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Research paper

Expression of *C/EBP* and *Kr-h1* transcription factors under immune stimulation in the noble crayfish

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ABSTRACT

Transcription factors (TFs) have an important role in the regulation of the gene expression network. The role of TFs in the immune response of freshwater crayfish is poorly understood, but leveraging the regulatory mechanisms of immune response could augment the resistance against the invasive oomycete pathogen, *Aphanomyces astaci*. Previous studies indicated that the TFs CCAAT/enhancer-binding protein (*C/EBP*) and putative Krüppel homolog-1 protein (*Kr-h1*) might play a role in immune and stress response of the noble crayfish (*Astacus astacus*). Here, we aimed to further characterise these two gene products to gain a better understanding of their evolutionary origin, domain organisation and expression patterns across different crayfish tissues. Furthermore, we conducted an immune stimulation experiment to observe the potential changes in the gene expression of *C/EBP* and *Kr-h1* under immune challenge in different crayfish tissues. Our results showed that both *C/EBP* and *Kr-h1* are closely related to other *C/EBPs* and *Kr-h1s* in Malacostraca. Gene expression analysis revealed that both TFs are present in all analysed tissues, with higher expression of *C/EBP* in the gills and *Kr-h1* in the abdominal muscle. Immune stimulation with laminarin (mimicking β -1–3-glucan in the oomycete cell wall) showed an activation of the crayfish immune system, with an overall increase in the total haemocyte count (THC) compared to untreated control and crayfish buffered saline (CBS) treatment. On the gene expression level, an up-regulation of the *C/EBP* gene was detected in the laminarin treated group in hepatopancreas and heart, while no changes were observed for the *Kr-h1* gene. Our results indicate an early change in *C/EBP* expression in multiple tissues during immune stimulation and suggest its involvement in the immune response of the noble crayfish.

1. Introduction

Freshwater crayfish have a substantial impact on the functioning of freshwater ecosystems and are of significant economic and cultural importance (Reynolds et al., 2013). Adverse effects of water regulation, pollution and climate change, together with the introduction of invasive

crayfish species and their pathogens, have ushered in an array of challenges for the declining European freshwater crayfish stocks (Jussila et al., 2015; Martín-Torrijos et al., 2019; Ungureanu et al., 2020). In Europe, the noble crayfish, *Astacus astacus* (Linnaeus, 1758), is a species of high concern protected by national and international laws (Jussila et al., 2021a; Annex III of the Bern Convention and Appendix V of EU

Abbreviations: CBS, crayfish buffered saline; C/EBP, CCAAT/enhancer-binding protein; EF1 α , elongation factor 1 α protein; Kr-h1, Krüppel homolog-1 protein; ML, maximum likelihood; THC, total haemocyte count; TFs, transcription factors; TSS, transcriptions start site; WSSV, white spot syndrome virus; PAMP, pathogen associated molecular pattern; PRR, pattern recognition receptor.

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Habitat Directive/92/43/EEC). Harvesting of the noble crayfish (and other native European freshwater crayfish species) collapsed after the spread of the invasive North American crayfish and their oomycete pathogen *Aphanomyces astaci* Schikora (1906) which caused an estimated loss of up to 50–70 % of the noble crayfish populations (Edsman et al., 2023; Richman et al., 2015). There is evidence for an ongoing co-evolution between the native European freshwater crayfish and the crayfish plague pathogen, possibly occurring on short time scales. Resistance to *Ap. astaci* has been reported for the populations of the noble crayfish (Jussila et al., 2021b, 2011), the narrow-clawed crayfish, *Pontastacus leptodactylus* (Eschscholtz, 1823) (Alvanou et al., 2024; Kokko et al., 2012), the stone crayfish, *Austropotamobius torrentium* (Schrank, 1803) (Jussila et al., 2017) and the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858) (Martín-Torrijos et al., 2017). Substantial efforts have been undertaken in the last decades to gain a better understanding of the immune system of freshwater crayfish and its interaction with *Ap. astaci* (Bouallegui, 2021). Freshwater crayfish co-evolved with the pathogens in their environment, which drove the evolution of a complex and highly diverse innate immune arsenal (Loker et al., 2004; Williams and Gilmore, 2022). The components of the innate immune system are tightly regulated, as the maintenance of the immune defence is a significant energetic burden, and miss-regulated components of the immunity can be self-destructive (Burnett and Burnett, 2015; Cerenius et al., 2008; Paludan et al., 2021). Still, current immunological studies have focused mainly on the immune response effectors, and immune response regulators are poorly investigated.

Transcription factors (TFs) serve as primary regulators of gene expression (Weidemüller et al., 2021). These proteins bind to specific gene regulatory sequences and recruit or interact with other proteins, thereby orchestrating the gene expression network within a cell (Mitsis et al., 2020). Consequently, TFs participate in regulating the chromatin structure of the DNA, making it accessible or inaccessible to transcriptional machinery (Weidemüller et al., 2021). TFs are integral to various biological processes, like cell differentiation and development, as well as the response to external stimuli including immune regulation (Smale, 2014; Spitz and Furlong, 2012). TFs are functionally very divergent between species and often demonstrate tissue specific expression patterns (Mitsis et al., 2020). While a small subset of TFs is well studied and their roles in signalling and gene regulation are well characterised, many TFs are considered understudied or “dark TFs” (Weidemüller et al., 2021). This knowledge gap is particularly pronounced in non-model invertebrates. Within Crustacea, the second largest subphylum of Arthropoda, over 130 000 TFs have been identified based on genome and transcriptome data (Qin et al., 2017). Despite this huge diversity, significant effort is still required to comprehensively characterise the function of these TFs.

Recently, by leveraging transcriptomic data we have identified two TFs, namely putative CCAAT/enhancer-binding protein (*C/EBP*) and putative Krüppel homolog-1 protein (*Kr-h1*), as differentially expressed in the immune response of the noble crayfish under *Ap. astaci* challenge (Boštjančić et al., 2022). While *C/EBP* is a well-studied immune response gene in vertebrates, *Kr-h1* is studied mostly in insects, where it is considered a cornerstone gene in their developmental cycle (He and Zhang, 2022; Ramji and Foka, 2002). Still, both their roles and functions in the immune response of decapods are not clear. Previously, *C/EBP* was observed as up-regulated and *Kr-h1* as down-regulated in the hepatopancreas of the *Ap. astaci* challenged noble crayfish (Boštjančić et al., 2022). Furthermore, our previous findings demonstrated that the gene expression of these TFs in the hepatopancreas of the noble crayfish challenged with the highly virulent strain of *Ap. astaci* is altered in the symptomatic stage of the crayfish plague disease (Boštjančić et al., 2023). Similarly, in haemolymph and gill tissue of the whiteleg shrimp *Penaeus vannamei* (Boone, 1931), *C/EBP* played a role in the response to a challenge with the white spot syndrome virus (WSSV) (Wang et al., 2023). In the marbled crayfish, *Procambarus virginalis* Lyko 2017, the expression of the *C/EBP* in the hepatopancreas was up-regulated under

stressful conditions of starvation and *Ap. astaci* challenge (Dobrovčić et al., 2022). This growing evidence suggests that both *C/EBP* and *Kr-h1* might play an important role in the regulation of the immune and stress response in freshwater crayfish.

As an alternative to the direct pathogen challenge, it has been shown that the injection with crayfish buffered saline (CBS) or laminarin (analogue of the β -1–3-glucan present in the oomycete cell wall) can alter the number of the crayfish immune cells (haemocytes), as well as modulate the expression of immune genes in the North American signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) (Ekblom et al., 2021). Early studies showed that inoculation of inactivated *Ap. astaci* spores in the noble crayfish can also alter its susceptibility to the challenge with *Ap. astaci*, effectively promoting immunisation and “acquired resistance” towards the pathogen (Unestam and Weiss, 1970). Furthermore, changes in the immune response parameters, such as enzyme activity or gene expression, are often linked to the changes in the total haemocyte count (THC), a fundamental measure of the overall immune status of the crayfish (Smith, 2016; Söderhäll, 2016). Based on the effects of the *Ap. astaci* pathogen challenge on the THC and expression of *C/EBP* and *Kr-h1* in previous studies, we could expect that the injection of an analogue immunostimulant, laminarin, affects the immune system similarly. In this context, we had the following study aims: (i) To verify the gene organisation, copy number, evolutionary origin and domain organisation of the noble crayfish *C/EBP* and *Kr-h1*; (ii) To investigate the tissue-specific expression patterns of the transcription factors *C/EBP* and *Kr-h1* in the noble crayfish; (iii) To assess the effects of immune stimulation with CBS and laminarin on the total haemocyte count in the noble crayfish and (iv) To determine the effect of CBS and laminarin immune stimulation on the expression of the transcription factors *C/EBP* and *Kr-h1* in the noble crayfish. Understanding the effects of laminarin on the noble crayfish immune system might be beneficial for its future application as an immunostimulant of the farmed crayfish populations.

2. Materials and methods

2.1. Genomic, phylogenetic and structural analysis of *C/EBP* and *Kr-h1*

Genomic sequences of *Kr-h1* and *C/EBP* were reconstructed based on the BLASTn search (Altschul et al., 1990) against the draft genome assembly of *Astacus astacus* (Rutz et al., unpublished results). The putative contigs were then manually inspected and assembled (all sequences are available in the Supplementary file 1). Genomic sequences were further compared with representative genome assemblies of decapods: *Cherax quadricarinatus* (GCF_026875155.1), *Procambarus clarkii* (GCF_020424385.1), *Homarus americanus* (GCF_018991925.1) and *Penaeus vannamei* (GCF_003789085.1). The promoter sequence and the transcription start site (TSS) were identified with NNPP version 2.2 (BDGP: Neural Network Promoter Prediction (fruitfly.org), accessed on the 10th of March 2024) and ElemeNT version 2023 (ElemeNT V2023 – Juven-Gershon Lab, accessed on the 10th of March 2024). CpG island location was predicted with the EMBOSS Cpgplot (EMBOSS Cpgplot < EMBL-EBI, accessed on the 10th of March 2024) and Meth Primer (MethPrimer-Design MSP/BSP primers and predict CpG islands – Li Lab, PUMCH (urogene.org), accessed on the 10th of March 2024), with the CpG island length set to > 100 bp. Dot-plot analysis was conducted on the maffw web server (MAFFT – a multiple sequence alignment program (cbrc.jp), accessed on the 10th of March 2024). We conducted a DIAMOND 2.0.4 BLASTx search (sensitive mode, e-value $\leq 1e-25$) (Buchfink et al., 2015) against the publicly available transcriptomes of 15 freshwater crayfish species obtained from the TSA database (GAFS01, GAFY01, GBEI01, GADE01, HACK01, HACB02, GBYW01, GARH01, GBEV01, GICG01, GEDF01, GIVA01, GJEC01, GJEB01) with the noble crayfish *C/EBP* (GJEB01120680.1) and *Kr-h1* (GJEB01090533.1) as bait sequences. Protein sequences were predicted using ORF Finder tool provided by NCBI (<https://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Furthermore, we enriched our sequence datasets with closely related

sequences from other species extracted from literature sources. All protein sequences, sequence lengths and accession numbers are available in [Supplementary table 1](#) and [Supplementary table 2](#) for *C/EBP* and *Kr-h1*, respectively. Multiple alignments of the protein sequences were conducted using ClustalO with default settings (Sievers et al., 2011). Multiple sequence alignments used for the generation of the phylogenetic trees are available in the [Supplementary file 2](#) and [Supplementary file 3](#). Maximum likelihood (ML) tree reconstruction was conducted in IQ-TREE (Nguyen et al., 2015) with 10 000 bootstrap replicates and automatic model selection. The selected protein evolution model for *C/EBP* phylogenetic reconstruction was Q.insect + F + I + G4 and for *Kr-h1* GTR20 + F + I + G4. The functional domains of *C/EBP* and *Kr-h1* were predicted using the SMART program (<https://smart.emblheidelberg.de>) and molecular weight and isoelectric point were predicted using the Expasy Molecular Biology server (https://web.expasy.org/compute_pi). Potential novel conserved protein regions were detected using the iterative PSI-BLAST search approach with the e-value set to < 0.005 (Altschul, 1997). The subcellular localisation of the noble crayfish *C/EBP* and *Kr-h1* proteins was predicted using Euk-mPLoc 2.0 (Chou and Shen, 2010).

2.2. Changes in the gene expression of *C/EBP* and *Kr-h1* under immune challenge

2.2.1. Noble crayfish keeping

In total, 13 noble crayfish were obtained from the breeder Dr. Harald Groß (Bad Münstereifel-Schönau, Germany). All crayfish used in the experiment were 3-year-old intermoult sexually-mature males. Carapace length and mass of each individual crayfish are available in the [Supplementary table 3](#). 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 h was maintained. All aquaria were equipped with an aerator. The initial acclimatisation period before the start of the experiment was 22 days. Water exchange was conducted twice a week, and crayfish were fed a green pea every 2 days. All animal experiments were conducted in accordance with 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.

2.2.2. Experimental design

Noble crayfish were divided into three experimental groups: untreated control group (n = 4), crayfish buffered saline (CBS) injected group (n = 4) and laminarin injected group (n = 5). Crayfish buffered saline injected group was used as an injection control, as we expected an immune reaction at the site of the puncture wound. Crayfish buffered saline (CBS) was prepared with the following composition 210 mM NaCl, 5.4 mM KCl, 10 mM CaCl₂·2H₂O, 2.4 mM MgCl₂·6H₂O and 2 mM NaHCO₃ in 1 L of water, and pH was adjusted to 6.8 with 1 mM KOH (Goldina et al., 2011). Laminarin (containing β-1–3-glucan) solution was prepared by dissolving (5 mg/mL) laminarin (L9634, Sigma-Aldrich, USA) in CBS according to Ekblom et al., 2021. Crayfish were injected using a 26-gauge needle directly into the dorsal cephalothoracic cavity with 100 μL of CBS or laminarin. Post injection, crayfish were monitored 30 min for any signs of distress (scratching, loss of limbs, loss of movement, mortality). Sampling of the crayfish from all experimental groups was conducted four hours post injection. This time point was chosen based on the observations in a previous study where changes in immunological parameters in the signal crayfish were observed from 30 min to 6 h (Ekblom et al., 2021).

2.2.3. Haemolymph sampling and total haemocyte count

Haemolymph was sampled from each crayfish (Söderhäll et al., 2022) from the dorsal cephalothoracic cavity using a 26-gauge needle. For total haemocyte count, 100 μL of haemolymph was immediately

mixed with 900 μL of the anticoagulant buffer (Söderhäll et al., 2022) and haemocytes were counted with an optical microscope (total magnification of 100x) using a Bürker chamber. For RNA isolation, 1 mL of haemolymph was mixed with 1 mL of anticoagulant buffer and centrifuged for five min on a table-top mini centrifuge (MiniStar, VWR, US) at 18 °C. The supernatant was removed and the haemocyte pellet was frozen at –80 °C. All sampling was done with plasticware and buffers precooled at + 4 °C. Haemolymph samples were kept on ice to prevent coagulation. No immediate mortalities were induced by haemolymph sampling in treatment or in control groups.

2.2.4. Euthanasia and tissue sampling

Post haemolymph sampling, all crayfish were placed at –20 °C and checked every five min for 20 to 25 min until nonresponsive to external stimuli (Becking et al., 2015; Boštjančić et al., 2023; Puri and Faulkes, 2015; Ross et al., 2019). No experimental crayfish showed signs of distress during this process. This was followed by euthanasia with swift splitting of the ventral nervous cord as per international guidelines for humane killing of crayfish (Conte et al., 2021) and immediate sampling of the tissues for RNA isolation. Tissue samples were taken from heart, hepatopancreas, abdominal muscle, hindgut, gills, green glands and testes. Immediately after sampling, tissues were frozen at –80 °C until RNA isolation.

2.2.5. RNA isolation and cDNA synthesis

Total RNA was isolated using the TRIzol Reagent (Invitrogen, USA). For extraction of the larger tissues (hepatopancreas, heart, abdominal muscle), 1 mL of TRIzol Reagent was added to the sample, while for the extraction of smaller tissues (hindgut, gills, testes and haemocytes), 500 μL of TRIzol Reagent was added to the sample. Whole tissue samples were first homogenised with sterile pestles. Samples were mixed for 10 min at 37 °C at 1 600 rpm on ThermoMixer C (Eppendorf, Germany), followed by centrifugation at 16 000 x g, for 3 min at 4 °C to remove cellular debris. The supernatant was transferred to a new tube containing 200 μL of chloroform, vortexed (15 s) and incubated at room temperature for three min. Samples were then centrifuged at 12 000 x g while cooled at 4 °C for 15 min. The upper aqueous phase was carefully transferred into a new tube and DNase treatment was conducted using the rDNase Set (Macherey-Nagel, Germany) according to the manufacturer's protocol for in solution DNA digestion. Incubation was conducted with 20 μL DNase solution at RT for 25 min. This was followed by RNA precipitation with 500 μL of ice-cold isopropanol. Tubes with samples were carefully inverted three times and incubated for 10 min at 4 °C. Samples were then centrifuged at 10 000 x g, 4 °C for 10 min. RNA pellet was washed with 700 μL of ice-cold 75 % ethanol, and centrifuged at 7 500 x g, 4 °C, 5 min. The ethanol wash was repeated two times. After discarding the supernatant, the RNA pellets were dried for 10 to 15 min at room temperature and dissolved in 100 μL of RNase-free water. The extracted RNA samples were stored at –80 °C until reverse transcription. RNA quality was assessed on the NanoVue Spectrophotometer (Biochrom, UK), 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 manufacturer's protocol.

2.2.6. Gene expression analysis

Target transcripts for the qPCR were CCAAT/enhancer-binding protein (*C/EBP*) and Krüppel homolog1 protein (*Kr-h1*). Elongation factor one alpha- *EF1α* was used as housekeeping gene. Target-specific primers were synthesised for the amplification of *C/EBP* and *EF1α* based on Dobrović et al., 2022 and for the amplification of *Kr-h1* based on Boštjančić et al., 2023 (Table 1). For the quantitative reverse transcription PCR (RT-qPCR), target transcripts 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

Table 1
Primer sequences used for qPCR analysis.

Primer name	Sequence (5'-3')	Exon spanning	Length (bp)	Tm (°C)	GC content (%)	Product length (bp)	Primer efficiency (%)
Kr-h1-F	AGTGTGAGGTTGTCGGTAAAG	yes	20	59.97	55	99	96
Kr-h1-R	GGCAGTACTCACAGGTGATGG	no	22	60.42	54.55	99	
EF1 α -F	TGGTGCTTATGAGTTTGTGACAC	no	23	59.18	43.48	99	91
EF1 α -R	ACATCTGCAGAGGAAGACG	yes	20	59.47	55	99	
C/EBP-F	AGTGGTTGAAAAGGCACGACG	no	20	61.16	55	99	108
C/EBP-R	AAACGCCAGCTCCGTACC	no	18	60.05	61.11	99	

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) (Livak and Schmittgen, 2001). Analysis of the gene expression was made for all sampled tissues from crayfish belonging to the control group. For the CBS and laminarin injected crayfish, gene expression analysis was only conducted for hepatopancreas, heart, hindgut, gills, abdominal muscle and haemolymph tissues, as we did not expect to see any statistical difference in the testes and green glands.

2.3. Data visualisation and statistical analyses

All statistical analyses were conducted in R 4.2.2. (R Core Team, 2022). All datasets were tested for normality using Shapiro–Wilk test, and appropriate statistical tests were applied (t-test for normally

distributed data, and a non-parametric Wilcoxon rank sum test for non-normally distributed datasets). As significance level we used $\alpha = 0.05$ and for multiple testing p-values were adjusted according to the Benjamini-Hochberg method (BH). Principal component analysis was conducted with the scaled and normalised data in the stats package (R Core Team, 2022). Data visualisation was conducted with ggplot2 (Wickham, 2016). Phylogenetic trees were visualised in FigTree v.1.4.4 (https://github.com/rambaut/figtree/). Resulting graphics were finally edited in Inkscape 1.2 (Inkscape Project, 2020). The graphical abstract was created with BioRender (BioRender.com).

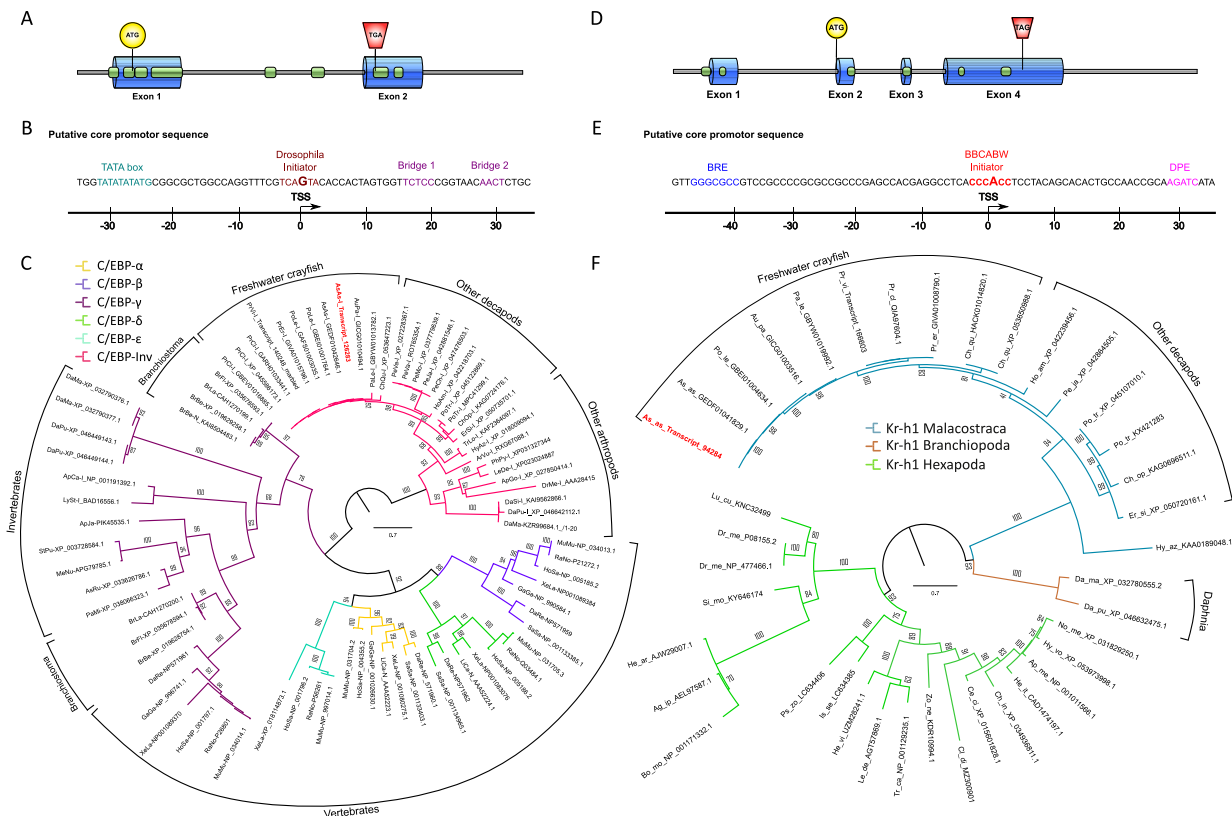


Fig. 1. Genomic organisation, putative promoter sequences and phylogeny of CCAAT/enhancer-binding protein (*C/EBP*) and Krüppel homolog-1 protein (*Kr-h1*). (A) Structure of the *C/EBP* genomic locus, (B) putative promoter sequences of *C/EBP* and (C) maximum likelihood phylogenetic tree of the *C/EBP* protein, (D) structure of the *Kr-h1* genomic locus, (E) putative promoter sequences of *Kr-h1*, (F) maximum likelihood phylogenetic tree of the *Kr-h1* protein. In the gene structure plots, blue boxes denote the position of exons, green boxes denote the position of CpG islands, ATG indicates the position of the translation start codon, TGA and TAG indicate the position of STOP codons. In the putative promoter sequence, the position of the initiator sequences is in red, with the putative transcription start site (TSS) nucleotide enlarged and in bold. Other structural sequences are coloured: TATA box in light blue, Bridge sequences in purple, B recognition element (BRE) in dark blue and downstream promoter element (DPE) in pink. In the phylogenetic tree, bootstrap support values are reported on the tree branches. Phylogenetic trees are unrooted. The noble crayfish *C/EBP* and *Kr-h1* protein sequences are marked in red. Species names are abbreviated to four letters. A complete list of protein sequences, species names and accession numbers used for the phylogenetic tree reconstructions are available in [Supplementary table 1](#) and [Supplementary table 2](#).

3. Results

3.1. Genomic organisation, copy number, evolutionary origin and domain organisation of the noble crayfish *C/EBP* and *Kr-h1* genes

We reconstructed the genomic sequences of the *C/EBP* and *Kr-h1* gene loci in the noble crayfish. *C/EBP* was present as a single copy gene in the noble crayfish. The *C/EBP* genomic locus consisted of two exons (exon 1 = 914 bp, exon 2 = 839 bp) separated by one intronic sequence (2543 bp, Fig. 1A). The position of the translation start codon was within exon 1, while the translation stop codon was located within exon 2. Comparative analysis of the genomic *C/EBP* loci showed that the structure of the locus is conserved among the analysed decapod species with the highest variation in the sequence length and base composition observed (Supplementary table 4). Dot-plot analysis of the *C/EBP* loci revealed two tandemly repeated regions located in the *C/EBP* intron (Supplementary Fig. 1). Eight CpG islands were predicted, one in the putative promoter region, three within exon 1, two within the intronic region and two within exon 2 (Fig. 1A, Supplementary table 5). The putative *C/EBP* promoter sequence consisted of the TATA box, followed by the *Drosophila* initiator sequence and two bridge sequences (Fig. 1B). The noble crayfish *C/EBP* protein was 285 amino acids long, with a molecular weight of 31.18 kDa and a theoretical isoelectric point of 8.17. Further, the noble crayfish *C/EBP* protein had a highly conserved bZIP domain (amino acids 192–257) at the C-terminus (Supplementary Fig. 2A). Based on the maximum likelihood phylogenetic tree reconstruction, the noble crayfish *C/EBP* grouped with invertebrate *C/EBP* clade, together with other *C/EBP* proteins from Decapoda (Fig. 1C). The predicted subcellular location of the noble crayfish *C/EBP* protein were nucleus and endoplasmic reticulum (Supplementary table 1).

The genomic locus of *Kr-h1* consisted of four exons (exon 1 = 493 bp, exon 2 = 314 bp, exon 3 = 172 bp, exon 4 = 2021 bp) separated by three introns (intron 1 = 1652 bp, intron 2 = 782 bp, intron 3 = 545 bp). The position of the translation start codon was within exon 2, while the translation stop codon was located within exon 4. Comparative analysis of the genomic *Kr-h1* loci showed that the structure of the locus is conserved among the analysed decapod species, although variation in length and base composition of the intronic regions was observed (Supplementary table 4). Dot-plot analysis of the *Kr-h1* loci revealed two tandemly repeated regions located in the intron 1 and one tandemly repeated region in the intron 2 (Supplementary Fig. 1). Six CpG islands were predicted: one within the putative promoter region, one within exon 1, one within exon 2, one within exon 3 and two within exon 4 (Fig. 1D, Supplementary table 5). The putative *Kr-h1* promoter sequence consists of the B recognition element (BRE), BBCABW initiator and downstream promoter element (DPE) (Fig. 1E). The *Kr-h1* of the noble

crayfish is a single copy gene, encoding a 608 amino acid long protein, with a molecular weight of 69.05 kDa and theoretical isoelectric point of 8.35. The noble crayfish *Kr-h1* possessed seven conserved zinc-finger C2H2 type domains at amino acid positions: 77–104 (Zn1), 105–132 (Zn2), 133–160 (Zn3), 161–188 (Zn4), 189–216 (Zn5), 217–244 (Zn6), 245–273 (Zn7) (Supplementary Fig. 2B). At the position 515–607 we detected a putative C-terminal conserved protein region that is present in all *Kr-h1* proteins of Malacostraca analysed in this study (Supplementary Fig. 2C), as well as in various C2H2 zinc finger proteins of Arthropoda (Supplementary file 4). Based on the maximum likelihood phylogenetic tree reconstruction, noble crayfish *Kr-h1* groups within the Malacostraca clade of *Kr-h1* (Fig. 1F). The predicted subcellular location of the noble crayfish *Kr-h1* protein was the nucleus (Supplementary table 1).

3.2. Tissue-specific expression patterns of the transcription factors *C/EBP* and *Kr-h1*

In the control noble crayfish ($n = 4$) tissues, the highest gene expression of the *C/EBP* was in the gills (Fig. 2A). For the *Kr-h1* the highest expression was in the abdominal muscle (Fig. 2B). All expression estimates were made on four biological replicates, except for the haemolymph tissue ($n = 2$), due to the insufficient RNA amount.

3.3. Effects of immune stimulation with CBS and laminarin on the total haemocyte count

No signs of distress were observed in response to the injection of the immunostimulant in the noble crayfish. The average total haemocyte count (THC) for the control noble crayfish in this study was 6.69×10^5 ($SD \pm 1.08 \times 10^5$). Noble crayfish injected with CBS showed a significantly increased in THC compared to the control group (t -test, T -value = -3.14 , p -value = 0.021), with an average of 9.29×10^5 ($SD \pm 1.25 \times 10^5$). The laminarin injected group of the noble crayfish showed a significant increase in the THC compared to the control group (t -test, T -value = -5.55 , p -value = 0.002), and CBS injected group (t -test, T -value = -3.26 , p -value = 0.016) with an average of 1.33×10^6 ($SD \pm 2.39 \times 10^5$) (Fig. 3).

3.4. Effect of the CBS and laminarin immune stimulation on the expression of the transcription factors *C/EBP* and *Kr-h1*

For the noble crayfish injected with CBS, there was no change in the expression of *Kr-h1* gene in all tested tissues, while *C/EBP* was significantly up-regulated in the heart (pairwise t -test, T -value = 4.25 , p -value = 0.044) (Fig. 4A). In the laminarin injected noble crayfish, significant

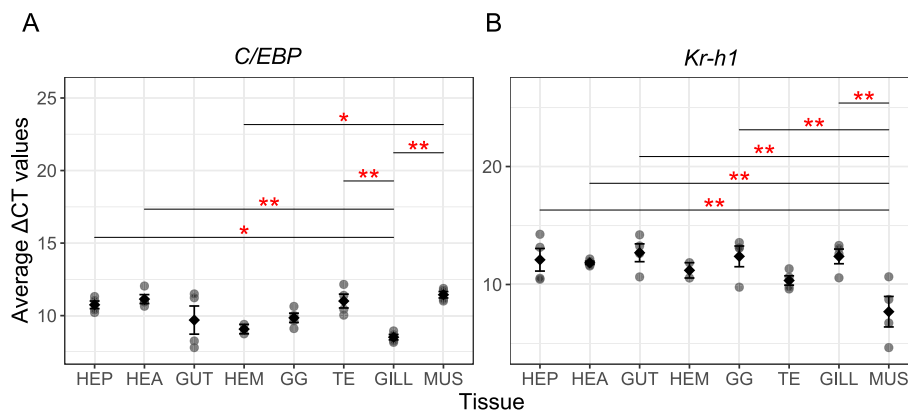


Fig. 2. Relative gene expression levels of the (A) CCAAT/enhancer-binding protein (*C/EBP*) and (B) Krüppel homolog-1 (*Kr-h1*) in different tissues of the noble crayfish expressed as average ΔCT values. HEP-hepatopancreas, HEA-heart, GUT-hindgut, HEM-haemolymph, GG-green glands, TE-testes, GILL-gills, MUS-muscle. Each dot represents a single replicate, diamonds represent means and error bars represent standard error of the mean. Statistical significance of the pairwise t -test is represented as * (p -value < 0.05) or ** (p -value < 0.01).

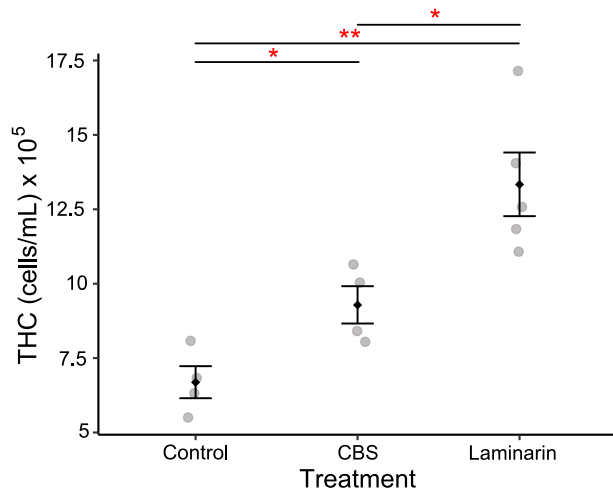


Fig. 3. Changes in the total haemocyte count (THC) of the noble crayfish four hours post injection with the crayfish buffered saline (CBS) and laminarin, and control. Each dot represents a single replicated, diamonds represent means and error bars represent standard error of the mean. Statistical significance is represented as * (p-value < 0.05) or ** (p-value < 0.01).

up-regulation of *C/EBP* gene expression compared to the control was detected in the hepatopancreas (pairwise *t*-test, T-value = 6.88, p-value = 0.001) and in the heart (pairwise *t*-test, T-value = 4.38, p-value = 0.002). When compared to the CBS injection, laminarin injected noble crayfish had a significant up-regulation of *C/EBP* gene expression in the hepatopancreas (pairwise *t*-test, T-value = 4.67, p-value = 0.015). There was no difference in the gene expression of *Kr-h1* gene in all tested tissues (Fig. 4B). For the haemolymph tissue, enough RNA for qPCR assay

was isolated only from two individuals per treatment group. Therefore, statistical analysis of the samples could not be conducted. The results for haemolymph are presented in the [Supplementary Fig. 3](#). Principal component analysis (PCA) showed the well-defined grouping of samples by treatment, as well as correlation between the THC and *C/EBP* gene expression in hepatopancreas ($r = 0.73$) and heart ($r = 0.74$) (Fig. 4C, [Supplementary Fig. 4](#)). No correlation was observed in PCA between THC and *Kr-h1* gene expression.

4. Discussion

Due to their ability to bind DNA, TFs play a major role in the regulation of gene expression and are key modulators of cellular processes (Weidemüller et al., 2021). However, their role in the regulation of the immune system dynamics in crayfish is not well understood. Here, we focused on the putative transcriptional activator *C/EBP* and putative transcriptional repressor *Kr-h1* which were shown to be promising candidates with a potential role in the crayfish immune system (Boštjančić et al., 2023; Dobrović et al., 2022).

4.1. Noble crayfish *C/EBP* and *Kr-h1*

C/EBP belongs to the family of transcription factors with the C-terminal basic region leucine zipper domain, well studied in vertebrates (Ramji and Foka, 2002). In contrast to *C/EBP*, *Kr-h1* is a transcriptional factor characterised by a central domain with eight C2H2 zinc fingers, well studied in hexapods (He and Zhang, 2022). Here, we conducted a comparative analysis of the *C/EBP* and *Kr-h1* genomic loci in representative decapod species. Both *C/EBP* and *Kr-h1* genes exhibit a conserved exon–intron structure across decapods (Fig. 1A, D). We observed a high variation in the intronic regions among the species, which might partly be explained due to the presence of tandem repeats

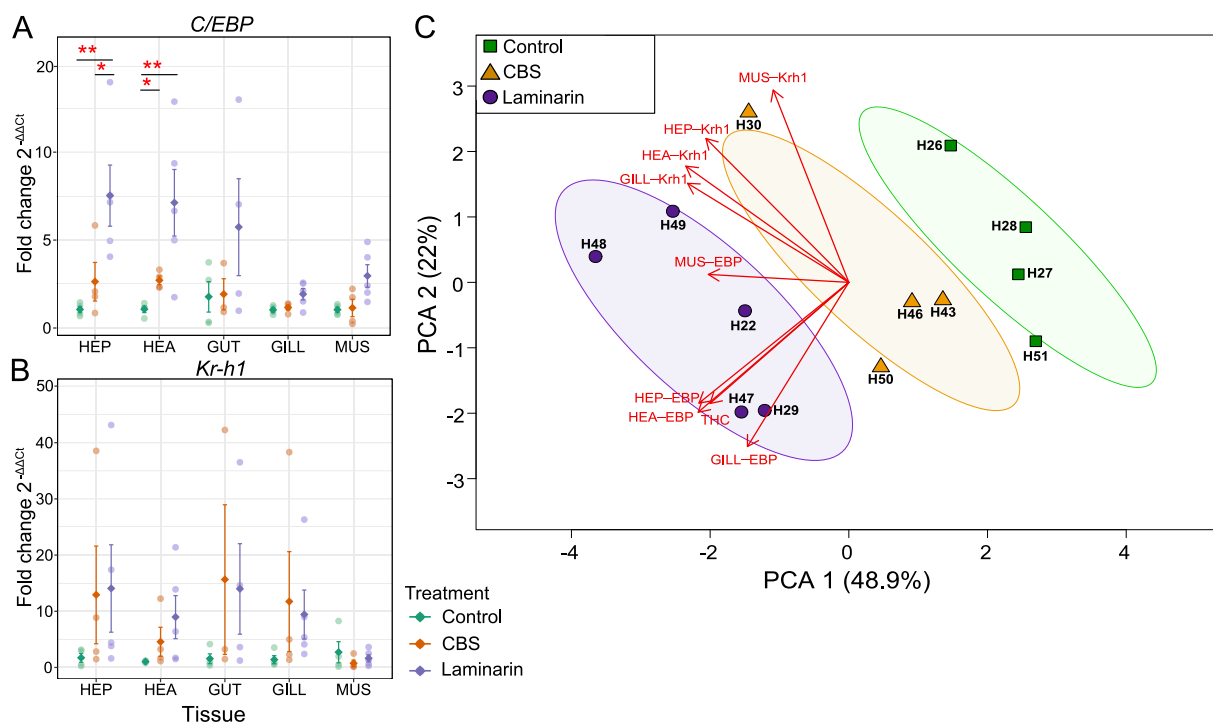


Fig. 4. Relative gene expression levels of the (A) CCAAT/enhancer-binding protein (*C/EBP*) and (B) Krüppel homolog-1 (*Kr-h1*) under the immune stimulation with crayfish buffered saline (CBS) and laminarin in the noble crayfish tissues. Each dot represents a single replicate (green – control group, orange – CBS injected, purple – laminarin injected), diamonds represent means and error bars represent standard error of the mean. HEP-hepatopancreas, HEA-heart, GUT-hindgut, GILL-gills, MUS-abdominal muscle. (C) Principal component analysis of the gene expression and total haemocytes count (THC) datasets. Shapes represent different experimental groups, green squares – control, orange triangle – CBS injected, purple circles – laminarin injected. Statistical significance of the multiple-pairwise comparison between sample conditions, Control, CBS and laminarin is shown as * (p-value < 0.05) or ** (p-value < 0.01). Statistically significant values were adjusted for multiple testing according to the Benjamini-Hochberg adjustment implemented in R.

(Supplementary table 4, Supplementary Fig. 1). Both *C/EBP* and *Kr-h1* have CpG islands preferentially present in the promoter and exon regions in the noble crayfish (Fig. 1A). CpG islands are often preferential targets of methylation, thus important for the regulation of gene expression. Epigenetic studies in the noble crayfish are a new frontier awaiting the challenging assembly of the repeat-rich genome (Rutz et al., 2023). To our knowledge, the only freshwater crayfish species whose methylome was characterised is the crayfish plague resistant marbled crayfish, *Procambarus virginalis* Lyko, 2017 (Dobrović et al., 2022; Gatzmann et al., 2018). In this triploid parthenogenetic crayfish species, DNA methylation preferentially occurred in the gene bodies of the CpG-poor and evolutionary conserved genes, characteristic of the housekeeping genes (Gatzmann et al., 2018). In the genetically uniform (clonal) marbled crayfish, epigenetic mechanisms contribute significantly to the phenotypic plasticity including coloration, spination, morphometric parameters, growth, food preference, population structure, trophic position, and niche width (Vogt, 2023). Epigenetic reprogramming could also be an underlying mechanism of the acquired and inheritable disease resistance in crustaceans (Low and Chong, 2020). Therefore, studying the effects of the immune stimulation on the noble crayfish epigenome, might be interesting in the context of promoting the increased disease resistance.

Domain organisation analysis showed that noble crayfish *C/EBP* and *Kr-h1* share similarities with the domain organisation previously observed in other crustaceans (Miyakawa et al., 2018; Wang et al., 2023; Xie et al., 2018). In addition, here we detected that all *Kr-h1* proteins of Malacostraca have a putative C-terminal conserved protein region of C2H2 zinc finger proteins, which is not present in the *Kr-h1* of hexapods or branchiopods (Supplementary Fig. 2, Supplementary file 3). The conserved nature of this region is indicative of its potential functional role, which requires further characterisation. Phylogenetic analyses suggest that invertebrate *C/EBP* is distinct from the *C/EBP* found in vertebrates (Fig. 1C, Supplementary Fig. 2), while *Kr-h1* of Malacostraca are distinct from the *Kr-h1* of hexapods and branchiopods (Fig. 1F). Finally, expression patterns of *C/EBP* and *Kr-h1* in the noble crayfish are consistent with previous observations in crustaceans (Fig. 2). *C/EBP* and *Kr-h1* were detected across all tested tissues in the noble crayfish, with the *C/EBP* transcript level highest in the gills, followed by the haemolymph and hindgut of the noble crayfish, while the *Kr-h1* gene had the highest expression in the abdominal muscle of the noble crayfish. A similar expression pattern was detected in the whiteleg shrimp, *Penaeus vannamei* Boone, 1931, where the highest expression of the *C/EBP* gene was observed in the gills and gut, followed by muscle, heart, hepatopancreas, stomach and finally haemocytes (Wang et al., 2023). Similarly, in the swimming crab, *Portunus trituberculatus* (Miers, 1876) the expression of *Kr-h1* was also highest in the abdominal muscle (Xie et al., 2018). While the high expression of *C/EBP* in these tissues could be explained by its role in the immune system, the reason behind the expression pattern of *Kr-h1* remains unclear and requires further investigations.

4.2. Changes in the noble crayfish immune system and expression of *C/EBP* and *Kr-h1* genes under immune stimulation

The potential of laminarin (containing β -1-3-glucan) as immunostimulant in freshwater crayfish has been known since the discovery of its involvement in the activation of the prophenoloxidase enzyme and clotting followed by subsequent phenoloxidase attachment (Söderhäll, 1981; Unestam and Söderhäll, 1977). Furthermore, it was also shown that the pathogen *Ap. astaci* stimulates the crayfish immune response through the presence of the laminarin (Söderhäll and Unestam, 1979; Unestam and Söderhäll, 1977). In this study, immune stimulation of the noble crayfish with CBS and laminarin led to an increase in the THC (4 h post injection, Fig. 3). In previous studies on the noble crayfish as well as on the signal crayfish, immune stimulation with CBS led to an increase in the THC (30 min post injection), whereas immune stimulation with

laminarin led to a drop in THC (30 min post injection) (Ekblom et al., 2021; Smith and Söderhäll, 1983). Additionally, previous studies report a gradual restoration of THC to baseline 24–48 h post immune stimulation with both CBS and laminarin (Ekblom et al., 2021; Smith and Söderhäll, 1983). Thus, an increase in THC following laminarin injection, as observed in this study, is in contrast with previous studies (Ekblom et al., 2021; Smith and Söderhäll, 1983). This difference could be attributed to the difference in dosage: while in this study each crayfish received a total of 0.5 mg of laminarin with an average of 0.0084 mg per g of crayfish (SD \pm 0.0012), it is not possible to evaluate how much laminarin the crayfish received in previous studies, because the size of the experimental animals is not reported (Ekblom et al., 2021; Smith and Söderhäll, 1983). A recent review showed that an effective dosage of supplementary polysaccharides is species-specific, with a wide range of applied concentrations and exposure procedures (Mohan et al., 2019).

Based on our experimental results presented herein, we can argue that immune stimulation with CBS and laminarin led to the enhanced immune cell recruitment in the haemolymph of the noble crayfish (Zheng et al., 2021). The increased number of haemocytes might be beneficial in the wound healing reactions from the injection itself, and the even higher increase in the THC in the laminarin immune stimulation group might be to promote an increased phagocytosis needed to clear the injected material (Lin and Söderhäll, 2011; Smith, 2016). Based on previous research, and considering that haemocyte regulation is a dynamic process, we can expect the haemocyte mobilisation in response to CBS or laminarin stimulation in the noble crayfish to be temporary, with the increased haemocyte numbers not persisting post-immune stimulation. Nonetheless, early studies have shown that immune stimulation with sub-lethal dose of the *Ap. astaci* spores may lead to the increased disease resistance in the susceptible noble crayfish (Unestam and Weiss, 1970). This approach would, however, not be applicable in aquacultural context, due to the risk of pathogen introduction in the closed system. To our knowledge, immune stimulation with laminarin has never been applied to increase the resistance of the susceptible European crayfish species to a challenge with the *Ap. astaci* and might be an interesting prospect for future research.

Exposure to the β -1,3-glucan activates the effector molecules of the humoral response of the crayfish, including the prophenoloxidase system (Lee et al., 2000; Smith and Söderhäll, 1983). β -1,3-glucan is a pathogen-associated molecular pattern (PAMP), recognised by the pattern recognition receptors (PRRs) of the host. Specifically, β -1,3-glucan is recognised by the β -1,3-glucan binding protein (β GBP) (Baracco et al., 1991) and LPS- and β -1,3-glucan-binding protein (Lee et al., 2000). In the insect *Locusta migratoria manilensis* Meyen, 1835, it was shown that the Toll pathway has an important role in the downstream signal transduction, anti-fungal response and β -1,3-glucan sensing (Zheng et al., 2020). Components of the Toll pathway are also conserved in the noble crayfish (Boštjančić et al., 2022), but their role in the response of the freshwater crayfish to *Ap. astaci* challenge is not yet explored. In invertebrates, the Toll signalling pathway activates the transcriptional factor NF- κ B and the subsequent transcription of genes for anti-microbial peptides (Kurata, 2010). In our study, the immune stimulation of the noble crayfish with laminarin caused an up-regulation of *C/EBP* in hepatopancreas and heart (Fig. 4A). These results are consistent with our previous observation of the *C/EBP* up-regulation in the hepatopancreas of the *Ap. astaci* challenged noble crayfish (Boštjančić et al., 2023, 2022), as well as in the marbled crayfish exposed to *Ap. astaci* under starvation (Dobrović et al., 2022). In mammals, *C/EBP β* and *C/EBP δ* , essential for the production of proinflammatory cytokines, are involved in Toll-like receptor (TLR) signalling pathways, which are homologous to the Toll pathway in invertebrates (Lu et al., 2009). This hints to the possibility of the invertebrate Toll pathway being involved in the activation of the *C/EBP* expression. There is need for more targeted studies to explore the potential interaction between the Toll pathway and *C/EBP* in invertebrates.

The up-regulation of *C/EBP* gene expression in the hepatopancreas can be connected to its role as central metabolic organ, as well as a site of synthesis for some humoral response factors (Boštjančić et al., 2022; Röszer, 2014). Furthermore, here we observed a positive correlation between the *C/EBP* gene expression (in hepatopancreas, heart, gills) and THC (Fig. 4C). It is known that crayfish hepatopancreas is infiltrated by the circulating haemocytes (Bouallegui, 2021), which could influence the individual gene expression profiles. Similarly, heart of crustaceans lies in a cavity called pericardial sinus which is filled with haemocytes (Smolowitz, 2021). It is possible that up-regulation of *C/EBP* expression is a result of an increased number of circulating haemocytes that are also present in and around the heart tissue. Alternatively, up-regulation of *C/EBP* expression could be a consequence of the tissue damage near the injection site, rather than a sign of direct involvement of the heart in the immune response mechanisms. Although differential expression of *C/EBP* was not observed in the gills, there was an overall increase in the *C/EBP* expression (Fig. 4A). Gills of freshwater crayfish have been recognised as haemocyte reservoirs, important in maintaining the haemocyte homeostasis and allowing the exchange between the sessile and the circulating pool of haemocytes (Zheng et al., 2021). Overall, we cannot exclude that the presence of haemocytes in the hepatopancreas, heart and gills had an influence on the *C/EBP* gene expression patterns. Moreover, based on our preliminary results, it is likely that *C/EBP* is up-regulated in the haemocytes (Supplementary Fig. 3). This would be expected, considering the higher expression of *C/EBP* in the gills (haemocyte reserve) and the increase in the THC under immune stimulation with CBS and laminarin (Fig. 2, Fig. 3). Future studies should test the significance of this observation with a higher number of biological replicates. Finally, our results give additional support for the involvement of *C/EBP* in the immune response of the noble crayfish.

On the other hand, gene expression of *Kr-h1* was not altered in any of the tested tissues under immune stimulation (Fig. 4B). Furthermore, we also did not observe a correlation between the *Kr-h1* gene expression and THC. It is possible that this is due to the high variability of *Kr-h1* gene expression, as observed in previous study on the noble crayfish (Boštjančić et al., 2023). Previously, *Kr-h1* expression was only analysed in the hepatopancreas of the *Ap. astaci* challenged noble crayfish, where it was found to be down-regulated under the pathogen challenge (Boštjančić et al., 2023, 2022). This could suggest that expression of *Kr-h1* is context-dependent, and if the immune stimulation is lower than a certain threshold (like in laminarin immune stimulation), it is favourable to have a higher number of *Kr-h1* transcripts. It is also possible that laminarin has no effect on the *Kr-h1* expression. Conversely, a lower number of *Kr-h1* transcripts might be favourable in the case of an acute infection, as we previously observed in the moribund noble crayfish under *Ap. astaci* challenge (Boštjančić et al., 2023, 2022). For now, the *Kr-h1* threshold hypothesis, as well as its actual involvement in the immune response, remain to be explored in future studies. Furthermore, it has been previously suggested that both *C/EBP* and *Kr-h1* might have a metabolic role, especially in the moribund *Ap. astaci* challenged noble crayfish or marbled crayfish (Boštjančić et al., 2023; Dobrović et al., 2022). While the results presented in this study provide evidence for the role of the *C/EBP* in the immune system, we cannot rule out the potential involvement of *C/EBP* or *Kr-h1* in the metabolism. Finally, immune response and metabolism are inevitably connected since every immune response has an energy cost for the organism.

5. Conclusions

Here we show that the noble crayfish TFs *C/EBP* and *Kr-h1* are conserved on the genomic and protein level. Both *C/EBP* and *Kr-h1* are single copy genes and exhibit a conserved exon–intron structure with other decapods. Their domain organisation resembles their homologues in Malacostraca, and they group together according to the established phylogeny. Based on the gene expression analysis, *C/EBP* has the highest expression in gills, while *Kr-h1* has the highest expression in the

abdominal muscle. Overall, both genes were ubiquitously expressed across all tested tissues. Immune stimulation with CBS and laminarin led to an overall increase of the THC. Moreover, immune stimulation with laminarin caused a change in the expression of *C/EBP* in hepatopancreas and heart. Therefore, a change in the expression of *C/EBP* under a non-lethal immune challenge echoes its role in the immune response. While this does not stand for *Kr-h1*, it is possible that the immune stimulation with laminarin was simply not enough to activate the pathways where *Kr-h1* is involved.

6. Declaration of generative AI in scientific writing

During the preparation of this work the author did not use generative AI in scientific writing.

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CRediT authorship contribution statement

Ljudevit Luka Boštjančić: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paula Dragičević:** Writing – review & editing, Validation, Methodology, Investigation. **Lena Bonassin:** Writing – review & editing, Validation, Investigation. **Caterina Francesconi:** Writing – review & editing, Validation, Methodology, Conceptualization. **Anita Tarandek:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Leonie Schardt:** Writing – review & editing, Resources, Methodology. **Christelle Rutz:** Writing – review & editing, Validation. **Sandra Hudina:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Klaus Schwenk:** Writing – review & editing, Validation. **Odile Lecompte:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Kathrin Theissinger:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Ljudevit Luka Boštjančić reports financial support was provided by German Research Foundation. Odile Lecompte reports financial support was provided by French National Research Agency. Sandra Hudina reports financial support was provided by Croatian Ministry of Science and Education. Kathrin Theissinger reports financial support was provided by German Academic Exchange Service. Kathrin Theissinger reports financial support was provided by German Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.].

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gene.2024.148813>.

References

- Altschul, S., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. <https://doi.org/10.1093/nar/25.17.3389>.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
- Alvanou, M.V., Lattos, A., Cheimonopoulou, M.T., Michaelidis, B., Apostolidis, A.P., Giantsis, I.A., 2024. First detection of *Aphanomyces astaci* and its potential responsibility for mass mortalities of *Pontastacus (Astacus) leptodactylus* in Greek lakes. *J. Biol. Res.* 31, 1–9. <https://doi.org/10.26262/jbrtv31i0.9777>.
- Barracco, M.A., Duvic, B., Söderhäll, K., 1991. The β -1,3-glucan-binding protein from the crayfish *Pacifastacus leniusculus*, when reacted with a β -1,3-glucan, induces spreading and degranulation of crayfish granular cells. *Cell Tissue Res.* 266, 491–497. <https://doi.org/10.1007/BF00318590>.
- Becking, T., Mrugała, A., Delaunay, C., Svoboda, J., Raimond, M., Viljamaa-Dirks, S., Petrussek, A., Grandjean, F., Braquart-Varnier, C., 2015. Effect of experimental exposure to differently virulent *Aphanomyces astaci* strains on the immune response of the noble crayfish *Astacus astacus*. *J. Invertebr. Pathol.* 132, 115–124. <https://doi.org/10.1016/j.jip.2015.08.007>.
- Boštjančić, L.L., Francesconi, C., Rutz, C., Hoffbeck, L., Poidevin, L., Kress, A., Jussila, J., Makkonen, J., Feldmeyer, B., Bálint, M., Schwenk, K., Lecompte, O., Theissinger, K., 2022. Host-pathogen coevolution drives innate immune response to *Aphanomyces astaci* infection in freshwater crayfish: transcriptomic evidence. *BMC Genomics* 23, 600. <https://doi.org/10.1186/s12864-022-08571-z>.
- Boštjančić, L.L., Francesconi, C., Bonassin, L., Hudina, S., Gračan, R., Maguire, I., Rutz, C., Beck, A., Dobrović, A., Lecompte, O., Theissinger, K., 2023. Temporal dynamics of the immune response in *Astacus astacus* (Linnaeus, 1758) challenged with *Aphanomyces astaci* Schikora, 1906. *Fish Shellfish Immunol.* 143, 109185. <https://doi.org/10.1016/j.fsi.2023.109185>.
- Bouallegui, Y., 2021. A Comprehensive Review on Crustaceans' Immune System With a Focus on Freshwater Crayfish in Relation to Crayfish Plague Disease. *Front. Immunol.* 12, 1–15. <https://doi.org/10.3389/fimmu.2021.667787>.
- Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–60. <https://doi.org/10.1038/nmeth.3176>.
- Burnett, K.G., Burnett, L.E., 2015. Integrative and Comparative Biology Respiratory and Metabolic Impacts of Crustacean Immunity. Are There Implications for the Insects? 55, 856–868. <https://doi.org/10.1093/icb/094>.
- Cerenius, L., Lee, B.L., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol.* 29, 263–271. <https://doi.org/10.1016/j.it.2008.02.009>.
- Chou, K.C., Shen, H.B., 2010. A new method for predicting the subcellular localization of eukaryotic proteins with both single and multiple sites: Euk-mPLoc 2.0. *PLoS One* 5, 1–9. <https://doi.org/10.1371/journal.pone.0009931>.
- Conte, F., Voslarova, E., Vecerek, V., Elwood, R.W., Coluccio, P., Pugliese, M., Passantino, A., 2021. Humane slaughter of edible decapod crustaceans. *Animals* 11, 1–13. <https://doi.org/10.3390/ani11041089>.
- Dobrović, A., Geček, S., Klanjšček, T., Haberle, I., Dragičević, P., Pavić, D., Petelinec, A., Boštjančić, L.L., 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>.
- Edsman, L., Füreder, L., Gherardi, F., Souty-Grosset, C., 2023. *Astacus astacus* [WWW Document]. IUCN Red List Threat. Species 2010 e.T2191A9338388. <https://dx.doi.org/10.2305/IUCN.UK.2010-3.RLTS.T2191A9338388.en>.
- Eklblom, C., Söderhäll, K., Söderhäll, I., 2021. Early changes in crayfish hemocyte proteins after infection with a β -1,3-glucan, compared to saline injected and naive animals. *Int. J. Mol. Sci.* 22, 1–20. <https://doi.org/10.3390/ijms22126464>.
- Gatzmann, F., Falckenhayn, C., Gutekunst, J., Hanna, K., Raddatz, G., Carneiro, V.C., Lyko, F., 2018. The methylome of the marbled crayfish links gene body methylation to stable expression of poorly accessible genes. *Epigenetics and Chromatin* 11, 1–12. <https://doi.org/10.1186/s13072-018-0229-6>.
- Goldina, A., Simms, T.M., Pitzer, T., Street, S.W., Fl, M., 2011. Once a loser always a loser? using crayfish to teach behavioral endocrinology. *Test Stud. Lab. Teachingproceedings Assoc. Biol. Lab. Educ.* 32, 354–359.
- He, Q., Zhang, Y., 2022. Kr-h1, a Cornerstone Gene in Insect Life. *History* 13, 1–14. <https://doi.org/10.3389/fphys.2022.905441>.
- Inkscape Project, 2020. Inkscape.
- Jussila, J., Makkonen, J., Vainikka, A., Kortet, R., Kokko, H., 2011. Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (*Astacus astacus*) population. *Aquaculture* 321, 17–20. <https://doi.org/10.1016/j.aquaculture.2011.08.026>.
- Jussila, J., Vrezec, A., Jaklič, T., Kukkonen, H., Makkonen, J., Kokko, H., 2017. *Aphanomyces astaci* isolate from latently infected stone crayfish (*Austropotamobius torrentium*) population is virulent. *J. Invertebr. Pathol.* 149, 15–20. <https://doi.org/10.1016/j.jip.2017.07.003>.
- Jussila, J., Edsman, L., Maguire, I., Diéguez-Urbeondo, J., Theissinger, K., 2021a. Money Kills Native Ecosystems: European Crayfish as an Example. *Front. Ecol. Evol.* 9. <https://doi.org/10.3389/fevo.2021.648495>.
- Jussila, J., Vrezec, A., Makkonen, J., Kortet, R., Kokko, H., 2015. Invasive crayfish and their invasive diseases in Europe with the focus on the virulence evolution of the crayfish plague. *Biol. Invasions Chang. Ecosyst. Vectors, Ecol. Impacts, Manag. Predict.* 183–211. 10.1515/9783110438666-013.
- Jussila, J., Francesconi, C., Theissinger, K., Kokko, H., Makkonen, J., 2021b. Is *Aphanomyces astaci* Losing its Stamina: A Latent Crayfish Plague Disease Agent From Lake Venesjärvi. Finland. *Freshw. Crayfish* 26, 139–144. <https://doi.org/10.5869/fc.2021.v26-2.139>.
- Kokko, H., Koistinen, L., Harlioglu, M.M., Makkonen, J., Aydın, H., Jussila, J., 2012. Recovering Turkish narrow clawed crayfish (*Astacus leptodactylus*) populations carry *Aphanomyces astaci*. *Knowl. Manag. Aquat. Ecosyst.* 12. <https://doi.org/10.1051/kmae/2012006>.
- Kurata, S., 2010. Extracellular and intracellular pathogen recognition by *Drosophila* PGRP-LE and PGRP-LC. *Int. Immunol.* 22, 143–148. <https://doi.org/10.1093/intimm/dxp128>.
- Lee, S.Y., Wang, R., Söderhäll, K., 2000. A lipopolysaccharide- and β -1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. Purification, characterization, and cDNA cloning. *J. Biol. Chem.* 275, 1337–1343. <https://doi.org/10.1074/jbc.275.2.1337>.
- Lin, X., Söderhäll, I., 2011. Crustacean hematopoiesis and the astakine cytokines. *Blood* 117, 6417–6424. <https://doi.org/10.1182/blood-2010-11-320614>.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2⁻ Δ CT Method. *Methods* 25, 402–408. <https://doi.org/10.1006/meth.2001.1262>.
- Loker, E.S., Adema, C.M., Zhang, S.M., Kepler, T.B., 2004. *Invertebrate immune systems – not homogeneous, not simple, not well understood - Loker - 2004 - Immunological Reviews - Wiley Online Library.* *Immunol. Rev.* 198, 10–24.
- Low, C.F., Chong, C.M., 2020. Peculiarities of innate immune memory in crustaceans. *Fish Shellfish Immunol.* 104, 605–612. <https://doi.org/10.1016/j.fsi.2020.06.047>.
- Lu, Y.-C., Kim, I., Lye, E., Shen, F., Suzuki, N., Suzuki, S., Gerondakis, S., Akira, S., Gaffen, S.L., Yeh, W.-C., Ohashi, P.S., 2009. Differential Role for c-Rel and C/EBP β in TLR-Mediated Induction of Proinflammatory Cytokines. *J. Immunol.* 182, 7212–7221. <https://doi.org/10.4049/jimmunol.0802971>.
- Martín-Torrijos, L., Campos Llach, M., Pou-Rovira, Q., Diéguez-Urbeondo, J., 2017. Resistance to the crayfish plague, *Aphanomyces astaci* (Oomycota) in the endangered freshwater crayfish species, *Austropotamobius pallipes*. *PLoS One* 12, 1–13. <https://doi.org/10.1371/journal.pone.0181226>.
- Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., Diéguez-Urbeondo, 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, 1–14. <https://doi.org/10.1371/journal.pone.0219223>.
- Mitsis, F., Efthimiadou, A., Bacopoulou, F., Vlachakis, D., Chrousos, G.P., Eliopoulos, E., 2020. Transcription factors and evolution: An integral part of gene expression (Review). *World Acad. Sci. J.* 2, 3–8. <https://doi.org/10.3892/wasj.2020.32>.
- Miyakawa, H., Watanabe, M., Araki, M., Ogino, Y., Miyagawa, S., Iguchi, T., 2018. Juvenile hormone-independent function of Krüppel homolog 1 in early development of water flea *Daphnia pulex*. *Insect Biochem. Mol. Biol.* <https://doi.org/10.1016/j.ibmb.2017.12.007>.
- Mohan, K., Ravichandran, S., Muralisankar, T., Uthayakumar, V., Chandrasekar, R., Seedeivi, P., Abirami, R.G., Rajan, D.K., 2019. Application of marine-derived polysaccharides as immunostimulants in aquaculture: A review of current knowledge and further perspectives. *Fish Shellfish Immunol.* 86, 1177–1193. <https://doi.org/10.1016/j.fsi.2018.12.072>.
- Nguyen, L.T., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32, 268–274. <https://doi.org/10.1093/molbev/msu300>.
- Paludan, S.R., Pradeu, T., Masters, S.L., Mogensen, T.H., 2021. Constitutive immune mechanisms: mediators of host defence and immune regulation. *Nat. Rev. Immunol.* 21. <https://doi.org/10.1038/s41577-020-0391-5>.
- Puri, S., Faulkes, Z., 2015. Can crayfish take the heat? *Procambarus clarkii* show nociceptive behaviour to high temperature stimuli, but not low temperature or chemical stimuli. *Biol. Open* 4, 441–448. <https://doi.org/10.1242/bio.20149654>.
- Qin, J., Hu, Y., Ma, K.Y., Jiang, X., Ho, C.H., Tsang, L.M., Yi, L., Leung, R.W.T., Chu, K.H., 2017. CrustTF: A comprehensive resource of transcriptomes for evolutionary and functional studies of crustacean transcription factors. *BMC Genomics* 18, 1–9. <https://doi.org/10.1186/s12864-017-4305-2>.
- R Core Team, 2022. R: A language and environment for statistical computing.
- Ramji, D.P., Foka, P., 2002. CCAAT/enhancer-binding proteins: Structure, function and regulation. *Biochem. J.* 365, 561–575. <https://doi.org/10.1042/BJ20020508>.
- Reynolds, J., Souty-Grosset, C., Richardson, A., 2013. Ecological roles of crayfish in freshwater and terrestrial habitats. *Freshw. Crayfish* 19, 197–218. <https://doi.org/10.5869/fc.2013.v19-2.197>.
- Richman, N.I., Bo, M., Adams, S.B., Alvarez, F., Bergey, E.A., Bunn, J.J.S., Burnham, Q., Cordeiro, J., Coughran, J., Crandall, K.A., Dawkins, K.L., Distefano, J., Doran, N.E., Edsman, L., Eversole, A.G., Furse, J.M., Gherardi, F., Hamr, P., Holdich, D.M., Horwitz, P., Johnston, K., Jones, C.M., Jones, J.P.G., Jones, R.L., Jones, T.G., Kawai, T., Miller, R.M., Pedraza, C., Lawler, S., Lo, M., Reynolds, J.D., Richardson, A.M.M., Schultz, M.B., Schuster, G.A., Sibley, P.J., Souty-grosset, C., Taylor, A., Thoma, R.F.,

- Walls, J., Walsh, T.S., Collen, B., 2015. Multiple drivers of decline in the global status of freshwater crayfish (Decapoda : Astacidea). 10.1098/rstb.2014.0060.
- Ross, E.P., Behringer, D.C., Muñoz, A., Díaz, D., Bojko, J., 2019. A histological atlas for the Palinuridae (Crustacea: Decapoda: Achelata): A guide to parasite discovery and spotting the abnormal in spiny lobsters. *J. Invertebr. Pathol.* 163, 21–33. <https://doi.org/10.1016/j.jip.2019.03.001>.
- Rószter, T., 2014. The invertebrate midintestinal gland (“hepatopancreas”) is an evolutionary forerunner in the integration of immunity and metabolism. *Cell Tissue Res.* 358, 685–695. <https://doi.org/10.1007/s00441-014-1985-7>.
- Rutz, C., Bonassin, L., Kress, A., Francesconi, C., Boštjančić, L.L., Merlat, D., Theissing, K., Lecompte, O., 2023. Abundance and Diversification of Repetitive Elements in Decapoda Genomes. *Genes (basel)*. 14 <https://doi.org/10.3390/genes14081627>.
- Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7 <https://doi.org/10.1038/msb.2011.75>.
- Smale, S.T., 2014. Transcriptional regulation in the immune system: A status report. *Trends Immunol.* 35, 190–194. <https://doi.org/10.1016/j.it.2014.03.003>.
- Smith, V.J., Söderhäll, K., 1983. β -1, 3 Glucan activation of Crustacean hemocytes in vitro and in vivo. *Biol. Bull.* 164, 299–314. <https://doi.org/10.2307/1541146>.
- Smith, V.J., 2016. Immunology of Invertebrates: Cellular. eLS 1–13. 10.1002/9780470015902.a0002344.pub3.
- Smolowitz, R., 2021. Arthropoda Gross Anatomy of Adults 277–299.
- Soderhall, K., 1981. Fungal cell wall β -1,3-glucans induce clotting and phenoloxidase attachment to foreign surfaces of crayfish hemocyte lysate. *Dev. Comp. Immunol.* 5, 565–573. [https://doi.org/10.1016/s0145-305x\(81\)80031-6](https://doi.org/10.1016/s0145-305x(81)80031-6).
- Söderhäll, I., 2016. Crustacean hematopoiesis. *Dev. Comp. Immunol.* 58, 129–141. <https://doi.org/10.1016/j.dci.2015.12.009>.
- Söderhäll, I., Fasterius, E., Ekblom, C., Söderhäll, K., 2022. Characterization of hemocytes and hematopoietic cells of a freshwater crayfish based on single-cell transcriptome analysis. *iScience* 25, 104850. <https://doi.org/10.1016/j.isci.2022.104850>.
- Söderhäll, K., Unestam, T., 1979. Activation of serum prophenoloxidase in arthropod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of crayfish phenoloxidase. *Can. J. Microbiol.* 25, 406–414. <https://doi.org/10.1139/m79-062>.
- Spitz, F., Furlong, E.E.M., 2012. Transcription factors: From enhancer binding to developmental control. *Nat. Rev. Genet.* 13, 613–626. <https://doi.org/10.1038/nrg3207>.
- Unestam, T., Söderhäll, K., 1977. Soluble fragments from fungal cell walls elicit defence reactions in crayfish. *Nature* 267, 45–46. <https://doi.org/10.1038/267045a0>.
- Unestam, T., Weiss, D.W., 1970. The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. *J. Gen. Microbiol.* 60, 77–90. <https://doi.org/10.1099/00221287-60-1-77>.
- Ungureanu, E., Mojžišová, M., Tangerman, M., Ion, M.C., Părvulescu, L., Petrussek, A., 2020. The spatial distribution of aphanomyces astaci genotypes across europe: Introducing the first data from Ukraine. *Freshw. Crayfish* 25, 77–87. <https://doi.org/10.5869/FC.2020.V25-1.077>.
- Vogt, G., 2023. Phenotypic plasticity in the monoclonal marbled crayfish is associated with very low genetic diversity but pronounced epigenetic diversity. *Curr. Zool.* 69, 426–441. <https://doi.org/10.1093/cz/zoac094>.
- Wang, C., Zhang, H., Zhu, J., Liu, H., Yang, Y., Sun, B., Wu, T., Zhang, Y., Yao, D., 2023. The transcription factor CEBP homolog of *Penaeus vannamei* contributes to WSSV replication. *Fish Shellfish Immunol.* 134, 108571 <https://doi.org/10.1016/j.fsi.2023.108571>.
- Weidemüller, P., Kholmatov, M., Petsalaki, E., Zaugg, J.B., 2021. Transcription factors: Bridge between cell signaling and gene regulation. *Proteomics* 21, 1–14. <https://doi.org/10.1002/pmic.202000034>.
- Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis.
- Williams, L.M., Gilmore, T.D., 2022. An innate ability: How do basal invertebrates manage their chronic exposure to microbes? *PLoS Pathog.* 18, 1–8. <https://doi.org/10.1371/journal.ppat.1010897>.
- Xie, X., Liu, M., Jiang, Q., Zheng, H., Zheng, L., Zhu, D., 2018. Role of Kruppel homolog 1 (Kr-h1) in methyl farnesoate-mediated vitellogenesis in the swimming crab *Portunus trituberculatus*. *Gene* 679, 260–265. <https://doi.org/10.1016/j.gene.2018.08.046>.
- Zheng, X., Li, S., Si, Y., Hu, J., Xia, Y., 2020. Locust can detect β -1, 3-glucan of the fungal pathogen before penetration and defend infection via the Toll signaling pathway. *Dev. Comp. Immunol.* 106, 103636 <https://doi.org/10.1016/j.dci.2020.103636>.
- Zheng, Z., Li, F., Li, H., Zhu, K., Xu, L., Yang, F., 2021. Rapid regulation of hemocyte homeostasis in crayfish and its manipulation by viral infection. *Fish Shellfish Immunol. Reports* 2, 100035. <https://doi.org/10.1016/j.fsirep.2021.100035>.