NeoBiota 77: 155–177 (2022)
doi: 10.3897/neobiota.77.87474
https://neobiota.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

Recurring infection by crayfish plague pathogen only marginally affects survival and growth of marbled crayfish

Ana Dobrović[1*], Sunčana Geček[2*], Tin Klanjšček[2], Ines Haberle[2], Paula Dragičević[1], Dora Pavić[3], Ana Petelinec[1,4], Ljudevit Luka Boštjančić[5,6], Lena Bonassin[5,6], Kathrin Theissinger[5], Sandra Hudina[1]

1 *Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia* **2** *Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička cesta 54, 10002 Zagreb, Croatia* **3** *Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia* **4** *Division of Molecular Biology, Ruđer Bošković Institute, Bijenička cesta 54, 10002 Zagreb, Croatia* **5** *LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), Senckenberg Biodiversity and Climate Research Centre, Senckenberganlage 25, D-60325 Frankfurt/Main, Germany* **6** *Department of Computer Science, ICube, UMR 7357, University of Strasbourg, CNRS, Centre de Recherche en Biomédecine de Strasbourg, 1 Rue Eugène Boeckel, 67000 Strasbourg, France*

Corresponding author: Sandra Hudina (sandra.hudina@biol.pmf.hr)

Academic editor: Eric Larson | Received 7 June 2022 | Accepted 8 October 2022 | Published 2 November 2022

Citation: Dobrović A, Geček S, Klanjšček T, Haberle I, Dragičević P, Pavić D, Petelinec A, Boštjančić LL, Bonassin L, Theissinger K, Hudina S (2022) Recurring infection by crayfish plague pathogen only marginally affects survival and growth of marbled crayfish. NeoBiota 77: 155–177. https://doi.org/10.3897/neobiota.77.87474

Abstract

Invasive alien crayfish threaten the diversity of freshwater ecosystems and native crayfish fauna. In Europe, this is largely due to transmission of the crayfish plague to susceptible native crayfish. Many invasive species tolerate crayfish plague, but the infection still has the potential to reduce the fitness of a tolerant host due to energy trade-offs between immune response maintenance and life-history traits, such as growth and reproduction. In combination with other unfavourable conditions, such a response could alter further invasion success of an otherwise successful crayfish invader. We examined whether repeated infection with one of the most virulent haplogroups of crayfish plague agent (*Aphanomyces astaci*) affects growth or survival of the juvenile marbled crayfish (*Procambarus virginalis*). Juveniles were exposed to i) two levels of pathogen concentrations, and ii) two different feeding regimes under the higher pathogen concentration. In all performed trials, repeated infection reduced growth rates, while the combination of recurring infection and food limitation significantly increased mortality. The average energy cost of the immune response was estimated at 12.07 J/day for individuals weighing 0.3 grams. Since infections were frequent

* These authors contributed equally to this work.

and pathogen concentrations high, results suggest that marbled crayfish is resistant to *A. astaci* pathogen and its survival is only affected by adding the stress of food limitation. The survival of almost half of the individuals exposed to high pathogen loads and extreme food limitation indicates that chronic infection by crayfish plague is unlikely to be an important factor impeding invasion success of the marbled crayfish, even under harsh conditions. Our results add to the growing body of evidence that marbled crayfish has potential to become one of the most successful freshwater invaders.

Keywords
food limitation, freshwater, immunity cost, infection, invasive species, trade-off

Introduction

Invasive alien species drive biodiversity loss and impair ecosystem services worldwide (Pyšek and Richardson 2010), partly because they carry pathogens to which native species lack resistance. In freshwater ecosystems, crayfish directly affect ecosystem structure and diversity (Dorn and Wojdak 2004). Many native crayfish, particularly in Europe, are on the brink of extinction due to introductions of invasive crayfish and their pathogens (Jussila et al. 2021; Theissinger et al. 2021). Invasive crayfish imported from North America into Europe introduced a novel and lethal disease, the crayfish plague, caused by the oomycete *Aphanomyces astaci* Schikora, 1906 (Jussila et al. 2021; Theissinger et al. 2021). The introduction and transmission of pathogen *A. astaci* is the main reason why native crayfish were displaced by invaders, and crayfish plague is solely responsible for decimation of numerous native European crayfish populations (Martín-Torrijos et al. 2019; Jussila et al. 2021; Theissinger et al. 2021).

North American crayfish species tolerate the crayfish plague due to their rapid immune reaction to the pathogen. The fast activation of the prophenoloxidase (ProPO) system encapsulates the pathogen's hyphae in melanin (Cerenius et al. 2003). However, even in North American crayfish, stressful conditions (i.e. chronic infections, unfavourable environmental conditions) may increase their susceptibility to pathogens (Chinchio et al. 2020) or negatively affect their fitness, growth and development (cf. Francesconi et al. 2021). Chronic infection requires continuous mounting of an immune response even in a tolerant species, thus incurring energy costs. In other species, covering these costs requires energy redistribution that results in trade-offs with life-history traits, such as growth and reproduction (e.g. Lochmiller and Deerenberg 2000; Martin et al. 2003, 2008; Rantala and Roff 2005; Lee 2006; van Der Most et al. 2011; Brown and Shine 2014; Schwenke et al. 2016; Kirschman et al. 2017; Körner et al. 2017; Miyashita et al. 2019). These trade-offs are most apparent under stressful conditions (i.e. limited resources or environmental/physiological stress; Kirschman et al. 2017; Rumschlag and Boone 2020) and may cause death (Garner et al. 2009) and ultimately adversely affect host population dynamics (Boots and Norman 2000; Boots et al. 2003; Campbell et al. 2019). Therefore, while chronic exposure to *A. astaci* may be sublethal in a resistant invasive crayfish species, it can cause mortality if individuals are under additional stress (Aydin et al. 2014). Such circumstances may lead to

population crashes even in tolerant invaders (e.g. as in signal crayfish; Edsman et al. 2015), alter their further invasion success, and even mitigate their negative effects on the ecosystem (Fincham et al. 2019; Chinchio et al. 2020).

We analyse whether chronic exposure to *A. astaci* may lead to significant sublethal or lethal effects in the marbled crayfish (*Procambarus virginalis* Lyko 2017, Cambaridae), a high-risk crayfish invader in Europe (Chucholl 2016; Kouba et al. 2021; Vogt 2021), listed amongst the Invasive Alien Species of Union concern (EU Regulation No. 1143/2014). Marbled crayfish is the only obligatory parthenogenetic freshwater crayfish (Scholtz et al. 2003) producing a large number of genetically identical offspring (Martin et al. 2007; Vogt et al. 2008), which makes it an ideal model organism. Although genealogically most closely related to North American species *Procambarus fallax* (Hagen, 1870), it has no known native habitat (Lyko 2017; Hossain et al. 2018). Its occurrence in Europe is significantly increasing due to its popularity in pet-trade (Chucholl 2016), as are contacts with other crayfish invaders that are known *A. astaci* carriers (cf. Linzmaier et al. 2020). Marbled crayfish can carry *A. astaci* haplogroup D, associated with another invader, *Procambarus clarkii* (Girard, 1852) (Keller et al. 2014; Mrugała et al. 2015). It is resistant to low concentrations of a highly virulent *A. astaci* haplogroup B, associated with invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) (Francesconi et al. 2021). However, the impact of chronic infection by crayfish plague and its combined effect with other stressors on survival and life history of marbled crayfish have not been explored yet, even though they could potentially affect its future expansion.

Methods

Experimental animals

Juvenile marbled crayfish were taken from an adult female transferred from Šoderica Lake in northern Croatia (46°14'20.9"N, 16°54'33.6"E) to the laboratory of the Department of Biology at the Faculty of Science in Zagreb. Adult marbled crayfish were kept in the laboratory for five months prior to oviposition, when juveniles for the experiments were obtained. For marbled crayfish keeping and research, we obtained a licence from the responsible Ministry of Environment and Energy, Croatia (Licence number: UP/I-612-07/19-43/01; 517-05-1-1-20-6), in accordance with the EU Regulation on Invasive Alien Species (EU Regulation No. 1143/2014).

Juvenile marbled crayfish were used in all experiments, since they moult often, allowing faster assessment of effects of exposure to *A. astaci* pathogen on growth and since juvenile growth is linear (McLay and van den Brink 2016).

Housing of experimental animals

Juvenile marbled crayfish were kept individually in 100 ml of aerated tap water in 250 ml plastic containers with a diameter of 90 mm. Containers were placed in cooled

incubators (ST 6 COMF, POL-EKO-APARATURA, Poland) 16–18 days before the start of the experiments to allow the juveniles to adapt to housing conditions (temperature regime 20 °C, photoperiod 12:12 light:dark). To avoid cross-contamination, each experimental group (described below) was housed in a separate incubator. The selected temperature results in the lowest mortality in the laboratory (Vogt et al. 2004). An aquarium pump (JK-AP9500, JK ANIMALS, Czech Republic) was placed between the containers and all containers were covered with a plastic lid to prevent water evaporation and escape of the crayfish. The crayfish were fed one pellet of JBL NovoPrawn food for shrimps with frequency depending on the feeding regime, and the water in all the containers was changed twice a week.

Aphanomyces astaci zoospore production

Haplogroup B strain of *A. astaci*, associated with the signal crayfish (Francesconi et al. 2021), was selected for the experiment because of its potential horizontal transmission from the signal crayfish to marbled crayfish. Šoderica Lake is close (ca. 450 m) to the Drava River, which contains signal crayfish infected with *A. astaci* (Maguire et al. 2016) and contact between the two species is expected to occur (Dobrović et al. 2021). Additionally, the signal crayfish is the most widespread invasive crayfish species in Europe, present in over 29 EU countries and in EU candidate countries (Dragičević et al. 2020), making transmission of this highly virulent haplogroup to the marbled crayfish likely.

A. astaci PEC8 isolate (haplogroup B) was obtained from Prof. Frédéric Grandjean from University of Poitiers, France. The isolate was grown on PG1 agar (Unestam 1965) with addition of antibiotics - oxolinic acid (SIGMA 0-00877) and ampicillin (SIGMA A 9518) at a concentration of 10 mg/l, to prevent bacterial growth. *A. astaci* zoospore production was induced using a protocol, based on Makkonen et al. (2012). A piece of PG1 agar (4 × 4 mm) containing *A. astaci* hyphae was used to inoculate 45 ml of liquid PG1 medium (Unestam 1965) with antibiotics in 12 replicates. The replicates were incubated at 18 °C for four days, after which the hyphae were cut into small fragments and agar was removed. Total hyphae biomass was divided into four portions, each portion was transferred into 50 ml of fresh liquid PG1 medium with antibiotics and grown at 18 °C for three days. Liquid PG1 medium was then removed and sporulation was induced by washing the hyphae in water from the stream Vrapčak in Gornje Vrapče, Zagreb (45°50'36.3"N, 15°53'44.8"E). Hyphae were washed four times with 200 ml of autoclaved natural water with mild linear shaking for 45 minutes at 18 °C, and were left in the shaker for 20 hours in the last (fourth) wash. Hyphae were then removed and zoospore concentration in the suspension was counted using a Thoma counting chamber. Crayfish infection was performed immediately after the zoospore production by adding zoospores to crayfish containers. Experimental zoospore concentrations (described below) in 100 ml of water were obtained by diluting the produced zoospore suspension with aerated tap water.

Experimental design

Prior to the infection experiment, virulence of the PEC8 isolate and sublethal load was tested in a pilot experiment to determine two sublethal concentrations of *A. astaci* zoospores and frequency of the repeated infections (Vukelić 2021). Infections were set to be repeated every two weeks at concentrations of 7500 zoospores/ml and 15000 zoospores/ml in Experiment 1, and 15000 zoospores/ml in Experiment 2 (described below). Dosages were higher than in other studies involving infection trials on marbled crayfish (i.e. Francesconi et al. 2021), but still within a range used for tolerant crayfish invaders (i.e. 10000 zoospores/ml in signal crayfish infection trials; Aydin et al. 2014) and considered realistic in the case of acute infection in the wild (Aydin et al. 2014).

During the experiments, crayfish from all experimental groups (control and infected groups) were photographed and weighed i) directly before each infection, ii) two weeks after the last infection (Experiment 1 and 2), and iii) six weeks after the last infection (Experiment 1). Due to the small size of the juveniles, their total length (TL; from the top of the rostrum to the end of the telson, in mm) was measured from photographs using the image processing programme, ImageJ (Schneider et al. 2012; http://rsbweb.nih.gov/ij/). Each crayfish was measured three times and the mean value represented TL. Before weighing, each crayfish was carefully dried on filter paper to remove excess water and weighed using a digital scale (accuracy: ± 0.0001 g). During the experiment, we also recorded mortality, disease symptoms (melanisation of the cuticle) and moulting of the crayfish. At the end of the experiments, surviving crayfish were euthanised by a rapid cut of the nerve cord through the thorax to the abdomen, in accordance with available guidelines for humane killing of crayfish (Conte et al. 2021). All specimens were stored in 96% ethanol for detection of *A. astaci* DNA from crayfish cuticle as described in Suppl. material 1: S1, while in surviving crayfish from Experiment 2, hepatopancreas was additionally dissected for gene expression analysis (described below).

Experiment 1: Repeated exposure of juvenile marbled crayfish to different *A. astaci* zoospore concentrations

A total of 55 juveniles were used in this experiment. For the trial, juvenile marbled crayfish were randomly divided into three experimental groups: 1) control group (15 individuals, non-infected), 2) group 7500 (20 individuals infected with 7500 zoospores/ml), and 3) group 15000 (20 individuals infected with 15000 zoospores/ml). The infection experiment was conducted for 18 weeks, with *A. astaci* infection performed every other week, six infections in total. In one case, the third infection was performed four weeks after the second infection due to unsuccessful *A. astaci* zoospore production at the time. Surviving crayfish were euthanised six weeks after the last infection and the cuticle of all crayfish was tested for presence of *A. astaci* (those that died during the experiment and those that survived until the end of the experiment; crayfish plague detection described in Suppl. material 1: S1).

Experiment 2: Repeated exposure of juvenile marbled crayfish to *A. astaci* under differing feeding regimes

A total of 60 individuals from a separate batch were used to test the interaction of chronic exposure to *A. astaci* with food availability. While food availability is unlikely to be a limiting factor in nature because crayfish are omnivores (Holdich 2002), it was used as a proxy for density-dependent effects. Juvenile crayfish form social dominance hierarchies (Issa et al. 1999; Sato and Nagayama 2012) and dominant individuals have increased access to food (Herberholz et al. 2007). Increasing population density is thus expected to limit resource availability, increase competition intensity (Capelli and Hamilton 1984), and induce stress.

In this experiment, crayfish were adapted to laboratory conditions for 16 days during which all individuals were fed twice a week. Then, four experimental groups of 15 individuals were used: 1) control - fed five times a week, non-infected, 2) control, food-restricted - fed once a week, non-infected, 3) infected - fed five times a week, and 4) infected, food-restricted - fed once a week. This experiment was conducted for 12 weeks, also with *A. astaci* infection intervals every other week (five in total).

Surviving crayfish were euthanised two weeks after the last infection and cuticle samples of all crayfish were tested for *A. astaci*, as in Experiment 1 (crayfish plague detection described in Suppl. material 1: S1). Additionally, euthanised crayfish were dissected and the hepatopancreas from each was carefully removed and stored in an RNA stabilising agent (RNA later; Sigma Aldrich, MO, USA) at -80 °C until RNA extraction for gene expression analysis. Five random individuals from each group were analysed for changes in expression of two genes related to crayfish innate immunity (prophenoloxidase - ProPO and CCAAT/enhancer-binding protein beta - C/EBP-β), and central metabolic pathways of glycolysis and citrate cycle (citrate synthase - CS and glyceraldehyde 3-phosphate dehydrogenase - GAPDH), selected based on Boštjančić et al. (2021) and Zheng et al. (2021). Total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). RNA quality was checked on the NanoVue Spectrophotometer, and RNA quantity with the QuantiFluor RNA System on the Quantus platform (Promega, USA). For the cDNA synthesis, 1 μg of total RNA was reversely transcribed with the iScript Select cDNA Synthesis Kit (Bio-Rad, USA) and Oligo(dT)15 primer. 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 input cDNA template on the CFX Opus 96 Real-Time PCR (Bio-Rad, USA). All samples were run in duplicates with the standard deviation of cycle threshold values < 0.5. Primer pairs for all target genes and endogenous control (elongation factor 1-α) can be found in Suppl. material 1: Table S2.1. The difference in the gene expression values between the target and control samples was expressed according to the delta-delta Ct method ($2^{-\Delta\Delta CT}$; Livak and Schmittgen 2001).

Estimating energy cost of the infection

We estimated energy cost of immunity response to chronic infection based on the differences in weight of non-infected and infected food-restricted groups in Experiment

2. Neither of the food-restricted groups grew appreciably in length, so differences in weight change between the two groups stem from differences in energy reserve dynamic: if size is the same, lighter individuals have smaller energy reserves. The reduction in energy reserves can be explained either by smaller energy input (e.g. reduction in food intake due to infection) or a higher basal metabolic rate related to the infection. Food intake was equal for both groups because all food had been eaten, so reduction in energy reserves must have been caused by increased metabolism due to the infection. Therefore, the energy content corresponding to the difference in weight represents the cost of the infection. In crayfish, changes in energy reserves are primarily reflected in change of hepatosomatic index, i.e. hepatopancreas weight (Jussila and Mannonen 1997; Sacristán et al. 2017). Energy content of hepatopancreas (E; kJ/g) of marbled crayfish was estimated using the correlation with moisture content of hepatopancreas (M; %) as suggested by Jussila and Mannonen (1997), using the regression for *Astacus astacus* (Linnaeus, 1758), for pooled sexes:

$$E = -0.336M + 54.189. \quad (1)$$

Average moisture content of hepatopancreas of marbled crayfish from our sampling site was reported to be 62% (Žižak 2015) and, therefore, this value was used to calculate energy reserve content. Finally, the difference in weight change between the groups was multiplied by the estimated energy content and divided by the duration of experiment to obtain cost of immunity response per day.

Statistical analysis

The effects of repeated infections using two *A. astaci* concentrations and interactive effects of repeated infections and food availability were examined through three major endpoints: 1) total growth (i.e. weight/length gain throughout the total duration of both experiments), 2) rate of growth (i.e. weight/length increment over time, in each experimental week, presented in Suppl. material 1: S3–S5), and 3) mortality (measured through the whole experimental period). In addition, the changes in immune and metabolic gene expression and metabolism were analysed in Experiment 2. Statistical analyses and graphical representation of results were performed in the R statistical environment (R version 3.6.3, R Studio 1.2.5033). The '*base*' and '*pastecs*' packages (Grosjean and Ibanez 2018; R Core Team 2020) were used for descriptive statistics and basic homoscedasticity (Levene test), normality (Shapiro-Wilks, QQ-plot) and extreme value tests, while the '*ggplot2* ' package was used for graphical display.

Total growth

Robust ANOVAs based on trimmed means (20% of trimming level; Wilcox 2012; Mair and Wilcox 2020; '*WRS2* ' package in R) were used to test (i) the effects of *A. astaci* infection (Experiment 1; one-way factorial design; three levels), and (ii) the joint effects of *A. astaci* infection and food availability (Experiment 2; two-way

factorial design; 2 × 2 levels) on total weight (length) gain. Total weight (length) gain was calculated as the difference between the final weight (length) at the end of the experiment and the initial weight (length) at the beginning of the experiment. Robust ANOVAs were used instead of classical ANOVAs to overcome problems associated with deviations from homoscedasticity and to reduce the influence of outliers observed in the data. Post-hoc tests were also performed in the robust '*WRS2*' environment (Mair and Wilcox 2020), while p-values were adjusted for multiple testing using the Benjamini-Hochberg (BH) method. The '*multcompView*' package (Graves et al. 2019) was used to convert the vector of p-values to a character-based display in which common characters denote levels or groups that are not significantly different.

Rate of growth

Growth rate analysis was performed to analyse questions such as: (a) what average growth trajectory best describes the rate of growth over time for all crayfish, (b) what is the variability in growth rates across crayfish, and finally, (c) does *A. astaci* infection and food availability explain variability in growth rates? Growth rate analysis, unlike total growth analysis, is based on complete longitudinal measurements of weight (and/or length) rather than just initial and final values. As the measurements at specific time points are clustered (nested) within individual crayfish, they imply a hierarchical structure of the data and intra-individual correlation. Therefore, the multilevel modelling (MLM) technique was used for data analysis (Peugh 2010; Monsalves et al. 2020). A detailed specification of all data levels, model variables and model equations can be found in Suppl. material 1: S3.

Mortality

Fisher's exact tests were used to determine the association between exposure to *A. astaci* and mortality in both experiments. Pairwise (post-hoc) Fisher test from the '*rstatix*' package in R (Kassambara 2020) with the Holm method of adjusting p-values was used for multiple comparisons of groups in Experiment 2. Kaplan-Meier estimates (curves) of survival probability and comparison of survival times between groups by the log-rank test were performed using the '*survival*' and '*survminer*' packages in R (Kassambara et al. 2020; Therneau 2022).

Gene expression

The Kruskal-Wallis test was used to determine whether the expression of immune and metabolic genes differed significantly between the groups in Experiment 2. Dunn's test from the '*FSA*' package in R (Ogle et al. 2021) was used for multiple pairwise comparisons of the groups. In the Dunn test, p-values were adjusted according to

the Holm method. To further investigate the relationships between gene expression variables, the '*FactoMineR*' and '*factoextra*' packages in R (Le et al. 2008; Kassambara and Mundt 2020) were used for principal component analysis (PCA) of the gene expression dataset.

Data availability

Data are permanently deposited in an open repository (Dryad: https://doi.org/10.5061/dryad.3xsj3txkp).

Results

Infection success

In both experiments, all crayfish that died (marked in red colour in Fig. 1) tested *A. astaci* positive, while all control animals tested to be pathogen-free (Fig. 1). Overall, the proportion of *A. astaci* positive individuals was higher in Experiment 2 (*A. astaci* detection two weeks since the last infection; Fig. 1B) than in Experiment 1 (*A. astaci* detection six weeks since the last infection; Fig. 1A).

Experiment 1: Repeated exposure of juvenile marbled crayfish to different *A. astaci* zoospore concentrations

Total growth

Repeated exposure to the crayfish plague pathogen (Fig. 2A) significantly reduced total weight gain ($F_{(2,17.06)} = 10.04$, $p = 0.001$, effect size $\xi = 0.84$, CI(ξ) = [0.64, 1.11]; Suppl. material 1: Table S4.1C). Both exposed groups weighed less than the control group, but did not differ from each other (Fig. 2A; post-hoc tests in Suppl. material 1: Table S4.1E). Total length gain (Suppl. material 1: Fig. S4.1A) was not significantly different between treatments ($F_{(2,17.19)} = 1.86$, $p = 0.185$; Suppl. material 1: Table S4.1D).

Rate of growth

Due to the complexity of the MLM model, a detailed description of the results on individual and group growth rates is given in Suppl. material 1: S3.2, S4, Figs S4.2, S4.3, Tables S4.2, S4.3). Here, we provide only a summary of the main results (fixed effects). *A. astaci* infection significantly reduced growth rates: the growth rate of weight in the control group (12.7 mg/wk) was significantly higher

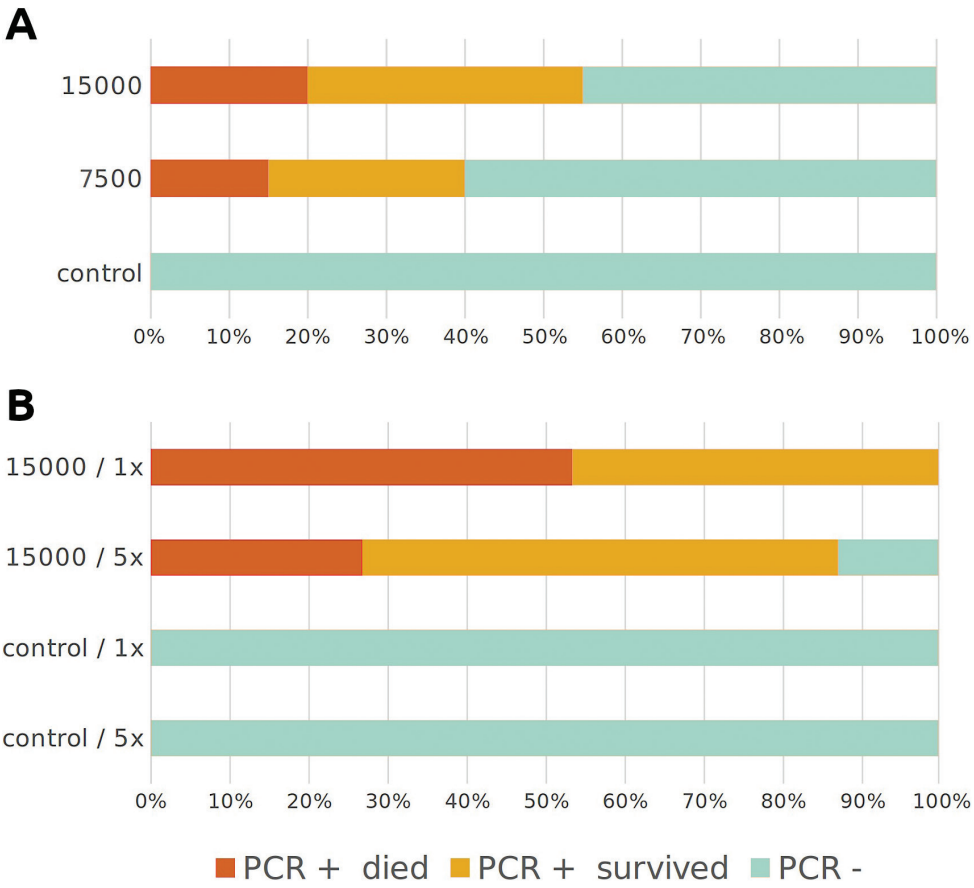

Figure 1. Detection of *A. astaci* in the cuticle of marbled crayfish in **A** Experiment 1 and **B** Experiment 2. In Experiment 1, surviving crayfish were euthanised six weeks after the last infection; in Experiment 2, they were euthanised after two weeks. PCR+ = *A. astaci* detected, PCR - = *A. astaci* not detected.

than the growth rates in both *A. astaci* infected groups (10.2 mg/wk and 9.6mg/wk; Suppl. material 1: Table S4.2D), while growth rate of length was significantly reduced only under higher *A. astaci* concentration (Suppl. material 1: Table S4.3D). Weight and length increased significantly over time in all groups.

Mortality

The effect of exposure to pathogens on mortality was not significant ($p = 0.26$, Fisher's exact test). A similar result, i.e. insignificant effect of infection, was also inferred from the overlapping confidence intervals of the Kaplan-Meier survival curves (Fig. 2C) and from the comparison of survival times between the groups using the log-rank test ($\chi^2(2) = 3.2$, $p = 0.20$).

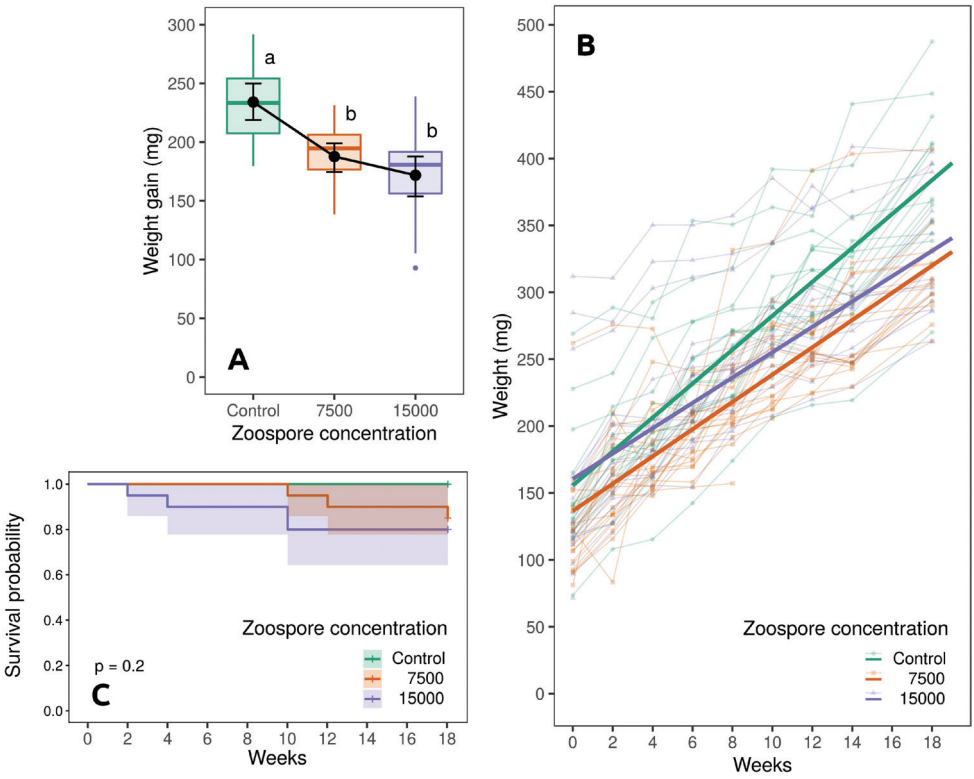

Figure 2. Effects of repeated infection of marbled crayfish juveniles using two *A. astaci* zoospore concentrations (7500 and 15000 zoospore/ml) on **A** total weight gain (total growth) **B** rate of weight increment (rate of growth) and **C** survival probability of individuals. Different letters in panel A denote significant differences, error bars represent 95% confidence intervals (CIs) around the mean. Results for total length gain and rates of length increment are presented in Suppl. material 1: S4.

Experiment 2: Repeated exposure of juvenile marbled crayfish to *A. astaci* under differing feeding regimes

Total growth

Food availability (i.e. different feeding regimes) significantly influenced crayfish growth (Fig. 3A). There was a significant effect of food ($Q = 652.22$, $p = 0.001$), *A. astaci* infection ($Q = 44.55$, $p = 0.001$), and their interaction ($Q = 11.92$, $p = 0.003$) on the total weight gain during the experiment (Suppl. material 1: Table S5.1C). Pairwise post-hoc comparisons revealed significant differences in weight gain between all groups (Suppl. material 1: Table S5.1D). Food-restricted groups gained significantly less weight - both in the control ($\hat{\psi} = 279.11$, $p < 10^{-5}$) and the infected groups ($\hat{\psi} = 212.62$, $p < 10^{-5}$). Weight gain of the control food-restricted group was approximately 90% lower than in the control group fed five times

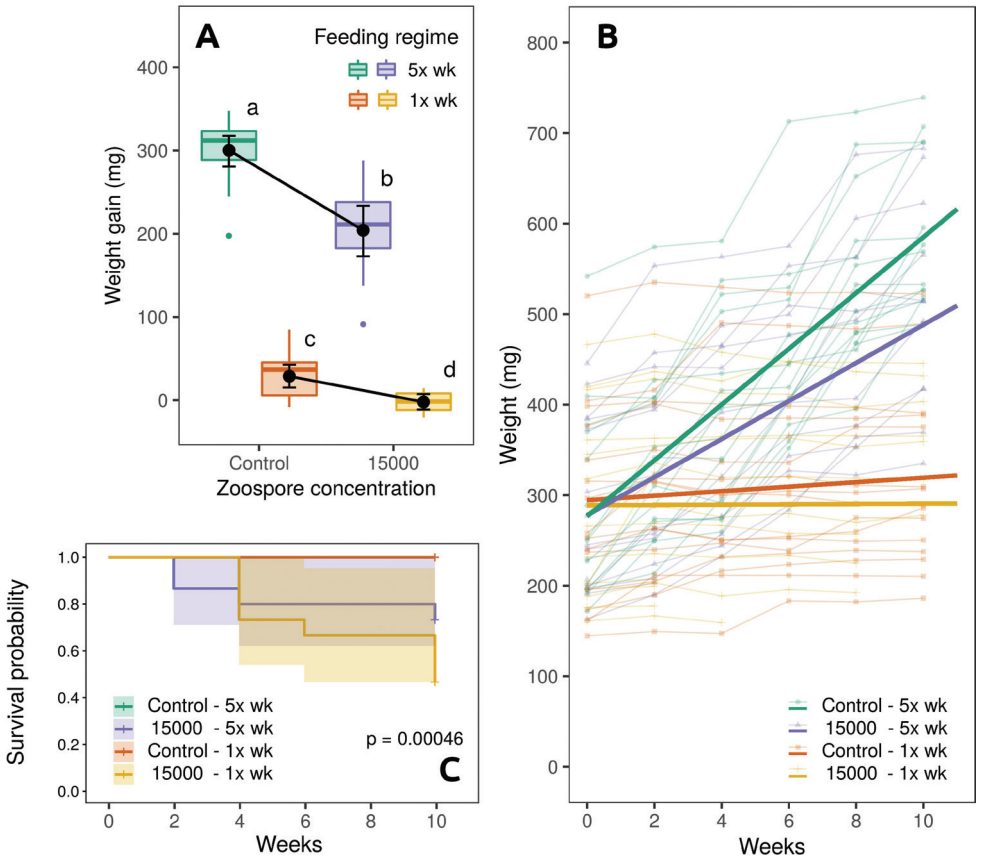

Figure 3. Effects of repeated infection of marbled crayfish juveniles with 15000 zoospore/ml of *A. astaci* under two feeding regimes (once a week and five times a week) on **A** total weight gain (total growth) **B** rate of weight increment (rate of growth) and **C** survival probability of individuals. Different letters in panel A denote significant differences, error bars represent 95% confidence intervals (CIs) around the mean. Results for total length gain and rates of length increment are presented in Suppl. material 1: S5.

a week (Suppl. material 1: Table S5.1A). Total weight gain was lower when crayfish were infected, compared to non-infected control groups (feeding regime five times per week: $\hat{\psi}$ = 97.50, p = 0.0002; feeding regime once a week: $\hat{\psi}$ = 31.01, p = 0.014; Suppl. material 1: Table S5.1D). In general, the effect of food on weight gain (ξ = 0.93, CI(ξ) = [0.89; 1]) was stronger than the effect of *A. astaci* infection (ξ = 0.21, CI(ξ) = [0.03; 0.56]). Furthermore, the effect of infection was stronger at high food than food-restricted regime due to significant interactions between food and weight (Suppl. material 1: Table S5.1C). Additional inferential analysis of length as a response variable leads to similar conclusions, but without significant differences in total length gain between infected and non-infected groups under food restriction (p = 0.07; Suppl. material 1: Fig. S5.1A, Table S5.1B, E, F).

Rate of growth

A detailed analysis of the results of the MLM model can be found in Suppl. material S3.3 and S5, Figs S5.2, S5.3, Tables S5.2, S5.3); here, a summary of the main results (fixed effects) is given. Both food restriction and infection with *A. astaci*, as well as their interactive effect, significantly reduced growth rate of weight (and length). The average growth rate of uninfected crayfish fed five times per week was 30.8 mg/wk, compared with an average growth rate of 21.0 mg/wk for the infected group under the same feeding regime, and an average growth rate of 2.5 mg/wk for the food-restricted control group. The average growth rate of the infected crayfish with food restriction was only 0.2 mg/wk. The infection-induced decrease in growth rate was greater when food was abundant than when food was restricted.

Mortality

Food limitation and *A. astaci* infection increased individual mortality. Mortality differed significantly amongst groups in Experiment 2 ($p < 10^{-3}$, Fisher's exact test), with the highest mortality occurring in the infected group fed once a week (8 deaths, i.e. 53% in 10 weeks; Fig. 3C). The statistically significant difference in mortality in pairwise comparisons was obtained only between this group and (i) the control (non-infected) group fed once per week ($p = 0.013$) and (ii) the control group fed five times per week ($p = 0.013$). The difference in mortality rate between the infected and non-infected groups fed five times a week was not significant ($p = 0.398$). Consistent with the result of Fisher's test, survival times estimated by Kaplan-Meier method (Fig. 3C) were significantly different between groups according to the log-rank test ($\chi^2(3) = 17.9$, $p < 10^{-3}$).

Expression of immune and metabolic genes

Repeated exposure to *A. astaci* and different food availability significantly affected the expression of the metabolic genes CS ($H(3) = 15.34$, $p = 0.002$) and GAPDH ($H(3) = 9.72$, $p = 0.021$), and the immune gene C/EBP-β ($H(3) = 14.54$, $p = 0.002$), with no significant effects on ProPO expression (Fig. 4A; Suppl. material 1: Table S5.4A, C, E, G). The expression of metabolic genes was reduced in groups fed once a week, but the significantly lower mean rank was confirmed by post-hoc analysis only for the infected group (CS: $p = 0.01$; GAPDH: $p = 0.038$; Suppl. material 1: Table S5.4B, D). Conversely, the expression of the immune gene C/EBP-β was elevated in the groups fed once a week, but again, a significantly higher mean rank was confirmed by post-hoc analysis only for the infected group ($p = 0.007$; Suppl. material 1: Table S5.4F).

Relationships amongst gene expression variables were further analysed using the principal component analysis. Two principal components captured most of the variability in the dataset (87.6%, Fig. 4B), justifying the reduction of the complexity of the data analysis from four dimensions to two dimensions. The first principal component

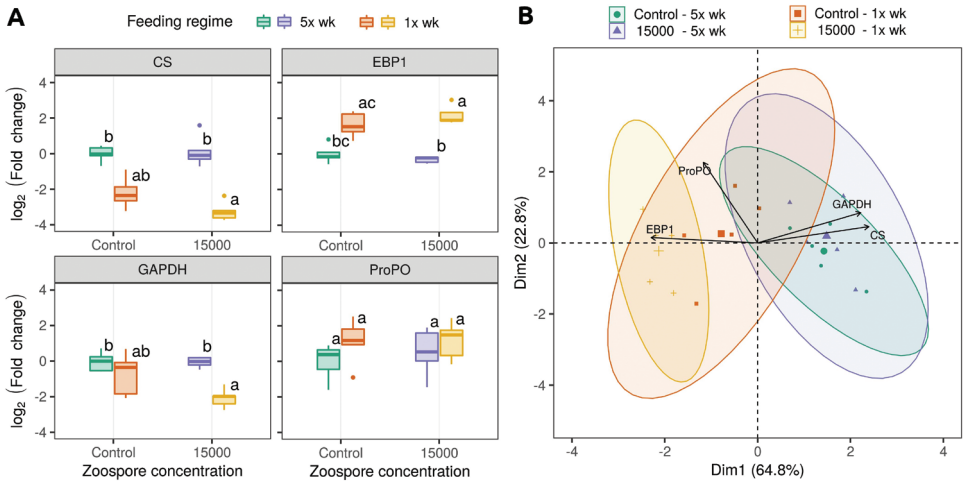

Figure 4. Differential expression of immune and metabolic genes in Experiment 2 analysed using **A** inferential comparison between groups and **B** principal component analysis (PCA). Different letters in panel A denote significant differences.

is strongly correlated with three of the gene expression variables CS, GAPDH and C/EBP-β, suggesting that these variables may vary together. PCA analysis also showed a high positive correlation between metabolic gene expressions, a positive correlation between immune gene expressions and negative correlations between metabolic and immune gene expressions. Consistent with the inferential analysis, experimental groups fed five times a week showed higher expression of GAPDH and CS genes.

Energy cost of infection

The food-restricted infected group of Experiment 2 had to activate and maintain immune response to the pathogen, inducing additional energy costs in contrast to the respective control (non-infected) group. Based on the average moisture content of the hepatopancreas, crayfish had 33.357 kJ/g in reserve. The comparison of total weight change between the two groups showed a total difference of 0.025 g, corresponding to 12.07 J/day of energy reserves used for immune response maintenance in infected individuals weighing on average 0.3 g.

Discussion

Chronic infection of invasive marbled crayfish juveniles with the *A. astaci* pathogen leads to trade-offs in energy use that reduces growth. Although this is the first such finding for crayfish, this is consistent with previous research on other invertebrates (i.e. arthropods: Rantala and Roff 2005; Bascuñán-García et al. 2010; Körner et al. 2017;

molluscs: O'Connell-Milne et al. 2016) and vertebrates (Lochmiller and Deerenberg 2000; Lee 2006; van Der Most et al. 2011; Bonneaud et al. 2012), which suggest that maintenance of an active immune system is energetically demanding, and trade-offs against growth, reproduction and development following infection are frequent. However, effects on growth observed here were achieved under repeated infections with high pathogen loads, while the observed sublethal effects (reduction of growth rates) translated into lethal effects (mortality) only under extreme food limitation. This shows that the marbled crayfish is (i) highly resistant to one of the most virulent haplogroups of *A. astaci* pathogen (haplogroup B) and can act as its carrier, and (ii) requires multiple stressors occurring simultaneously to suffer increased mortality.

Growth was reduced under both pathogen concentrations in Experiment 1 and under both feeding regimes in Experiment 2. Even though not statistically significant in some scenarios, the omnipresence of effects on growth suggest that exposure to pathogens increases allocation of energy to the immune system. Alternatively, the observed slower growth of infected groups could be the consequence of suppressed food intake suggested to occur during immune challenges (Lochmiller and Deerenberg 2000; Moberg 2000). We deem this unlikely because food was entirely consumed by crayfish in our experiments, suggesting that observed effects on growth are, indeed, related to the energy allocation to immune response.

A larger effect size of repeated infections on weight was observed in both experiments and became especially evident in Experiment 2. Here, the extreme food limitation (feeding once a week) almost completely ceased growth in both control (non-infected) and infected groups. The miniscule (if any) weight increase of the control food-restricted group suggests that those individuals were very near or at the point of starvation. Additional need to fuel the immune response of infected individuals further depleted energy reserves, thus increasing the severity of starvation resulting in increased mortality of the infected group fed once a week.

Starvation, as well as the difference in severity of starvation, was reflected in the down-regulation of genes involved in the central metabolic pathways of glycolysis and citrate cycle in both infected and non-infected group fed once a week. This is in line with previous studies on freshwater species which have shown that the expression of both CS and GAPDH decrease during starvation and fasting (Tripathi and Verma 2003; Salem et al. 2007). The most pronounced reduction in metabolic gene expression was observed in the food-restricted infected group, corroborating our assumptions that immune response to a pathogen resulted in additional depletion of energy reserves.

Food limitation and the resulting starvation induced up-regulation of the C/EBP-β transcription factor, belonging to the CCAAT/enhancer-binding protein (C/EBP) family, which are involved in the regulation of the metabolism, cell growth, differentiation, death, immune and inflammatory processes (Ramji and Foka 2002; Wang et al. 2019). C/EBP-β up-regulation is crucial in the acute phase of the *A. astaci* infection in the native noble crayfish (Boštjančić et al. 2021). In our study, C/EBP-β was up-regulated in both control and infected food-restricted groups in comparison to groups fed five times a week, with the highest change in C/EBP-β expression observed in the food-restricted

infected group. This indicates that stressful conditions of both starvation and pathogen infection increase the activity of C/EBP-β gene, signifying its role in regulation of both metabolism and immune response in crayfish. The expression of the ProPO gene, which belongs to the core immune response mechanism in crustaceans engaged in the encapsulation of the pathogen (ProPO pathway; Cerenius et al. 2003), did not differ significantly between infected and non-infected individuals in any of the groups in Experiment 2. This is consistent with previous reports from infection trials that applied a single infection using lower pathogen concentration of the same *A. astaci* haplogroup (Boštjančić et al. 2021), suggesting that there was no acute immune response and that energy was not invested into synthesis of immune effectors in hepatocytes. Rather, it probably went into the reparation of the damaged epicuticle, as the first line in the defence against the pathogen (Cerenius et al. 2003), which is energetically costly.

Lower mortality in the group fed five times a week indicates that these individuals were more able to cover the additional energy costs of fighting the disease, including the potential reparation of damage, as opposed to the group fed once a week. The scale of the cost is well represented by the differences in weight gain between the infected and non-infected food-restricted groups: the 'missing' weight can be solely attributed to energy costs of fighting the infection (immune response maintenance costs) and related consequences.

Chronic exposure to the *A. astaci* pathogen is unlikely to have a long-term effect on marbled crayfish populations, except under extreme limitation of food availability, which was used in this study as a proxy for density-dependent effects. In all experimental groups, infected individuals grew more slowly than non-infected. In nature, the slower growth could translate into slower maturation rates and/or lower fecundity of infected individuals due to size-dependence of these traits (Hossain et al. 2019) and reduction in energy available for reproduction. However, we do not expect that such growth reduction would have high implications for the invasion success of this species due to its parthenogenetic mode of reproduction and high fecundity in comparison to other native and invasive crayfish (Hossain et al. 2018).

Furthermore, a lower proportion (40 - 55%) of individuals tested positive for *A. astaci* in Experiment 1 (analyses performed six weeks after last infection) compared to Experiment 2 (analyses performed two weeks after the last infection), where almost all individuals (87 - 100%) were *A. astaci* positive. This either means that: i) infections were less successful in Experiment 1 or that ii) some individuals from Experiment 1 were able to contain the infection during the six weeks since the last *A. astaci* infection, unlike in Experiment 2 where *A. astaci* detection was performed two weeks after the last infection. We consider the former unlikely, as the procedures were the same in both experimental trials and number of infections was higher in Experiment 1 (6 repeated infections in Experiment 1 vs. 5 in Experiment 2). If the latter is true, this suggests that at least some proportion of marbled crayfish that survived repeated infections could potentially efficiently contain the pathogen and minimise further trade-offs with growth.

Our results add to the growing knowledge regarding the high tolerance of marbled crayfish to multiple single and combined stressors (Kaldre et al. 2015; Guo et al.

2019; Hossain et al. 2021; Stara et al. 2021). Such tolerance, in combination with fast growth, high fecundity and parthenogenetic mode of reproduction, prime it for becoming one of the most successful crayfish invaders.

Acknowledgements

This research has been supported by Croatian Science Foundation (HRZZ) [installation grant HRZZ-UIP-2017-05-1720 to SH, PD and TK, DOK-2018-09-4671 to IH and DOK-09-2018 to AD]. We thank Ana Bielen, Anđela Miljanović and Filip Ložek for their help in design of experimental procedures and help in crayfish plague cultivation. Additionally, we thank all students (Mara Vukelić, Dominik Guzalić, Marina Malnar, Jana Zekirovski, Mihaela Šurina, Iva Žulj) and Mirjana Hudina for their help with crayfish maintenance and help in the laboratory during infection trials. We also thank two reviewers and the editor (Eric R. Larson) for their helpful comments and suggestions.

References

Aydin H, Kokko H, Makkonen J, Kortet R, Kukkonen H, Jussila J (2014) The signal crayfish is vulnerable to both the As and the PsI-isolates of the crayfish plague. Knowledge and Management of Aquatic Ecosystems 413(413): 3. https://doi.org/10.1051/kmae/2014004

Bascuñán-García AP, Lara C, Córdoba-Aguilar A (2010) Immune investment impairs growth, female reproduction and survival in the house cricket, *Acheta domesticus*. Journal of Insect Physiology 56(2): 204–211. https://doi.org/10.1016/j.jinsphys.2009.10.005

Bonneaud C, Balenger SL, Hill GE, Russell AF (2012) Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. Molecular Ecology 21(19): 4787–4796. https://doi.org/10.1111/j.1365-294X.2012.05736.x

Boots M, Norman R (2000) Sublethal infection and the population dynamics of host-microparasite interactions. Journal of Animal Ecology 69(3): 517–524. https://doi.org/10.1046/j.1365-2656.2000.00417.x

Boots M, Greenman J, Ross D, Norman R, Hails R, Sait S (2003) The population dynamical implications of covert infections in host-microparasite interactions. Journal of Animal Ecology 72(6): 1064–1072. https://doi.org/10.1046/j.1365-2656.2003.00777.x

Boštjančić LL, Francesconi C, Rutz C, Hoffbeck L, Poidevin L, Kress A, Jussila J, Makkonen J, Feldmeyer B, Bálint M, Lecompte O, Theissinger K (2021) Comparative transcriptome analysis of noble crayfish and marbled crayfish immune response to *Aphanomyces astaci* challenges. bioRxiv. https://doi.org/10.1101/2021.05.25.445163

Brown GP, Shine R (2014) Immune response varies with rate of dispersal in invasive cane toads (*Rhinella marina*). PLoS ONE 9(6): e99734. https://doi.org/10.1371/journal.pone.0099734

Campbell L, Bower DS, Clulow S, Stockwell M, Clulow J, Mahony M (2019) Interaction between temperature and sublethal infection with the amphibian chytrid fungus impacts

a susceptible frog species. Scientific Reports 9(1): 1–8. https://doi.org/10.1038/s41598-018-35874-7

Capelli GM, Hamilton PA (1984) Effects of food and shelter on aggressive activity in the crayfish *Orconectes rusticus* (Girard). Journal of Crustacean Biology 4(2): 252. https://doi.org/10.2307/1548022

Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I, Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. Cellular Microbiology 5(5): 353–357. https://doi.org/10.1046/j.1462-5822.2003.00282.x

Chinchio E, Crotta M, Romeo C, Drewe JA, Guitian J, Ferrari N (2020) Invasive alien species and disease risk: An open challenge in public and animal health. PLoS Pathogens 16(10): e1008922. https://doi.org/10.1371/journal.ppat.1008922

Chucholl C (2016) Marbled crayfish gaining ground in Europe: the role of the pet trade as invasion pathway. In: Kawai T, Faulkes Z, Scholtz G (Eds) Freshwater Crayfish: Global Overview. CRC Press, Boca Raton, 83–114. https://doi.org/10.1201/b18723-8

Conte F, Voslarova E, Vecerek V, Elwood RW, Coluccio P, Pugliese M, Passantino A (2021) Humane Slaughter of Edible Decapod Crustaceans. Animals (Basel) 11(4): 1089. https://doi.org/10.3390/ani11041089

Dobrović A, Maguire I, Boban M, Grbin D, Hudina S (2021) Reproduction dynamics of the marbled crayfish *Procambarus virginalis* Lyko, 2017 from an anthropogenic lake in northern Croatia. Aquatic Invasions 16(3): 482–498. https://doi.org/10.3391/ai.2021.16.3.06

Dorn NJ, Wojdak JM (2004) The role of omnivorous crayfish in littoral communities. Oecologia 140(1): 150–159. https://doi.org/10.1007/s00442-004-1548-9

Dragičević P, Faller M, Kutleša P, Hudina S (2020) Update on the signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) range expansion in Croatia: A 10-year report. BioInvasions Records 9(4): 793–807. https://doi.org/10.3391/bir.2020.9.4.13

Edsman L, Nyström P, Sandström A, Stenberg M, Kokko H, Tiitinen V, Makkonen J, Jussila J (2015) Eroded swimmeret syndrome in female crayfish *Pacifastacus leniusculus* associated with *Aphanomyces astaci* and *Fusarium* spp. infections. Diseases of Aquatic Organisms 112(3): 219–228. https://doi.org/10.3354/dao02811

EU (2014) Regulation No. 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species. Official Journal of the European Union L317: 35–55. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%5C%3A32014R1143

Fincham WNW, Dunn AM, Brown LE, Hesketh H, Roy HE (2019) Invasion success of a widespread invasive predator may be explained by a high predatory efficacy but may be influenced by pathogen infection. Biological Invasions 21(12): 3545–3560. https://doi.org/10.1007/s10530-019-02067-w

Francesconi C, Makkonen J, Schrimpf A, Jussila J, Kokko H, Theissinger K (2021) Controlled Infection Experiment With *Aphanomyces astaci* Provides Additional Evidence for Latent Infections and Resistance in Freshwater Crayfish. Frontiers in Ecology and Evolution 9: 1–12. https://doi.org/10.3389/fevo.2021.647037

Garner TWJ, Walker S, Bosch J, Leech S, Marcus Rowcliffe J, Cunningham AA, Fisher MC (2009) Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. Oikos 118(5): 783–791. https://doi.org/10.1111/j.1600-0706.2008.17202.x

Graves S, Piepho HP, Selzer L Dorai-RajS (2019) multcompView: Visualizations of Paired Comparisons. R package version 0.1-8. https://CRAN.R-project.org/package=multcompView

Grosjean P, Ibanez F (2018) pastecs: Package for Analysis of Space-Time Ecological Series. R package version 1.3.21. https://CRAN.R-project.org/package=pastecs

Guo W, Kubec J, Veselý L, Hossain MS, Buřič M, McClain R, Kouba A (2019) High air humidity is sufficient for successful egg incubation and early post-embryonic development in the marbled crayfish (*Procambarus virginalis*). Freshwater Biology 64(9): 1603–1612. https://doi.org/10.1111/fwb.13357

Herberholz J, Mccurdy C, Edwards DH (2007) Direct benefits of social dominance in juvenile crayfish. The Biological Bulletin 213(1): 21–27. https://doi.org/10.2307/25066615

Holdich DM (2002) Biology of Freshwater Crayfish. Blackwell Science, Oxford, 720 pp.

Hossain MS, Patoka J, Kouba A, Buřič M (2018) Clonal crayfish as biological model: A review on marbled crayfish. Biologia 73(9): 841–855. https://doi.org/10.2478/s11756-018-0098-2

Hossain MS, Kouba A, Buřič M (2019) Morphometry, size at maturity, and fecundity of marbled crayfish (*Procambarus virginalis*). Zoologischer Anzeiger 281: 68–75. https://doi.org/10.1016/j.jcz.2019.06.005

Hossain MS, Kubec J, Guo W, Roje S, Ložek F, Grabicová K, Randák T, Kouba A, Buřič M (2021) A combination of six psychoactive pharmaceuticals at environmental concentrations alter the locomotory behavior of clonal marbled crayfish. The Science of the Total Environment 751: e141383. https://doi.org/10.1016/j.scitotenv.2020.141383

Issa FA, Adamson DJ, Edwards DH (1999) Dominance hierarchy formation in juvenile crayfish *Procambarus clarkii*. The Journal of Experimental Biology 202(24): 3497–3506. https://doi.org/10.1242/jeb.202.24.3497

Jussila J, Mannonen A (1997) Marron (*Cherax tenuimanus*) and noble crayfish (*Astacus astacus*) hepatopancreas energy and its relationship to moisture content. Aquaculture (Amsterdam, Netherlands) 149(1–2): 157–161. https://doi.org/10.1016/S0044-8486(96)01425-1

Jussila J, Edsman L, Maguire I, Diéguez-Uribeondo J, Theissinger K (2021) Money Kills Native Ecosystems: European Crayfish as an Example. Frontiers in Ecology and Evolution 9: e648495. https://doi.org/10.3389/fevo.2021.648495

Kaldre K, Meženin A, Paaver T, Kawai T (2015) A Preliminary Study on the Tolerance of Marble Crayfish *Procambarus fallax* f. *virginalis* to Low Temperature in Nordic Climate. In: Kawai T, Faulkes Z, Scholtz G (Eds), Freshwater Crayfish: A Global Overview. CRC Press, Boca Raton, 54–62. https://doi.org/10.1201/b18723-6

Kassambara A (2020) rstatix: Pipe-Friendly Framework for Basic Statistical Tests. R package version 0.6.0. https://CRAN.R-project.org/package=rstatix

Kassambara A, Mundt F (2020) factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.7. https://CRAN.R-project.org/package=factoextra

Kassambara A, Kosinski M, Biecek P (2020) survminer: Drawing Survival Curves using 'ggplot2'. R package version 0.4.8. https://CRAN.R-project.org/package=survminer

Keller NS, Pfeiffer M, Roessink I, Schulz R, Schrimpf A (2014) First evidence of crayfish plague agent in populations of the marbled crayfish (*Procambarus fallax* forma *virginalis*). Knowledge and Management of Aquatic Ecosystems 414(414): 15. https://doi.org/10.1051/kmae/2014032

Kirschman LJ, Quade AH, Zera AJ, Warne RW (2017) Immune function trade-offs in response to parasite threats. Journal of Insect Physiology 98: 199–204. https://doi.org/10.1016/j.jinsphys.2017.01.009

Körner M, Vogelweith F, Foitzik S, Meunier J (2017) Condition-Dependent Trade-Off Between Weapon Size and Immunity in Males of the European Earwig. Scientific Reports 7(1): 7988. https://doi.org/10.1038/s41598-017-08339-6

Kouba A, Lipták B, Kubec J, Bláha M, Veselý L, Haubrock PJ, Oficialdegui FJ, Niksirat H, Patoka J, Buřič M (2021) Survival, growth, and reproduction: Comparison of marbled crayfish with four prominent crayfish invaders. Biology (Basel) 10(5): 422. https://doi.org/10.3390/biology10050422

Le S, Josse J, Husson F (2008) FactoMineR: An R Package for Multivariate Analysis. Journal of Statistical Software 25(1): 1–18. https://doi.org/10.18637/jss.v025.i01

Lee KA (2006) Linking immune defenses and life history at the levels of the individual and the species. Integrative and Comparative Biology 46(6): 1000–1015. https://doi.org/10.1093/icb/icl049

Linzmaier SM, Musseau C, Matern S, Jeschke JM (2020) Trophic ecology of invasive marbled and spiny-cheek crayfish populations. Biological Invasions 22(11): 3339–3356. https://doi.org/10.1007/s10530-020-02328-z

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods (San Diego, Calif.) 25(4): 402–408. https://doi.org/10.1006/meth.2001.1262

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: Just what is the cost of immunity? Oikos 88(1): 87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x

Lyko F (2017) The marbled crayfish (Decapoda: Cambaridae) represents an independent new species. Zootaxa 4363(4): 544–552. https://doi.org/10.11646/zootaxa.4363.4.6

Maguire I, Jelić M, Klobučar G, Delpy M, Delaunay C, Grandjean F (2016) Prevalence of the pathogen *Aphanomyces astaci* in freshwater crayfish populations in Croatia. Diseases of Aquatic Organisms 118(1): 45–53. https://doi.org/10.3354/dao02955

Mair P, Wilcox R (2020) Robust statistical methods in R using the WRS2 package. Behavior Research Methods 52(2): 464–488. https://doi.org/10.3758/s13428-019-01246-w

Makkonen J, Jussila J, Kortet R, Vainikka A, Kokko H (2012) Differing virulence of *Aphanomyces astaci* isolates and elevated resistance of noble crayfish *Astacus astacus* against crayfish plague. Diseases of Aquatic Organisms 102(2): 129–136. https://doi.org/10.3354/dao02547

Martin II LB, Scheuerlein A, Wikelski M (2003) Immune activity elevates energy expenditure of house sparrows: A link between direct and indirect costs? Proceedings. Biological Sciences 270(1511): 153–158. https://doi.org/10.1098/rspb.2002.2185

Martin P, Kohlman K, Scholtz G (2007) The parthenogenetic Marmorkrebs (marbled crayfish) produces genetically uniform offspring. Naturwissenschaften 94(10): 843–846. https://doi.org/10.1007/s00114-007-0260-0

Martin LB, Weil ZM, Nelson RJ (2008) Seasonal changes in vertebrate immune activity: Mediation by physiological trade-offs. Philosophical Transactions of the Royal Society of London: Series B, Biological Sciences 363(1490): 321–339. https://doi.org/10.1098/rstb.2007.2142

Martín-Torrijos L, Kokko H, Makkonen J, Jussila J, Diéguez-Uribeondo J (2019) Mapping 15 years of crayfish plague in the Iberian Peninsula: The impact of two invasive species on the endangered native crayfish. PLoS ONE 14(8): e0219223. https://doi.org/10.1371/journal.pone.0219223

McLay CL, van den Brink AM (2016) Crayfish Growth and Reproduction. In: Longshaw M, Stebbing PD (Eds) Biology and Ecology of Crafish. CRC Press, Boca Raton, 62–116.

Miyashita A, Lee TYM, McMillan LE, Easy R, Adamo SA (2019) Immunity for nothing and the eggs for free: Apparent lack of both physiological trade-offs and terminal reproductive investment in female crickets (*Gryllus texensis*). PLoS ONE 14(5): e0209957. https://doi.org/10.1371/journal.pone.0209957

Moberg GP (2000) Biological response to stress: implications for animal welfare. In: Moberg GP, Mench JA (Eds) The biology of animal stress: basic principles and implications for animal welfare. CABI, Wallingford, 1–21. https://doi.org/10.1079/9780851993591.0001

Monsalves MJ, Bangdiwala AS, Thabane A, Bangdiwala SI (2020) LEVEL (Logical Explanations & Visualizations of Estimates in Linear mixed models): Recommendations for reporting multilevel data and analyses. BMC Medical Research Methodology 20(1): 1–9. https://doi.org/10.1186/s12874-019-0876-8

Mrugała A, Kozubíková-Balcarová E, Chucholl C, Cabanillas Resino S, Viljamaa-Dirks S, Vukić J, Petrusek A (2015) Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: Crayfish plague and white spot syndrome. Biological Invasions 17(5): 1313–1326. https://doi.org/10.1007/s10530-014-0795-x

O'Connell-Milne SA, Poulin R, Savage C, Rayment W (2016) Reduced growth, body condition and foot length of the bivalve *Austrovenus stutchburyi* in response to parasite infection. Journal of Experimental Marine Biology and Ecology 474: 23–28. https://doi.org/10.1016/j.jembe.2015.09.012

Ogle DH, Doll JC, Wheeler P, Dinno A (2021) FSA: Fisheries Stock Analysis. R package version 0.9.1, https://github.com/droglenc/FSA

Peugh JL (2010) A practical guide to multilevel modeling. Journal of School Psychology 48(1): 85–112. https://doi.org/10.1016/j.jsp.2009.09.002

Pyšek P, Richardson DM (2010) Invasive Species, Environmental Change and Management, and Health. Annual Review of Environment and Resources 35: 25–55. https://doi.org/10.1146/annurev-environ-033009-095548

R Core Team (2020) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Ramji DP, Foka P (2002) CCAAT/enhancer-binding proteins: Structure, function and regulation. The Biochemical Journal 365(3): 561–575. https://doi.org/10.1042/bj20020508

Rantala MJ, Roff DA (2005) An analysis of trade-offs in immune function, body size and development time in the Mediterranean Field Cricket, *Gryllus bimaculatus*. Functional Ecology 19(2): 323–330. https://doi.org/10.1111/j.1365-2435.2005.00979.x

Rumschlag SL, Boone MD (2020) Lethal and sublethal amphibian host responses to *Batrachochytrium dendrobatidis* exposure are determined by the additive influence of host resource availability. Journal of Wildlife Diseases 56(2): 338–349. https://doi.org/10.7589/2019-01-021

Sacristán HJ, Rodríguez YE, los Angeles Pereira N, Lopez Greco LS, Lovrich GA, Fernandez Gimenez AV (2017) Energy reserves mobilization: Strategies of three decapod species. PLoS ONE 12(9): e0184060. https://doi.org/10.1371/journal.pone.0184060

Salem M, Silverstein J, Rexroad CE III, Yao J (2007) Effect of starvation on global gene expression and proteolysis in rainbow trout (*Oncorhynchus mykiss*). BMC Genomics 8(1): 1–16. https://doi.org/10.1186/1471-2164-8-328

Sato D, Nagayama T (2012) Development of agonistic encounters in dominance hierarchy formation in juvenile crayfish. The Journal of Experimental Biology 215(7): 1210–1217. https://doi.org/10.1242/jeb.066191

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9(7): 671–675. https://doi.org/10.1038/nmeth.2089

Scholtz G, Braband A, Tolley L, Reimann A, Mittmann B, Lukhaup C, Steuerwald F, Vogt G (2003) Ecology: Parthenogenesis in an outsider crayfish. Nature 421(6925): 806. https://doi.org/10.1038/421806a

Schwenke RA, Lazzaro BP, Wolfner MF (2016) Reproduction-Immunity Trade-Offs in Insects. Annual Review of Entomology 61(1): 239–256. https://doi.org/10.1146/annurev-ento-010715-023924

Stara A, Zuskova E, Vesely L, Kouba A, Velisek J (2021) Single and combined effects of thiacloprid concentration, exposure duration, and water temperature on marbled crayfish *Procambarus virginalis*. Chemosphere 273: e128463. https://doi.org/10.1016/j.chemosphere.2020.128463

Theissinger K, Edsman L, Maguire I, Diéguez-Uribeondo J, Jussila J (2021) Editorial: Conservation of European Freshwater Crayfish. Frontiers in Ecology and Evolution 9: e804629. https://doi.org/10.3389/fevo.2021.804629

Therneau T (2022) A Package for Survival Analysis in R. R package version 3.4-0. https://CRAN.R-project.org/package=survival

Tripathi G, Verma P (2003) Starvation-induced impairment of metabolism in a freshwater catfish. Zeitschrift für Naturforschung. C, A Journal of Biosciences 58(5–6): 446–451. https://doi.org/10.1515/znc-2003-5-626

Unestam T (1965) Studies on the Crayfish Plague Fungus *Aphanomyces astaci* I. Some Factors Affecting Growth in vitro. Physiologia Plantarum 18(2): 483–505. https://doi.org/10.1111/j.1399-3054.1965.tb06911.x

van Der Most PJ, De Jong B, Parmentier HK, Verhulst S (2011) Trade-off between growth and immune function: A meta-analysis of selection experiments. Functional Ecology 25(1): 74–80. https://doi.org/10.1111/j.1365-2435.2010.01800.x

Vogt G (2021) Evaluation of the suitability of the parthenogenetic marbled crayfish for aquaculture: Potential benefits versus conservation concerns. Hydrobiologia 848(2): 285–298. https://doi.org/10.1007/s10750-020-04395-8

Vogt G, Tolley L, Scholtz G (2004) Life stages and reproductive components of the marmorkrebs (marbled crayfish), the first parthenogenetic decapod Crustacean. Journal of Morphology 261(3): 286–311. https://doi.org/10.1002/jmor.10250

Vogt G, Huber M, Thiemann M, van den Boogaart G, Schmitz OJ, Schubart CD (2008) Production of different phenotypes from the same genotype in the same environment by developmental variation. The Journal of Experimental Biology 211(4): 510–523. https://doi.org/10.1242/jeb.008755

Vukelić M (2021) The effect of infection with crayfish plague pathogen *Aphanomyces astaci* (Schikora, 1906) on mortality and growth of marbled crayfish *Procambarus virginalis* (Lyko, 2017). Master's Thesis. University of Zagreb, Faculty of Science, Department of Biology.

Wang W, Xia X, Mao L, Wang S (2019) The CCAAT/Enhancer-Binding Protein Family: Its Roles in MDSC Expansion and Function. Frontiers in Immunology 10: 1804. https://doi.org/10.3389/fimmu.2019.01804

Wilcox RR (2012) Introduction to robust estimation and hypothesis testing (3rd Edn.). Elsevier, USA, 1–690. https://doi.org/10.1016/B978-0-12-386983-8.00001-9

Zheng X, Jiang W, Zhang L, Espe M, Abasubong KP, Zhang C, Zhang D, Li X, Jiang G, Chi C, Wenbin L (2021) Investigation of the regulatory effect of icariin on the hepatopancreas glycolipid metabolism and energy metabolism of female *Eriocheir sinensis* during exogenous vitellogenesis based on the transcriptomics and metabolomics. Aquaculture Nutrition 27(6): 2066–2083. https://doi.org/10.1111/anu.13341

Žižak A (2015) Organosomatic indices of invasive species *Procambarus fallax* (Hagen, 1870) f. *virginalis* from the Šoderica Lake (Koprivnica). Master's Thesis. University of Zagreb. Faculty of Science. Department of Biology.

Supplementary material 1

Protocols, primers, statistical support data, images

Authors: Ana Dobrović, Sunčana Geček, Tin Klanjšček, Ines Haberle, Paula Dragičević, Dora Pavić, Ana Petelinec, Ljudevit Luka Boštjančić, Lena Bonassin, Kathrin Theissinger, Sandra Hudina

Explanation note: The file contains addtional detailed descriptions of used protocols and applied statistical analyses, additional statistical support data and images.

Link: https://doi.org/10.3897/neobiota.77.87474.suppl1