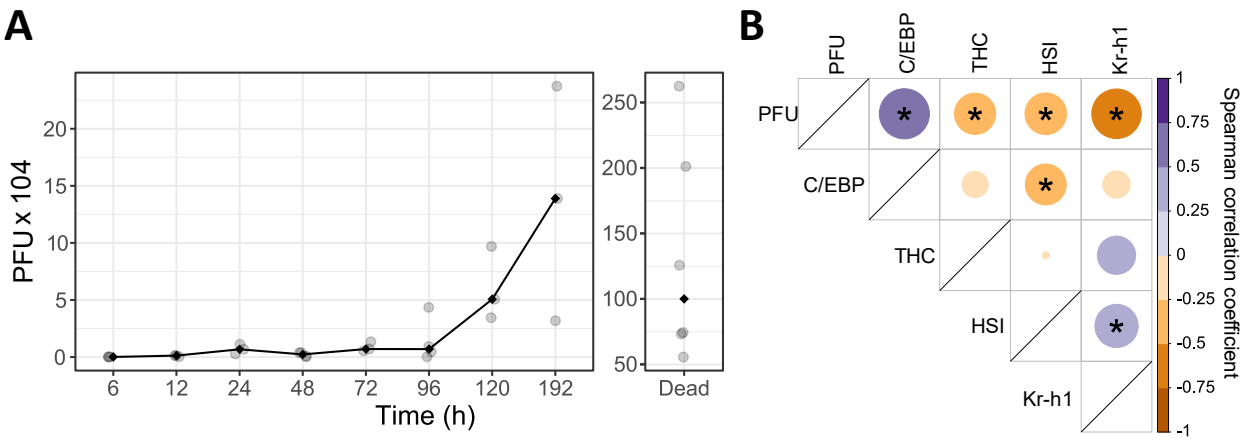

Figure 4. Changes in the *Ap. astaci* load in noble crayfish during a challenge with the highly virulent strain. (**A**) Changes in the PCR performing unit (PFU) values of *Ap. astaci* DNA were detected with the pathogen specific qPCR assay. (**B**) Spearman correlation between the pathogen load (PFU) and measured immunological parameters: CCAAT/enhancer-binding protein (*C/EBP*) gene expression, total haemocyte count (THC), hepatosomatic index (HSI), Krüppel homolog-1 protein *(Kr-h1*) gene expression in the *Ap. astaci* challenged noble crayfish. Positive correlation is indicated in the shades of purple and negative correlation in the shades of orange, size of the circle corresponds to the strength of the correlation. Statistically significant correlations are marked with an asterisk (*).

Presence of *Ap. astaci* in noble crayfish tissues

Based on the Grocott's methenamine silver staining, *Ap. astaci* hyphal infiltrates were observed in the gills (64 %), heart muscle (16 %), and abdominal muscle (20 %) (**Figure 3**). Presence of the *Ap. astaci* hyphal infiltrates in gills were recorded in all time points except 24 h and 120 h. While the overall quality of the sections from the gill tissue was poor. In abdominal muscle and heart tissue, hyphal infiltrates were recorded in samples from 96 h, 120 h and 192 h. Hyphal infiltrates were completely absent from hepatopancreas of the analysed *Ap. astaci* challenged crayfish.

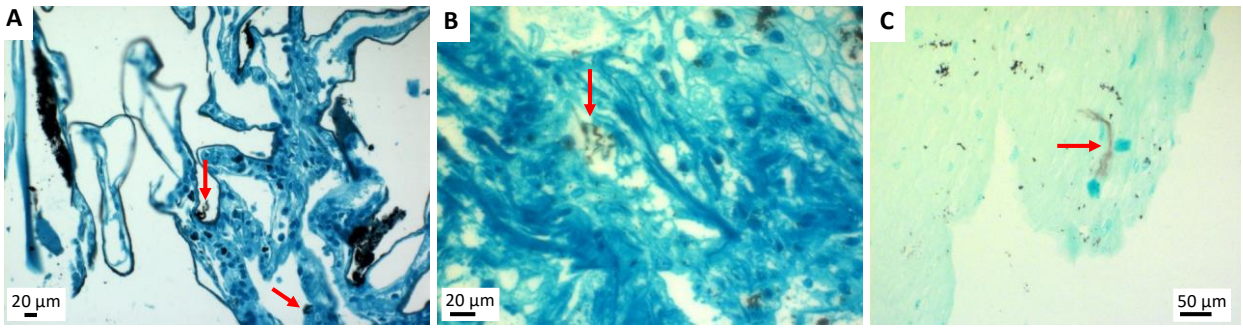

Figure 5. Grocott's methenamine silver staining showing *Ap. astaci* hyphal infiltrates (red arrows) in (**A**) gills (96 h), (**B**) heart muscle (192 h) and (**C**) abdominal muscle (192 h) of the infected noble crayfish.

Discussion

Multiple stages of the crayfish plague disease

Based on the exhibited gross symptoms in the *Ap. astaci* challenged noble crayfish we can divide the progression of the crayfish plague disease into three stages: asymptomatic stage, symptomatic stage and ultimately death (**Figure 6**). High susceptibility of the noble crayfish to the haplogroup B strain of *Ap. astaci* was previously observed in several independent controlled infection experiments [12–15]. This was also observed in the mass mortality events among wild noble crayfish populations [11,37]. Multiple gross symptoms of the disease can be observed in the infected noble crayfish before their death. The initial symptoms were behavioural changes, autotomy of pereiopods and chelipeds, ataxia and finally death. The scratching suggests that the noble crayfish is capable of sensing the invading pathogen growing within its cuticle and eyes. Furthermore, it has been suggested that *Ap. astaci* has a neurotoxic effect on its host, thus inducing behavioural changes and ataxia [27,38]. Previously, studies analysing *Ap. astaci* secreted effectors have mainly focused on the chitinases and other enzymes responsible for the degradation of the cuticle of the crayfish host [39,40]. It is, however, known that plant specific oomycete pathogens can produce widely diverse secreted effectors that are involved in manipulation, suppression and modification of the plant host defence mechanisms [41]. The chemical composition and existence of the specific neurotoxic compounds excreted by *Ap. astaci*, to our knowledge, still remains unconfirmed.

Autotomy, or loss of limbs, is a known defence mechanism in crustaceans. Autotomy increases the chances of surviving a predatory encounter, and a limb injury is usually the triggering stimuli [42]. When self-induced, autotomy occurs at the base of the limb along the fracture plane of the basi-ischium transition, as a result of the muscle contraction within a limb [43]. In response to the infection with *Ap. astaci*, self-inflicted autotomy, observed at the same fracture plane in our study, is probably linked to the increased stress of the animal. However, we cannot rule out that the growing hyphae of *Ap. astaci* caused a disturbance in the cuticular and/or muscular integrity, thus activating the autotomy reflex. Protrusion of *Ap. astaci* hyphae into the exoskeleton, joints of antennae and pereiopods, and in the soft cuticle of the abdomen, was observed in the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858) [44] and Australian red claw crayfish, *Cherax quadricatinatus* (von Martens, 1868) [45]. Alternatively, since autotomy of limbs is directly controlled by interneurons and motoneurons, it is possible that *Ap. astaci* is causing direct damage to neural tissue by growing within. Although we could not directly evaluate the damage on the nervous system (specifically brain and nerve cord), nerve cord and crayfish eyes were previously reported as target foci for *Ap.*

astaci [27,38,45–47]. Therefore, it is likely that *Ap. astaci* growth might contribute to disturbance of the neural pathways that control autotomy.

Our pilot attempt to identify potential foci of the pathogen growth led to observation of the disseminations of the *Ap. astaci* hyphae (based on Grocott's staining) within the gills, abdominal muscle and hearth of the noble crayfish. Hyphal infiltrates within the abdominal muscle and gills have been previously observed in *C. quadricarinatus* [45]. The significance of these results in the context of direct damage to the host and the host tissues remains to be explored. For now, it seems that the occurrence of the hyphal infiltrates in the heart and abdominal muscle (96 h) coincides with the onset of symptoms (96 h) in the infected noble crayfish. Higher number of biological replicates and analysis of a larger number of tissues would be beneficial to conduct a conclusive histological analysis and gain better understanding of the pathological changes that occur in the infected noble crayfish. Furthermore, in our experiment we observed that the hepatopancreas of the challenged noble crayfish was seemingly not affected by hyphal infiltrates of *Ap. astaci*. Although HSI was not significantly different in the infected crayfish compared to control, we observed a correlation between the HSI and gene expression (negative for *C/EBP* and positive for *Kr-h1*), as well as HSI and PFU (see below). This suggests a possible involvement of the hepatopancreas in the immune response, either through direct synthesis of immune molecules or energy investment of this central metabolic organ [21]. Afterall, hepatopancreas is an integrated organ of the crayfish immunity and metabolism [21]. Previously, high energy investment was observed in the invasive marbled crayfish, *Procambarus virginalis* Lyko, 2017, under multiple challenges with the highly virulent strain of *Ap. astaci* [23]. Further histological investigations might shed more light on the involvement of hepatopancreas in the immune response of *Ap. astaci* challenged noble crayfish.

Characteristics of the noble crayfish immune response

The increase in the THC (96 h) in the challenged group occurs simultaneously with the onset of gross symptoms of *Ap. astaci* infection (scratching and loss of limb). At the same time point *Ap. astaci* hyphae can be detected in the abdominal muscle and heart (**Figure 6**). A significant drop in the THC happened soon after, at 120 h, in the challenged group. Similar observations in the noble crayfish have been previously made in response to *Ap. astaci* challenge [12]. The average THC in the control noble crayfish had a high variance, but the overall THC was within the expected ranges for the European freshwater crayfish species [22,24]. A drop in THC might be indicative of the immune exhaustion in the challenged noble

crayfish. In fact, soon after the drop in the THC (at 120 h and 192 h), the crayfish showed loss of balance (120 h) and the first crayfish died (from 216 h). Circulating haemocytes play a crucial role in the host response to pathogen challenges. They are involved in phagocytosis, pathogen recognition, pathogen trapping and other inflammatory responses such as the release of antimicrobial peptides, components of the proPO system and other humoral response factors directly in the infection site [49]. Upon degranulation, crayfish haemocytes are lysed [50]. Furthermore, the loss of circulating haemocytes could be a consequence of their recruitment to the infection sites [51]. Thus, systemic haemocytes lost from circulation must be replaced by the release of new haemocytes from hematopoietic tissues [52]. This generates a state of hemocytopenia where crayfish are highly vulnerable, and their pathogen control capabilities are decreased [53,54]. Further studies focusing on the pathohistological changes in the noble crayfish infected with the *Ap. astaci* might provide more clarity. Nonetheless, it seems that the survival of the noble crayfish is connected to its capability of retaining normal THC levels. However, although THC might be a good indicator of the immune system activity, haemocytes are not the sole factor involved in the immune response.

Simultaneously with the drop in the THC, we recorded a down-regulation of *Kr-h1* (120 h). Similarly, an up-regulation of the *C/EBP* was observed soon after (192 h). Changes in the expression of these transcriptional factors appears rather delayed, as the major symptoms of infection already started, and the death of the infected mortality group animals occurred 3 days later [9]. In Crustaceans the function of *Kr-h1* is largely unexplored and was not clearly linked to the immune response previously. In the water flea, *Daphnia pulex* (De Geer, 1778), it was suggested to be essential in embryonic development [56]. In the swimming crab *Portunus trituberculatus* (Miers, 1876), it has been suggested that *Kr-h1* is involved in the regulation of vitellogenin synthesis in hepatopancreas [57]. *Kr-h1* is well explored in insects, where it has a role in development, vitellogenesis and embryogenesis, cast identity, neural morphogenesis, maturation of sexual behaviour and metabolism [55]. However, it has to be noted that on the level of integration in the regulatory networks mediated by juvenile hormones, as well as protein structure, *Kr-h1* in Crustaceans seems to be distinct from the *Kr-h1* in insects [56,57]. Conversely, an up-regulation of *C/EBP* has been observed in the marbled crayfish, challenged with *Ap. astaci* and under food restricting feeding regime [24]. Furthermore, in whiteleg shrimp, an up-regulation of the host *C/EBP* was confirmed in response to a challenge with the white spot syndrome virus (WSSV) [59]. In this context, changes in the expression of the *C/EBP* and *Kr-h1* in hepatopancreas might be a consequence of the altered metabolism in the moribund noble crayfish. This seems to be supported by a correlation between the changes in the gene

expression and HSI. An early down-regulation of *C/EBP* was also observed 12 h post challenge. However, to the best of our current knowledge, it was not possible to link this early change in the expression of C/EBP to any known process in the noble crayfish. On the other hand, down-regulation of *Kr-h1* and up-regulation of *C/EBP* in response to a highly virulent *Ap. astaci* strain has already been observed in the transcriptomic data of noble crayfish 3-days post infection [8]. Unfortunately, due to the lack of detailed histopathological analysis, neither in the aforementioned study nor in this study it was possible to distinguish if the observed changes in the gene expression are occurring in the hepatocytes and/or hepatopancreas associated immune cells or in infiltrated systemic haemocytes. Thus, conclusive evidence for the role of *C/EBP* and *Kr-h1* in the immune response of the noble crayfish is still lacking. The overall impact of these transcriptional factors on the transcriptional network remains unknown. Detailed functional characterization of these factors in healthy and immune challenged crayfish is required.

Three phases of pathogen growth

Based on the pathogen load analysis, we could identify three *Ap. astaci* growth phases within the tissues of challenged noble crayfish: an initial lag phase, followed by an exponential growth phase and finally a sporulation phase (**Figure 6**). During the initial lag phase (96 h), we did not detect any significant increase of the pathogen load inside the crayfish tissues. In these first hours of the infection, the activity of *Ap. astaci* is mainly aimed at overcoming the host immune defences. In fact, upon attachment to the host, germination of the *Ap. astaci* spores occurs within 2-6 h [58]. Zoospores lyse the surface layer of the crayfish epicuticle and form the germ tubes that penetrate through the epicuticle [17]. In this period, the germinating *Ap. astaci* hyphae have a proteolytic, hyaluronidase, and esterase activity [59]. Expression of the major chitinase gene *AaCht1*, responsible for chitin degradation, starts ~10h after spore germination [40]. Chitinase activity and synthesis slowly increases as the pathogen reaches the endocuticle of the host [40]. The germ tube starts protruding within the endocuticle of the crayfish ~24-48 h after infection [39]. The hyphal growth of the pathogen occurs along the chitin fibres [60]. Therefore, it is not surprising that our observed changes in the pathogen load during the initial 96 h post challenge are minimal, since pathogen growth is hindered by the host's cuticular barrier.

Finally, it seems that the end of the lag phase (48-96 h) occurs when *Ap. astaci* starts growing through the crayfish cuticle, where hyphae come in contact with the crayfish haemocoel [60] and can be soon after (96 h) observed in the abdominal muscle and heart.

Within the hemocoel, haemocytes circulating in the haemolymph of the noble crayfish slow down the *Ap. astaci* growth by specific inhibition towards the pathogen's proteases [61]. Haemocytes are also mobilised towards the infection site, and the immune system of the noble crayfish is now actively engaged in the defence reactions against the protruding pathogen [60]. This equilibrium between the invading pathogen and infected host seems to last until the immune system of the noble crayfish is exhausted (drop in THC), soon after which the lag phase ends and the exponential growth of *Ap. astaci* starts. Preliminary histological analysis showed that the invading hyphae are now growing withing the noble crayfish host (heart, abdominal muscle), which ultimately leads to the host death. In the dead crayfish, a 100-fold increase in the *Ap. astaci* load was detected. This change, indicative of the rapid growth that leads to spore generation, corresponds to the previous observations of the massive sporulation from infected noble crayfish after the death of the crayfish host [62]. Although three pathogen growth phases were observed in this study, they might not be universal and apply to each strain or crayfish host. Previous observations show that some *Ap. astaci* strain do not cause mortality in infected noble crayfish and the pathogen load decreases over time [13]. Similarly, in some resistant invasive crayfish species it has been shown that the *Ap. astaci* spores are constantly released [63] and there is no exponential *Ap. astaci* growth phase [13].

Study limitations and future perspectives

Our study design allowed us to explore the interaction of the crayfish plague pathogen with the immune system of the noble crayfish, nonetheless there are several limitations of the study that might hinder the generalisation of the obtained results. Firstly, all experiments have been conducted on the uniform cohort of intermoult adult male crayfish. Although there is no evidence for differences in the resistance of male and female noble crayfish, the expression of immune related genes might be influenced by the critical processes of the life cycle such as reproduction and moulting [64–66]. Furthermore, juvenile crayfish might be more susceptible to pathogen challenge compared to the sexually mature crayfish used in this study [67]. Therefore, our study design allowed us to limit these potential sources of noise in the biological signal. Moreover, based on the co-evolutionary theory and observations in the wild, it has been proposed that different noble crayfish populations might have different levels of resistance towards *Ap. astaci* [14]. The influence of the presented parameters on the outcome of *Ap. astaci* infections remains to be further explored in future. Secondly, we analysed the immune response of the noble crayfish to a single dose of a highly virulent pathogen's strain. However, it has been shown that different pathogen doses and strains can elicit variable immune responses in

the infected crayfish, both regarding the kinetics of disease progression and severity of the symptoms [12,15]. Multiple pathogenic strains of *Ap. astaci* are present in Europe [11], and understanding the factors that underlie their virulence and their influence on the disease progression in the native European crayfish species is of high conservation interest. Lastly, the analysis of the gene expression was conducted on hepatopancreas tissue. Due to the ease of RNA isolation and interplay of immunity and metabolism, the hepatopancreas is suitable for analysing the immune response of freshwater crayfish. On the other hand, this also makes the interpretation of the obtained results less straightforward, especially in the case of *C/EBP* and *Kr-h1* whose roles in freshwater crayfish are not yet fully explored. The characterisation of their role in terms of structure, origin, expression patterns and interaction partners is pending. Furthermore, the hepatopancreas is also highly influenced by other factors, such as diet, environment and various potentially pathogenic microorganisms present in the tissue [21]. It remains to be explored to which degree the hepatopancreas is infiltrated by haemocytes during *Ap. astaci* challenge, as well as the changes in the gene expression occurring in the other tissues of the infected noble crayfish.

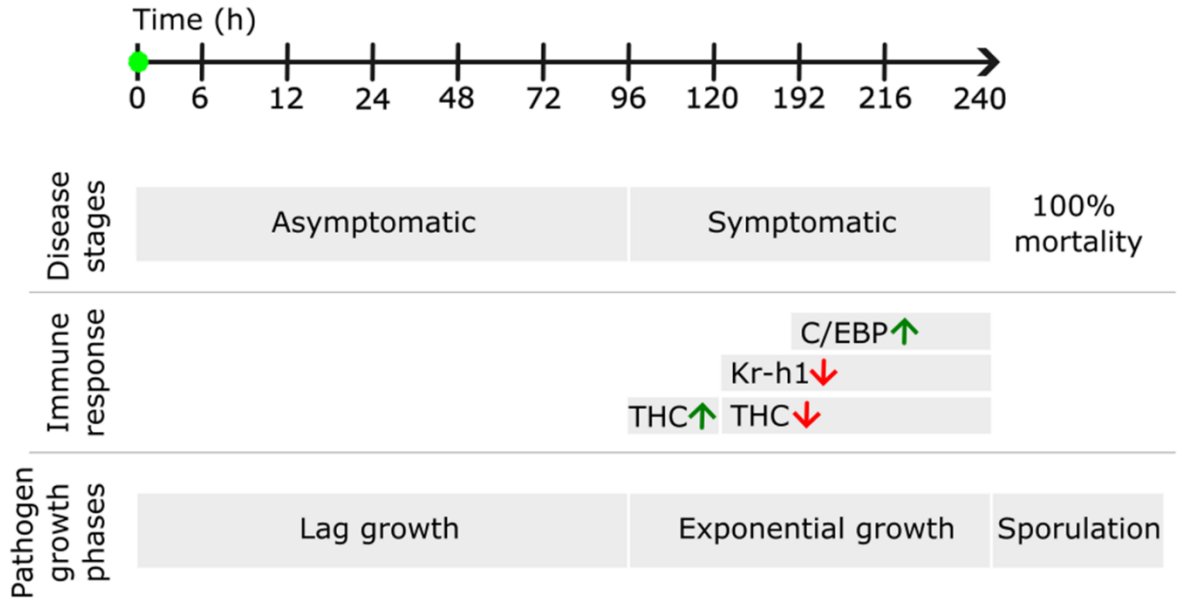

Figure 6. Summary of the crayfish plague disease progression in the noble crayfish challenged with the highly virulent strain of *Ap. astaci* belonging to haplogroup B, host immune response and *Ap. astaci* growth in the challenged noble crayfish. Based on the crayfish gross symptoms of infection three stages of the disease can be observed: asymptomatic stage (0-6 h), symptomatic stage (96-240 h) and onset of death. First changes in the immune parameters are observed 96 h post challenge. In the immune response, increase in total haemocyte count (THC,

96 h) is followed by the down-regulation of the Krüppel homolog-1 protein (*Kr-h1*, 120 h), decrease of THC (120 h) and up-regulation of CCAAT/enhancer-binding protein (*C/EBP*, 192 h) gene expression. Three phases of the pathogen growth can be distinguished: lag growth phase (0-96 h), exponential growth phase (96-240 h) and finally sporulation/rapid growth phase which occurs *post mortem*. Green dot marks the start of the infection challenge.

Conclusions

Based on our results, the interaction between the susceptible noble crayfish host and the highly virulent pathogen *Ap. astaci* is a dynamic process. It is characterised by multiple stages of the disease, changes in the immune response parameters, and multiple pathogen growth phases. Ultimately, *Ap. astaci* posed a too high challenge for the immune system of the challenged noble crayfish. Capabilities of the noble crayfish to actively engage in the clearance of the crayfish plague pathogen seemed to be limited. Reduction in the systemic count of the haemocytes precede the death of the noble crayfish. Nonetheless, the outcome and progression of the crayfish plague disease might differ based on the combination of the pathogen strain and crayfish population. We might expect that further research efforts in the field of pathogen toxicology, detailed histological analysis of the host under *Ap. astaci* challenge and comprehensive immunological studies will reveal key processes that precede the death of the noble crayfish. In this context, our research represents a stepping stone towards a better understanding of the host-pathogen relationship between freshwater crayfish and their oomycete pathogen, *Ap. astaci*.

Author Contributions

Lj.L.B., C.F., K.T. Conceptualization; Lj.L.B. Data curation; Lj.L.B. Formal analysis; K.T., O.L., R.G. Funding acquisition; Lj.L.B., C.F., L.B., A.D., R.G., A.B. Investigation; Lj.L.B., C.F., R.G. Methodology; K.T., O.L. Project administration; K.T., O.L., R.G. Resources; Lj.L.B Software; O.L., K.T., R.G., S.H., I.M. Supervision; O.L., K.T., R.G., A.B., I.M., S.H., C.F., Lj.L.B., L.B., C.R. Validation; Lj.L.B. Visualization; Lj.L.B. Writing-original draft. All authors read and approved the final version of the manuscript.

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Supplementary material

Supplementary table 1: Carapace length (CL), Weight of crayfish (W_t), weight of hepatopancreas (W_{hep}) and hepatosomatic index (HSI) for all crayfish used in the experiment.

Conflict of Interest Statement

Authors express no conflict of interest.

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